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(54) **Title:** BIOPROCESSING AND BIOPRODUCTION USING AVIAN CELL LINES

(57) **Abstract:** The invention relates to compositions and methods of modulating the status, activity or expression of avian transcripts in the process of bioproduction. In one aspect, the invention provides for a method for producing a biological product from an avian host cell.



BIOPROCESSING AND BIOPRODUCTION USING AVIAN CELL LINES**CROSS REFERENCE TO RELATED APPLICATIONS**

This application claims the benefit of U.S. Provisional Patent Application No. 61/499,757, filed June 22, 2011, entitled "BIOPROCESSING AND BIOPRODUCTION USING AVIAN CELL LINES" and U.S. Provisional Patent Application No. 61/639,333, filed April 27, 2012, entitled "BIOPROCESSING AND BIOPRODUCTION USING AVIAN CELL LINES" the contents, each of which is incorporated by reference in its entirety.

REFERENCE TO SEQUENCE LISTING

The instant application contains a "lengthy" Sequence Listing which has been submitted via CD-R in lieu of a printed paper copy, and is hereby incorporated by reference in its entirety. This sequence listing text file which is part of the originally filed subject matter has the following features: File name: 200212pc.TXT; File size: 622,641,152 Bytes; Date created: May 22, 2012. These CD-Rs are labeled "CRF," "Copy 1," "Copy 2," and "Copy 3" respectively, and each contains only one identical file, as identified immediately above.

REFERENCE TO LENGTHY TABLE

The specification includes Lengthy Table 1. Lengthy Table 1 has been submitted via EFS-Web in electronic format as follows: File name: AvianTranscripts.PDF; Date created: June 19, 2012; File size: 1,928,702 Bytes, and is incorporated herein by reference in its entirety. Please refer to the end of the specification for access instructions.

FIELD OF THE INVENTION

The invention relates generally to avian transcriptomes, organized transcriptomes and systems and methods (including methods of doing business) using transcriptomes for the design of targeting constructs useful in the production of biomolecules in avian cells

and avian cell cultures. The invention further relates to engineering avian cells and cell lines for more effective and efficient production of biological products and biomolecules.

BACKGROUND OF THE INVENTION

Cell culture and bioprocessing techniques are used to manufacture a wide range of biological products, including biopharmaceuticals, biofuels, metabolites, vitamins and nutraceuticals. A number of strategies have been developed to enhance productivity, yield, efficiency, and other aspects of cell culture bioprocesses in order to facilitate industrial scale production and meet applicable standards for product quality and consistency. Traditional strategies for optimizing cell culture bioprocesses involve adjusting physical and biochemical parameters, such as culture media (e.g., pH, nutrients) and conditions (e.g., temperature, duration), and selecting host cells having desirable phenotypes. More recently, genetic approaches have been developed for optimizing cell culture bioprocesses by introducing recombinant DNA into host cells, where the DNA encodes an exogenous protein that influences production of a biological product or regulates expression of an endogenous protein that influences production of the biological product. However, such methods require costly and time-consuming laboratory manipulations and can be incompatible with certain genes, proteins, host cells, and biological products.

Avian cells have been used in the production of biomolecules, particularly complex proteins such as antibodies. However, little is known about the coordinate expression and modulation of genes in these cells, and thus, optimized and targeted modulation of bioprocesses of these cells is unavailable presently.

Accordingly, there is a need in the art for new genetic approaches for optimizing cell culture bioprocesses involving a wide range of host cells, including avian cells, and biological products produced in these cells.

SUMMARY OF THE INVENTION

The invention is based at least in part on the surprising discovery that Targeting Constructs (TCs) can be applied to cells in culture to effect potent, durable modulation of gene expression, such that the quality and quantity of biological product that is produced

by an avian host cell can be improved without the need for extensive cell line engineering. As such, in a first aspect, the invention provides compositions and methods for producing a biological product from a host cell. In one embodiment the host cell is an avian cell or cell derived from avian cells. In various embodiments, the biological
5 product is a polypeptide (including enzymes), a metabolite, a nutraceutical, a chemical intermediate, a biofuel, a food additive, or an antibiotic (e.g., antiviral, antibacterial, antifungal, and the like, etc). The methods of the present invention also find utility in the fields of chemical manufacture, textiles and electronics.

The details of various embodiments of the invention are set forth in the
10 description below. Other features, objects, and advantages of the invention will be apparent from the description and the drawings, and from the claims.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides methods for producing a biological product in an avian
15 host cell, the methods including the steps of contacting the cell with at least one or more Targeting Construct (TC), maintaining the avian cell for a time sufficient to modulate expression of the target gene or alter a cellular process, wherein the modulation and/or alteration enhances production of the biological product, and recovering the biological product from the cell, cell culture or supernatant. The description provided herein
20 discloses how to make and use targeting constructs (TCs) to produce a biological product in an avian host cell or cell culture according to methods provided herein. Also disclosed are cell culture reagents and compositions comprising the targeting constructs and kits for carrying out the disclosed methods.

Also described herein are compositions and methods of modulation the status,
25 activity, or expression of Avian Transcripts (ATs) in an avian cell (or cell culture), tissue or organism. Further provided are compositions and methods for treating pathological conditions and diseases in a mammal using a biological product or targeting construct of the present invention. Further disclosed are screening methods, kits and assays which are designed to utilize the cellular pathways and systems associated with avian transcripts
30 and their manipulation in bioprocessing and bioproduction.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the targeting constructs (TC) and methods featured in the invention, suitable methods and materials are described below.

The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims.

As used herein and in the claims, the singular forms include the plural reference and vice versa unless the context clearly indicates otherwise. Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about."

All patents, oligonucleotide sequences identified by gene identification numbers (whether GeneID, SEQ ID, transcript identifier, etc), and other publications identified herein are expressly incorporated by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the present invention. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as those commonly understood to one of ordinary skill in the art to which this invention pertains. Although human gene symbols are typically designated by upper-case letters, in the present specification the use of either upper-case or lower-case gene symbols may be used interchangeably and include both human and/or non-human

species. In other words, the upper-case or lower-case letters in a particular gene symbol do not limit the scope of the gene or gene target to human or non-human species.

This application describes a variety of genes, transcripts, proteins, etc. using known names for the nucleic acid sequence. To the extent a specific sequence identifier is not cross-referenced to such a name, the artisan can readily do so by known means. For example, there are numerous searchable sites such as GeneCards (a collaborative searchable, integrated, database of human genes that provides concise genomic, transcriptomic, genetic, proteomic, functional and disease related information on all known and predicted human genes; database developed at the Crown Human Genome Center, Department of Molecular Genetics, the Weizmann Institute of Science), and publications that form the basis of such sites. One can readily use the name to locate the sequence and using such sequence cross-reference the Sequence ID No. used herein.

Throughout the specification, in some cases we have given the gene abbreviation or alias of the avian target gene and corresponding SEQ ID NOs for that gene or transcript. In some cases we have given the full gene name of the target gene, the corresponding SEQ ID No for the target gene (e.g., transcript sequence) as well as example nucleic acid based targeting construct SEQ ID NOs directed against the target gene. In various embodiments of the invention, the targeting construct is a siRNA that comprises an antisense strand comprising at least 16 contiguous nucleotides of a siRNA nucleotide sequence of any of the siRNA sequences identified herein by SEQ ID NO.

I. Avian transcript targets

Targets of the present invention include nucleic acid transcripts of avian cells. The avian nucleic acid transcripts include the genomic DNA genes and RNA transcripts (whether transcribed from either strand of chromosome and whether or not translated).

The avian transcript targets may be derived from the cells, cell cultures, tissue or hybridomas of any Superorder or Order of birds (Aves). Superorders include both the Palaeognathae and Neognathae. Orders of birds within the Palaeognathae include the Struthioniformes (ostriches, emus, kiwis) and Tinamiformes (tinamous) any of whose cells may encode avian transcripts as targets of the invention. The superorder

Neognathae includes the orders *Anseriformes* (waterfowl), *Galliformes* (fowl), *Charadriiformes* (gulls, button-quails, plover), *Gaviiformes* (loons), *Podicipediformes* (grebes), *Procellariiformes* (albatrosses, petrels), *Sphenisciformes* (penguins), *Pelecaniformes* (pelicans), *Phaethontiformes* (tropicbirds), *Ciconiiformes* (storks),
 5 *Cathartiformes* (New World vultures), *Phoenicopteriformes* (flamingos), *Falconiformes* (falcons, eagles, hawks), *Gruiformes* (cranes), *Pteroclidiformes* (sandgrouse), *Columbiformes* (doves and pigeons), *Psittaciformes* (parrots), *Cuculiformes* (cuckoos and turacos), *Opisthocomiformes* (hoatzin), *Strigiformes* (owls), *Caprimulgiformes* (nightjars), *Apodiformes* (swifts and hummingbirds), *Coraciiformes* (kingfishers),
 10 *Piciformes* (woodpeckers), *Trogoniformes* (trogons), *Coliiformes* (mousebirds) and *Passeriformes* (passerines).

In one embodiment, the avian transcript targets are those of the Order *Anseriformes* (waterfowl) and in particular those of ducks. Ducks whose transcripts, cells, cell cultures or tissues which may be targeted by the targeting constructs of the present
 15 invention include Abacot Ranger (also known as Streicher), Allier Duck (Blanc d'Allier), Ancona duck, Aylesbury Duck, Bali Duck, Black East Indian, Blue Swedish duck, Buff Orpington Duck, Call Duck, Challans, Cayuga Duck, Chara Chemballi Duck, Crested Duck, Danish Duck, Duclair, Dutch Hookbill (kromsnaveleend), East Indie Duck, Forest Duck (Eend van Vorst), Gimbsheimer, Golden Cascade, Gressingham Duck (Wild
 20 Mallard crossed with Pekin), Huttengem Duck, Indian Runner Duck, Magpie Duck, Majorcan Duck, Muscovy duck, Orpington duck, Pekin Duck (also known as Long Island duck), Rouen Duck, Saxony Duck, Semois, Silver Appleyard Duck, Silver Bantam Duck, Termonde Duck, Venetian Duck (Germanata Veneta), Welsh Harlequin Duck, Wood Duck and the Swedish Duck.

25 In one embodiment the transcripts, cells or cell cultures are from the Pekin duck. Cells of the Pekin duck can be obtained from ATCC (Mannassas, VA; number CCL-141; *Anas platyrhynchos domesticus*). Other duck cells which may be used in the methods of the present invention include those disclosed in US Publications 20090081251, 20100062489 and 20100226912, each of which is incorporated herein in its entirety. For
 30 example, cells derived by the methods in these publications such as EB12, EB24, or

EB66 may serve as suitable cell lines for the present invention. Targeting constructs which are useful in, for example, the CCL-1 line may also be applied to any of the EB-series of cell lines, or derivatives or hybrids thereof.

Avian genes may be as small as 1kb (kilobase) or as large as 100kb (kilobases)
5 while avian transcripts may range in size from 50 nucleotides to 20kb.

Avian transcripts may be at least 50, 75, 100, 150, 200 nucleotides, at least 300 nucleotides, at least 400 nucleotides, at least 500 nucleotides, at least 600 nucleotides, at least 700 nucleotides, at least 800 nucleotides, at least 900 nucleotides, at least 1000 nucleotides, at least 5000 nucleotides, at least 10,000 nucleotides or at least 20,000
10 nucleotides, and range from 250-300 nucleotides, 300-400 nucleotides, 400-500 nucleotides, 500-600 nucleotides, 600-700 nucleotides, 700-800 nucleotides, 800-900 nucleotides, 900-1000 nucleotides, 1000-5000 nucleotides, 5000-10,000 nucleotides, or 10,000-20,000 nucleotides in length.

As used herein, the term "avian gene" refers to a gene which is encoded within an
15 avian genome or in a genomic construct (whether natural or synthetic). Endogenous avian genes, e.g., those encoded within or engineered to be encoded by a host cell genome, may be intronic or intergenic and may encode proteins or other structural or functional RNAs. Avian genes include non-coding genes including those for lincRNA (long non coding RNAs) and the like.

20 "Intronic avian genes" are those found to be encoded substantially within an intron of a gene.

"Intergenic avian genes" are those found to be encoded between two different genes.

As used herein, the term "avian transcript," or "avian transcript target" refers to
25 an RNA transcript encoded by an "avian gene." Avian transcripts may encode peptides of 50 amino acids or less. It should be understood that avian transcripts may be synthesized as avian transcript variants, which may be engineered to encode a variety of peptides or proteins.

Other avian transcripts which may be targets of the invention includes primary
30 microRNAs (pri-miRs) which are processed to produce micro-RNAs or small RNA

species. In this context, either the pri-miRs or the pre-miRs may be avian transcript targets.

Representative avian transcript targets are listed in Lengthy Table 1. Certain avian genes of the present invention were identified from the Ensembl database (www.ensembl.org) and their respective RNA transcripts were extracted (SEQ ID NOs: 1-17169, having transcript names ENSAPLT000000000001 through ENSAPLT00000017169). Other microRNA avian transcripts were identified via internal sequencing efforts and these are also listed in Lengthy Table 1 (SEQ ID NOs: 17170 through 18039). To be clear, Table 1 lists the Ensembl gene identifier of each avian transcript with the prefix, ENSAPLT.

Also reported in Lengthy Table 1 are the length of the transcript, type and description of the transcript, SEQ ID NO of the transcript and the SEQ ID NOs of the Targeting Constructs designed to target each avian transcript. Where no targeting constructs were designed, the entry "NA" appears in the table. However, the selection of nucleic acid targeting constructs which would hybridize to the avian transcripts provided in Table 1 (and the sequence listing) is within the skill of one in the art.

As used herein, "target gene" refers to a gene that affects one or more aspects of the production of a biological product by a host cell, such that modulating expression of the gene enhances production of the biological product. Target genes may or may not encode proteins. Target genes can be derived from the host cell, may be latent in the host cell, may be endogenous to the host cell (present in the host cell genome), may be transgenes (gene constructs inserted at ectopic sites in the host cell genome), or may be derived from a pathogen (e.g., a virus, fungus or bacterium) that is capable of infecting the host cell or the subject who will use the biological product or derivatives thereof (e.g., humans). Additionally, in some embodiments, a "target gene" refers to a gene that regulates expression of a nucleic acid (i.e., non-encoding genes) that affects one or more aspects of the production of a biological product by a cell, such that modulating expression of the gene enhances production of the biological product.

In one embodiment, target genes are avian transcripts.

By “target gene RNA” or “target RNA” is meant RNA transcribed from the target gene. Hence, a target RNA may comprise a coding region, an introns, an exon, an introns-exon junction, a promoter region, a 3' untranslated region (3'-UTR), and/or a 5'-UTR of the target gene.

5 A target gene RNA that encodes a polypeptide is more commonly known as messenger RNA (mRNA). In some embodiments, the target gene encodes a protein that affects one or more aspects of post-translational modification, e.g., peptide glycosylation, by an avian host cell.

10 In some embodiments, the target gene encodes an avian non-coding RNA (ncRNA), such as an untranslated region. As used herein, a ncRNA refers to a target gene RNA that is not translated into a protein. The ncRNA can also be referred to as non-protein-coding RNA (npcRNA), non-messenger RNA (nmRNA), small non-messenger RNA (snmRNA), and functional RNA (fRNA) in the art. The target gene from which a ncRNA is transcribed as the end product is also referred to as a RNA gene or ncRNA
15 gene. ncRNA genes include highly abundant and functionally important RNAs such as transfer RNA (tRNA) and ribosomal RNA (rRNA), long intervening or intergenic RNAs (lncRNAs), as well as RNAs such as snoRNAs, microRNAs, siRNAs, and piRNAs.

20 In some embodiments, the target gene is an endogenous gene of the avian host cell. For example, the target gene can encode the biological product or a portion thereof when the biological product is a polypeptide. The target gene can also encode an avian host cell protein that directly or indirectly affects one or more aspects of the production of the biological product. Examples of target genes that affect the production of polypeptides include genes encoding proteins involved in the secretion, folding or post-translational modification of polypeptides (e.g., glycosylation, deamidation, disulfide
25 bond formation, methionine oxidation, or pyroglutamation); genes encoding proteins that influence a property or phenotype of the host cell (e.g., growth, viability, cellular pH, cell cycle progression, apoptosis, carbon metabolism or transport, lactate formation, cytoskeletal structure (e.g., actin dynamics), susceptibility to viral infection or RNAi uptake, activity or efficacy); and genes encoding proteins that impair the production of a

biological product by the host cell (e.g., a protein that binds or co-purifies with the biological product).

In some embodiments, production of a biological product is enhanced by targeting the expression of a protein that binds to the biological product. For example, in producing a growth factor, a hormone, or a cell signaling protein, it can be advantageous to reduce or inhibit expression of its receptor/ligand so that its production in the cell does not elicit a biological response. A receptor can be a cell surface receptor or an internal (e.g., nuclear) receptor. The expression of the binding partner can be modulated by contacting the host cell with a targeting construct targeting the receptor target gene according to methods described herein.

In some embodiments, the target gene encodes an avian host cell protein that indirectly affects the production of the biological product such that inhibiting expression of the target gene enhances production of the biological product. For example, the target gene can encode an abundantly expressed avian host cell protein that does not directly influence production of the biological product, but indirectly decreases its production, for example by utilizing cellular resources that could otherwise enhance production of the biological product.

Consequently, the production of biomolecules and biological products can be affected by modulation of the expression of an avian transcript target. The term “modulates expression of” and the like, in so far as it refers to a target gene, herein refers to the alteration of expression of a target gene, as manifested by a change (e.g., an increase or a decrease) in the amount of target gene RNA, mRNA, or protein that can be isolated from or detected in cells in which a target gene is transcribed (and/or translated) and that have been treated such that the expression of a target gene is modulated, as compared to a second cell or group of cells substantially identical to the first cell or group of cells but that have not been so treated (control cells). Avian cells are preferred cells of the invention.

The degree of modulation can be given in terms of a percent change of control or in terms of a parameter that is functionally linked to target gene expression, e.g., the amount of protein encoded by a target gene, or the number of cells displaying a certain

phenotype, e.g., stabilization of microtubules. In principle, target gene modulation can be determined in any avian host cell expressing the target gene, either constitutively or by genomic engineering, and by any appropriate assay known in the art.

In certain instances, expression of a target gene is inhibited. For example,
5 expression of a target gene is inhibited by at least about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% by administration of a targeting construct provided herein. In some embodiments, a target gene is inhibited by at least about 60%, 70%, or 80% by administration of a targeting construct. In some embodiments, a target gene is inhibited by at least about 85%, 90%, or 95% or more by administration of a targeting construct as
10 described herein. In other instances, expression of a target gene is activated by at least about 10%, 20%, 25%, 50%, 100%, 200%, 400% or more by administration of a targeting construct provided herein. In some embodiments, the modulation of expression is a partial inhibition. In some aspects, the partial inhibition is no greater than a percent inhibition selected from the group consisting of 10%, 15%, 20%, 25%, 30%, 35%, 40%,
15 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, and 85%.

It is expected that modulation or alteration of avian transcript function or levels (modulation of expression) can be used to regulate chromatin status, gene expression, transcription, translation, post-translational events and global biomolecular trafficking in the cell.

20 Methods of designing, modulating or targeting avian transcripts may be either structure-based or sequence based. Traditionally, methods of targeting nucleic acid molecules in the cell have been sequence based and have depended in some form on harnessing the hybridization or base pairing of two complementary molecules. Sequence-based methods of modulating or altering avian transcript function and levels
25 are described herein.

Also described are herein structure-based methods. As used herein, “structure based methods” are those methods of altering or modulating an avian transcript function or level that depends on the determination or knowledge of the higher order structure of at least a portion of an avian transcript target. “Higher order structures” include but are
30 not limited to the overall secondary, tertiary or quaternary structure of a molecule, e.g.,

hairpin structures, bulges, etc. These structures may be determined informatically with prediction algorithms based on thermodynamic parameters and energy calculations. Preferably, secondary structure prediction is performed using either M-fold or RNA Structure algorithm. Programs for secondary structure determination are freely available
5 online. Structures may also be determined by NMR, Mass Spectroscopy or by crystallographic methods. For RNA molecules, methods of determining overall structure or structures of portions of the RNA molecule are known in the art.

In US Patent 6,221,587, incorporated herein by reference in its entirety, are methods of identifying secondary structures in eukaryotic and prokaryotic RNA
10 molecules termed "molecular interaction sites." Molecular interaction sites are small, usually less than 30 nucleotides, independently folded, functional subdomains contained within a larger RNA molecule. These methods may be used to determine molecular interaction sites on avian transcripts.

Avian transcript targets may also be subjected to mimicry design. Disclosed in US
15 Patent 6,368,863 incorporated herein by reference in its entirety, are methods of identifying protein interacting sites on an RNA molecule and then designing an oligonucleotide that mimics that portion of the larger RNA molecules. These methods may be used in the present invention to design small avian transcript target mimics which will bind proteins.

20 Unlike sequence-based or hybridization driven targeting, which must rely on access of the targeting molecule to the target in order for base pairing to occur, structure-based targeting embraces a larger portion of the avian transcript target such as one or more features.

"Features," when referring to avian transcripts, are defined as distinct nucleic
25 acid-based components of the molecule. Features of the avian transcripts of the present invention may be structural features and may include surface manifestations, local conformational shape, folds, loops, half-loops, domains, half-domains, sites, termini or any combination thereof. When designing avian transcript variant molecules, the starting molecule may be one selected from Table 1 or known in the cell as the wild type
30 molecule. Alternatively, a series of modifications may be made in which the starting

molecule may be referred to simply as the parent molecule. Structural features of the present invention may be at least 200 nucleotides in length or from about 200 to about 500 nucleotides in length or from about 200 to about 300 nucleotides in length or from about 50 to about 100 nucleotides in length. They may also comprise the whole or any
5 part of a defined structural feature. Structural features may be 4-10, 5-15, 10-20, 10-30, or 20-50 nucleotides in length. These may be represented in increments of the triplet code and therefore may be any multiple of three. For example, features may be from 15-18, 15-30, 15-36, 15-60, 30-60, 30-90, 30-120 or larger.

As used herein when referring to avian transcripts the term "surface
10 manifestation" refers to a nucleic acid based component of an avian transcript appearing on an outermost surface of the avian transcript.

As used herein when referring to avian transcripts the term "local conformational shape" means a nucleic acid based structural manifestation of an avian transcript which is located within a definable space of the avian transcript.

15 As used herein when referring to avian transcripts the term "fold" means the resultant conformation of a nucleic acid sequence upon energy minimization. A fold may occur at the secondary or tertiary level of the folding process. Examples of secondary level folds include hairpins, loops and bulges. Examples of tertiary folds include domains and regions formed due to aggregation or separation of energetic forces. Regions formed
20 in this way include hydrophobic and hydrophilic pockets, and the like.

As used herein the term "turn" as it relates to avian transcript conformation means a bend which alters the direction of the backbone of a poly- or oligonucleotide and may involve one, two, three or more nucleotides.

As used herein when referring to avian transcripts the term "loop" refers to a
25 structural feature of a poly- or oligonucleotide which reverses the direction of the backbone of a poly- or oligonucleotide and comprises four or more nucleotides.

As used herein when referring to avian transcripts the term "half-loop" refers to a portion of an identified loop having at least half the number of nucleotides as the loop from which it is derived. It is understood that loops may not always contain an even
30 number of nucleotides. Therefore, in those cases where a loop contains or is identified to

comprise an odd number of nucleotides, a half-loop of the odd-numbered loop will comprise the whole number portion or next whole number portion of the loop (number of nucleotides of the loop/2+/-0.5 nucleotides). For example, a loop identified as a 7 nucleotide loop could produce half-loops of 3 nucleotides or 4 nucleotides ($7/2=3.5\pm0.5$ being 3 or 4).

As used herein when referring to avian transcripts the term "domain" refers to a motif of a poly- or oligonucleotide having one or more identifiable structural or functional characteristics or properties (e.g., binding capacity, serving as a site for protein-protein interactions, etc).

As used herein when referring to avian transcripts the term "half-domain" means portion of an identified domain having at least half the number of nucleotides as the domain from which it is derived. It is understood that domains may not always contain an even number of nucleotides. Therefore, in those cases where a domain contains or is identified to comprise an odd number of nucleotides, a half-domain of the odd-numbered domain will comprise the whole number portion or next whole number portion of the domain (number of nucleotides of the domain/2+/-0.5 nucleotides). It is also understood that sub-domains may be identified within domains or half-domains, these subdomains possessing less than all of the structural or functional properties identified in the domains or half domains from which they were derived. It is also understood that the nucleotides that comprise any of the domain types herein need not be contiguous along the backbone of the poly- or oligonucleotide (i.e., nonadjacent nucleotides may fold structurally to produce a domain, half-domain or subdomain).

As used herein when referring to avian transcripts the term "site" represents a location for targeting an avian transcript. A site represents a position within a poly- or oligonucleotide that may be modified, manipulated, altered, derivatized or varied within the polypeptide based molecules of the present invention. In one embodiment, "sites" of targeting can represent hundreds to thousands of nucleotides and may include nucleotides very distal in sequence location. For example, upon folding, avian transcripts may present surfaces, domains or sites which comprise nucleotides which only appear

juxtaposed due to the folded nature of the avian transcript. In one embodiment of the invention, targeting constructs may target any site on an avian transcript.

As used herein the terms "termini or terminus" when referring to avian transcripts refers to an extremity of a poly- or oligonucleotide. Such extremity is not limited only to the first or final site of the poly- or oligonucleotide but may include additional nucleotides in the terminal regions. The poly- or oligonucleotide based molecules of the present invention may be characterized as having both a 5' and a 3' terminus. Poly- or oligonucleotides of the invention are in some cases made up of multiple chains brought together by disulfide bonds or by non-covalent forces (multimers, oligomers or dendrimers). These sorts of poly- or oligonucleotides will have multiple 5' and 3'-termini. Alternatively, the termini of the poly- or oligonucleotide may be modified such that they begin or end, as the case may be, with a non- poly- or oligonucleotide based moiety such as a conjugate.

Once any of the features have been identified or defined as a component of an avian transcript of the invention, any of several manipulations and/or modifications of these features may be performed by moving, swapping, inverting, deleting, randomizing or duplicating them to produce additional avian transcript variants.

In one embodiment an avian transcript transcript variant is designed to encode a targeting construct. For example, an avian transcript may comprise the sequence of a shRNA or other nucleic acid based targeting construct. When encoded in the avian transcript variant the targeting construct (nucleic acid based) may be one that targets a different site on the avian transcript in which is is encoded or it may target the RNA transcript of a coding gene or any nucleic acid based transcript to which it will either hybridize (sequence based targeting) or form an interaction with (structure based targeting).

Furthermore, it is understood that manipulation of features may result in the same outcome as a modification to the molecules of the invention. For example, a manipulation which involved deleting a domain would result in the alteration of the length of a molecule just as modification of a nucleic acid to encode less than a full length molecule would.

Modifications and manipulations can be accomplished by methods known in the art. The resulting modified molecules may then be tested for activity using in vitro or in vivo assays such as those described herein or any other suitable screening assay known in the art.

5 In one embodiment of the invention, a feature of an avian transcript is removed to produce an avian transcript variant.

 In one embodiment of the invention, a feature of an avian transcript is duplicated to produce an avian transcript variant.

10 In one embodiment of the invention, a feature of an avian transcript is swapped with a second feature of an avian transcript to produce an avian transcript variant. In some embodiments the second feature is from the same or a different avian transcript.

 In one embodiment, hairpin features of an avian transcript are altered or modified. Hairpin structures of a first avian transcript may be inserted into a second avian transcript. They may also be removed from an avian transcript. Where a feature is found
15 to have a biological activity such as an interface for binding, or as a signal for localization, the feature may be reproduced in isolation by chemical or synthetic methods and used as targeting construct of the invention.

 In one embodiment of the invention, avian transcripts may be targeted to alter cellular memory, or cell identity. Without wishing to be bound by theory, it is believed
20 that the avian transcripts may contribute to cellular memory and play a determinative role in the cells ability to produce daughter cells of the same lineage or RNA population signature thereby maintaining the identity of the cells during cell divisions. As used herein, the “RNA population signature” of a cell is the qualitative complement of RNA transcripts present in a cell at a particular time or timeframe that distinguishes the cell
25 from other cell types. It should be understood that an RNA population signature of a cell does not necessarily comprise the sum total of all RNAs present in a cell but a set or subset of transcripts which may be used to identify one cell type from another cell type. RNA population signatures may comprise “nuclear signatures”, “cytoplasmic signatures”, “organelle associated signatures,” “tissue-associated signatures,” or combinations thereof.
30 They may also comprise the set of avian genes or transcripts or subsets thereof.

For example, the RNA nuclear population signature of an avian cell may comprise the set or subset of RNA transcripts present in the nucleus of the cell at a particular time or developmental phase such that this signature can be compared to other cells in order to determine whether the cells are of the same type or along the same path of lineage. Methods of measuring the presence of RNA in a cell are well known in the art. Methods of determining the cell type of a specific cell are known in the art and include for example the measurement of cell type specific markers such as proteins or protein or ligand expression, receptor or ligand presence or secretion.

In one embodiment of the present invention, the RNA population signature of an avian cell may be measured or identified and compared to another cell. The comparison will reveal differences between the two signatures. Differences in the avian transcript components of the signature may then be assessed and a cellular RNA population may be supplemented or reduced to effect a similar signature in the target cell population.

Avian cells of the present invention include, but are not limited to, cells that are derived primarily from endoderm (gland cells, exocrine secretory epithelial cells, hormone secreting cells, epithelial cells lining closed internal body cavities); cells derived primarily from ectoderm (integumentary system, keratinizing epithelial cells, wet stratified barrier epithelial cells, nervous system cells, sensory transducer cells, autonomic neuron cells, sense organ and peripheral neuron supporting cells, central nervous system neurons and glial cells, lens cells); cells derived primarily from mesoderm (metabolism and storage cells, barrier function cells (Lung, Gut, Exocrine Glands and Urogenital Tract), kidney; extracellular matrix secretion cells, contractile cells, blood and immune system cells, pigment cells, nurse cells and interstitial cells). Avian stem cells include, but are not limited to adult, embryonic, pluripotent, totipotent, and induced pluripotent.

In one embodiment of the invention are methods of controlling or reprogramming the cellular memory of a cell by administering to said cell or introducing into said cell a targeting construct which alters the function of an avian transcript. Alternatively, the RNA population signature may be altered by adding back one or more avian genes or transcripts. By altering the level of avian transcripts, cellular processes are altered such

that the cell may differentiate along a different path to alter the phenotype of the cell or to mirror the RNA population signature of a target cell. In one embodiment, alteration of cellular processes improves production of a biological product from an avian cell.

Alternatively, it is contemplated that the RNA population signature of an avian
5 cell may be altered by administering a targeting construct or an exogenously prepared avian gene or transcript, the outcomes of which would result in the alteration of the cellular phenotype. In one example, avian cells may be treated to alter the lineage or differentiation state of for example stem cells or cells of early developmental lineage.

These methods and compositions have utility in the areas of not only cellular
10 regulation but in the field of stem cell technology and the guided evolution of avian cellular phenotypes including the processes of bioproduction.

In one embodiment of the invention, the RNA population signature of an avian cell, cell line or tissue may be used in screening applications. According to the present invention, methods are provided for the use of the RNA population signature, more
15 specifically the avian transcript RNA population signature of an avian cell or tissue, in screening applications. It is also the case that avian transcripts may be supplemented in cells or cell populations that lack the normative complement of avian genes or transcripts. Supplementation may be from a donor cell which is avian. The donor cell may also be non-avian.

20 In one embodiment, the milieu of a cell or tissue may be used to provide a signaling environment for the study or exploitation of avian transcript regulation. As used herein, the term "milieu of a cell or tissue" means the supernatant or "soup" of a cell population or an extract of the cell system.

According to the present invention, a first population of cells may be incubated in
25 the milieu of a second population of cells in order to provide an environment which alters the expression levels or RNA population signature of the first population of cells. At least one of the first or second cell populations may be avian. To the milieu may be added one or more avian genes, transcripts or targeting constructs of the present invention. As a consequence of this addition the development, differentiation or overall gene expression
30 profile of the first population of cells may be changed. Cells which may be used to

provide the incubating milieu or which may be incubated in the milieu include but are not limited to somatic or gamete, stem cells, pluripotent cells, cells of primary origin, cells of any avian tissue, etc.

In one embodiment of the invention, chromatin inactivation or activation may be effected by the administration of one or more targeting constructs (whether sequence based or structure based) or the administration of one or more avian genes, avian transcripts or variant avian transcripts. According to the present invention, avian transcripts may be added to cells or cell systems to alter the epigenetic landscape of a cell or tissue.

In one embodiment of the invention, avian transcript cassettes may be added to or administered to a cell or tissue. As used herein an “avian transcript cassette” is a polynucleotide that encodes one or more avian transcripts. Avian transcript cassettes may encode a full length wild type avian transcript or may be designed to encode a modified avian transcript. The term “modified avian transcript” means an avian transcript which differs from the wild type sequence of the avian transcript in question. Modifications to avian transcripts include those modifications to the exonic structure of the avian transcript and include those having shuffled exon structures, omitted exons and additional exons.

According to the present invention, the avian transcript cassette may also contain a modified promoter from that which is found in the wild type avian transcript. Promoters may be swapped with those of protein coding genes or other avian genes. They may also be modified by addition, deletion or shuffling of promoter components. Synthetic avian genes or transcripts may be modified before contacting or administration to cells and the promoters may have temporary or permanent tags or transcription factors pre-associated with them. Targeting constructs of the present invention may also be designed to target the promoters of avian genes. In this design, the targeting constructs may be linked to, conjugated, associated or complexed with factors that target the targeting construct to the site of the avian transcript in the cell.

Where avian transcripts are found to encode or engineered to encode proteins or peptides, these proteins or peptides or their locus within the avian transcript may be a

target of the invention. Consequently, methods of regulating avian transcripts of the present invention may also regulate their encoded peptides.

As used herein “synthetic” refers to a state of having been created or man-made, e.g., not of natural origin. The targeting constructs or avian transcripts of the present invention may be synthesized using chemical or enzymatic or recombinant methods. 5 They may then be isolated from the synthetic mixture. Compounds of the present invention may also be isolated from a natural source.

Targeting constructs or avian transcripts of the present invention may be associated with chromatin modifying complexes, nucleosome components, proteins or 10 enzymes. They may also be modified to localize to either the nucleus or cytoplasm of the cells. In this manner, the targeting constructs or avian transcripts of the present invention may be guided to specific sites in a cell or tissue and may affect cellular processes such as gene expression, imprinting, aging, epigenetic signatures and the like.

15

II. Targeting constructs

The compositions of the present invention are those which may be used to regulate, control, manipulate, perturb or otherwise alter the expression, levels, activity or status of avian transcripts which in turn may alter processes in avian cells, cell culture or avian tissues. As such, the compositions of the present invention are termed “targeting constructs” or “TC.”

The targeting constructs of the present invention broadly include, but are not limited to, oligonucleotides, polynucleotides, iRNA agents, antisense molecules, ribozymes, aptamers, small molecules, amino acid-based constructs (antibodies, peptides, proteins, enzymes or fragments thereof), and vitamins.

Preferably, the targeting constructs useful for the methods and compositions featured in the invention specifically target RNAs (primary or processed) of the avian transcript target. Compositions and methods for inhibiting the expression of avian transcripts using iRNAs can be prepared and performed as described herein. In one embodiment, a composition containing a targeting construct, where the targeting construct includes a nucleotide sequence that is complementary to at least a part of an RNA or DNA transcript of the avian transcript is administered to an avian cell.

In one embodiment, a targeting construct as described herein effects modulation, inhibition or activation of avian transcript expression. As used herein, the term "modulate the expression of," refers to an at least partial "inhibition" or partial "activation" of an avian gene or transcript expression in a cell contacted with a targeting construct composition as described herein compared to the expression of an avian gene or transcript in an untreated cell. Modulation of expression may be determined not only by direct measurement of an avian transcript level after contacting with the targeting construct, but also be inference by associating a known phenotypic outcome which correlates to said contacting.

The term “expression” as used herein is intended to mean the transcription to a RNA and/or translation to one or more polypeptides from a target gene (e.g., the avian transcripts) coding for the sequence of the RNA and/or the polypeptide.

The terms “silence,” “inhibit the expression of,” “down-regulate the expression of,” “suppress the expression of,” and the like, in so far as they refer to an avian gene or transcript, herein refer to the at least partial suppression of the expression of avian transcript, as manifested by a reduction of the amount of the avian transcript in whole or in part which may be isolated from or detected in a first cell or group of cells in which an avian transcript is transcribed and which has or have been treated such that the expression of an avian transcript is inhibited, as compared to a second cell or group of cells substantially identical to the first cell or group of cells but which has or have not been so treated (control cells).

Alternatively, inhibition or the degree of inhibition may be given in terms of a reduction of a parameter that is functionally linked to avian transcript expression, *e.g.*, the amount of protein encoded by a mRNA that itself is controlled by an avian transcript, or the number of cells displaying a certain phenotype, *e.g.*, lack of or decreased cytokine production or the status of a cell, *e.g.*, the epigenetic profile or signature of a cell which is altered upon modulation of one or more avian transcript targets.

In principle, avian transcript silencing may be determined in any cell expressing avian transcripts, either constitutively or by genomic engineering, and by any appropriate assay.

For example, in certain instances, expression of an avian transcript is suppressed by a targeting construct by at least about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% by administration of a targeting construct featured in the invention. In some embodiments, an avian transcript is suppressed by at least about 60%, 70%, or 80% by administration of a targeting construct featured in the invention. In some embodiments, an avian transcript is suppressed by at least about 85%, 90%, 95%, 98%, 99%, or more by administration of a targeting construct as described herein.

The terms “activate,” “enhance,” “up-regulate the expression of,” “increase the expression of,” and the like, in so far as they refer to an avian gene or transcript, herein refer to the at least partial activation of the expression of an avian transcript, as manifested by an increase in the amount of avian transcript in whole or in part, which may be isolated from or detected in a first cell or group of cells in which an avian

transcript is transcribed and which has or have been treated such that the expression of an avian transcript is increased, as compared to a second cell or group of cells substantially identical to the first cell or group of cells but which has or have not been so treated (control cells).

5 In one embodiment, expression of an avian transcript is activated by a targeting construct by at least about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% by administration of a targeting construct as described herein. In some embodiments, an avian transcript is activated by at least about 60%, 70%, or 80% by administration of a targeting construct featured in the invention. In some embodiments, expression of an
10 avian transcript is activated by at least about 85%, 90%, or 95% or more by administration of a targeting construct as described herein. In some embodiments, the avian transcript expression is increased by at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 50-fold, at least 100-fold, at least 500-fold, at least 1000 fold or more in cells treated with a targeting construct as described herein compared to the
15 expression in an untreated cell. Activation of expression of coding mRNAs by small dsRNAs is described, for example, in Li et al., 2006 *Proc. Natl. Acad. Sci. U.S.A.* 103:17337-42, and in US20070111963 and US2005226848, each of which is incorporated herein by reference. It is believed that constructs that activate the expression of coding RNA transcripts will also activate the expression of non-coding RNA
20 transcripts such as the avian transcripts of the present invention.

Targeting constructs may be designed to target regions or sites along an avian transcript which correlate to hypersensitivity sites (HS) found on the corresponding DNA encoding the avian transcript. Hypersensitivity sites (HS) and methods of identification are described in PCT Publication WO/2004/053106, the contents of which are
25 incorporated by reference herein in its entirety.

In some embodiments, the “region” when used in reference to a targeting construct comprises a portion of a targeting construct. Where the targeting construct is an oligonucleotide, one of skill in the art can vary the length of the “region” that is complementary to the target gene or arranged in a duplex, such that a targeting construct
30 having desired characteristics (e.g., inhibition of a target gene or stability) is produced.

In one embodiment, the targeting constructs of the present invention may target, mimic, bind to, replace or alter the levels or function of a product of an avian transcript. As used herein “avian transcript products” include any molecule engineered to be a product of an avian transcript either by transcription, translation, cleavage, splicing, or
5 other mechanism that produces a derivative of an avian transcript. Examples of avian transcript products include, but are not limited to, peptides or proteins engineered to be coded by the avian transcript or fragments of the avian transcript.

Targeting constructs may be oligonucleotides or nucleic acid molecules. In the context of this invention, the term “oligonucleotide” or “nucleic acid molecule”
10 encompasses not only nucleic acid molecules as expressed or found in nature, but also analogs and derivatives of nucleic acids comprising one or more ribo- or deoxyribo-nucleotide/nucleoside analogs or derivatives as described herein or as known in the art. Such analogs or derivatives (including modified and/or substituted oligonucleotides) are often used over natural (native or wild type) forms because of properties such as, for
15 example, enhanced cellular uptake, increased stability in the presence of nucleases, and the like, discussed further herein. A “nucleoside” includes a nucleoside base and a ribose sugar, and a “nucleotide” is a nucleoside with one, two or three phosphate moieties. The terms “nucleoside” and “nucleotide” can be considered to be equivalent as used herein. An oligonucleotide can be modified in the nucleobase structure or in the sugar-phosphate
20 backbone structure, e.g., as described herein, including the modification of a RNA nucleotide into a DNA nucleotide.

As non-limiting examples, an oligonucleotide can also include at least one modified nucleoside including but not limited to a 2'-O-methyl modified nucleoside, a nucleoside comprising a 5' phosphorothioate group, a terminal nucleoside linked to a
25 cholesterol derivative or dodecanoic acid bisdecylamide group, a locked nucleoside, an abasic nucleoside, a 2'-deoxy-2'-fluoro modified nucleoside, a 2'-amino-modified nucleoside, 2'-alkyl-modified nucleoside, morpholino nucleoside, a phosphoramidate or a non-natural base comprising nucleoside, or any combination thereof. Alternatively, an oligonucleotide can comprise at least two modified nucleosides, at least 3, at least 4, at
30 least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, or

more, up to the entire length of the oligonucleotide. The modifications need not be the same for each of such a plurality of modified nucleosides in an oligonucleotide. When the targeting construct (TC) is double stranded oligonucleotide, each strand can be independently modified as to number, type and/or location of the modified nucleosides.

5 In one embodiment, modified oligonucleotides contemplated for use in methods and compositions described herein are peptide nucleic acids (PNAs) that have the ability to form the required duplex structure and that permit or mediate the specific degradation of a target RNA via a RISC pathway.

The terms “ribonucleoside”, “ribonucleotide”, “nucleotide”, or
10 “deoxyribonucleotide” can also refer to a modified nucleotide, as further detailed herein, or a surrogate replacement moiety. A ribonucleotide comprising a thymine base is also referred to as 5-methyl uridine and a deoxyribonucleotide comprising a uracil base is also referred to as deoxy-Uridine in the art. Guanine, cytosine, adenine, thymine and uracil can be replaced by other moieties without substantially altering the base pairing
15 properties of an oligonucleotide comprising a nucleotide bearing such replacement moiety. For example, without limitation, a nucleotide comprising inosine as its base can base pair with nucleotides containing adenine, cytosine, or uracil. Hence, nucleotides containing uracil, guanine, or adenine can be replaced in the nucleotide sequences of dsRNA featured in the invention by a nucleotide containing, for example, inosine. In
20 another example, adenine and cytosine anywhere in the oligonucleotide can be replaced with guanine and uracil, respectively to form G-U Wobble base pairing with the target mRNA. Sequences containing such replacement moieties are suitable for the compositions and methods featured in the invention.

Similarly, the skilled artisan will recognize that the term “RNA molecule” or
25 “ribonucleic acid molecule” encompasses not only RNA molecules as expressed or found in nature, but also analogs and derivatives of RNA comprising one or more ribonucleotide or ribonucleoside analogs or derivatives as described herein or as known in the art.

In one aspect, a targeting construct can include a deoxyribonucleoside residue. In
30 such an instance, a targeting construct agent can comprise one or more deoxynucleosides,

including, for example, a deoxynucleoside overhang(s), or one or more deoxynucleosides within the double stranded portion of a dsRNA.

In some embodiments, a plurality of targeting constructs is used to modulate expression of one or more target genes. A “plurality” refers to at least 2 or more targeting constructs e.g., 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 80, 100 targeting constructs or more. “Plurality” can also refer to at least 2 or more target genes, e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100 target genes or more.

Targeting constructs of the present invention are administered to the avian host cell in a variety of ways. Cells may be simply contacted and/or the targeting constructs may be taken up by the cells. As used herein the term “contacting a host cell” refers to the treatment of a host cell with an agent such that the agent is introduced into the cell. Typically the host cell is in culture, e.g., using at least one Targeting Construct (e.g., an siRNA), often prepared in a composition comprising a delivery agent that facilitates targeting construct uptake into the cell e.g., to contact the cell in culture by adding the composition to the culture medium. In one embodiment the host cell is contacted with a vector that encodes a targeting construct, e.g., an integrating or non-integrating vector. In one embodiment the cell is contacted with a vector that encodes a targeting construct prior to culturing the host cell for biological production, e.g., by transfection or transduction.

In one embodiment, contacting a host cell includes contacting an avian host cell with a vector that encodes a targeting construct, e.g., added to the host cell culture during the process of producing a biological product. The step of contacting a host cell in culture with a targeting construct(s) can be repeated more than once (e.g., twice, 3x, 4x, 5x, 6x, 7x, 8x, 9x, 10x, 11x, 12x, 13x, 14x, 15x, 16x, 17x, 18x, 19x, 20x, 30x, 40x, 50x, 60x, 70x, 80x, 90x, 100x or more). In one embodiment, the cell is contacted such that the target gene is modulated only transiently, e.g., by addition of a targeting construct composition to the cell culture medium used for the production of a biological product where the presence of the targeting constructs dissipates over time, i.e., the targeting construct is not constitutively expressed in the cell.

“Introducing into a cell”, when referring to a targeting construct, means facilitating or effecting uptake or absorption into the avian cell, as is understood by those skilled in the art. Absorption or uptake of a targeting construct can occur through unaided diffusive or active cellular processes, or by auxiliary agents or devices. For example, introducing into a cell means contacting a host cell with at least one targeting construct, or means the treatment of a cell with at least one targeting construct and an agent that facilitates or effects uptake or absorption into the cell, often prepared in a composition comprising the targeting construct and delivery agent that facilitates targeting construct uptake (e.g., a transfection reagent, an emulsion, a cationic lipid, a non-cationic lipid, a charged lipid, a liposome, an anionic lipid, a penetration enhancer, or a modification to the targeting construct to attach, e.g., a ligand, a targeting moiety, a peptide, a lipophilic group etc.). *In vitro* introduction into a cell includes methods known in the art such as electroporation and lipofection. Further approaches are described herein below or known in the art.

Compositions comprising the targeting constructs of the present invention are also provided. As used herein, a “targeting construct composition” includes an effective amount of a targeting construct and an acceptable carrier. As used herein, “effective amount” refers to that amount of a targeting construct effective to produce an effect (e.g., modulatory effect) on a bioprocess for the production of a biological product. In one embodiment, the targeting construct composition comprises a reagent that facilitates targeting construct uptake (e.g., a transfection reagent, an emulsion, a cationic lipid, a non-cationic lipid, a charged lipid, a liposome, an anionic lipid, a penetration enhancer, or a modification to the targeting construct to attach e.g., a ligand, a targeting moiety, a peptide, a lipophilic group, etc.).

The term “acceptable carrier” refers to a carrier for administration of a targeting construct to avian cells. Such carriers include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. In one embodiment the term “acceptable carrier” specifically excludes cell culture medium.

In one embodiment, the method includes administering a targeting construct composition as described herein to an avian cell such that expression of the avian

transcript is increased by e.g., at least 10% compared to an untreated cells. In some embodiments, the activation of avian transcripts occurs over an extended duration, e.g., at least two, three, four days or more, e.g., one week, two weeks, three weeks, four weeks, or more. Without wishing to be bound by theory, a targeting construct can activate avian transcript expression by stabilizing the avian transcript, interacting with a promoter in the genome, and/or inhibiting an inhibitor of avian transcript expression.

Amino acid-based targeting constructs

The targeting constructs of the present invention may also be amino acid based molecules. These molecules may be "peptides," "polypeptides," or "proteins" whether structural (antibodies) or catalytic (enzymes). While it is known in the art that these terms imply relative size, these terms as used herein should not be considered limiting with respect to the size of the various polypeptide based molecules referred to herein and which are encompassed within this invention.

The terms "amino acid" and "amino acids" refer to all naturally occurring L-alpha-amino acids. The amino acids are identified by either the one-letter or three-letter designations as follows: aspartic acid (Asp:D), isoleucine (Ile:I), threonine (Thr:T), leucine (Leu:L), serine (Ser:S), tyrosine (Tyr:Y), glutamic acid (Glu:E), phenylalanine (Phe:F), proline (Pro:P), histidine (His:H), glycine (Gly:G), lysine (Lys:K), alanine (Ala:A), arginine (Arg:R), cysteine (Cys:C), tryptophan (Trp:W), valine (Val:V), glutamine (Gln:Q) methionine (Met:M), asparagines (Asn:N), where the amino acid is listed first followed parenthetically by the three and one letter codes, respectively.

The amino acid sequences of the targeting constructs of the invention may comprise naturally occurring amino acids and as such may be considered to be proteins, peptides, polypeptides, or fragments thereof. Alternatively, the targeting constructs may comprise both naturally and non-naturally occurring amino acids or only non-naturally occurring amino acids.

The term "amino acid sequence variant" refers to molecules with some differences in their amino acid sequences as compared to a native sequence. The amino acid sequence variants may possess substitutions, deletions, and/or insertions at certain positions within the amino acid sequence. Ordinarily, variants will possess at least about

70% homology to a native sequence, and preferably, they will be at least about 80%, more preferably at least about 90% homologous to a native sequence.

"Homology" as it applies to amino acid sequences is defined as the percentage of residues in the candidate amino acid sequence that are identical with the residues in the amino acid sequence of a second sequence after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology. Methods and computer programs for the alignment are well known in the art. It is understood that homology depends on a calculation of percent identity but may differ in value due to gaps and penalties introduced in the calculation.

By "homologs" as it applies to amino acid sequences is meant the corresponding sequence of other species having substantial identity to a second sequence of a second species.

"Analog" is meant to include polypeptide variants which differ by one or more amino acid alterations, e.g., substitutions, additions or deletions of amino acid residues that still maintain the properties of the parent polypeptide.

The term "derivative" is used synonymously with the term "variant" and refers to a molecule that has been modified or changed in any way relative to a reference molecule or starting molecule.

The present invention contemplates several types of targeting constructs which are amino acid based including variants and derivatives. These include substitutional, insertional, deletion and covalent variants and derivatives. As such, included within the scope of this invention are polypeptide based molecules containing substitutions, insertions and/or additions, deletions and covalently modifications. For example, sequence tags or amino acids, such as one or more lysines, can be added to the peptide sequences of the invention (e.g., at the N-terminal or C-terminal ends). Sequence tags can be used for peptide purification or localization. Lysines can be used to increase peptide solubility or to allow for biotinylation. Alternatively, amino acid residues located at the carboxy and amino terminal regions of the amino acid sequence of a peptide or protein may optionally be deleted providing for truncated sequences. Certain amino acids (e.g., C-terminal or N-terminal residues) may alternatively be deleted depending on the use of

the sequence, as for example, expression of the sequence as part of a larger sequence which is soluble, or linked to a solid support.

"Substitutional variants" when referring to proteins are those that have at least one amino acid residue in a native or starting sequence removed and a different amino acid
5 inserted in its place at the same position. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule.

As used herein the term "conservative amino acid substitution" refers to the substitution of an amino acid that is normally present in the sequence with a different
10 amino acid of similar size, charge, or polarity. Examples of conservative substitutions include the substitution of a non-polar (hydrophobic) residue such as isoleucine, valine and leucine for another non-polar residue. Likewise, examples of conservative substitutions include the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, and between glycine
15 and serine. Additionally, the substitution of a basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue such as aspartic acid or glutamic acid for another acidic residue are additional examples of conservative substitutions. Examples of non-conservative substitutions include the substitution of a non-polar (hydrophobic) amino acid residue such as isoleucine, valine, leucine, alanine,
20 methionine for a polar (hydrophilic) residue such as cysteine, glutamine, glutamic acid or lysine and/or a polar residue for a non-polar residue.

"Insertional variants" when referring to proteins are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a native or starting sequence. "Immediately adjacent" to an amino acid means connected to either the
25 alpha-carboxy or alpha-amino functional group of the amino acid.

"Deletional variants" when referring to proteins are those with one or more amino acids in the native or starting amino acid sequence removed. Ordinarily, deletional variants will have one or more amino acids deleted in a particular region of the molecule.

"Covalent derivatives" when referring to proteins include modifications of a
30 native or starting protein with an organic proteinaceous or non-proteinaceous derivatizing

agent, and post-translational modifications. Covalent modifications are traditionally introduced by reacting targeted amino acid residues of the protein with an organic derivatizing agent that is capable of reacting with selected side-chains or terminal residues, or by harnessing mechanisms of post-translational modifications that function in selected recombinant host cells. The resultant covalent derivatives are useful in programs directed at identifying residues important for biological activity, for immunoassays, or for the preparation of anti-protein antibodies for immunoaffinity purification of the recombinant glycoprotein. Such modifications are within the ordinary skill in the art and are performed without undue experimentation.

Certain post-translational modifications are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues may be present in the proteins used in accordance with the present invention.

Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the .alpha.-amino groups of lysine, arginine, and histidine side chains (T. E. Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)).

Covalent derivatives specifically include fusion molecules in which proteins of the invention are covalently bonded to a non-proteinaceous polymer. The non-proteinaceous polymer ordinarily is a hydrophilic synthetic polymer, i.e. a polymer not otherwise found in nature. However, polymers which exist in nature and are produced by recombinant or in vitro methods are useful, as are polymers which are isolated from nature. Hydrophilic polyvinyl polymers fall within the scope of this invention, e.g. polyvinylalcohol and polyvinylpyrrolidone. Particularly useful are polyvinylalkylene ethers such as polyethylene glycol, polypropylene glycol. The proteins may be linked to various non-proteinaceous polymers, such as polyethylene glycol, polypropylene glycol

or polyoxyalkylenes, in the manner set forth in U.S. Pat. No. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

"Features" when referring to proteins are defined as distinct amino acid sequence-based components of a molecule. Features of the proteins of the present invention include
5 surface manifestations, local conformational shape, folds, loops, half-loops, domains, half-domains, sites, termini or any combination thereof.

As used herein when referring to proteins the term "surface manifestation" refers to a polypeptide based component of a protein appearing on an outermost surface.

As used herein when referring to proteins the term "local conformational shape"
10 means a polypeptide based structural manifestation of a protein which is located within a definable space of the protein.

As used herein when referring to proteins the term "fold" means the resultant conformation of an amino acid sequence upon energy minimization. A fold may occur at the secondary or tertiary level of the folding process. Examples of secondary level folds
15 include beta sheets and alpha helices. Examples of tertiary folds include domains and regions formed due to aggregation or separation of energetic forces. Regions formed in this way include hydrophobic and hydrophilic pockets, and the like.

As used herein the term "turn" as it relates to protein conformation means a bend which alters the direction of the backbone of a peptide or polypeptide and may involve
20 one, two, three or more amino acid residues.

As used herein when referring to proteins the term "loop" refers to a structural feature of a peptide or polypeptide which reverses the direction of the backbone of a peptide or polypeptide and comprises four or more amino acid residues. Oliva et al. have identified at least 5 classes of protein loops (J. Mol Biol 266 (4): 814-830; 1997).

25 As used herein when referring to proteins the term "half-loop" refers to a portion of an identified loop having at least half the number of amino acid residues as the loop from which it is derived. It is understood that loops may not always contain an even number of amino acid residues. Therefore, in those cases where a loop contains or is identified to comprise an odd number of amino acids, a half-loop of the odd-numbered
30 loop will comprise the whole number portion or next whole number portion of the loop

(number of amino acids of the loop/2+/-0.5 amino acids). For example, a loop identified as a 7 amino acid loop could produce half-loops of 3 amino acids or 4 amino acids ($7/2=3.5\pm0.5$ being 3 or 4).

As used herein when referring to proteins the term "domain" refers to a motif of a polypeptide having one or more identifiable structural or functional characteristics or properties (e.g., binding capacity, serving as a site for protein-protein interactions).

As used herein when referring to proteins the term "half-domain" means portion of an identified domain having at least half the number of amino acid residues as the domain from which it is derived. It is understood that domains may not always contain an even number of amino acid residues. Therefore, in those cases where a domain contains or is identified to comprise an odd number of amino acids, a half-domain of the odd-numbered domain will comprise the whole number portion or next whole number portion of the domain (number of amino acids of the domain/2+/-0.5 amino acids). For example, a domain identified as a 7 amino acid domain could produce half-domains of 3 amino acids or 4 amino acids ($7/2=3.5\pm0.5$ being 3 or 4). It is also understood that sub-domains may be identified within domains or half-domains, these subdomains possessing less than all of the structural or functional properties identified in the domains or half domains from which they were derived. It is also understood that the amino acids that comprise any of the domain types herein need not be contiguous along the backbone of the polypeptide (i.e., nonadjacent amino acids may fold structurally to produce a domain, half-domain or subdomain).

As used herein when referring to proteins the terms "site" as it pertains to amino acid based embodiments is used synonymous with "amino acid residue" and "amino acid side chain". A site represents a position within a peptide or polypeptide that may be modified, manipulated, altered, derivatized or varied within the polypeptide based molecules of the present invention.

As used herein the terms "termini or terminus" when referring to proteins refers to an extremity of a peptide or polypeptide. Such extremity is not limited only to the first or final site of the peptide or polypeptide but may include additional amino acids in the terminal regions. The polypeptide based molecules of the present invention may be

characterized as having both an N-terminus (terminated by an amino acid with a free amino group (NH₂)) and a C-terminus (terminated by an amino acid with a free carboxyl group (COOH)). Proteins of the invention are in some cases made up of multiple polypeptide chains brought together by disulfide bonds or by non-covalent forces (multimers, oligomers). These sorts of proteins will have multiple N- and C-termini. Alternatively, the termini of the polypeptides may be modified such that they begin or end, as the case may be, with a non-polypeptide based moiety such as an organic conjugate.

Once any of the features have been identified or defined as a component of a molecule of the invention, any of several manipulations and/or modifications of these features may be performed by moving, swapping, inverting, deleting, randomizing or duplicating. Furthermore, it is understood that manipulation of features may result in the same outcome as a modification to the molecules of the invention. For example, a manipulation which involved deleting a domain would result in the alteration of the length of a molecule just as modification of a nucleic acid to encode less than a full length molecule would.

Modifications and manipulations can be accomplished by methods known in the art such as site directed mutagenesis. The resulting modified molecules may then be tested for activity using in vitro or in vivo assays such as those described herein or any other suitable screening assay known in the art.

Antibodies

In one embodiment of the present invention, antibodies and antibody technology may be employed to affect the expression or role of avian transcripts in a cell. As discussed elsewhere herein, antibodies may also represent the biological product or biomolecule to be produced by the methods of the present invention.

As used herein, the term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of an intact antibody, preferably comprising the

antigen binding region thereof. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments,
5 each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an F(ab')₂ fragment that has two antigen-binding sites and is still capable of cross-linking antigen. For the purposes herein, an "intact antibody" is one comprising heavy and light variable domains as well as an Fc region.

10 "Native antibodies" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide
15 bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed
20 to form an interface between the light chain and heavy chain variable domains.

The term "variable domains" in reference to antibodies refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen.

25 "Fv" is the minimum antibody fragment that contains a complete antigen-recognition and antigen-binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa and lambda based on
30 the amino acid sequences of their constant domains. Depending on the amino acid

sequence of the constant domain of their heavy chains, antibodies can be assigned to different classes. There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2.

- 5 "Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. In some embodiments, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains that enables the scFv to form the desired structure for antigen binding.

The term "diabodies" refers to small antibody fragments with two antigen-binding
10 sites, which fragments comprise a heavy chain variable domain V_H connected to a light chain variable domain V_L in the same polypeptide chain. By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites.

- 15 The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variants that may arise during production of the monoclonal antibody, such variants generally being present in minor amounts. In contrast to polyclonal antibody preparations
20 that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen

The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be
25 construed as requiring production of the antibody by any particular method. The monoclonal antibodies herein include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or

homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies.

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity.

The term "hypervariable region" when used herein in reference to antibodies refers to the amino acid residues of an antibody that are responsible for antigen binding. The hypervariable region comprises amino acid residues from a "complementarity determining region.

In one embodiment of the invention, antibodies which alter the levels or function of avian transcripts may be designed to directly or indirectly affect avian transcript targets. The preparation of antibodies, whether monoclonal or polyclonal, is known in the art. Techniques for the production of antibodies are well known in the art and described, e.g. in Harlow and Lane "Antibodies, A Laboratory Manual", Cold Spring Harbor Laboratory Press, 1988 and Harlow and Lane "Using Antibodies: A Laboratory Manual" Cold Spring Harbor Laboratory Press, 1999.

Antibodies of the present invention may be designed to directly affect the levels of avian transcript targets by binding directly to the avian transcripts. Alternatively, antibodies can be designed to target the proteins encoded by the nearest neighbor (those immediately upstream or downstream on a chromosome) genes of avian transcripts, thereby altering the expression of the avian transcript.

Other proteins as targeting constructs

In one embodiment, artificial avian nucleosome proteins are provided. These proteins are only "artificial" in the sense that they are not identical to the wild-type proteins of the nucleosomes of the cells, or histones.

It is known in the art that nucleosomes undergo dynamic and necessary changes in order to facilitate presentation of genomic loci to the transcriptional machinery. This

opening and closing of this chromosome packaging structure or “nucleosome respiration” is also accompanied by changes in the histone proteins at the nucleosome core.

Taking advantage of this cellular process, the present invention provides compositions and methods for the manipulation and/or control of avian gene expression and cellular regulation via the substitution or alteration of the protein components of avian nucleosomes.

More specifically, the present invention provides amino acid based compositions which replace one or more of the proteins contained in an avian nucleosome, but in doing so, alter the overall function of the nucleosome in a manner that alters the expression of one or more avian genes. The avian gene affected in this manner can be one whose DNA is packaged around the artificial nucleosome (e.g., the nucleosome harboring the protein variant) or one lying up or downstream of the artificial nucleosome whose transcription is altered as a result of the changes in the wild-type nucleosome.

In one embodiment are methods of altering the gene expression pattern of avian genes within a cell comprising contacting the cells with artificial nucleosome proteins.

In one embodiment are methods of altering the epigenetic signature of avian cells comprising contacting the cell with one or more artificial nucleosome proteins.

The artificial nucleosome proteins of the present invention include, but are not limited to analogs, homologs, variants or derivatives of wild-type avian histone proteins.

In one embodiment, the avian epigenetic signature, a component of which is the modification pattern of the histones, e.g, methylation, may be changed by replacing wild type nucleosome proteins or histones with artificial histones engineered to have sites which are blocked such that cellular enzymes may no longer access or effect modifications to the proteins. These modifications may be permanent or transient. For example, blocking access of histones by histone methyl transferases, or other modifying enzymes, can alter the status of a cells resulting in gene expression being “transcriptionally on” or “transcriptionally off”. This embodiment is an attractive alternative to the small molecules being developed to modulate cellular enzymes which act to acetylate, methylate, ubiquitinate, etc, DNA or nucleosome components.

Other artificial proteins, not contained within the nucleosome, are also provided which may alter the expression of an avian gene. These include variants or derivatives of nucleic acid binding proteins (e.g., RNA binding proteins, DNA binding proteins, chaperones, and the like) either localized in or which traffic to or within the nucleus.

5 RNA agents

“RNA agents” of the present invention include, but are not limited to, small interfering RNAs (siRNA), double stranded RNAs (dsRNAs), inverted repeats, short hairpin RNAs (shRNAs), small temporally regulated RNAs (stRNA), clustered inhibitory RNAs (cRNAs), including radial clustered inhibitory RNA, asymmetric clustered inhibitory RNA, linear clustered inhibitory RNA, and complex or compound clustered inhibitory RNA, dicer substrates, DNA-directed RNAi (ddRNAi), single-stranded RNAi (ssRNAi), microRNA (miRNA) antagonists, microRNA mimics, microRNA agonists, blockmirs (a.k.a. Xmirs), microRNA mimetics, microRNA addbacks, supermiRs, the oligomeric constructs disclosed in PCT Publication WO/2005/013901 the contents of which are incorporated herein in its entirety, tripartite RNAi constructs such as those disclosed in US Publication 20090131360, the contents of which are incorporated herein in its entirety, the solo-rxRNA constructs disclosed in PCT Publication WO/2010/011346, the contents of which are incorporated herein by reference in its entirety; the sd-rxRNA constructs disclosed in PCT Publication WO/2010/033247 the contents of which are incorporated herein by reference in its entirety, dual acting RNAi constructs which reduce RNA levels and also modulate the immune response as disclosed in PCT Publications WO/2010/002851 and WO/2009/141146 the contents of which are incorporated herein by reference in their entirety and antigenic RNAs (agRNA) or small activating RNAs (saRNAs) which increase expression of the target to which they are designed disclosed in PCT Publications WO/2006/130201, WO/2007/086990, WO/2009/046397, WO/2009/149182, WO/2009/086428 the contents of which are incorporated herein by reference in their entirety.

As used herein, the term “RNA” refers to an agent that comprises at least an oligonucleotide component (e.g., nucleic acid, either RNA or DNA or modifications thereof), and which is capable of functioning one or more hybridization and/or binding

mechanisms. In some embodiments, the iRNA agent mediates the targeted cleavage of an RNA transcript via an RNA-induced silencing complex (RISC) pathway. In one embodiment, an iRNA agent as described herein effects inhibition of avian transcript expression. Alternatively, in another embodiment, an iRNA agent as described herein
5 activates avian transcript expression. Alternatively, in one embodiment, an iRNA agent sterically blocks access to at least a portion of the avian transcript target. Such blocking can result in the modulation of avian transcript expression, levels or function.

It is also understood that iRNA agents may act via binding but not trigger any cleavage event, but exert an effect on the function of the avian transcript target by steric
10 means. For example, the agent may block the site of another moiety which normally would bind to the avian transcript to itself effect cleavage.

In one embodiment, the iRNA agent will comprise nucleic acid and non-nucleic acid components and the nucleic acid component may be responsible for the binding but not directly for the alteration in function of the avian transcript target. For example,
15 conjugates of iRNA agents may have two or more functions with the nucleic acid component providing at least the hybridization function, while second, third or additional components provide functional effect to the targeting construct.

As used herein when referring to iRNA agents, "target sequence" refers to a contiguous portion of the nucleotide sequence of a DNA molecule of an avian gene or
20 RNA sequence formed during the transcription of an avian transcript, including the avian transcript that is a product of RNA processing of a primary transcription product. Where the targeting construct is an iRNA agent, the target sequence will be at least long enough to serve as a substrate for iRNA-directed activity (e.g., cleavage, blocking, etc) at or near that portion. For example, the target sequence will generally be from 9-36 nucleotides in
25 length, e.g., 15-30 nucleotides in length, including all sub-ranges therebetween. As non-limiting examples, the target sequence can be from 15-30 nucleotides, 15-26 nucleotides, 15-23 nucleotides, 15-22 nucleotides, 15-21 nucleotides, 15-20 nucleotides, 15-19 nucleotides, 15-18 nucleotides, 15-17 nucleotides, 18-30 nucleotides, 18-26 nucleotides, 18-23 nucleotides, 18-22 nucleotides, 18-21 nucleotides, 18-20 nucleotides, 19-30
30 nucleotides, 19-26 nucleotides, 19-23 nucleotides, 19-22 nucleotides, 19-21 nucleotides,

19-20 nucleotides, 20-30 nucleotides, 20-26 nucleotides, 20-25 nucleotides, 20-24 nucleotides, 20-23 nucleotides, 20-22 nucleotides, 20-21 nucleotides, 21-30 nucleotides, 21-26 nucleotides, 21-25 nucleotides, 21-24 nucleotides, 21-23 nucleotides, or 21-22 nucleotides.

5 Smaller target sequences are also contemplated by the present invention. In one embodiment the target sequence can be from 9-15 nucleotides, 10-12 nucleotides, 9 nucleotides, 10 nucleotides, 11 nucleotides, 12 nucleotides, 13 nucleotides, 14 nucleotides, or 15 nucleotides.

 As used herein, the term “strand comprising a sequence” refers to an
10 oligonucleotide comprising a chain of nucleotides that is described by the sequence referred to using the standard nucleotide nomenclature.

 As used herein, and unless otherwise indicated, the term “complementary,” when used to describe a first nucleotide sequence in relation to a second nucleotide sequence, refers to the ability of an oligonucleotide or polynucleotide comprising the first
15 nucleotide sequence to hybridize and form a duplex structure under certain conditions with an oligonucleotide or polynucleotide comprising the second nucleotide sequence, as will be understood by the skilled person. Such conditions can, for example, be stringent conditions, where stringent conditions may include: 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C for 12-16 hours followed by washing. Other conditions,
20 such as physiologically relevant conditions as may be encountered inside an organism, or cell or tissue or cell culture can apply. The skilled person will be able to determine the set of conditions most appropriate for a test of complementarity of two sequences in accordance with the ultimate application of the hybridized nucleotides.

 Complementary sequences within a targeting construct, e.g., within an iRNA
25 agent as described herein, include base-pairing of the oligonucleotide or polynucleotide comprising a first nucleotide sequence to an oligonucleotide or polynucleotide comprising a second nucleotide sequence over a portion of one or both nucleotide sequences. When base-pairing is over the entire length of both sequences, such sequences can be referred to as “fully complementary” with respect to each other herein.
30 However, where a first sequence is referred to as “substantially complementary” with

respect to a second sequence herein, the two sequences can be fully complementary, or they may form one or more, but generally not more than 5, 4, 3 or 2 mismatched base pairs upon hybridization for a duplex up to 30 base pairs, while retaining the ability to hybridize under the conditions most relevant to their ultimate application, e.g.,

5 modulation of gene expression via a RNAi pathway. For the case of longer sequences, (>30nt), mismatches may be as many as 10, 20, 30 or more up to 25% of the molecule. However, where two oligonucleotides are designed to form, upon hybridization, one or more single stranded overhangs, such overhangs shall not be regarded as mismatches with regard to the determination of complementarity. For example, an iRNA agent (e.g.,
10 dsRNA) comprising one oligonucleotide 21 nucleotides in length and another oligonucleotide 23 nucleotides in length, wherein the longer oligonucleotide comprises a sequence of 21 nucleotides that is fully complementary to the shorter oligonucleotide, may yet be referred to as “fully complementary” for the purposes described herein.

“Complementary” sequences, as used herein, may also include, or be formed
15 entirely from, non-Watson-Crick base pairs and/or base pairs formed from non-natural and modified nucleotides, in as far as the above requirements with respect to their ability to hybridize are fulfilled. Such non-Watson-Crick base pairs includes, but are not limited to, G:U Wobble or Hoogstein base pairing.

The terms “complementary,” “fully complementary” and “substantially
20 complementary” herein may be used with respect to the base matching between the sense strand and the antisense strand of a dsRNA or siRNA, or between the antisense strand of a targeting construct and a target sequence, as will be understood from the context of their use.

As used herein, a polynucleotide that is “substantially complementary to at least
25 part of” an avian transcript refers to a polynucleotide that is substantially complementary to a contiguous portion of the avian transcript of interest (e.g., an avian transcript or gene). For example, a polynucleotide is complementary to at least a part of an avian transcript if the sequence is substantially complementary to a non-interrupted portion of an avian transcript or gene.

“G,” “C,” “A,” “T” and “U” each generally stand for a nucleotide that contains guanine, cytosine, adenine, thymidine and uracil as a base, respectively. The skilled person is well aware that guanine, cytosine, adenine, and uracil may be replaced by other moieties without substantially altering the base pairing properties of an oligonucleotide comprising a nucleotide bearing such replacement moiety. For example, without limitation, a nucleotide comprising inosine as its base may base pair with nucleotides containing adenine, cytosine, or uracil. Hence, nucleotides containing uracil, guanine, or adenine may be replaced in the nucleotide sequences of dsRNA featured in the invention by a nucleotide containing, for example, inosine. The skilled person is also aware that a representation of an oligonucleotide as DNA may also be construed as RNA if the “T” nucleotides of the DNA are replaced in the sequence representation by “U”.

In another example, adenine and cytosine anywhere in the oligonucleotide can be replaced with guanine and uracil, respectively to form G-U Wobble base pairing with the target avian transcript. US Patent 7,732,593 describes constructs forming G-Uwobble base pairs and is incorporated herein by reference in its entirety. Sequences containing such replacement moieties are suitable for the compositions and methods featured in the invention.

ssRNAi

In one aspect, an RNA interference agent (iRNA agent) includes a single stranded RNA that interacts with an avian transcript to direct the cleavage of the avian transcript. Thus, in one aspect the invention relates to a single stranded RNA that promotes silencing of the avian transcript, i.e., ssRNAi.

A “single strand iRNA agent” as used herein, is an iRNA agent which is made up of a single molecule. It may include a duplexed region, formed by intra-strand pairing, *e.g.*, it may be, or include, a hairpin or pan-handle structure. Single strand iRNA agents are preferably antisense with regard to the target molecule. In preferred embodiments single strand iRNA agents are 5' phosphorylated or include a phosphoryl analog at the 5' prime terminus. 5'-phosphate modifications include those which are compatible with RISC mediated gene silencing. Suitable modifications include: 5'-monophosphate ((HO)₂(O)P-O-5'); 5'-diphosphate ((HO)₂(O)P-O-P(HO)(O)-O-5'); 5'-triphosphate

((HO)₂(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-guanosine cap (7-methylated or non-methylated) (7m-G-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-adenosine cap (Appp), and any modified or unmodified nucleotide cap structure (N-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-monothiophosphate (phosphorothioate; (HO)₂(S)P-O-5'); 5'-monodithiophosphate (phosphorodithioate; (HO)(HS)(S)P-O-5'), 5'-phosphorothiolate ((HO)₂(O)P-S-5'); any additional combination of oxygen/sulfur replaced monophosphate, diphosphate and triphosphates (*e.g.* 5'-alpha-thiotriphosphate, 5'-gamma-thiotriphosphate, etc.), 5'-phosphoramidates ((HO)₂(O)P-NH-5', (HO)(NH₂)(O)P-O-5'), 5'-alkylphosphonates (R=alkyl=methyl, ethyl, isopropyl, propyl, etc., *e.g.* RP(OH)(O)-O-5'-, (OH)₂(O)P-5'-CH₂-), 5'-alkyletherphosphonates (R=alkylether=methoxymethyl (MeOCH₂-), ethoxymethyl, etc., *e.g.* RP(OH)(O)-O-5'-). (These modifications can also be used with the antisense strand of a double stranded iRNA.) If the iRNA agent is a single strand it is particularly preferred that it include a 5' modification which includes one or more phosphate groups or one or more analogs of a phosphate group.

A single strand iRNA agent should be sufficiently long that it can enter the RISC and participate in RISC mediated cleavage of a target avian transcript. A single strand iRNA agent is at least 14, and more preferably at least 15, 20, 25, 29, 35, 40, or 50 nucleotides in length. It is preferably less than 200, 100, or 60 nucleotides in length.

Hairpin i single strand RNA agents will have a duplex region equal to or at least 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotide pairs. The duplex region will preferably be equal to or less than 200, 100, or 50, in length. Preferred ranges for the duplex region are 15-30, 17 to 23, 19 to 23, and 19 to 21 nucleotides pairs in length. The hairpin will preferably have a single strand overhang or terminal unpaired region, preferably the 3', and preferably of the antisense side of the hairpin. Preferred overhangs are 2-3 nucleotides in length.

pdRNA

In some embodiments, the targeting construct is a promoter-directed RNA (pdRNA) which is substantially complementary to at least a portion of a noncoding region of an mRNA transcript of an avian target gene. The pdRNA can be substantially

complementary to at least a portion of the promoter region of the avian target gene mRNA at a site located upstream from the transcription start site, e.g., more than 100, more than 200, or more than 1,000 bases upstream from the transcription start site. Also, the pdRNA can be substantially complementary to at least a portion of the 3'-UTR of an avian target gene mRNA transcript. For example, the pdRNA comprises dsRNA of 18 to 28 bases optionally having 3' di- or tri-nucleotide overhangs on each strand. The dsRNA is substantially complementary to at least a portion of the promoter region or the 3' UTR region of a target gene mRNA transcript. In another embodiment, the pdRNA comprises a gapmer consisting of a single stranded polynucleotide comprising a DNA sequence which is substantially complementary to at least a portion of the promoter or the 3'-UTR of a target gene mRNA transcript, and flanking the polynucleotide sequences (e.g., comprising the five terminal bases at each of the 5' and 3' ends of the gapmer) comprising one or more modified nucleotides, such as 2'MOE, 2'OMe, or Locked Nucleic Acid bases (LNA), which protect the gapmer from cellular nucleases.

pdRNA can be used to selectively increase, decrease, or otherwise modulate expression of an avian target gene. Without being limited to theory, pdRNAs may modulate expression of target genes by binding to endogenous antisense RNA transcripts which overlap with noncoding regions of a target gene mRNA transcript, and recruiting Argonaute proteins (in the case of dsRNA) or host cell nucleases (e.g., RNase H) (in the case of gapmers) to selectively degrade the endogenous antisense RNAs. In some embodiments, the endogenous antisense RNA negatively regulates expression of the target gene and the pdtargeting construct activates expression of the avian target gene. Thus, in some embodiments, pdRNAs can be used to selectively activate the expression of an avian target gene by inhibiting the negative regulation of target gene expression by endogenous antisense RNA. Methods for identifying antisense transcripts encoded by promoter sequences of target genes and for making and using promoter-directed RNAs are known. See, e.g., WO 2009/046397; the contents of which are incorporated herein by reference in their entirety.

ciRNA

Expressed interfering RNA (eiRNA) can be used to selectively increase, decrease, or otherwise modulate expression of an avian target gene. Typically with eiRNA, the dsRNA is expressed in the first transfected cell from an expression vector. In such a vector, the sense strand and the antisense strand of the dsRNA can be transcribed from the same nucleic acid sequence using e.g., two convergent promoters at either end of the nucleic acid sequence or separate promoters transcribing either a sense or antisense sequence. Alternatively, two plasmids can be cotransfected, with one of the plasmids designed to transcribe one strand of the dsRNA while the other is designed to transcribe the other strand. Methods for making and using eiRNA targeting constructs are known in the art. See, e.g., WO 2006/033756; U.S. Patent Pubs. No. 2005/0239728 and No. 2006/0035344.

piRNA

In some embodiments, the targeting construct comprises a small single-stranded Piwi-interacting RNA (piRNA) which is substantially complementary to at least a portion of an avian target gene, as defined herein, and which selectively binds to proteins of the Piwi or Aubergine subclasses of Argonaute proteins. Without being limited to a particular theory, it is believed that piRNA interact with RNA transcripts of target genes and recruit Piwi and/or Aubergine proteins to form a ribonucleoprotein (RNP) complex that induces transcriptional and/or post-transcriptional gene silencing of target genes. A piRNA can be about 10 to 50 nucleotides in length, about 25 to 39 nucleotides in length, or about 26 to 31 nucleotides in length. See, e.g., U.S. Patent Application Pub. No. 2009/0062228.

miRNA

MicroRNAs are a highly conserved class of small RNA molecules that are transcribed from DNA in the genomes of plants and animals, but are not translated into protein. Pre-microRNAs are processed into miRNAs. Processed microRNAs are single stranded ~17 to 25 nucleotide (nt) RNA molecules that become incorporated into the RNA-induced silencing complex (RISC) and have been identified as key regulators of development, cell proliferation, apoptosis and differentiation. They are believed to play a role in regulation of gene expression by binding to the 3'-untranslated region of specific mRNAs. MicroRNAs cause post-transcriptional silencing of specific target genes, e.g.,

by inhibiting translation or initiating degradation of the targeted mRNA. In some embodiments, the miRNA is completely complementary with the avian target nucleic acid. In other embodiments, the miRNA has a region of noncomplementarity with the target nucleic acid, resulting in a “bulge” at the region of non-complementarity. In some
5 embodiments, the region of noncomplementarity (the bulge) is flanked by regions of sufficient complementarity, e.g., complete complementarity, to allow duplex formation. For example, the regions of complementarity are at least 8 to 10 nucleotides long (e.g., 8, 9, or 10 nucleotides long).

miRNA can inhibit gene expression by, e.g., repressing translation, such as when
10 the miRNA is not completely complementary to the target nucleic acid, or by causing target RNA degradation, when the miRNA binds its target with perfect or a high degree of complementarity. In further embodiments, the targeting construct can include an oligonucleotide agent which targets an endogenous avian miRNA or pre-miRNA. For example, the targeting construct can target an endogenous miRNA which negatively
15 regulates expression of an avian target gene, such that the targeting construct alleviates miRNA-based inhibition of the target gene. The targeting construct can include naturally occurring nucleobases, sugars, and covalent internucleotide (backbone) linkages and/or oligonucleotides having one or more non-naturally-occurring features that confer desirable properties, such as enhanced cellular uptake, enhanced affinity for the
20 endogenous miRNA target, and/or increased stability in the presence of nucleases. In some embodiments, a targeting construct designed to bind to a specific endogenous miRNA has substantial complementarity, e.g., at least 70%, 80%, 90%, or 100% complementary, with at least 10, 20, or 25 or more bases of the target miRNA.

Exemplary targeting constructs that target miRNAs and pre-miRNAs are described, for
25 example, in U.S. Patent Pubs. No. 20090317907, No. 20090298174, No. 20090291907, No. 20090291906, No. 20090286969, No. 20090236225, No. 20090221685, No. 20090203893, No. 20070049547, No. 20050261218, No. 20090275729, No. 20090043082, No. 20070287179, No. 20060212950, No. 20060166910, No. 20050227934, No. 20050222067, No. 20050221490, No. 20050221293, No.
30 20050182005, and No. 20050059005.

Antagomirs

In some embodiments, the targeting construct comprises an antagomir.

Antagomirs are single stranded, double stranded, partially double stranded or hairpin structures that target a microRNA. An antagomir consists essentially of or comprises at least 10 or more contiguous nucleotides substantially complementary to an endogenous miRNA and more particularly a target sequence of an miRNA or pre-miRNA nucleotide sequence. Antagomirs preferably have a nucleotide sequence sufficiently complementary to a miRNA target sequence of about 12 to 25 nucleotides, such as about 15 to 23 nucleotides, to allow the antagomir to hybridize to the target sequence. More preferably, the target sequence differs by no more than 1, 2, or 3 nucleotides from the sequence of the antagomir. In some embodiments, the antagomir includes a non-nucleotide moiety, e.g., a cholesterol moiety, which can be attached, e.g., to the 3' or 5' end of the oligonucleotide agent.

In some embodiments, antagomirs are stabilized against nucleolytic degradation by the incorporation of a modification, e.g., a nucleotide modification. For example, in some embodiments, antagomirs contain a phosphorothioate comprising at least the first, second, and/or third internucleotide linkages at the 5' or 3' end of the nucleotide sequence. In further embodiments, antagomirs include a 2'-modified nucleotide, e.g., a 2'-deoxy, 2'-deoxy-2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), 2'-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE), or 2'-O-N-methylacetamido (2'-O-NMA). In some embodiments, antagomirs include at least one 2'-O-methyl-modified nucleotide.

Antisense

In one embodiment of the present invention, antisense molecules or compounds are used as targeting constructs. The design, synthesis and use of compounds utilizing RNaseH as a cleavage mechanisms are known in the art and described in "Antisense drug technology: principles, strategies, and applications" by Stanley T. Crooke, 2007, Marcel Dekker, New York.

Given a coding strand sequence (e.g., the sequence of a sense strand of a cDNA molecule), antisense nucleic acids can be designed according to the rules of Watson-Crick base pairing. The antisense nucleic acid can be complementary to a portion of the coding or noncoding region of a RNA, e.g., the region surrounding the translation start site of a pre-mRNA or mRNA, e.g., the 5' UTR. An antisense oligonucleotide can be, for example, about 10 to 25 nucleotides in length (e.g., 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 nucleotides in length). In some embodiments, the antisense oligonucleotide comprises one or more modified nucleotides, e.g., phosphorothioate derivatives and/or acridine substituted nucleotides, designed to increase its biological stability of the molecule and/or the physical stability of the duplexes formed between the antisense and target nucleic acids. Antisense oligonucleotides can comprise ribonucleotides only, deoxyribonucleotides only (e.g., oligodeoxynucleotides), or both deoxyribonucleotides and ribonucleotides. For example, an antisense agent consisting only of ribonucleotides can hybridize to a complementary RNA and prevent access of the translation machinery to the target RNA transcript, thereby preventing protein synthesis. An antisense molecule including only deoxyribonucleotides, or deoxyribonucleotides and ribonucleotides, can hybridize to a complementary RNA and the RNA target can be subsequently cleaved by an enzyme, e.g., RNase H, to prevent translation. The flanking RNA sequences can include 2' O methylated nucleotides, and phosphorothioate linkages, and the internal DNA sequence can include phosphorothioate internucleotide linkages. The internal DNA sequence is preferably at least five nucleotides in length when targeting by RNaseH activity is desired.

Ribozymes

Targeting constructs of the present invention may also be designed to be catalytic nucleic acids or ribozymes. The design, synthesis and use of ribozyme technology is known in the art and described in "Intracellular Ribozyme Applications: Principles and Protocols", by Rossi and Couture, 1999; Taylor & Francis, Inc. In one embodiment avian transcript variants are designed to encode one or more ribozymes or ribozyme moieties. These ribozyme or ribozyme moieties are either active as encoded in the avian transcript variant or when cleaved from the parent avian transcript variant.

Aptamers

In some embodiments, the targeting construct comprises an aptamer which binds to a non-nucleic acid ligand, such as a small organic molecule or protein, e.g., a transcription or translation factor, and subsequently modifies (e.g., inhibits) activity. The design, synthesis and use of aptamer technology is known in the art and described in “The Aptamer Handbook: Functional Oligonucleotides and Their Applications” by Klussman, 2006, Wiley VCH. In one embodiment, aptamers may be designed to target one or more features of an avian transcript, transcript variant or biological product. An aptamer can fold into a specific structure that directs the recognition of a targeted binding site on the non-nucleic acid ligand. Aptamers can contain any of the modifications described herein.

Double-stranded ribonucleic acid (dsRNA)

Described herein are targeting constructs that inhibit the expression of one or more avian transcripts. In one embodiment, the targeting construct includes double-stranded ribonucleic acid (dsRNA) molecules for inhibiting or activating the expression of an avian gene or transcript in a cell. Expression of an avian transcript in cell culture can be assayed by measuring avian RNA levels, such as by bDNA or TaqMan assay, or by measuring protein levels of an associated protein coding gene (e.g., one indicative of avian transcript levels), such as by immunofluorescence analysis, using, for example, Western Blotting or flowcytometric techniques.

The term “double-stranded RNA” or “dsRNA,” as used herein, refers to an targeting construct that includes an RNA molecule or complex of molecules having a hybridized duplex region that comprises two anti-parallel and substantially complementary nucleic acid strands, which will be referred to as having “sense” and “antisense” orientations with respect to a target RNA. The duplex region can be of any length that permits specific degradation of a desired target RNA through a RISC pathway, but will typically range from 9 to 50 base pairs in length, e.g., 15-30 base pairs in length. Where relevant, a “part” of an avian transcript target is a contiguous sequence of an avian transcript of sufficient length to be a substrate for RNAi-directed cleavage

(i.e., cleavage through a RISC pathway). dsRNAs having duplexes as short as 9 base pairs can, under some circumstances, mediate RNAi-directed RNA cleavage.

In another embodiment, a targeting construct agent useful to target avian transcript expression is generated in the target cell by cleavage of a larger dsRNA.

5 Considering a duplex between 9 and 50 base pairs, the duplex can be any length in this range, for example, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 and any sub-range therein between, including, but not limited to 15-30 base pairs, 15-26 base pairs, 15-23 base pairs, 15-22 base pairs, 15-21 base pairs, 15-20 base
10 pairs, 15-19 base pairs, 15-18 base pairs, 15-17 base pairs, 18-30 base pairs, 18-26 base pairs, 18-23 base pairs, 18-22 base pairs, 18-21 base pairs, 18-20 base pairs, 19-30 base pairs, 19-26 base pairs, 19-23 base pairs, 19-22 base pairs, 19-21 base pairs, 19-20 base pairs, 20-30 base pairs, 20-26 base pairs, 20-25 base pairs, 20-24 base pairs, 20-23 base pairs, 20-22 base pairs, 20-21 base pairs, 21-30 base pairs, 21-26 base pairs, 21-25 base
15 pairs, 21-24 base pairs, 21-23 base pairs, or 21-22 base pairs. The two strands forming the duplex structure can be from a single RNA molecule having at least one self-complementary region, or can be formed from two or more separate RNA molecules. Where the duplex region is formed from two strands of a single molecule, the molecule can have a duplex region separated by a single stranded chain of nucleotides (herein
20 referred to as a "hairpin loop") between the 3'-end of one strand and the 5'-end of the respective other strand forming the duplex structure. The hairpin loop can comprise at least one unpaired nucleotide; in some embodiments the hairpin loop can comprise at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 20, at least 23 or more unpaired nucleotides. Where the two substantially complementary
25 strands of a dsRNA are comprised by separate RNA molecules, those molecules need not, but can be covalently connected. Where the two strands are connected covalently by means other than a hairpin loop, the connecting structure is referred to as a "linker." The term "siRNA" while being an iRNA agent may also be used herein to refer to a dsRNA as described above.

A dsRNA as described herein may further include one or more single-stranded nucleotide overhangs. The dsRNA can be synthesized by standard methods known in the art as further discussed below, *e.g.*, by use of an automated DNA synthesizer, such as are commercially available from, for example, Biosearch, Applied Biosystems, Inc.

5 As used herein, the term “nucleotide overhang” refers to at least one unpaired nucleotide that protrudes from the duplex structure of a targeting construct, *e.g.*, a dsRNA, siRNA or generally an iRNA agent. For example, when a 3'-end of one strand of a dsRNA extends beyond the 5'-end of the other strand, or vice versa, there is a nucleotide overhang. A dsRNA can comprise an overhang of at least one nucleotide;
10 alternatively the overhang can comprise at least two nucleotides, at least three nucleotides, at least four nucleotides, at least five nucleotides or more. A nucleotide overhang can comprise or consist of a nucleotide/nucleoside analog, including a deoxynucleotide/nucleoside. The overhang(s) may be on the sense strand, the antisense strand or any combination thereof. Furthermore, the nucleotide(s) of an overhang can be
15 present on the 5' end, 3' end or both ends of either an antisense or sense strand of a dsRNA.

In one embodiment, the antisense strand of a dsRNA has a 1-10 nucleotide overhang at the 3' end and/or the 5' end. In one embodiment, the sense strand of a dsRNA has a 1-10 nucleotide overhang at the 3' end and/or the 5' end. In another
20 embodiment, one or more of the nucleotides in the overhang is replaced with a nucleoside thiophosphate.

In one embodiment, the sense strand of a dsRNA is connected with a biocleavable or biostable 1-25 nucleotide overhang at the 3' end and/or the 5' end capable of activating RNase H. In another embodiment, one or more of the nucleotides in the
25 overhang is replaced with a nucleotide sequence functioning as an immunostimulatory agent or as an aptamer. In another embodiment, the 5'-end of the sense strand or antisense strand or both strands carry a triphosphate capable of activating RIG-I protein.

The terms “blunt” or “blunt ended” as used herein in reference to a dsRNA mean that there are no unpaired nucleotides or nucleotide analogs at a given terminal end of a
30 dsRNA, *i.e.*, no nucleotide overhang. One or both ends of a dsRNA can be blunt. Where

both ends of a dsRNA are blunt, the dsRNA is said to be blunt ended. To be clear, a “blunt ended” dsRNA is a dsRNA that is blunt at both ends, *i.e.*, no nucleotide overhang at either end of the molecule. Most often such a molecule will be double-stranded over its entire length.

5 The term “antisense strand” or “guide strand” refers to the strand of an iRNA agent, e.g., a dsRNA or siRNA, which includes a region that is substantially complementary to a target sequence. As used herein, the term “region of complementarity” refers to the region on the antisense strand that is substantially complementary to a sequence, for example a target sequence, as defined herein. Where
10 the region of complementarity is not fully complementary to the target sequence, the mismatches may be in the internal or terminal regions of the molecule. Generally, the most tolerated mismatches are in the terminal regions, *e.g.*, within 5, 4, 3, or 2 nucleotides of the 5’ and/or 3’ terminus. However, mismatches may be located in the internal positions of the molecule and on either strand of a dsRNA molecule.

15 The term “sense strand” or “passenger strand” as used herein, refers to the strand of a iRNA agent that includes a region that is substantially complementary to a region of the antisense strand as that term is defined herein.

 In specific embodiments, the first sequence is a sense strand of a dsRNA that includes a sense sequence disclosed herein, and the second sequence is selected from the
20 group consisting of the corresponding antisense sequences disclosed herein, the pairs of which are reported along with SEQ ID Nos of each sense:antisense pair in the accompanying sequence listing. Pairs are listed with the sense strand first and then the antisense strand. For example, for the sense:antisense pair, (18040,18041), SEQ ID NO: 18040 is the sense strand and SEQ ID NO: 18041 is the antisense strand. Each avian
25 transcript from which the dsRNA are designed are also disclosed in Lengthy Table 1.

 Alternative dsRNA agents that target elsewhere in the target sequence provided in Table 1 can readily be determined using the target sequence and the flanking avian sequence.

 In addition, the avian transcript targeting constructs provided identify a site that is
30 susceptible to RISC-mediated cleavage. As such, the present invention further features

dsRNAs that target within one of such sequences. As used herein, an iRNA agent (e.g., dsRNA) is said to target within a particular site of an avian RNA transcript if the iRNA agent promotes cleavage of the transcript anywhere within that particular site. Such an iRNA agent will generally include at least 15 contiguous nucleotides from one of the sequences provided here coupled to additional nucleotide sequences taken from the region contiguous to the selected sequence in an avian transcript. Given the identification of the sites for targeting provided by the sequences disclosed here, it is also within the scope of the present invention for a dsRNA to target substantially the same location or site.

While an avian target site is generally 15-30 nucleotides in length, there is wide variation in the suitability of particular sequences in this range for directing cleavage of any given avian target RNA. Various software packages and the guidelines set out herein provide guidance for the identification of optimal target sequences for any given gene target, but an empirical approach can also be taken in which a “window” or “mask” of a given size (as a non-limiting example, 21 nucleotides) is literally or figuratively (including, e.g., *in silico*) placed on the target sequence to identify sequences in the size range that may serve as target sequences. By moving the sequence “window” progressively one nucleotide upstream or downstream of an initial target sequence location, the next potential target sequence can be identified, until the complete set of possible sequences is identified for any given target size selected. This process, coupled with systematic synthesis and testing of the identified sequences (using assays as described herein or as known in the art) to identify those sequences that perform optimally can identify those sequences that, when targeted with a dsRNA, mediate the best inhibition of target gene expression. Thus, it is contemplated that further optimization of inhibition efficiency can be achieved by progressively “walking the window” one nucleotide upstream or downstream of the given sequences to identify sequences with equal or better inhibition characteristics.

Further, it is contemplated that for any sequence identified, further optimization could be achieved by systematically either adding or removing nucleotides to generate longer or shorter sequences and testing those and sequences generated by walking a

window of the longer or shorter size up or down the target avian transcript from that point. Again, coupling this approach to generating new candidate targets with testing for effectiveness of dsRNAs based on those target sequences in an inhibition assay as known in the art or as described herein can lead to further improvements in the efficiency of inhibition.

Further still, such optimized sequences can be adjusted by, e.g., the introduction of modified nucleotides as described herein or as known in the art, addition or changes in overhang, or other modifications as known in the art and/or discussed herein to further optimize the molecule (e.g., increasing serum stability or circulating half-life, increasing thermal stability, enhancing transmembrane delivery, targeting to a particular location or cell type, increasing interaction with silencing pathway enzymes, increasing release from endosomes, etc.) as an expression inhibitor.

A dsRNA as described herein can contain one or more mismatches to the target sequence. In one embodiment, a dsRNA as described herein contains no more than 3 mismatches. If the antisense strand of the dsRNA contains mismatches to a target sequence, it is preferable that the area of mismatch not be located in the center of the region of complementarity. If the antisense strand of the dsRNA contains mismatches to the target sequence, it is preferable that the mismatch be restricted to be within the last 5 nucleotides from either the 5' or 3' end of the region of complementarity. For example, for a 23 nucleotide antisense strand which is complementary to a region of an avian transcript, the antisense strand generally does not contain any mismatch within the central 13 nucleotides.

The methods described herein or methods known in the art can be used to determine whether an iRNA agent containing a mismatch to a target sequence is effective in inhibiting the expression of an avian transcript. Consideration of the efficacy of dsRNAs with mismatches in inhibiting expression of an avian transcript is important, especially if the particular region of complementarity in an avian transcript is known to have polymorphic sequence variation within the population.

Vector encoded dsRNAs

In another aspect, targeting constructs (e.g., iRNAs, proteins or peptides) targeting the avian transcript can be expressed from transcription units inserted into DNA or RNA vectors (see, e.g., Couture, A, *et al.*, *TIG.* (1996), 12:5-10; Skillern, A., *et al.*, International PCT Publication No. WO 00/22113, Conrad, International PCT Publication No. WO 00/22114, and Conrad, U.S. Pat. No. 6,054,299). Expression can be transient (on the order of hours to weeks) or sustained (weeks to months or longer), depending upon the specific construct used and the target tissue or cell type. These transgenes can be introduced as a linear construct, a circular plasmid, or a viral vector, which can be an integrating or non-integrating vector. The transgene can also be constructed to permit it to be inherited as an extrachromosomal plasmid (Gassmann, *et al.*, *Proc. Natl. Acad. Sci. USA* (1995) 92:1292).

The individual strand or strands of a targeting construct (when RNA or DNA) can be transcribed from a promoter on an expression vector. Where two separate strands are to be expressed to generate, for example, a dsRNA, two separate expression vectors can be co-introduced (e.g., by transfection or infection) into a target cell. Alternatively each individual strand of a dsRNA can be transcribed by promoters both of which are located on the same expression plasmid. In one embodiment, a dsRNA is expressed as an inverted repeat joined by a linker polynucleotide sequence such that the dsRNA has a stem and loop structure.

Expression vectors are generally DNA plasmids or viral vectors. Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells, can be used to produce recombinant constructs for the expression of a targeting construct as described herein. Eukaryotic cell expression vectors are well known in the art and are available from a number of commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired nucleic acid segment. Delivery of targeting construct expressing vectors can be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that allows for introduction into a desired target cell.

Targeting construct expression plasmids can be transfected into target cells as a complex with cationic lipid carriers (*e.g.*, Oligofectamine) or non-cationic lipid-based carriers (*e.g.*, Transit-TKOTM). Multiple lipid transfections for targeting construct-mediated knockdowns targeting different regions of an avian transcript target over a period of a week or more are also contemplated by the invention. Successful introduction of vectors into host cells can be monitored using various known methods. For example, transient transfection can be signaled with a reporter, such as a fluorescent marker, such as Green Fluorescent Protein (GFP). Stable transfection of cells *ex vivo* can be ensured using markers that provide the transfected cell with resistance to specific environmental factors (*e.g.*, antibiotics and drugs), such as hygromycin B resistance.

Viral vector systems which can be utilized with the methods and compositions described herein include, but are not limited to, (a) adenovirus vectors; (b) retrovirus vectors, including but not limited to lentiviral vectors, moloney murine leukemia virus, etc.; (c) adeno-associated virus vectors; (d) herpes simplex virus vectors; (e) SV 40 vectors; (f) polyoma virus vectors; (g) papilloma virus vectors; (h) picornavirus vectors; (i) pox virus vectors such as an orthopox, *e.g.*, vaccinia virus vectors or avipox, *e.g.* canary pox or fowl pox; and (j) a helper-dependent or gutless adenovirus. Replication-defective viruses can also be advantageous. Different vectors will or will not become incorporated into the cells' genome. The constructs can include viral sequences for transfection, if desired. Alternatively, the construct may be incorporated into vectors capable of episomal replication, *e.g.* EPV and EBV vectors. Constructs for the recombinant expression of a targeting construct will generally require regulatory elements, *e.g.*, promoters, enhancers, etc., to ensure the expression of the targeting construct in target cells. Other aspects to consider for vectors and constructs are further described below.

Vectors useful for the delivery of a targeting construct will include regulatory elements (promoter, enhancer, etc.) sufficient for expression of the targeting construct in the desired target cell or tissue. The regulatory elements can be chosen to provide either constitutive or regulated/inducible expression.

Expression of the targeting construct can be precisely regulated, for example, by using an inducible regulatory sequence that is sensitive to certain physiological regulators, *e.g.*, circulating glucose levels, or hormones (Docherty *et al.*, 1994, FASEB J. 8:20-24). Such inducible expression systems, suitable for the control of expression in
5 cells or in mammals include, for example, regulation by ecdysone, by estrogen, progesterone, tetracycline, chemical inducers of dimerization, and isopropyl-beta-D1 - thiogalactopyranoside (IPTG). A person skilled in the art would be able to choose the appropriate regulatory/promoter sequence based on the intended use of the transgene.

In a specific embodiment, viral vectors that contain nucleic acid sequences
10 encoding a targeting construct can be used. For example, a retroviral vector can be used (see Miller *et al.*, Meth. Enzymol. 217:581-599 (1993)). These retroviral vectors contain the components necessary for the correct packaging of the viral genome and integration into the host cell DNA. The nucleic acid sequences encoding a targeting construct are cloned into one or more vectors, which facilitates delivery of the nucleic acid into a cell,
15 tissue or patient. More detail about retroviral vectors can be found, for example, in Boesen *et al.*, Biotherapy 6:291-302 (1994), which describes the use of a retroviral vector to deliver the *mdr1* gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes *et al.*, J. Clin. Invest. 93:644-651 (1994); Kiem *et al.*, Blood
20 83:1467-1473 (1994); Salmons and Gunzberg, Human Gene Therapy 4:129-141 (1993); and Grossman and Wilson, Curr. Opin. in Genetics and Devel. 3:110-114 (1993). Lentiviral vectors contemplated for use include, for example, the HIV based vectors described in U.S. Patent Nos. 6,143,520; 5,665,557; and 5,981,276, which are herein incorporated by reference.

25 In one embodiment, the targeting constructs of the present invention may be delivered via a bacterial delivery approach as disclosed in PCT Publication WO/2008/156702, the contents of which are incorporated herein in its entirety.

Adenoviruses are also contemplated for use in delivery of nucleic acid based targeting constructs. Adenoviruses are especially attractive vehicles. Adenoviruses have
30 the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson,

Current Opinion in Genetics and Development 3:499-503 (1993) present a review of adenovirus-based gene therapy. A suitable AV vector for expressing a targeting construct featured in the invention, a method for constructing the recombinant AV vector, and a method for delivering the vector into target cells, are described in Xia H *et al.* (2002),
5 *Nat. Biotech.* 20: 1006-1010.

Use of Adeno-associated virus (AAV) vectors is also contemplated (Walsh et al., Proc. Soc. Exp. Biol. Med. 204:289-300 (1993); U.S. Pat. No. 5,436,146). In one embodiment, the targeting construct can be expressed as two separate, complementary single-stranded RNA molecules from a recombinant AAV vector having, for example,
10 either the U6 or H1 RNA promoters, or the cytomegalovirus (CMV) promoter. Suitable AAV vectors for expressing the dsRNA featured in the invention, methods for constructing the recombinant AV vector, and methods for delivering the vectors into target cells are described in Samulski R *et al.* (1987), J. Virol. 61: 3096-3101; Fisher K J *et al.* (1996), J. Virol. 70: 520-532; Samulski R *et al.* (1989), J. Virol. 63: 3822-3826;
15 U.S. Pat. No. 5,252,479; U.S. Pat. No. 5,139,941; International Patent Application No. WO 94/13788; and International Patent Application No. WO 93/24641, the entire disclosures of which are herein incorporated by reference.

Another preferred viral vector is a pox virus such as a vaccinia virus, for example an attenuated vaccinia such as Modified Virus Ankara (MVA) or NYVAC, an avipox
20 such as fowl pox or canary pox.

The tropism of viral vectors can be modified by pseudotyping the vectors with envelope proteins or other surface antigens from other viruses, or by substituting different viral capsid proteins, as appropriate. For example, lentiviral vectors can be pseudotyped with surface proteins from vesicular stomatitis virus (VSV), rabies, Ebola, Mokola, and
25 the like. AAV vectors can be made to target different cells by engineering the vectors to express different capsid protein serotypes; see, *e.g.*, Rabinowitz J E *et al.* (2002), J Virol 76:791-801, the entire disclosure of which is herein incorporated by reference.

The pharmaceutical preparation of a vector can include the vector in an acceptable diluent, or can include a slow release matrix in which the gene delivery vehicle is
30 imbedded. Alternatively, where the complete gene delivery vector can be produced intact

from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

Modifications

The nucleic acid based molecules featured in the invention may be synthesized
5 and/or modified by methods well established in the art, such as those described in “Current protocols in nucleic acid chemistry,” Beaucage, S.L. *et al.* (Eds.), John Wiley & Sons, Inc., New York, NY, USA, which is hereby incorporated herein by reference.

Modifications to nucleic acid based targeting constructs are discussed in more detail below under iRNA agent structure. Briefly, modifications may include, for
10 example, (a) end modifications, *e.g.*, 5' end modifications (phosphorylation (mono-, di- and tri-), conjugation, inverted linkages, etc.) 3' end modifications (conjugation, DNA nucleotides, inverted linkages, etc.), (b) base modifications, *e.g.*, replacement with stabilizing bases, destabilizing bases, or bases that base pair with an expanded repertoire of partners, removal of bases (abasic nucleotides), or conjugated bases, (c) sugar
15 modifications (*e.g.*, at the 2' position or 4' position) or replacement of the sugar, as well as (d) backbone modifications, including modification or replacement of the phosphodiester linkages. Specific examples of modified compounds useful in this invention include, but are not limited to RNAs containing modified backbones or no natural internucleoside linkages. RNAs having modified backbones include, among
20 others, those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified RNAs that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides. In particular embodiments, the modified RNA will have a phosphorus atom in its internucleoside backbone.

25 Modified RNA backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates,
30 thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5'

linked analogs of these, and those) having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative U.S. patents that teach the preparation of the above phosphorus-
5 containing linkages include, but are not limited to, U.S. Pat. Nos. 3,687,808; 4,469,863;
4,476,301; 5,023,243; 5,177,195; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717;
5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126;
5,536,821; 5,541,316; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,625,050; 6,028,188;
6,124,445; 6,160,109; 6,169,170; 6,172,209; 6,239,265; 6,277,603; 6,326,199;
10 6,346,614; 6,444,423; 6,531,590; 6,534,639; 6,608,035; 6,683,167; 6,858,715; 6,867,294;
6,878,805; 7,015,315; 7,041,816; 7,273,933; 7,321,029; and US Pat RE39464, each of
which is herein incorporated by reference.

Modified RNA backbones that do not include a phosphorus atom therein have
backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages,
15 mixed heteroatoms and alkyl or cycloalkyl internucleoside linkages, or one or more short
chain heteroatomic or heterocyclic internucleoside linkages. These include those having
morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane
backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl
backbones; methylene formacetyl and thioformacetyl backbones; alkene containing
20 backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones;
sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O,
S and CH₂ component parts.

Representative U.S. patents that teach the preparation of the above
oligonucleosides include, but are not limited to, U.S. Pat. Nos. 5,034,506; 5,166,315;
25 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,64,562; 5,264,564; 5,405,938; 5,434,257;
5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,608,046;
5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and, 5,677,439, each
of which is herein incorporated by reference.

In other RNA mimetics suitable or contemplated for use in iRNAs, both the sugar
30 and the internucleoside linkage, *i.e.*, the backbone, of the nucleotide units are replaced

with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an RNA mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar backbone of an RNA is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative U.S. patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found, for example, in Nielsen *et al.*, Science, 1991, 254, 1497-1500.

Some embodiments featured in the invention include RNAs with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular --CH₂--NH--CH₂--, --CH₂--N(CH₃)--O--CH₂--[known as a methylene (methylinino) or MMI backbone], --CH₂--O--N(CH₃)--CH₂--, --CH₂--N(CH₃)--N(CH₃)--CH₂-- and --N(CH₃)--CH₂--CH₂--[wherein the native phosphodiester backbone is represented as --O--P--O--CH₂--] of the above-referenced U.S. Pat. No. 5,489,677, and the amide backbones of the above-referenced U.S. Pat. No. 5,602,240. In some embodiments, the RNAs featured herein have morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506.

Modified RNAs may also contain one or more substituted sugar moieties. The targeting constructs, e.g., dsRNAs, featured herein can include one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C₁ to C₁₀ alkyl or C₂ to C₁₀ alkenyl and alkynyl. Exemplary suitable modifications include O[(CH₂)_nO]_mCH₃, O(CH₂)_nOCH₃, O(CH₂)_nNH₂, O(CH₂)_nCH₃, O(CH₂)_nONH₂, and O(CH₂)_nON[(CH₂)_nCH₃]₂, where n and m are from 1 to about 10. In other embodiments, dsRNAs include one of the following at the 2' position: C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving

group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an iRNA agent, or a group for improving the pharmacodynamic properties of an iRNA agent, and other substituents having similar properties. In some embodiments, the modification includes a 2'-methoxyethoxy (2'-O--CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.*, *Helv. Chim. Acta*, 1995, 78:486-504) *i.e.*, an alkoxy-alkoxy group. Another exemplary modification is 2'-dimethylaminoethoxy, *i.e.*, a O(CH₂)₂ON(CH₃)₂ group, also known as 2'-DMAOE, as described in examples herein below, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), *i.e.*, 2'-O--CH₂--O--CH₂--N(CH₃)₂, also described in examples herein below.

Other modifications include 2'-methoxy (2'-OCH₃), 2'-aminopropoxy (2'-OCH₂CH₂CH₂NH₂) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the RNA of a targeting construct, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked dsRNAs and the 5' position of 5' terminal nucleotide. iRNAs may also have sugar mimetics such as cyclobutyl, cyclohexenyl (CeNA), Hexose (HNA), FHNA moieties in place of the pentofuranosyl sugar. Representative U.S. patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

A modified compounds may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-

propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo, particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-daazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in *Modified Nucleosides in Biochemistry, Biotechnology and Medicine*, Herdewijn, P. ed. Wiley-VCH, 2008; those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J. L, ed. John Wiley & Sons, 1990, those disclosed by Englisch *et al.*, *Angewandte Chemie*, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y S., Chapter 15, *dsRNA Research and Applications*, pages 289-302, Crooke, S. T. and Lebleu, B., Ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds featured in the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y. S., Crooke, S. T. and Lebleu, B., Eds., *dsRNA Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are exemplary base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative U.S. patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Pat. No. 3,687,808, as well as U.S. Pat. Nos. 4,845,205; 5,130,30; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,681,941; 6,015,886; 6,147,200; 6,166,197; 6,222,025; 6,235,887; 6,380,368; 6,528,640; 6,639,062; 6,617,438; 7,045,610; 7,427,672; and 7,495,088, each of which is herein incorporated by reference, and U.S. Pat. No. 5,750,692, also herein incorporated by reference.

Nucleic acid based targeting construct can also be modified to include one or more locked nucleic acids (LNA). A locked nucleic acid is a nucleotide having a modified ribose moiety in which the ribose moiety comprises an extra bridge connecting the 2' and 4' carbons. This structure effectively "locks" the ribose in the 3'-endo structural conformation. Also within the present invention are the use of bicyclic nucleic acids, carbocyclic LNAs and amino LNAs. The addition of locked nucleic acids to siRNAs has been shown to increase siRNA stability in serum, and to reduce off-target effects (Elmen, J. et al., (2005) *Nucleic Acids Research* 33(1):439-447; Mook, OR. et al., (2007) *Mol Canc Ther* 6(3):833-843; Grunweller, A. et al., (2003) *Nucleic Acids Research* 31(12):3185-3193).

Representative U.S. Patents that teach the preparation of locked nucleic acid nucleotides include, but are not limited to, the following: U.S. Pat. Nos. 6,268,490; 6,670,461; 6,794,499; 6,998,484; 7,053,207; 7,084,125; and 7,399,845, each of which is herein incorporated by reference in its entirety.

iRNA agent structure

An iRNA agent can include a single strand or can include more than one strands, e.g., it can be a double stranded iRNA agent. If the iRNA agent is a single strand it is particularly preferred that it include a 5' modification which includes one or more phosphate groups or one or more analogs of a phosphate group.

In addition to homology to target RNA and the ability to down regulate a target gene, an iRNA agent will preferably have one or more of the following properties:

- (1) it will be of the Formula 1, 2, 3, or 4 set out below;
- (2) if single stranded it will have a 5' modification which includes one or more phosphate groups or one or more analogs of a phosphate group;
- (3) it will, despite modifications, even to a very large number, or all of the nucleosides, have an antisense strand that can present bases (or modified bases) in the proper three dimensional framework so as to be able to form correct base pairing and form a duplex structure with a homologous target RNA which is sufficient to allow down regulation of the target, e.g., by cleavage of the target RNA;

(4) it will, despite modifications, even to a very large number, or all of the nucleosides, still have "RNA-like" properties, *i.e.*, it will possess the overall structural, chemical and physical properties of an RNA molecule, even though not exclusively, or even partly, of ribonucleotide-based content. For example, an iRNA agent can contain, *e.g.*, a sense and/or an antisense strand in which all of the nucleotide sugars contain *e.g.*, 2' fluoro in place of 2' hydroxyl. This deoxyribonucleotide-containing agent can still be expected to exhibit RNA-like properties. While not wishing to be bound by theory, the electronegative fluorine prefers an axial orientation when attached to the C2' position of ribose. This spatial preference of fluorine can, in turn, force the sugars to adopt a C_{3'}-*endo* pucker. This is the same puckering mode as observed in RNA molecules and gives rise to the RNA-characteristic A-family-type helix. Further, since fluorine is a good hydrogen bond acceptor, it can participate in the same hydrogen bonding interactions with water molecules that are known to stabilize RNA structures. (Generally, it is preferred that a modified moiety at the 2' sugar position will be able to enter into H-bonding which is more characteristic of the OH moiety of a ribonucleotide than the H moiety of a deoxyribonucleotide. A preferred iRNA agent will: exhibit a C_{3'}-*endo* pucker in all, or at least 50, 75, 80, 85, 90, or 95 % of its sugars; exhibit a C_{3'}-*endo* pucker in a sufficient amount of its sugars that it can give rise to a the RNA-characteristic A-family-type helix; will have no more than 20, 10, 5, 4, 3, 2, or 1 sugar which is not a C_{3'}-*endo* pucker structure. These limitations are particularly preferably in the antisense strand;

(5) regardless of the nature of the modification, and even though the RNA agent can contain deoxynucleotides or modified deoxynucleotides, particularly in overhang or other single strand regions, it is preferred that DNA molecules, or any molecule in which more than 50, 60, or 70 % of the nucleotides in the molecule, or more than 50, 60, or 70 % of the nucleotides in a duplexed region are deoxyribonucleotides, or modified deoxyribonucleotides which are deoxy at the 2' position, are excluded from the definition of RNA agent.

RNA agents discussed herein include otherwise unmodified RNA as well as RNA which have been modified, *e.g.*, to improve efficacy, and polymers of nucleoside

surrogates. Unmodified RNA refers to a molecule in which the components of the nucleic acid, namely sugars, bases, and phosphate moieties, are the same or essentially the same as that which occur in nature, preferably as occur naturally in the human body. The art has referred to rare or unusual, but naturally occurring, RNAs as modified RNAs, see, *e.g.*, Limbach *et al.*, (1994) Summary: the modified nucleosides of RNA, Nucleic Acids Res. 22: 2183-2196. Such rare or unusual RNAs, often termed modified RNAs (apparently because they are typically the result of a post transcriptional modification) are within the term unmodified RNA, as used herein. Modified RNA as used herein refers to a molecule in which one or more of the components of the nucleic acid, namely sugars, bases, and phosphate moieties, are different from that which occur in nature, preferably different from that which occurs in the human body. While they are referred to as modified "RNAs," they will of course, because of the modification, include molecules which are not RNAs. Nucleoside surrogates are molecules in which the ribophosphate backbone is replaced with a non-ribophosphate construct that allows the bases to be presented in the correct spatial relationship such that hybridization is substantially similar to what is seen with a ribophosphate backbone, *e.g.*, non-charged mimics of the ribophosphate backbone. Examples of all of the above are discussed herein.

Much of the discussion below refers to single strand molecules. In many embodiments of the invention a double stranded iRNA agent, *e.g.*, a partially double stranded iRNA agent, is required or preferred. Thus, it is understood that that double stranded structures (*e.g.* where two separate molecules are contacted to form the double stranded region or where the double stranded region is formed by intramolecular pairing (*e.g.*, a hairpin structure)) made of the single stranded structures described below are within the invention. Preferred lengths are described elsewhere herein.

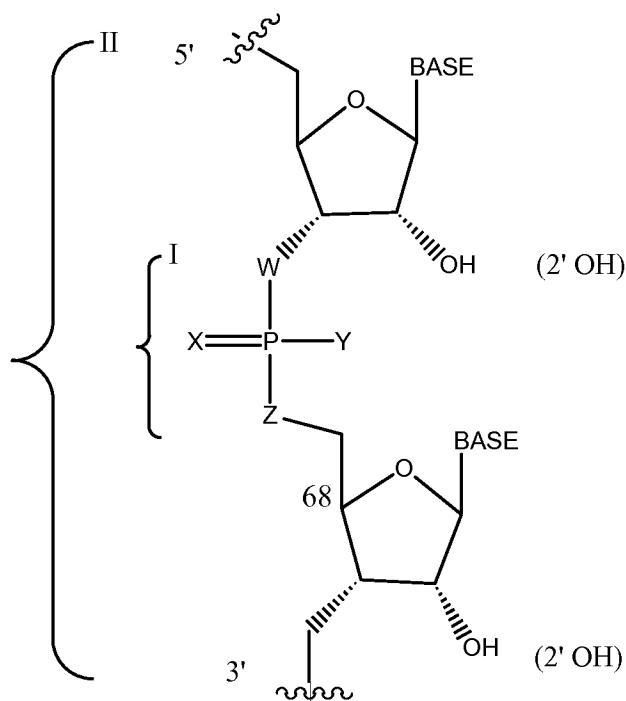
As nucleic acids are polymers of subunits or monomers, many of the modifications described below occur at a position which is repeated within a nucleic acid, *e.g.*, a modification of a base, or a phosphate moiety, or the a non-linking O of a phosphate moiety. In some cases the modification will occur at all of the subject positions in the nucleic acid but in many, and in fact in most cases it will not. By way of

example, a modification may only occur at a 3' or 5' terminal position, may only occur in a terminal regions, *e.g.* at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand. A modification may occur in a double strand region, a single strand region, or in both. A modification may occur only in the double strand region of an RNA or may only occur in a single strand region of an RNA. *E.g.*, a phosphorothioate modification at a non-linking O position may only occur at one or both termini, may only occur in a terminal regions, *e.g.*, at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand, or may occur in double strand and single strand regions, particularly at termini. The 5' end or ends can be phosphorylated.

In some embodiments it is particularly preferred, *e.g.*, to enhance stability, to include particular bases in overhangs, or to include modified nucleotides or nucleotide surrogates, in single strand overhangs, *e.g.*, in a 5' or 3' overhang, or in both. *E.g.*, it can be desirable to include purine nucleotides in overhangs. In some embodiments all or some of the bases in a 3' or 5' overhang will be modified, *e.g.*, with a modification described herein. Modifications can include, *e.g.*, the use of modifications at the 2' OH group of the ribose sugar, *e.g.*, the use of deoxyribonucleotides, *e.g.*, deoxythymidine, instead of ribonucleotides, and modifications in the phosphate group, *e.g.*, phosphothioate modifications. Overhangs need not be homologous with the target sequence.

Further modifications and nucleotide surrogates are discussed below.

FORMULA 1



The scaffold presented above in Formula 1 represents a portion of a ribonucleic acid. The basic components are the ribose sugar, the base, the terminal phosphates, and phosphate internucleotide linkers. Where the bases are naturally occurring bases, *e.g.*, adenine, uracil, guanine or cytosine, the sugars are the unmodified 2' hydroxyl ribose sugar (as depicted) and W, X, Y, and Z are all O, Formula 1 represents a naturally occurring unmodified oligoribonucleotide.

Unmodified oligoribonucleotides may be less than optimal in some applications, *e.g.*, unmodified oligoribonucleotides can be prone to degradation by *e.g.*, cellular nucleases. Nucleases can hydrolyze nucleic acid phosphodiester bonds. However, chemical modifications to one or more of the above RNA components can confer improved properties, and, *e.g.*, can render oligoribonucleotides more stable to nucleases. Unmodified oligoribonucleotides may also be less than optimal in terms of offering tethering points for attaching ligands or other moieties to an iRNA agent.

Modified nucleic acids and nucleotide surrogates can include one or more of:

(i) alteration, *e.g.*, replacement, of one or both of the non-linking (X and Y) phosphate oxygens and/or of one or more of the linking (W and Z) phosphate oxygens (When the phosphate is in the terminal position, one of the positions W or Z will not link the phosphate to an additional element in a naturally occurring ribonucleic acid. However, for simplicity of terminology, except where otherwise noted, the W position at the 5' end of a nucleic acid and the terminal Z position at the 3' end of a nucleic acid, are within the term "linking phosphate oxygens" as used herein.);

(ii) alteration, *e.g.*, replacement, of a constituent of the ribose sugar, *e.g.*, of the 2' hydroxyl on the ribose sugar, or wholesale replacement of the ribose sugar with a structure other than ribose, *e.g.*, as described herein;

(iii) wholesale replacement of the phosphate moiety (bracket I) with "dephospho" linkers;

(iv) modification or replacement of a naturally occurring base;

(v) replacement or modification of the ribose-phosphate backbone (bracket II);

(vi) modification of the 3' end or 5' end of the RNA, *e.g.*, removal, modification or replacement of a terminal phosphate group or conjugation of a moiety, *e.g.* a fluorescently labeled moiety, to either the 3' or 5' end of RNA.

5 The terms replacement, modification, alteration, and the like, as used in this context, do not imply any process limitation, *e.g.*, modification does not mean that one must start with a reference or naturally occurring ribonucleic acid and modify it to produce a modified ribonucleic acid but rather modified simply indicates a difference from a naturally occurring molecule.

10 It is understood that the actual electronic structure of some chemical entities cannot be adequately represented by only one canonical form (*i.e.* Lewis structure). While not wishing to be bound by theory, the actual structure can instead be some hybrid or weighted average of two or more canonical forms, known collectively as resonance forms or structures. Resonance structures are not discrete chemical entities and exist only on paper. They differ from one another only in the placement or "localization" of the bonding and nonbonding electrons for a particular chemical entity. It can be possible for
15 one resonance structure to contribute to a greater extent to the hybrid than the others. Thus, the written and graphical descriptions of the embodiments of the present invention are made in terms of what the art recognizes as the predominant resonance form for a particular species. For example, any phosphoroamidate (replacement of a nonlinking oxygen with nitrogen) would be represented by $X = O$ and $Y = N$ in the above figure.
20 Specific modifications are discussed in more detail below.

The Phosphate Group

The phosphate group is a negatively charged species. The charge is distributed equally over the two non-linking oxygen atoms (*i.e.*, X and Y in Formula 1 above).
25 However, the phosphate group can be modified by replacing one of the oxygens with a different substituent. One result of this modification to RNA phosphate backbones can be increased resistance of the oligoribonucleotide to nucleolytic breakdown. Thus, while not wishing to be bound by theory, it can be desirable in some embodiments to introduce

alterations which result in either an uncharged linker or a charged linker with unsymmetrical charge distribution.

Examples of modified phosphate groups include phosphorothioate, phosphoroselenates, borano phosphates, borano phosphate esters, hydrogen phosphonates, phosphoroamidates, alkyl or aryl phosphonates and phosphotriesters. Phosphorodithioates have both non-linking oxygens replaced by sulfur. Unlike the situation where only one of X or Y is altered, the phosphorus center in the phosphorodithioates is achiral which precludes the formation of oligoribonucleotides diastereomers. Diastereomer formation can result in a preparation in which the individual diastereomers exhibit varying resistance to nucleases. Further, the hybridization affinity of RNA containing chiral phosphate groups can be lower relative to the corresponding unmodified RNA species. Thus, while not wishing to be bound by theory, modifications to both X and Y which eliminate the chiral center, *e.g.* phosphorodithioate formation, may be desirable in that they cannot produce diastereomer mixtures. Thus, X can be any one of S, Se, B, C, H, N, or OR (R is alkyl or aryl). Thus Y can be any one of S, Se, B, C, H, N, or OR (R is alkyl or aryl). Replacement of X and/or Y with sulfur is preferred.

The phosphate linker can also be modified by replacement of a linking oxygen (*i.e.*, W or Z in Formula 1) with nitrogen (bridged phosphoroamidates), sulfur (bridged phosphorothioates) and carbon (bridged methylenephosphonates). The replacement can occur at a terminal oxygen (position W (3') or position Z (5')). Replacement of W with carbon or Z with nitrogen is preferred.

The Sugar Group

A modified RNA can include modification of all or some of the sugar groups of the ribonucleic acid. *E.g.*, the 2' hydroxyl group (OH) can be modified or replaced with a number of different "oxy" or "deoxy" substituents. While not being bound by theory, enhanced stability is expected since the hydroxyl can no longer be deprotonated to form a 2' alkoxide ion. The 2' alkoxide can catalyze degradation by intramolecular nucleophilic attack on the linker phosphorus atom. Again, while not wishing to be bound by theory, it

can be desirable to some embodiments to introduce alterations in which alkoxide formation at the 2' position is not possible.

Examples of "oxy"-2' hydroxyl group modifications include alkoxy or aryloxy (OR, *e.g.*, R = H, alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar);

5 polyethyleneglycols (PEG), $O(CH_2CH_2O)_nCH_2CH_2OR$; "locked" nucleic acids (LNA) in which the 2' hydroxyl is connected, *e.g.*, by a methylene bridge, to the 4' carbon of the same ribose sugar; O-AMINE (AMINE = NH_2 ; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino) and aminoalkoxy, $O(CH_2)_nAMINE$, (*e.g.*, AMINE = NH_2 ; alkylamino, 10 dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino). It is noteworthy that oligonucleotides containing only the methoxyethyl group (MOE), $(OCH_2CH_2OCH_3)$, a PEG derivative), exhibit nuclease stabilities comparable to those modified with the robust phosphorothioate modification.

15 "Deoxy" modifications include hydrogen (*i.e.* deoxyribose sugars, which are of particular relevance to the overhang portions of partially ds RNA); halo (*e.g.*, fluoro); amino (*e.g.* NH_2 ; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid); $NH(CH_2CH_2NH)_nCH_2CH_2-$ AMINE (AMINE = NH_2 ; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl 20 amino, heteroaryl amino, or diheteroaryl amino), $-NHC(O)R$ (R = alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar), cyano; mercapto; alkyl-thio-alkyl; thioalkoxy; and alkyl, cycloalkyl, aryl, alkenyl and alkynyl, which may be optionally substituted with *e.g.*, an amino functionality. Preferred substituents are 2'-methoxyethyl, 2'-OCH₃, 2'-O-allyl, 2'-C- allyl, and 2'-fluoro.

25 The sugar group can also contain one or more carbons that possess the opposite stereochemical configuration than that of the corresponding carbon in ribose. Thus, a modified RNA can include nucleotides containing *e.g.*, arabinose, as the sugar.

Modified RNA's can also include "abasic" sugars, which lack a nucleobase at C-1'. These abasic sugars can also be further contain modifications at one or more of the constituent sugar atoms.

To maximize nuclease resistance, the 2' modifications can be used in combination with one or more phosphate linker modifications (*e.g.*, phosphorothioate). The so-called "chimeric" oligonucleotides are those that contain two or more different modifications.

The modification can also entail the wholesale replacement of a ribose structure with another entity at one or more sites in the iRNA agent. These modifications are described in section entitled Ribose Replacements for RRMSs.

10 Replacement of the Phosphate Group

The phosphate group can be replaced by non-phosphorus containing connectors (*cf.* Bracket I in Formula 1 above). While not wishing to be bound by theory, it is believed that since the charged phosphodiester group is the reaction center in nucleolytic degradation, its replacement with neutral structural mimics should impart enhanced nuclease stability. Again, while not wishing to be bound by theory, it can be desirable, in some embodiment, to introduce alterations in which the charged phosphate group is replaced by a neutral moiety.

Examples of moieties which can replace the phosphate group include siloxane, carbonate, carboxymethyl, carbamate, amide, thioether, ethylene oxide linker, sulfonate, sulfonamide, thioformacetal, formacetal, oxime, methyleneimino, methylenemethylimino, methylenehydrazo, methylenedimethylhydrazo and methyleneoxymethylimino. Preferred replacements include the methylenecarbonylamino and methylenemethylimino groups.

Replacement of Ribophosphate Backbone

25 Oligonucleotide- mimicking scaffolds can also be constructed wherein the phosphate linker and ribose sugar are replaced by nuclease resistant nucleoside or nucleotide surrogates (see Bracket II of Formula 1 above). While not wishing to be bound by theory, it is believed that the absence of a repetitively charged backbone

diminishes binding to proteins that recognize polyanions (*e.g.* nucleases). Again, while not wishing to be bound by theory, it can be desirable in some embodiment, to introduce alterations in which the bases are tethered by a neutral surrogate backbone.

Examples include the morpholino, cyclobutyl, pyrrolidine and peptide nucleic acid (PNA) nucleoside surrogates. A preferred surrogate is a PNA surrogate.

Terminal Modifications

The 3' and 5' ends of an oligonucleotide can be modified. Such modifications can be at the 3' end, 5' end or both ends of the molecule. They can include modification or replacement of an entire terminal phosphate or of one or more of the atoms of the phosphate group. *E.g.*, the 3' and 5' ends of an oligonucleotide can be conjugated to other functional molecular entities such as labeling moieties, *e.g.*, fluorophores (*e.g.*, pyrene, TAMRA, fluorescein, Cy3 or Cy5 dyes) or protecting groups (based *e.g.*, on sulfur, silicon, boron or ester). The functional molecular entities can be attached to the sugar through a phosphate group and/or a spacer. The terminal atom of the spacer can connect to or replace the linking atom of the phosphate group or the C-3' or C-5' O, N, S or C group of the sugar. Alternatively, the spacer can connect to or replace the terminal atom of a nucleotide surrogate (*e.g.*, PNAs). These spacers or linkers can include *e.g.*, $-(CH_2)_n-$, $-(CH_2)_nN-$, $-(CH_2)_nO-$, $-(CH_2)_nS-$, $O(CH_2CH_2O)_nCH_2CH_2OH$ (*e.g.*, $n = 3$ or 6), abasic sugars, amide, carboxy, amine, oxyamine, oximine, thioether, disulfide, thiourea, sulfonamide, or morpholino, or biotin and fluorescein reagents. When a spacer/phosphate-functional molecular entity-spacer/phosphate array is interposed between two strands of iRNA agents, this array can substitute for a hairpin RNA loop in a hairpin-type RNA agent. The 3' end can be an $-OH$ group. While not wishing to be bound by theory, it is believed that conjugation of certain moieties can improve transport, hybridization, and specificity properties. Again, while not wishing to be bound by theory, it may be desirable to introduce terminal alterations that improve nuclease resistance. Other examples of terminal modifications include dyes, intercalating agents (*e.g.* acridines), cross-linkers (*e.g.* psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (*e.g.*, phenazine,

dihydrophenazine), artificial endonucleases (*e.g.* EDTA), lipophilic carriers (*e.g.*, cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis-O(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid, O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine) and peptide conjugates (*e.g.*, antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (*e.g.*, PEG-40K), MPEG, [MPEG]₂, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (*e.g.* biotin), transport/absorption facilitators (*e.g.*, aspirin, vitamin E, folic acid), synthetic ribonucleases (*e.g.*, imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu³⁺ complexes of tetraazamacrocycles).

Terminal modifications can be added for a number of reasons, including as discussed elsewhere herein to modulate activity or to modulate resistance to degradation. Terminal modifications useful for modulating activity include modification of the 5' end with phosphate or phosphate analogs. *E.g.*, in preferred embodiments iRNA agents, especially antisense strands, are 5' phosphorylated or include a phosphoryl analog at the 5' prime terminus. 5'-phosphate modifications include those which are compatible with RISC mediated gene silencing. Suitable modifications include: 5'-monophosphate ((HO)₂(O)P-O-5'); 5'-diphosphate ((HO)₂(O)P-O-P(HO)(O)-O-5'); 5'-triphosphate ((HO)₂(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-guanosine cap (7-methylated or non-methylated) (7m-G-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-adenosine cap (Appp), and any modified or unmodified nucleotide cap structure (N-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-monothiophosphate (phosphorothioate; (HO)₂(S)P-O-5'); 5'-monodithiophosphate (phosphorodithioate; (HO)(HS)(S)P-O-5'), 5'-phosphorothiolate ((HO)₂(O)P-S-5'); any additional combination of oxygen/sulfur replaced monophosphate, diphosphate and triphosphates (*e.g.* 5'-alpha-thiotriphosphate, 5'-gamma-thiotriphosphate, etc.), 5'-phosphoramidates ((HO)₂(O)P-NH-5', (HO)(NH₂)(O)P-O-5'), 5'-alkylphosphonates (R=alkyl=methyl, ethyl, isopropyl, propyl, etc., *e.g.* RP(OH)(O)-O-5', (OH)₂(O)P-5'-CH₂-), 5'-alkyletherphosphonates (R=alkylether=methoxymethyl (MeOCH₂-), ethoxymethyl, etc., *e.g.* RP(OH)(O)-O-5').

Terminal modifications useful for increasing resistance to degradation include

Terminal modifications can also be useful for monitoring distribution, and in such cases the preferred groups to be added include fluorophores, *e.g.*, fluorescein or an Alexa dye, *e.g.*, Alexa 488. Terminal modifications can also be useful for enhancing uptake,
 5 useful modifications for this include cholesterol. Terminal modifications can also be useful for cross-linking an RNA agent to another moiety; modifications useful for this include mitomycin C.

The Bases

Adenine, guanine, cytosine and uracil are the most common bases found in RNA.

10 These bases can be modified or replaced to provide RNA's having improved properties. *E.g.*, nuclease resistant oligoribonucleotides can be prepared with these bases or with synthetic and natural nucleobases (*e.g.*, inosine, thymine, xanthine, hypoxanthine, nubularine, isoguanisine, or tubercidine) and any one of the above modifications. Alternatively, substituted or modified analogs of any of the above bases, *e.g.*, "unusual
 15 bases" and "universal bases," can be employed. Examples include without limitation 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-halouracil, 5-(2-aminopropyl)uracil, 5-amino allyl uracil, 8-halo, amino,
 20 thiol, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine, 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine, dihydrouracil, 3-deaza-5-azacytosine, 2-aminopurine, 5-alkyluracil, 7-alkylguanine, 5-
 25 alkyl cytosine, 7-deazaadenine, N6, N6-dimethyladenine, 2,6-diaminopurine, 5-amino-allyl-uracil, N3-methyluracil, substituted 1,2,4-triazoles, 2-pyridinone, 5-nitroindole, 3-nitropyrrole, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-methoxycarbonylmethyluracil, 5-methyl-2-thiouracil, 5-methoxycarbonylmethyl-2-thiouracil, 5-methylaminomethyl-2-thiouracil, 3-(3-amino-3-carboxypropyl)uracil, 3-methylcytosine, 5-methylcytosine, N⁴-

acetyl cytosine, 2-thiocytosine, N6-methyladenine, N6-isopentyladenine, 2-methylthio-N6-isopentenyladenine, N-methylguanines, or O-alkylated bases. Further purines and pyrimidines include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in the Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859,

5 Kroschwitz, J. I., ed. John Wiley & Sons, 1990, and those disclosed by Englisch *et al.*, Angewandte Chemie, International Edition, 1991, 30, 613.

Generally, base changes are less preferred for promoting stability, but they can be useful for other reasons, *e.g.*, some, *e.g.*, 2,6-diaminopurine and 2 amino purine, are fluorescent. Modified bases can reduce target specificity. This should be taken into
10 consideration in the design of iRNA agents.

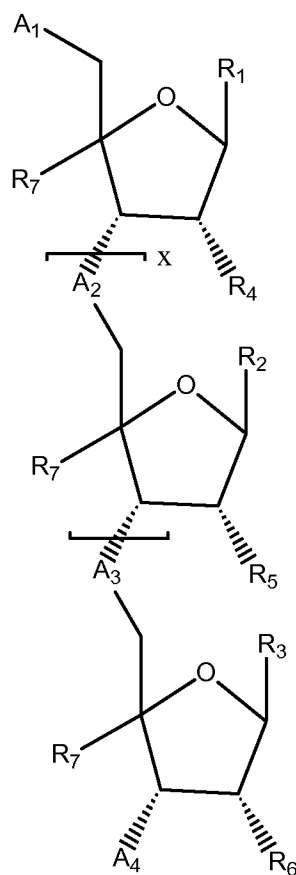
Evaluation of iRNA agents

One can evaluate a candidate iRNA agent, *e.g.*, a modified RNA, for a selected property by exposing the agent or modified molecule and a control molecule to the appropriate conditions and evaluating for the presence of the selected property. For
15 example, resistance to a degradant can be evaluated as follows. A candidate modified iRNA (and preferably a control molecule, usually the unmodified form) can be exposed to degradative conditions, *e.g.*, exposed to a milieu, which includes a degradative agent, *e.g.*, a nuclease. *E.g.*, one can use a biological sample, *e.g.*, one that is similar to a milieu, which might be encountered, in therapeutic use, *e.g.*, blood or a cellular fraction, *e.g.*, a
20 cell-free homogenate or disrupted cells. The candidate and control could then be evaluated for resistance to degradation by any of a number of approaches. For example, the candidate and control could be labeled, preferably prior to exposure, with, *e.g.*, a radioactive or enzymatic label, or a fluorescent label, such as Cy3 or Cy5. Control and modified iRNA's can be incubated with the degradative agent, and optionally a control,
25 *e.g.*, an inactivated, *e.g.*, heat inactivated, degradative agent. A physical parameter, *e.g.*, size, of the modified and control molecules are then determined. They can be determined by a physical method, *e.g.*, by polyacrylamide gel electrophoresis or a sizing column, to assess whether the molecule has maintained its original length, or assessed functionally. Alternatively, Northern blot analysis can be used to assay the length of an unlabeled
30 modified molecule.

A functional assay can also be used to evaluate the candidate iRNA agent. A functional assay can be applied initially or after an earlier non-functional assay, (*e.g.*, assay for resistance to degradation) to determine if the modification alters the ability of the molecule to silence avian transcript expression. For example, a cell, *e.g.*, a mammalian cell, such as a mouse or human cell, can be co-transfected with a plasmid expressing a fluorescent protein, *e.g.*, GFP, and a candidate RNA agent homologous to the transcript encoding the fluorescent protein (see, *e.g.*, WO 00/44914). For example, a modified dsRNA homologous to the GFP mRNA can be assayed for the ability to inhibit GFP expression by monitoring for a decrease in cell fluorescence, as compared to a control cell, in which the transfection did not include the candidate dsRNA, *e.g.*, controls with no agent added and/or controls with a non-modified RNA added. Efficacy of the candidate agent on gene expression can be assessed by comparing cell fluorescence in the presence of the modified and unmodified dsRNA agents.

Preferred iRNA Agents

Preferred RNA agents have the following structure (see Formula 2 below):



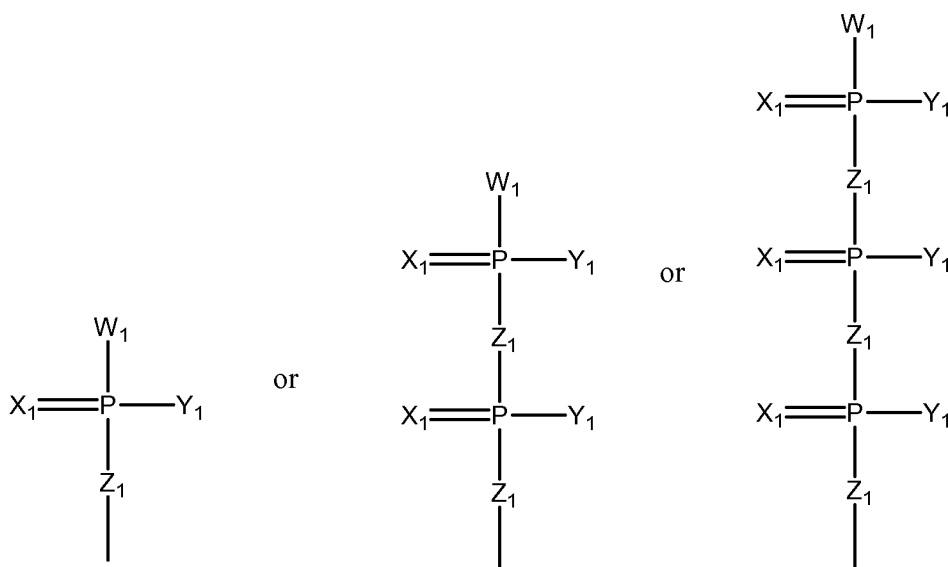
FORMULA 2

Referring to Formula 2 above, R¹, R², and R³ are each, independently, H, (*i.e.* abasic nucleotides), adenine, guanine, cytosine and uracil, inosine, thymine, xanthine, hypoxanthine, nubularine, tubercidine, isoguanisine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-halouracil, 5-(2-aminopropyl)uracil, 5-amino allyl uracil, 8-halo, amino, thiol, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine, 5-substituted pyrimidines, 6-azapyrimidines and

N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylecytosine, dihydrouracil, 3-deaza-5-azacytosine, 2-aminopurine, 5-alkyluracil, 7-alkylguanine, 5-alkyl cytosine, 7-deazaadenine, 7-deazaguanine, N6, N6-dimethyladenine, 2,6-diaminopurine, 5-amino-allyl-uracil, N3-methyluracil, substituted
 5 1,2,4-triazoles, 2-pyridinone, 5-nitroindole, 3-nitropyrrole, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-methoxycarbonylmethyluracil, 5-methyl-2-thiouracil, 5-methoxycarbonylmethyl-2-thiouracil, 5-methylaminomethyl-2-thiouracil, 3-(3-amino-3carboxypropyl)uracil, 3-methylcytosine, 5-methylcytosine, N⁴-acetyl cytosine, 2-thiocytosine, N6-methyladenine, N6-isopentyladenine, 2-methylthio-N6-
 10 isopentenyladenine, N-methylguanines, or O-alkylated bases.

R⁴, R⁵, and R⁶ are each, independently, OR⁸, O(CH₂CH₂O)_mCH₂CH₂OR⁸; O(CH₂)_nR⁹; O(CH₂)_nOR⁹, H; halo; NH₂; NHR⁸; N(R⁸)₂; NH(CH₂CH₂NH)_mCH₂CH₂NHR⁹; NHC(O)R⁸; ; cyano; mercapto, SR⁸; alkyl-thio-alkyl; alkyl, aralkyl, cycloalkyl, aryl, heteroaryl, alkenyl, alkynyl, each of which may be
 15 optionally substituted with halo, hydroxy, oxo, nitro, haloalkyl, alkyl, alkaryl, aryl, aralkyl, alkoxy, aryloxy, amino, alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, acylamino, alkylcarbamoyl, arylcarbamoyl, aminoalkyl, alkoxycarbonyl, carboxy, hydroxyalkyl, alkanesulfonyl, alkanesulfonamido, arenesulfonamido, aralkylsulfonamido, alkylcarbonyl, acyloxy,
 20 cyano, or ureido; or R⁴, R⁵, or R⁶ together combine with R⁷ to form an [-O-CH₂-] covalently bound bridge between the sugar 2' and 4' carbons.

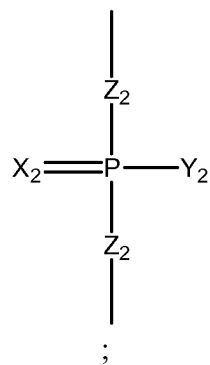
A¹ is:



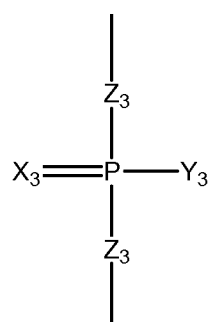
; H; OH; OCH_3 ; W^1 ; an abasic nucleotide; or absent;

- (a preferred $A1$, especially with regard to anti-sense strands, is chosen from 5'-monophosphate $((HO)_2(O)P-O-5')$, 5'-diphosphate $((HO)_2(O)P-O-P(HO)(O)-O-5')$, 5'-triphosphate $((HO)_2(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5')$, 5'-guanosine cap (7-methylated or non-methylated) $(7m-G-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5')$, 5'-adenosine cap (Appp), and any modified or unmodified nucleotide cap structure (N-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'), 5'-monothiophosphate (phosphorothioate; $(HO)_2(S)P-O-5'$), 5'-monodithiophosphate (phosphorodithioate; $(HO)(HS)(S)P-O-5'$), 5'-phosphorothiolate $((HO)_2(O)P-S-5')$; any additional combination of oxygen/sulfur replaced monophosphate, diphosphate and triphosphates (*e.g.* 5'-alpha-thiotriphosphate, 5'-gamma-thiotriphosphate, etc.), 5'-phosphoramidates $((HO)_2(O)P-NH-5'$, $(HO)(NH_2)(O)P-O-5'$), 5'-alkylphosphonates ($R=alkyl=methyl$, ethyl, isopropyl, propyl, etc., *e.g.* $RP(OH)(O)-O-5'$, $(OH)_2(O)P-5'-CH_2-$), 5'-alkyletherphosphonates ($R=alkylether=$ methoxymethyl ($MeOCH_2-$), ethoxymethyl, etc., *e.g.* $RP(OH)(O)-O-5'-$)).

A^2 is:

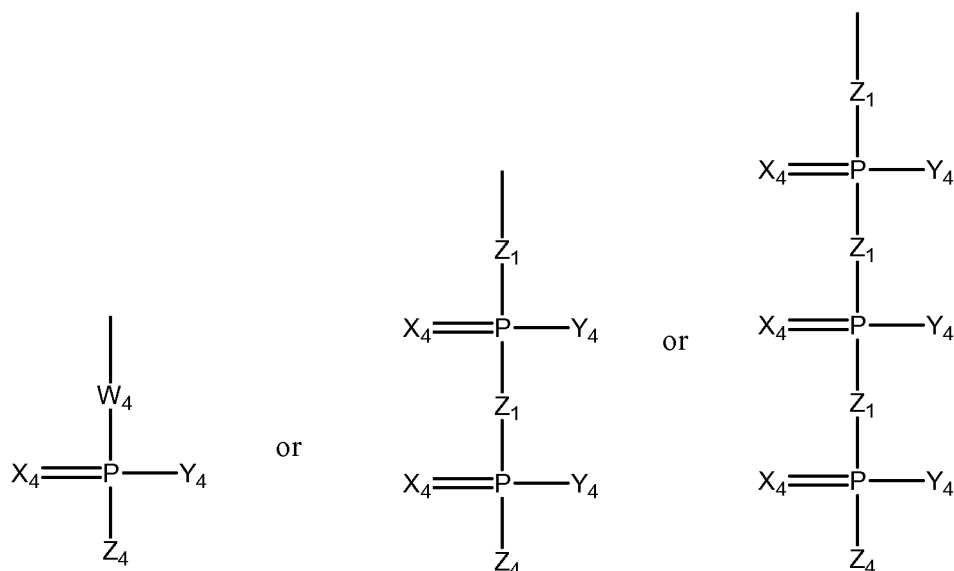


5 A^3 is:



; and

A⁴ is:



5 ; H; Z⁴; an inverted nucleotide; an abasic nucleotide; or absent.

W¹ is OH, (CH₂)_nR¹⁰, (CH₂)_nNHR¹⁰, (CH₂)_nOR¹⁰, (CH₂)_nSR¹⁰; O(CH₂)_nR¹⁰;
 O(CH₂)_nOR¹⁰, O(CH₂)_nNR¹⁰, O(CH₂)_nSR¹⁰; O(CH₂)_nSS(CH₂)_nOR¹⁰, O(CH₂)_nC(O)OR¹⁰,
 NH(CH₂)_nR¹⁰; NH(CH₂)_nNR¹⁰; NH(CH₂)_nOR¹⁰, NH(CH₂)_nSR¹⁰; S(CH₂)_nR¹⁰,
 S(CH₂)_nNR¹⁰, S(CH₂)_nOR¹⁰, S(CH₂)_nSR¹⁰ O(CH₂CH₂O)_mCH₂CH₂OR¹⁰;

10 O(CH₂CH₂O)_mCH₂CH₂NHR¹⁰, NH(CH₂CH₂NH)_mCH₂CH₂NHR¹⁰; Q-R¹⁰, O-Q-R¹⁰ N-Q-R¹⁰, S-Q-R¹⁰ or -O-. W⁴ is O, CH₂, NH, or S.

X¹, X², X³, and X⁴ are each, independently, O or S.

Y¹, Y², Y³, and Y⁴ are each, independently, OH, O⁻, OR⁸, S, Se, BH₃⁻, H, NHR⁹,
 N(R⁹)₂ alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally
 15 substituted.

Z¹, Z², and Z³ are each independently O, CH₂, NH, or S. Z⁴ is OH, (CH₂)_nR¹⁰,
 (CH₂)_nNHR¹⁰, (CH₂)_nOR¹⁰, (CH₂)_nSR¹⁰; O(CH₂)_nR¹⁰; O(CH₂)_nOR¹⁰, O(CH₂)_nNR¹⁰,
 O(CH₂)_nSR¹⁰, O(CH₂)_nSS(CH₂)_nOR¹⁰, O(CH₂)_nC(O)OR¹⁰; NH(CH₂)_nR¹⁰;
 NH(CH₂)_nNR¹⁰; NH(CH₂)_nOR¹⁰, NH(CH₂)_nSR¹⁰; S(CH₂)_nR¹⁰, S(CH₂)_nNR¹⁰,

$S(CH_2)_nOR^{10}$, $S(CH_2)_nSR^{10}O(CH_2CH_2O)_mCH_2CH_2OR^{10}$, $O(CH_2CH_2O)_mCH_2CH_2NHR^{10}$, $NH(CH_2CH_2NH)_mCH_2CH_2NHR^{10}$; $Q-R^{10}$, $O-Q-R^{10}$, $N-Q-R^{10}$, $S-Q-R^{10}$.

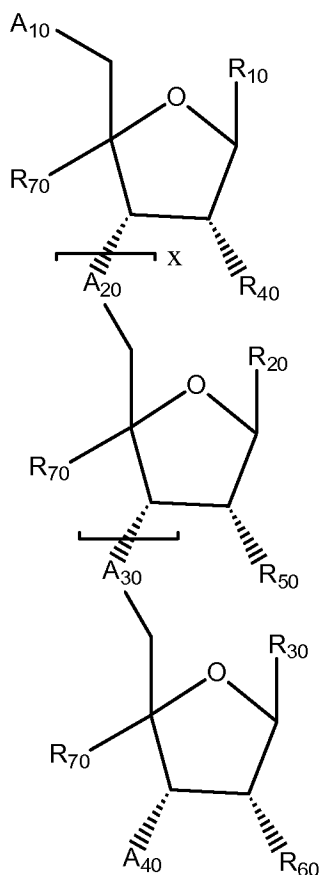
x is 5-100, chosen to comply with a length for an RNA agent described herein.

R^7 is H; or is together combined with R^4 , R^5 , or R^6 to form an [-O-CH₂-]

5 covalently bound bridge between the sugar 2' and 4' carbons.

R^8 is alkyl, cycloalkyl, aryl, aralkyl, heterocyclyl, heteroaryl, amino acid, or sugar; R^9 is NH₂, alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid; and R^{10} is H; fluorophore (pyrene, TAMRA, fluorescein, Cy3 or Cy5 dyes); sulfur, silicon, boron or ester protecting group; 10 intercalating agents (*e.g.* acridines), cross-linkers (*e.g.* psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (*e.g.*, phenazine, dihydrophenazine), artificial endonucleases (*e.g.* EDTA), lipophilic carriers (cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis-O(hexadecyl)glycerol, geranyloxyhexyl group, 15 hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid, O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine) and peptide conjugates (*e.g.*, antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (*e.g.*, PEG-40K), MPEG, [MPEG]₂, polyamino; alkyl, cycloalkyl, aryl, aralkyl, heteroaryl; radiolabelled markers, enzymes, 20 haptens (*e.g.* biotin), transport/absorption facilitators (*e.g.*, aspirin, vitamin E, folic acid), synthetic ribonucleases (*e.g.*, imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu³⁺ complexes of tetraazamacrocycles); or an RNA agent. m is 0-1,000,000, and n is 0-20. Q is a spacer selected from the group consisting of abasic sugar, amide, carboxy, oxyamine, oxyimine, thioether, disulfide, thiourea, 25 sulfonamide, or morpholino, biotin or fluorescein reagents.

Preferred iRNA agents in which the entire phosphate group has been replaced have the following structure (see Formula 3 below):



5

FORMULA 3

Referring to Formula 3, A^{10} - A^{40} is L-G-L; A^{10} and/or A^{40} may be absent, in which L is a linker, wherein one or both L may be present or absent and is selected from the group consisting of $CH_2(CH_2)_g$; $N(CH_2)_g$; $O(CH_2)_g$; $S(CH_2)_g$. G is a functional group selected from the group consisting of siloxane, carbonate, carboxymethyl, carbamate, amide, thioether, ethylene oxide linker, sulfonate, sulfonamide, thioformacetal,

10

formacetal, oxime, methyleneimino, methylenemethylimino, methylenehydrazo, methylenedimethylhydrazo and methyleneoxymethylimino.

R^{10} , R^{20} , and R^{30} are each, independently, H, (*i.e.* abasic nucleotides), adenine, guanine, cytosine and uracil, inosine, thymine, xanthine, hypoxanthine, nubularine, tubercidine, isoguanisine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-halouracil, 5-(2-aminopropyl)uracil, 5-amino allyl uracil, 8-halo, amino, thiol, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine, 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine, dihydrouracil, 3-deaza-5-azacytosine, 2-aminopurine, 5-alkyluracil, 7-alkylguanine, 5-alkyl cytosine, 7-deazaadenine, 7-deazaguanine, N6, N6-dimethyladenine, 2,6-diaminopurine, 5-amino-allyl-uracil, N3-methyluracil substituted 1,2,4-triazoles, 2-pyridinone, 5-nitroindole, 3-nitropyrrole, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-methoxycarbonylmethyluracil, 5-methyl-2-thiouracil, 5-methoxycarbonylmethyl-2-thiouracil, 5-methylaminomethyl-2-thiouracil, 3-(3-amino-3carboxypropyl)uracil, 3-methylcytosine, 5-methylcytosine, N^4 -acetyl cytosine, 2-thiocytosine, N6-methyladenine, N6-isopentyladenine, 2-methylthio-N6-isopentenyladenine, N-methylguanines, or O-alkylated bases.

R^{40} , R^{50} , and R^{60} are each, independently, OR^8 , $O(CH_2CH_2O)_mCH_2CH_2OR^8$; $O(CH_2)_nR^9$; $O(CH_2)_nOR^9$, H; halo; NH_2 ; NHR^8 ; $N(R^8)_2$; $NH(CH_2CH_2NH)_mCH_2CH_2R^9$; $NHC(O)R^8$; cyano; mercapto, SR^7 ; alkyl-thio-alkyl; alkyl, aralkyl, cycloalkyl, aryl, heteroaryl, alkenyl, alkynyl, each of which may be optionally substituted with halo, hydroxy, oxo, nitro, haloalkyl, alkyl, alkaryl, aryl, aralkyl, alkoxy, aryloxy, amino, alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, acylamino, alkylcarbamoyle, arylcarbamoyle, aminoalkyl, alkoxycarbonyl, carboxy, hydroxyalkyl, alkanesulfonyl, alkanesulfonamido, arenesulfonamido, aralkylsulfonamido, alkylcarbonyl, acyloxy, cyano, and ureido groups;

or R⁴⁰, R⁵⁰, or R⁶⁰ together combine with R⁷⁰ to form an [-O-CH₂-] covalently bound bridge between the sugar 2' and 4' carbons.

x is 5-100 or chosen to comply with a length for an RNA agent described herein.

R⁷⁰ is H; or is together combined with R⁴⁰, R⁵⁰, or R⁶⁰ to form an [-O-CH₂-]
 5 covalently bound bridge between the sugar 2' and 4' carbons.

R⁸ is alkyl, cycloalkyl, aryl, aralkyl, heterocyclyl, heteroaryl, amino acid, or sugar; and R⁹ is NH₂, alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid. m is 0-1,000,000, n is 0-20, and g is 0-2.

10 Preferred nucleoside surrogates have the following structure (see Formula 4 below):



FORMULA 4

15

S is a nucleoside surrogate selected from the group consisting of mophilino, cyclobutyl, pyrrolidine and peptide nucleic acid. L is a linker and is selected from the group consisting of CH₂(CH₂)_g; N(CH₂)_g; O(CH₂)_g; S(CH₂)_g; -C(O)(CH₂)_n-or may be absent. M is an amide bond; sulfonamide; sulfinate; phosphate group; modified

20 phosphate group as described herein; or may be absent.

R¹⁰⁰, R²⁰⁰, and R³⁰⁰ are each, independently, H (*i.e.*, abasic nucleotides), adenine, guanine, cytosine and uracil, inosine, thymine, xanthine, hypoxanthine, nubularine, tubercidine, isoguanisine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-
 25 halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-halouracil, 5-(2-aminopropyl)uracil, 5-amino allyl uracil, 8-halo, amino, thiol, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine, 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6

substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylecytosine, dihydrouracil, 3-deaza-5-azacytosine, 2-aminopurine, 5-alkyluracil, 7-alkylguanine, 5-alkyl cytosine, 7-deazaadenine, 7-deazaguanine, N6, N6-dimethyladenine, 2,6-diaminopurine, 5-amino-allyl-uracil, N3-methyluracil substituted 1,
5 2, 4,-triazoles, 2-pyridinones, 5-nitroindole, 3-nitropyrrole, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-methoxycarbonylmethyluracil, 5-methyl-2-thiouracil, 5-methoxycarbonylmethyl-2-thiouracil, 5-methylaminomethyl-2-thiouracil, 3-(3-amino-3carboxypropyl)uracil, 3-methylcytosine, 5-methylcytosine, N⁴-acetyl cytosine, 2-thiocytosine, N6-methyladenine, N6-isopentyladenine, 2-methylthio-N6-
10 isopentenyladenine, N-methylguanines, or O-alkylated bases.

x is 5-100, or chosen to comply with a length for an RNA agent described herein; and g is 0-2.

Nuclease resistant monomers

In one aspect, the invention features a nuclease resistant monomer, or an iRNA
15 agent which incorporates a nuclease resistant monomer (NRM), such as those described herein and those described in copending, co-owned United States Provisional Application Serial No. 60/469,612, filed on May 9, 2003, which is hereby incorporated by reference.

In addition, the invention includes iRNA agents having a NRM and another element described herein. E.g., the invention includes an iRNA agent described herein,
20 e.g., a palindromic iRNA agent, an iRNA agent having a non canonical pairing, an iRNA agent which targets a gene described herein, e.g., a gene active in the liver, an iRNA agent having an architecture or structure described herein, an iRNA associated with an amphipathic delivery agent described herein, an iRNA associated with a drug delivery module described herein, an iRNA agent administered as described herein, or an iRNA
25 agent formulated as described herein, which also incorporates a NRM.

An iRNA agent can include monomers which have been modified so as to inhibit degradation, e.g., by nucleases, e.g., endonucleases or exonucleases, found in the body of a subject. These monomers are referred to herein as NRM's, or nuclease resistance promoting monomers or modifications. In many cases these modifications will modulate
30 other properties of the iRNA agent as well, e.g., the ability to interact with a protein, e.g.,

a transport protein, e.g., serum albumin, or a member of the RISC (RNA-induced Silencing Complex), or the ability of the first and second sequences to form a duplex with one another or to form a duplex with another sequence, e.g., a target molecule.

While not wishing to be bound by theory, it is believed that modifications of the sugar, base, and/or phosphate backbone in an iRNA agent can enhance endonuclease and exonuclease resistance, and can enhance interactions with transporter proteins and one or more of the functional components of the RISC complex. Preferred modifications are those that increase exonuclease and endonuclease resistance and thus prolong the halflife of the iRNA agent prior to interaction with the RISC complex, but at the same time do not render the iRNA agent resistant to endonuclease activity in the RISC complex.

Again, while not wishing to be bound by any theory, it is believed that placement of the modifications at or near the 3' and/or 5' end of antisense strands can result in iRNA agents that meet the preferred nuclease resistance criteria delineated above. Again, still while not wishing to be bound by any theory, it is believed that placement of the modifications at e.g., the middle of a sense strand can result in iRNA agents that are relatively less likely to undergo off-targeting.

Modifications described herein can be incorporated into any double-stranded RNA and RNA-like molecule described herein, e.g., an iRNA agent. An iRNA agent may include a duplex comprising a hybridized sense and antisense strand, in which the antisense strand and/or the sense strand may include one or more of the modifications described herein. The anti sense strand may include modifications at the 3' end and/or the 5' end and/or at one or more positions that occur 1-6 (e.g., 1-5, 1-4, 1-3, 1-2) nucleotides from either end of the strand. The sense strand may include modifications at the 3' end and/or the 5' end and/or at any one of the intervening positions between the two ends of the strand. The iRNA agent may also include a duplex comprising two hybridized antisense strands. The first and/or the second antisense strand may include one or more of the modifications described herein. Thus, one and/or both antisense strands may include modifications at the 3' end and/or the 5' end and/or at one or more positions that occur 1-6 (e.g., 1-5, 1-4, 1-3, 1-2) nucleotides from either end of the strand. Particular configurations are discussed below.

Modifications that can be useful for producing iRNA agents that meet the preferred nuclease resistance criteria delineated above can include one or more of the following chemical and/or stereochemical modifications of the sugar, base, and/or phosphate backbone:

5 (i) chiral (S_P) thioates. Thus, preferred NRM's include nucleotide dimers with an enriched or pure for a particular chiral form of a modified phosphate group containing a heteroatom at the nonbridging position, e.g., S_P or R_P , at the position X, where this is the position normally occupied by the oxygen. The atom at X can also be S, Se, Nr_2 , or Br_3 . When X is S, enriched or chirally pure S_P linkage is preferred. Enriched means at least
10 70, 80, 90, 95, or 99% of the preferred form. Such NRM's are discussed in more detail below;

(ii) attachment of one or more cationic groups to the sugar, base, and/or the phosphorus atom of a phosphate or modified phosphate backbone moiety. Thus, preferred NRM's include monomers at the terminal position derivitized at a cationic
15 group. As the 5' end of an antisense sequence should have a terminal -OH or phosphate group this NRM is preferraly not used at th 5' end of an anti-sense sequence. The group should be attached at a position on the base which minimizes intererence with H bond formation and hybridization, e.g., away form the face which intereacts with the complementary base on the other strand, e.g, at the 5' position of a pyrimidine or a 7-
20 position of a purine. These are discussed in more detail below;

(iii) nonphosphate linkages at the termini. Thus, preferred NRM's include Non-phosphate linkages, e.g., a linkage of 4 atoms which confers greater resistance to cleavage than does a phosphate bond. Examples include 3' $CH_2-NCH_3-O-CH_2-5'$ and 3' $CH_2-NH-(O=)-CH_2-5'$;

25 (iv) 3'-bridging thiophosphates and 5'-bridging thiophosphates. Thus, preferred NRM's can inlcuded these structures;

(v) L-RNA, 2'-5' likages, inverted linkages, a-nucleosides. Thus, other preferred NRM's include: L nucleosides and dimeric nucleotides derived from L-nucleosides; 2'-5' phosphate, non-phosphate and modified phosphate linkages (e.g., thiophospahtes,
30 phosphoramidates and boronophosphates); dimers having inverted linkages, e.g., 3'-3' or

5'-5' linkages; monomers having an alpha linkage at the 1' site on the sugar, e.g., the structures described herein having an alpha linkage;

(vi) conjugate groups. Thus, preferred NRM's can include e.g., a targeting moiety or a conjugated ligand described herein conjugated with the monomer, e.g.,
5 through the sugar, base, or backbone ;

(vi) abasic linkages. Thus, preferred NRM's can include an abasic monomer, e.g., an abasic monomer as described herein (e.g., a nucleobaseless monomer); an aromatic or heterocyclic or polyheterocyclic aromatic monomer as described herein.; and

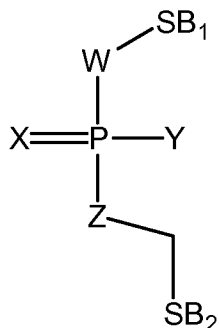
(vii) 5'-phosphonates and 5'-phosphate prodrugs. Thus, preferred NRM's include
10 monomers, preferably at the terminal position, e.g., the 5' position, in which one or more atoms of the phosphate group is derivatized with a protecting group, which protecting group or groups, are removed as a result of the action of a component in the subject's body, e.g, a carboxyesterase or an enzyme present in the subject's body. E.g., a
phosphate prodrug in which a carboxy esterase cleaves the protected molecule resulting
15 in the production of a thioate anion which attacks a carbon adjacent to the O of a phosphate and resulting in the production of an unprotected phosphate.

One or more different NRM modifications can be introduced into an iRNA agent or into a sequence of an iRNA agent. An NRM modification can be used more than once in a sequence or in an iRNA agent. As some NRM's interfere with hybridization the total
20 number incorporated, should be such that acceptable levels of iRNA agent duplex formation are maintained.

In some embodiments NRM modifications are introduced into the terminal the cleavage site or in the cleavage region of a sequence (a sense strand or sequence) which does not target a desired sequence or gene in the subject. This can reduce off-target
25 silencing.

Chiral S_P Thioates

A modification can include the alteration, *e.g.*, replacement, of one or both of the non-linking (X and Y) phosphate oxygens and/or of one or more of the linking (W and Z) phosphate oxygens. Formula X below depicts a phosphate moiety linking two
 5 sugar/sugar surrogate-base moities, SB₁ and SB₂.

FORMULA X

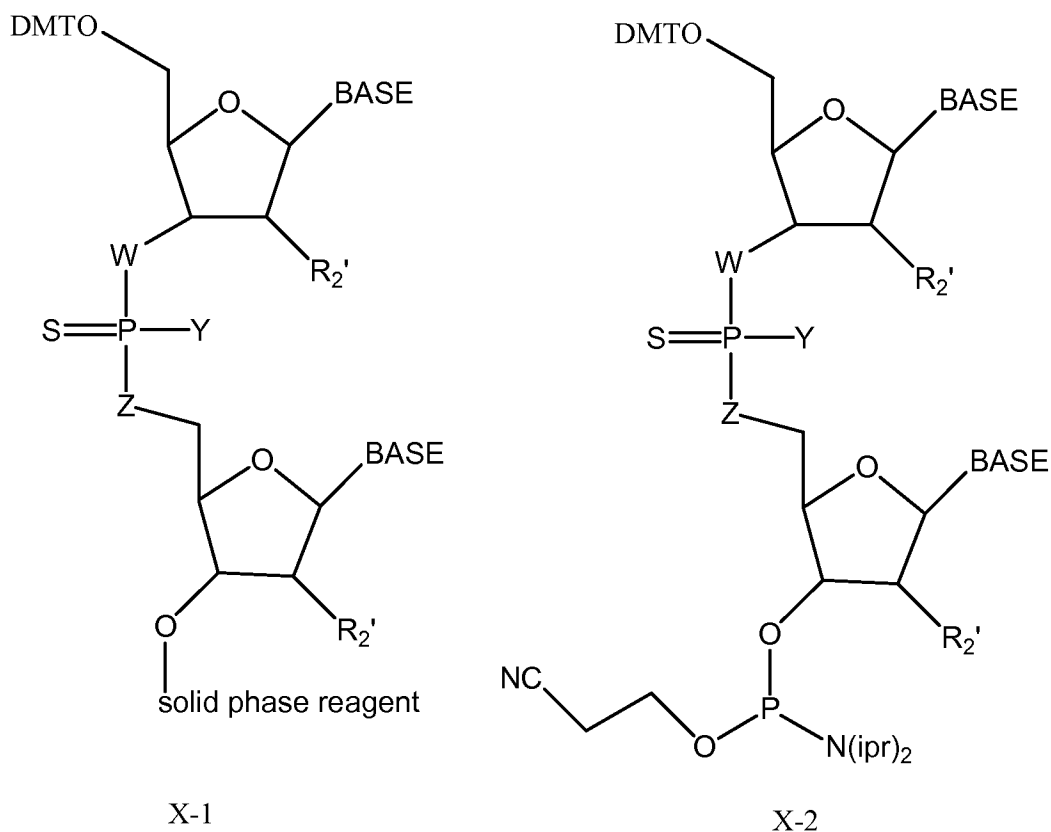
In certain embodiments, one of the non-linking phosphate oxygens in the phosphate backbone moiety (X and Y) can be replaced by any one of the following: S, Se, BR₃ (R is hydrogen, alkyl, aryl, etc.), C (i.e., an alkyl group, an aryl group, etc.), H, NR₂ (R is hydrogen, alkyl, aryl, etc.), or OR (R is alkyl or aryl). The phosphorus atom in
 15 an unmodified phosphate group is achiral. However, replacement of one of the non-linking oxygens with one of the above atoms or groups of atoms renders the phosphorus atom chiral; in other words a phosphorus atom in a phosphate group modified in this way is a stereogenic center. The stereogenic phosphorus atom can possess either the “R” configuration (herein R_P) or the “S” configuration (herein S_P). Thus if 60% of a
 20 population of stereogenic phosphorus atoms have the R_P configuration, then the remaining 40% of the population of stereogenic phosphorus atoms have the S_P configuration.

In some embodiments, iRNA agents, having phosphate groups in which a phosphate non-linking oxygen has been replaced by another atom or group of atoms, may

contain a population of stereogenic phosphorus atoms in which at least about 50% of these atoms (e.g., at least about 60% of these atoms, at least about 70% of these atoms, at least about 80% of these atoms, at least about 90% of these atoms, at least about 95% of these atoms, at least about 98% of these atoms, at least about 99% of these atoms) have the S_P configuration. Alternatively, iRNA agents having phosphate groups in which a phosphate non-linking oxygen has been replaced by another atom or group of atoms may contain a population of stereogenic phosphorus atoms in which at least about 50% of these atoms (e.g., at least about 60% of these atoms, at least about 70% of these atoms, at least about 80% of these atoms, at least about 90% of these atoms, at least about 95% of these atoms, at least about 98% of these atoms, at least about 99% of these atoms) have the R_P configuration. In other embodiments, the population of stereogenic phosphorus atoms may have the S_P configuration and may be substantially free of stereogenic phosphorus atoms having the R_P configuration. In still other embodiments, the population of stereogenic phosphorus atoms may have the R_P configuration and may be substantially free of stereogenic phosphorus atoms having the S_P configuration. As used herein, the phrase “substantially free of stereogenic phosphorus atoms having the R_P configuration” means that moieties containing stereogenic phosphorus atoms having the R_P configuration cannot be detected by conventional methods known in the art (chiral HPLC, ¹H NMR analysis using chiral shift reagents, etc.). As used herein, the phrase “substantially free of stereogenic phosphorus atoms having the S_P configuration” means that moieties containing stereogenic phosphorus atoms having the S_P configuration cannot be detected by conventional methods known in the art (chiral HPLC, ¹H NMR analysis using chiral shift reagents, etc.).

In a preferred embodiment, modified iRNA agents contain a phosphorothioate group, i.e., a phosphate groups in which a phosphate non-linking oxygen has been replaced by a sulfur atom. In an especially preferred embodiment, the population of phosphorothioate stereogenic phosphorus atoms may have the S_P configuration and be substantially free of stereogenic phosphorus atoms having the R_P configuration.

Phosphorothioates may be incorporated into iRNA agents using dimers e.g., formulas **X-1** and **X-2**. The former can be used to introduce phosphorothioate



at the 3' end of a strand, while the latter can be used to introduce this modification at the 5' end or at a position that occurs e.g., 1, 2, 3, 4, 5, or 6 nucleotides from either end of the strand. In the above formulas, Y can be 2-cyanoethoxy, W and Z can be O, R₂' can be, e.g., a substituent that can impart the C-3 endo configuration to the sugar (e.g., OH, F, OCH₃), DMT is dimethoxytrityl, and "BASE" can be a natural, unusual, or a universal base.

X-1 and X-2 can be prepared using chiral reagents or directing groups that can result in phosphorothioate-containing dimers having a population of stereogenic phosphorus atoms having essentially only the R_P configuration (i.e., being substantially free of the S_P configuration) or only the S_P configuration (i.e., being substantially free of the R_P configuration). Alternatively, dimers can be prepared having a population of

stereogenic phosphorus atoms in which about 50% of the atoms have the R_P configuration and about 50% of the atoms have the S_P configuration. Dimers having stereogenic phosphorus atoms with the R_P configuration can be identified and separated from dimers having stereogenic phosphorus atoms with the S_P configuration using e.g., enzymatic degradation and/or conventional chromatography techniques.

Cationic Groups

Modifications can also include attachment of one or more cationic groups to the sugar, base, and/or the phosphorus atom of a phosphate or modified phosphate backbone moiety. A cationic group can be attached to any atom capable of substitution on a natural, unusual or universal base. A preferred position is one that does not interfere with hybridization, i.e., does not interfere with the hydrogen bonding interactions needed for base pairing. A cationic group can be attached e.g., through the C2' position of a sugar or analogous position in a cyclic or acyclic sugar surrogate. Cationic groups can include e.g., protonated amino groups, derived from e.g., O-AMINE (AMINE = NH₂; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino); aminoalkoxy, e.g., O(CH₂)_nAMINE, (e.g., AMINE = NH₂; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino); amino (e.g. NH₂; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid); or NH(CH₂CH₂NH)_nCH₂CH₂-AMINE (AMINE = NH₂; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino).

Nonphosphate Linkages

Modifications can also include the incorporation of nonphosphate linkages at the 5' and/or 3' end of a strand. Examples of nonphosphate linkages which can replace the phosphate group include methyl phosphonate, hydroxylamino, siloxane, carbonate, carboxymethyl, carbamate, amide, thioether, ethylene oxide linker, sulfonate, sulfonamide, thioformacetal, formacetal, oxime, methyleneimino,

methylenemethylimino, methylenehydrazo, methylenedimethylhydrazo and methyleneoxymethylimino. Preferred replacements include the methyl phosphonate and hydroxylamino groups.

5 *3'-bridging thiophosphates and 5'-bridging thiophosphates; locked-RNA, 2'-5' linkages, inverted linkages, α -nucleosides; conjugate groups; abasic linkages; and 5'-phosphonates and 5'-phosphate prodrugs*

Referring to formula **X** above, modifications can include replacement of one of the bridging or linking phosphate oxygens in the phosphate backbone moiety (W and Z).

10 Unlike the situation where only one of X or Y is altered, the phosphorus center in the phosphorodithioates is achiral which precludes the formation of iRNA agents containing a stereogenic phosphorus atom..

Modifications can also include linking two sugars via a phosphate or modified phosphate group through the 2' position of a first sugar and the 5' position of a second sugar. Also contemplated are inverted linkages in which both a first and second sugar are each linked through the respective 3' positions. Modified RNA's can also include "abasic" sugars, which lack a nucleobase at C-1'. The sugar group can also contain one or more carbons that possess the opposite stereochemical configuration than that of the corresponding carbon in ribose. Thus, a modified iRNA agent can include nucleotides containing *e.g.*, arabinose, as the sugar. In another subset of this modification, the natural, unusual, or universal base may have the α -configuration. Modifications can also include L-RNA.

Modifications can also include 5'-phosphonates, *e.g.*, $P(O)(O^-)_2-X-C^{5'}$ -sugar ($X=CH_2, CF_2, CHF$ and 5'-phosphate prodrugs, *e.g.*, $P(O)[OCH_2CH_2SC(O)R]_2CH_2C^{5'}$ -sugar. In the latter case, the prodrug groups may be decomposed *via* reaction first with carboxy esterases. The remaining ethyl thiolate group via intramolecular S_N2 displacement can depart as episulfide to afford the underivatized phosphate group.

Modification can also include the addition of conjugating groups described elsewhere herein, which are preferably attached to an iRNA agent through any amino group available for conjugation.

Nuclease resistant modifications include some which can be placed only at the terminus and others which can go at any position. Generally the modifications that can inhibit hybridization so it is preferably to use them only in terminal regions, and preferable to not use them at the cleavage site or in the cleavage region of a sequence which targets a subject sequence or gene. The can be used anywhere in a sense sequence, provided that sufficient hybridization between the two sequences of the iRNA agent is maintained. In some embodiments it is desirable to put the NRM at the cleavage site or in the cleavage region of a sequence which does not target a subject sequence or gene, as it can minimize off-target silencing.

In most cases, the nuclease-resistance promoting modifications will be distributed differently depending on whether the sequence will target a sequence in the subject (often referred to as an anti-sense sequence) or will not target a sequence in the subject (often referred to as a sense sequence). If a sequence is to target a sequence in the subject, modifications which interfere with or inhibit endonuclease cleavage should not be inserted in the region which is subject to RISC mediated cleavage, e.g., the cleavage site or the cleavage region (As described in Elbashir *et al.*, 2001, Genes and Dev. 15: 188, hereby incorporated by reference, cleavage of the target occurs about in the middle of a 20 or 21 nt guide RNA, or about 10 or 11 nucleotides upstream of the first nucleotide which is complementary to the guide sequence. As used herein cleavage site refers to the nucleotide on either side of the cleavage site, on the target or on the iRNA agent strand which hybridizes to it. Cleavage region means a nucleotide with 1, 2, or 3 nucleotides of the cleavage site, in either direction.)

Such modifications can be introduced into the terminal regions, e.g., at the terminal position or with 2, 3, 4, or 5 positions of the terminus, of a sequence which targets or a sequence which does not target a sequence in the subject.

An iRNA agent can have a first and a second strand chosen from the following:
a first strand which does not target a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 3' end;

a first strand which does not target a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 5' end;

a first strand which does not target a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 3' end and which has a NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 5' end;

5 a first strand which does not target a sequence and which has an NRM modification at the cleavage site or in the cleavage region;

a first strand which does not target a sequence and which has an NRM modification at the cleavage site or in the cleavage region and one or more of an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 3' end, a NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 5' end, or NRM
10 modifications at or within 1, 2, 3, 4, 5, or 6 positions from both the 3' and the 5' end;
and

a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 3' end;

a second strand which targets a sequence and which has an NRM modification at
15 or within 1, 2, 3, 4, 5, or 6 positions from the 5' end (5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5, or 6 away from the 5' terminus of an antisense strand);

a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 3' end and which has a NRM modification
20 at or within 1, 2, 3, 4, 5, or 6 positions from the 5' end;

a second strand which targets a sequence and which preferably does not have an NRM modification at the cleavage site or in the cleavage region;

a second strand which targets a sequence and which does not have an NRM modification at the cleavage site or in the cleavage region and one or more of an NRM
25 modification at or within 1, 2, 3, 4, 5, or 6 positions from the 3' end, a NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 5' end, or NRM modifications at or within 1, 2, 3, 4, 5, or 6 positions from both the 3' and the 5' end (5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5, or 6 away from the 5' terminus of an antisense strand).

An iRNA agent can also target two sequences and can have a first and second strand chosen from:

a first strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end;

5 a first strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end (5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5 , or 6 away from the 5' terminus of an antisense strand);

10 a first strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end and which has a NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end;

a first strand which targets a sequence and which preferably does not have an NRM modification at the cleavage site or in the cleavage region;

15 a first strand which targets a sequence and which dose not have an NRM modification at the cleavage site or in the cleavage region and one or more of an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end, a NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end, or NRM modifications at or within 1, 2, 3, 4, 5 , or 6 positions from both the 3' and the 5' end(5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5 , or 6 away from the 5' terminus of an antisense strand) and

20 a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end;

25 a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end (5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5 , or 6 away from the 5' terminus of an antisense strand);

a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end and which has a NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end;

a second strand which targets a sequence and which preferably does not have an NRM modification at the cleavage site or in the cleavage region;

a second strand which targets a sequence and which dose not have an NRM modification at the cleavage site or in the cleavage region and one or more of an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end, a NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end, or NRM modifications at or within 1, 2, 3, 4, 5 , or 6 positions from both the 3' and the 5' end(5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5 , or 6 away from the 5' terminus of an antisense strand).

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Ribose Mimics

In one aspect, the invention features a ribose mimic, or an iRNA agent which incorporates a ribose mimic, such as those described herein and those described in copending co-owned United States Provisional Application Serial No. 60/454,962, filed on March 13, 2003, which is hereby incorporated by reference.

In addition, the invention includes iRNA agents having a ribose mimic and another element described herein. E.g., the invention includes an iRNA agent described herein, e.g., a palindromic iRNA agent, an iRNA agent having a non canonical pairing, an iRNA agent which targets a gene described herein, e.g., a gene active in the liver, an iRNA agent having an architecture or structure described herein, an iRNA associated with an amphipathic delivery agent described herein, an iRNA associated with a drug delivery module described herein, an iRNA agent administered as described herein, or an iRNA agent formulated as described herein, which also incorporates a ribose mimic.

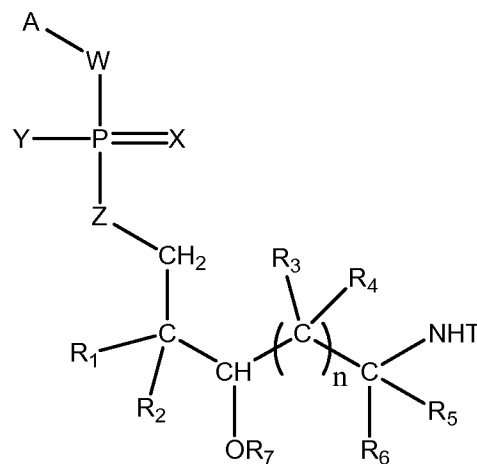
Thus, an aspect of the invention features an iRNA agent that includes a secondary hydroxyl group, which can increase efficacy and/or confer nuclease resistance to the agent. Nucleases, e.g., cellular nucleases, can hydrolyze nucleic acid phosphodiester bonds, resulting in partial or complete degradation of the nucleic acid. The secondary hydroxy group confers nuclease resistance to an iRNA agent by rendering the iRNA agent less prone to nuclease degradation relative to an iRNA which lacks the modification. While not wishing to be bound by theory, it is believed that the presence of

30

a secondary hydroxyl group on the iRNA agent can act as a structural mimic of a 3' ribose hydroxyl group, thereby causing it to be less susceptible to degradation.

The secondary hydroxyl group refers to an "OH" radical that is attached to a carbon atom substituted by two other carbons and a hydrogen. The secondary hydroxyl group that confers nuclease resistance as described above can be part of any acyclic carbon-containing group. The hydroxyl may also be part of any cyclic carbon-containing group, and preferably one or more of the following conditions is met (1) there is no ribose moiety between the hydroxyl group and the terminal phosphate group or (2) the hydroxyl group is not on a sugar moiety which is coupled to a base.. The hydroxyl group is located at least two bonds (e.g., at least three bonds away, at least four bonds away, at least five bonds away, at least six bonds away, at least seven bonds away, at least eight bonds away, at least nine bonds away, at least ten bonds away, etc.) from the terminal phosphate group phosphorus of the iRNA agent. In preferred embodiments, there are five intervening bonds between the terminal phosphate group phosphorus and the secondary hydroxyl group.

Preferred iRNA agent delivery modules with five intervening bonds between the terminal phosphate group phosphorus and the secondary hydroxyl group have the following structure (see formula Y below):



(Y)

Referring to formula Y, A is an iRNA agent, including any iRNA agent described herein. The iRNA agent may be connected directly or indirectly (e.g., through a spacer or linker) to “W” of the phosphate group. These spacers or linkers can include *e.g.*, -
 5 (CH₂)_n-, -(CH₂)_nN-, -(CH₂)_nO-, -(CH₂)_nS-, O(CH₂CH₂O)_nCH₂CH₂OH (*e.g.*, n = 3 or 6),
 abasic sugars, amide, carboxy, amine, oxyamine, oximine, thioether, disulfide, thiourea, sulfonamide, or morpholino, or biotin and fluorescein reagents.

The iRNA agents can have a terminal phosphate group that is unmodified (e.g., W, X, Y, and Z are O) or modified. In a modified phosphate group, W and Z can be
 10 independently NH, O, or S; and X and Y can be independently S, Se, BH₃⁻, C₁-C₆ alkyl, C₆-C₁₀ aryl, H, O, O⁻, alkoxy or amino (including alkylamino, arylamino, etc.).
 Preferably, W, X and Z are O and Y is S.

R₁ and R₃ are each, independently, hydrogen; or C₁-C₁₀₀ alkyl, optionally substituted with hydroxyl, amino, halo, phosphate or sulfate and/or may be optionally
 15 inserted with N, O, S, alkenyl or alkynyl.

R₂ is hydrogen; C₁-C₁₀₀ alkyl, optionally substituted with hydroxyl, amino, halo, phosphate or sulfate and/or may be optionally inserted with N, O, S, alkenyl or alkynyl;
 or, when n is 1, R₂ may be taken together with with R₄ or R₆ to form a ring of 5-12 atoms.

R₄ is hydrogen; C₁-C₁₀₀ alkyl, optionally substituted with hydroxyl, amino, halo, phosphate or sulfate and/or may be optionally inserted with N, O, S, alkenyl or alkynyl;
 20 or, when n is 1, R₄ may be taken together with with R₂ or R₅ to form a ring of 5-12 atoms.

R₅ is hydrogen, C₁-C₁₀₀ alkyl optionally substituted with hydroxyl, amino, halo, phosphate or sulfate and/or may be optionally inserted with N, O, S, alkenyl or alkynyl;
 or, when n is 1, R₅ may be taken together with with R₄ to form a ring of 5-12 atoms.

25 R₆ is hydrogen, C₁-C₁₀₀ alkyl, optionally substituted with hydroxyl, amino, halo, phosphate or sulfate and/or may be optionally inserted with N, O, S, alkenyl or alkynyl,
 or, when n is 1, R₆ may be taken together with with R₂ to form a ring of 6-10 atoms;

R₇ is hydrogen, C₁-C₁₀₀ alkyl, or C(O)(CH₂)_qC(O)NHR₉; T is hydrogen or a functional group; n and q are each independently 1-100; R₈ is C₁-C₁₀ alkyl or C₆-C₁₀ aryl;
 30 and R₉ is hydrogen, C₁-C₁₀ alkyl, C₆-C₁₀ aryl or a solid support agent.

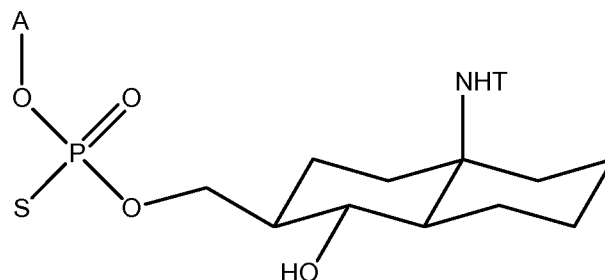
Preferred embodiments may include one of more of the following subsets of iRNA agent delivery modules.

In one subset of RNAi agent delivery modules, A can be connected directly or indirectly through a terminal 3' or 5' ribose sugar carbon of the RNA agent.

5 In another subset of RNAi agent delivery modules, X, W, and Z are O and Y is S.

In still yet another subset of RNAi agent delivery modules, n is 1, and R₂ and R₆ are taken together to form a ring containing six atoms and R₄ and R₅ are taken together to form a ring containing six atoms. Preferably, the ring system is a *trans*-decalin. For example, the RNAi agent delivery module of this subset can include a compound of

10 Formula (Y-1):



The functional group can be, for example, a targeting group (e.g., a steroid or a carbohydrate), a reporter group (e.g., a fluorophore), or a label (an isotopically labelled moiety). The targeting group can further include protein binding agents, endothelial cell targeting groups (e.g., RGD peptides and mimetics), cancer cell targeting groups (e.g., folate Vitamin B12, Biotin), bone cell targeting groups (e.g., bisphosphonates, polyglutamates, polyaspartates), multivalent mannose (for e.g., macrophage testing), lactose, galactose, N-acetyl-galactosamine, monoclonal antibodies, glycoproteins, lectins, melanotropin, or thyrotropin.

As can be appreciated by the skilled artisan, methods of synthesizing the compounds of the formulae herein will be evident to those of ordinary skill in the art. The synthesized compounds can be separated from a reaction mixture and further purified by a method such as column chromatography, high pressure liquid chromatography, or

recrystallization. Additionally, the various synthetic steps may be performed in an alternate sequence or order to give the desired compounds. Synthetic chemistry transformations and protecting group methodologies (protection and deprotection) useful in synthesizing the compounds described herein are known in the art and include, for example, those such as described in R. Larock, *Comprehensive Organic Transformations*, VCH Publishers (1989); T.W. Greene and P.G.M. Wuts, *Protective Groups in Organic Synthesis*, 2d. Ed., John Wiley and Sons (1991); L. Fieser and M. Fieser, *Fieser and Fieser's Reagents for Organic Synthesis*, John Wiley and Sons (1994); and L. Paquette, ed., *Encyclopedia of Reagents for Organic Synthesis*, John Wiley and Sons (1995), and subsequent editions thereof.

Ribose Replacement Monomer Subunits (RRMS)

iRNA agents can be modified in a number of ways which can optimize one or more characteristics of the iRNA agent. In one aspect, the invention features a ribose replacement monomer subunit (RRMS), or a an iRNA agent which incorporates a RRMS, such as those described herein and those described in one or more of United States Provisional Application Serial No. 60/493,986, filed on August 8, 2003, which is hereby incorporated by reference; United States Provisional Application Serial No. 60/494,597, filed on August 11, 2003, which is hereby incorporated by reference; United States Provisional Application Serial No. 60/506,341, filed on September 26, 2003, which is hereby incorporated by reference; and in United States Provisional Application Serial No. 60/158,453, filed on November 7, 2003, which is hereby incorporated by reference.

In addition, the invention includes iRNA agents having a RRMS and another element described herein. E.g., the invention includes an iRNA agent described herein, e.g., a palindromic iRNA agent, an iRNA agent having a non canonical pairing, an iRNA agent which targets a gene described herein, e.g., a gene active in the liver, an iRNA agent having an architecture or structure described herein, an iRNA associated with an amphipathic delivery agent described herein, an iRNA associated with a drug delivery module described herein, an iRNA agent administered as described herein, or an iRNA agent formulated as described herein, which also incorporates a RRMS.

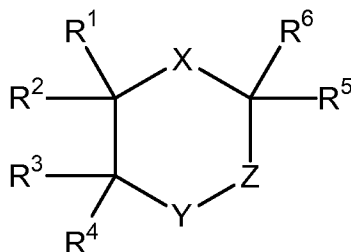
The ribose sugar of one or more ribonucleotide subunits of an iRNA agent can be replaced with another moiety, e.g., a non-carbohydrate (preferably cyclic) carrier. A ribonucleotide subunit in which the ribose sugar of the subunit has been so replaced is referred to herein as a ribose replacement modification subunit (RRMS). A cyclic carrier
5 may be a carbocyclic ring system, i.e., all ring atoms are carbon atoms, or a heterocyclic ring system, i.e., one or more ring atoms may be a heteroatom, e.g., nitrogen, oxygen, sulfur. The cyclic carrier may be a monocyclic ring system, or may contain two or more rings, e.g. fused rings. The cyclic carrier may be a fully saturated ring system, or it may contain one or more double bonds.

10 The carriers further include (i) at least two “backbone attachment points” and (ii) at least one “tethering attachment point.” A “backbone attachment point” as used herein refers to a functional group, e.g. a hydroxyl group, or generally, a bond available for, and that is suitable for incorporation of the carrier into the backbone, e.g., the phosphate, or modified phosphate, e.g., sulfur containing, backbone, of a ribonucleic acid. A “tethering
15 attachment point” as used herein refers to a constituent ring atom of the cyclic carrier, e.g., a carbon atom or a heteroatom (distinct from an atom which provides a backbone attachment point), that connects a selected moiety. The moiety can be, e.g., a ligand, e.g., a targeting or delivery moiety, or a moiety which alters a physical property, e.g., lipophilicity, of an iRNA agent. Optionally, the selected moiety is connected by an
20 intervening tether to the cyclic carrier. Thus, it will include a functional group, e.g., an amino group, or generally, provide a bond, that is suitable for incorporation or tethering of another chemical entity, e.g., a ligand to the constituent ring.

Incorporation of one or more RRMSs described herein into an RNA agent, e.g., an iRNA agent, particularly when tethered to an appropriate entity, can confer one or
25 more new properties to the RNA agent and/or alter, enhance or modulate one or more existing properties in the RNA molecule. E.g., it can alter one or more of lipophilicity or nuclease resistance. Incorporation of one or more RRMSs described herein into an iRNA agent can, particularly when the RRMS is tethered to an appropriate entity, modulate, e.g., increase, binding affinity of an iRNA agent to a target avian transcripts, change the
30 geometry of the duplex form of the iRNA agent, alter distribution or target the iRNA

agent to a particular part of the body, or modify the interaction with nucleic acid binding proteins (e.g., during RISC formation and strand separation).

Accordingly, in one aspect, the invention features, an iRNA agent preferably comprising a first strand and a second strand, wherein at least one subunit having a
5 formula (R-1) is incorporated into at least one of said strands.



(R-1)

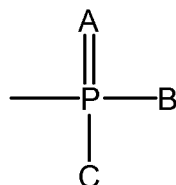
Referring to formula (R-1), X is $\text{N}(\text{CO})\text{R}^7$, NR^7 or CH_2 ; Y is NR^8 , O, S, CR^9R^{10} ,
10 or absent; and Z is $\text{CR}^{11}\text{R}^{12}$ or absent.

Each of R^1 , R^2 , R^3 , R^4 , R^9 , and R^{10} is, independently, H, OR^a , OR^b , $(\text{CH}_2)_n\text{OR}^a$, or $(\text{CH}_2)_n\text{OR}^b$, provided that at least one of R^1 , R^2 , R^3 , R^4 , R^9 , and R^{10} is OR^a or OR^b and that at least one of R^1 , R^2 , R^3 , R^4 , R^9 , and R^{10} is $(\text{CH}_2)_n\text{OR}^a$, or $(\text{CH}_2)_n\text{OR}^b$ (when the RRMS is terminal, one of R^1 , R^2 , R^3 , R^4 , R^9 , and R^{10} will include R^a and one will include
15 R^b ; when the RRMS is internal, two of R^1 , R^2 , R^3 , R^4 , R^9 , and R^{10} will each include an R^b); further provided that preferably OR^a may only be present with $(\text{CH}_2)_n\text{OR}^b$ and $(\text{CH}_2)_n\text{OR}^a$ may only be present with OR^b .

Each of R^5 , R^6 , R^{11} , and R^{12} is, independently, H, $\text{C}_1\text{-C}_6$ alkyl optionally substituted with 1-3 R^{13} , or $\text{C}(\text{O})\text{NHR}^7$; or R^5 and R^{11} together are $\text{C}_3\text{-C}_8$ cycloalkyl
20 optionally substituted with R^{14} .

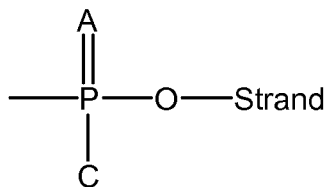
R^7 is $\text{C}_1\text{-C}_{20}$ alkyl substituted with NR^cR^d ; R^8 is $\text{C}_1\text{-C}_6$ alkyl; R^{13} is hydroxy, $\text{C}_1\text{-C}_4$ alkoxy, or halo; and R^{14} is NR^cR^7 .

R^a is:



; and

R^b is:



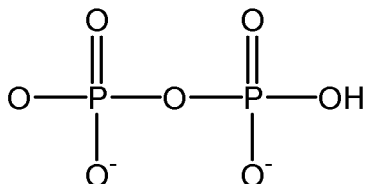
5

.

Each of A and C is, independently, O or S.

B is OH, O^- , or

10



.

R^c is H or C1-C6 alkyl; R^d is H or a ligand; and n is 1-4.

15 In a preferred embodiment the ribose is replaced with a pyrroline scaffold, and X is $N(\text{CO})R^7$ or NR^7 , Y is CR^9R^{10} , and Z is absent.

In other preferred embodiments the ribose is replaced with a piperidine scaffold, and X is $N(\text{CO})R^7$ or NR^7 , Y is CR^9R^{10} , and Z is $CR^{11}R^{12}$.

In other preferred embodiments the ribose is replaced with a piperazine scaffold, and X is $N(\text{CO})R^7$ or NR^7 , Y is NR^8 , and Z is $CR^{11}R^{12}$.

In other preferred embodiments the ribose is replaced with a morpholino scaffold, and X is $N(CO)R^7$ or NR^7 , Y is O, and Z is $CR^{11}R^{12}$.

In other preferred embodiments the ribose is replaced with a decalin scaffold, and X is CH_2 ; Y is CR^9R^{10} ; and Z is $CR^{11}R^{12}$; and R^5 and R^{11} together are C^6 cycloalkyl.

5 In other preferred embodiments the ribose is replaced with a decalin/indane scaffold and, and X is CH_2 ; Y is CR^9R^{10} ; and Z is $CR^{11}R^{12}$; and R^5 and R^{11} together are C^5 cycloalkyl.

In other preferred embodiments, the ribose is replaced with a hydroxyproline scaffold.

10 RRMSs described herein may be incorporated into any double-stranded RNA-like molecule described herein, e.g., an iRNA agent. An iRNA agent may include a duplex comprising a hybridized sense and antisense strand, in which the antisense strand and/or the sense strand may include one or more of the RRMSs described herein. An RRMS can be introduced at one or more points in one or both strands of a double-stranded iRNA agent. An RRMS can be placed at or near (within 1, 2, or 3 positions) of the 3' or 5' end of the sense strand or at near (within 2 or 3 positions of) the 3' end of the antisense strand. In some embodiments it is preferred to not have an RRMS at or near (within 1, 2, or 3 positions of) the 5' end of the antisense strand. An RRMS can be internal, and will preferably be positioned in regions not critical for antisense binding to the target.

20 In an embodiment, an iRNA agent may have an RRMS at (or within 1, 2, or 3 positions of) the 3' end of the antisense strand. In an embodiment, an iRNA agent may have an RRMS at (or within 1, 2, or 3 positions of) the 3' end of the antisense strand and at (or within 1, 2, or 3 positions of) the 3' end of the sense strand. In an embodiment, an iRNA agent may have an RRMS at (or within 1, 2, or 3 positions of) the 3' end of the antisense strand and an RRMS at the 5' end of the sense strand, in which both ligands are located at the same end of the iRNA agent.

25 In certain embodiments, two ligands are tethered, preferably, one on each strand and are hydrophobic moieties. While not wishing to be bound by theory, it is believed that pairing of the hydrophobic ligands can stabilize the iRNA agent *via* intermolecular van der Waals interactions.

30

In an embodiment, an iRNA agent may have an RRMS at (or within 1, 2, or 3 positions of) the 3' end of the antisense strand and an RRMS at the 5' end of the sense strand, in which both RRMSs may share the same ligand (e.g., cholic acid) via connection of their individual tethers to separate positions on the ligand. A ligand shared
 5 between two proximal RRMSs is referred to herein as a "hairpin ligand."

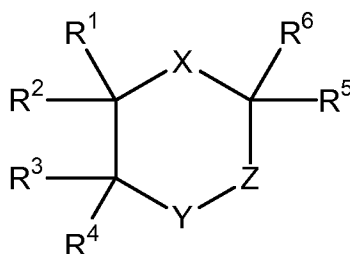
In other embodiments, an iRNA agent may have an RRMS at the 3' end of the sense strand and an RRMS at an internal position of the sense strand. An iRNA agent may have an RRMS at an internal position of the sense strand; or may have an RRMS at an internal position of the antisense strand; or may have an RRMS at an internal position
 10 of the sense strand and an RRMS at an internal position of the antisense strand.

In preferred embodiments the iRNA agent includes a first and second sequences, which are preferably two separate molecules as opposed to two sequences located on the same strand, have sufficient complementarity to each other to hybridize (and thereby form a duplex region), e.g., under physiological conditions, e.g., under physiological
 15 conditions but not in contact with a helicase or other unwinding enzyme.

It is preferred that the first and second sequences be chosen such that the ds iRNA agent includes a single strand or unpaired region at one or both ends of the molecule. Thus, a ds iRNA agent contains first and second sequences, preferable paired to contain an overhang, e.g., one or two 5' or 3' overhangs but preferably a 3' overhang of 2-3
 20 nucleotides. Most embodiments will have a 3' overhang. Preferred sRNA agents will have single-stranded overhangs, preferably 3' overhangs, of 1 or preferably 2 or 3 nucleotides in length at each end. The overhangs can be the result of one strand being longer than the other, or the result of two strands of the same length being staggered. 5' ends are preferably phosphorylated.

Preferred carriers have the general formula (R-3) provided below. (In that structure preferred backbone attachment points can be chosen from R^1 or R^2 ; R^3 or R^4 ; or R^9 and R^{10} if Y is CR^9R^{10} (two positions are chosen to give two backbone attachment points, e.g., R^1 and R^4 , or R^4 and R^9 . Preferred tethering attachment points include R^7 ; R^5 or R^6 when X is CH_2 . The carriers are described below as an entity, which can be
 30 incorporated into a strand. Thus, it is understood that the structures also encompass the

situations wherein one (in the case of a terminal position) or two (in the case of an internal position) of the attachment points, e.g., R^1 or R^2 ; R^3 or R^4 ; or R^9 or R^{10} (when Y is CR^9R^{10}), is connected to the phosphate, or modified phosphate, e.g., sulfur containing, backbone. E.g., one of the above-named R groups can be $-CH_2-$, wherein one bond is
 5 connected to the carrier and one to a backbone atom, e.g., a linking oxygen or a central phosphorus atom.)



10

(R-3)

X is $N(CO)R^7$, NR^7 or CH_2 ; Y is NR^8 , O, S, CR^9R^{10} ; and Z is $CR^{11}R^{12}$ or absent.
 Each of R^1 , R^2 , R^3 , R^4 , R^9 , and R^{10} is, independently, H, OR^a , or $(CH_2)_nOR^b$,
 15 provided that at least two of R^1 , R^2 , R^3 , R^4 , R^9 , and R^{10} are OR^a and/or $(CH_2)_nOR^b$.

Each of R^5 , R^6 , R^{11} , and R^{12} is, independently, a ligand, H, C_1 - C_6 alkyl optionally substituted with 1-3 R^{13} , or $C(O)NHR^7$; or R^5 and R^{11} together are C_3 - C_8 cycloalkyl optionally substituted with R^{14} .

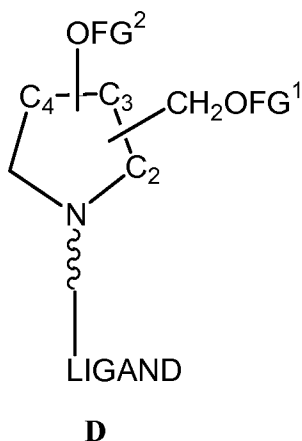
R^7 is H, a ligand, or C_1 - C_{20} alkyl substituted with NR^cR^d ; R^8 is H or C_1 - C_6 alkyl;
 20 R^{13} is hydroxy, C_1 - C_4 alkoxy, or halo; R^{14} is NR^cR^7 ; R^{15} is C_1 - C_6 alkyl optionally substituted with cyano, or C_2 - C_6 alkenyl; R^{16} is C_1 - C_{10} alkyl; and R^{17} is a liquid or solid phase support reagent.

L is $-C(O)(CH_2)_qC(O)-$, or $-C(O)(CH_2)_qS-$; R^a is CAr_3 ; R^b is $P(O)(O^-)H$, $P(OR^{15})N(R^{16})_2$ or $L-R^{17}$; R^c is H or C_1 - C_6 alkyl; and R^d is H or a ligand.

Each Ar is, independently, C₆-C₁₀ aryl optionally substituted with C₁-C₄ alkoxy; n is 1-4; and q is 0-4.

Exemplary carriers include those in which, e.g., X is N(CO)R⁷ or NR⁷, Y is CR⁹R¹⁰, and Z is absent; or X is N(CO)R⁷ or NR⁷, Y is CR⁹R¹⁰, and Z is CR¹¹R¹²; or X is N(CO)R⁷ or NR⁷, Y is NR⁸, and Z is CR¹¹R¹²; or X is N(CO)R⁷ or NR⁷, Y is O, and Z is CR¹¹R¹²; or X is CH₂; Y is CR⁹R¹⁰; Z is CR¹¹R¹², and R⁵ and R¹¹ together form C₆ cycloalkyl (**H**, z = 2), or the indane ring system, e.g., X is CH₂; Y is CR⁹R¹⁰; Z is CR¹¹R¹², and R⁵ and R¹¹ together form C₅ cycloalkyl (**H**, z = 1).

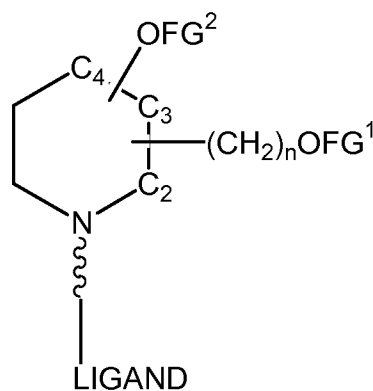
In certain embodiments, the carrier may be based on the pyrroline ring system or the 3-hydroxyproline ring system, e.g., X is N(CO)R⁷ or NR⁷, Y is CR⁹R¹⁰, and Z is absent (**D**). OFG¹ is preferably attached to a primary carbon, e.g., an exocyclic alkylene



group, e.g., a methylene group, connected to one of the carbons in the five-membered ring (-CH₂OFG¹ in **D**). OFG² is preferably attached directly to one of the carbons in the five-membered ring (-OFG² in **D**). For the pyrroline-based carriers, -CH₂OFG¹ may be attached to C-2 and OFG² may be attached to C-3; or -CH₂OFG¹ may be attached to C-3 and OFG² may be attached to C-4. . In certain embodiments, CH₂OFG¹ and OFG² may be geminally substituted to one of the above-referenced carbons. For the 3-hydroxyproline-based carriers, -CH₂OFG¹ may be attached to C-2 and OFG² may be attached to C-4. The pyrroline- and 3-hydroxyproline-based monomers may therefore contain linkages (e.g., carbon-carbon bonds) wherein bond rotation is restricted about that

particular linkage, e.g. restriction resulting from the presence of a ring. Thus, CH_2OFG^1 and OFG^2 may be *cis* or *trans* with respect to one another in any of the pairings delineated above. Accordingly, all *cis/trans* isomers are expressly included. The monomers may also contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of the monomers are expressly included. The tethering attachment point is preferably nitrogen.

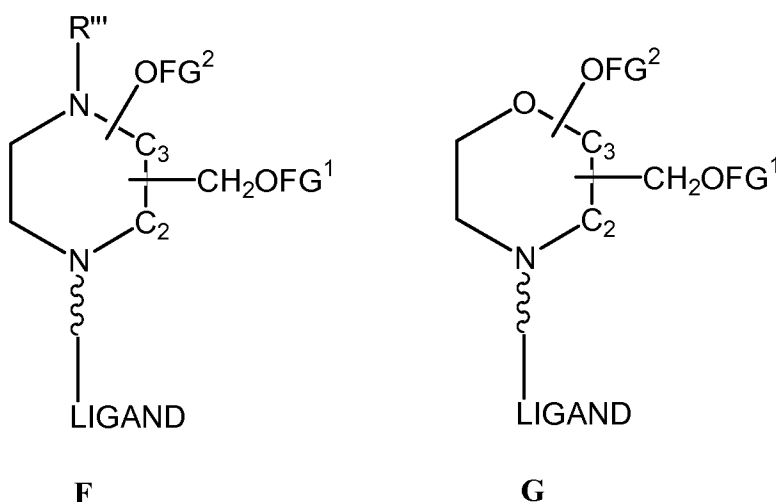
In certain embodiments, the carrier may be based on the piperidine ring system (**E**), e.g., X is $\text{N}(\text{CO})\text{R}^7$ or NR^7 , Y is CR^9R^{10} , and Z is $\text{CR}^{11}\text{R}^{12}$. OFG^1 is preferably



attached to a primary carbon, e.g., an exocyclic alkylene group, e.g., a methylene group ($n=1$) or ethylene group ($n=2$), connected to one of the carbons in the six-membered ring [$-(\text{CH}_2)_n\text{OFG}^1$ in **E**]. OFG^2 is preferably attached directly to one of the carbons in the six-membered ring ($-\text{OFG}^2$ in **E**). $-(\text{CH}_2)_n\text{OFG}^1$ and OFG^2 may be disposed in a geminal manner on the ring, i.e., both groups may be attached to the same carbon, e.g., at C-2, C-3, or C-4. Alternatively, $-(\text{CH}_2)_n\text{OFG}^1$ and OFG^2 may be disposed in a vicinal manner on the ring, i.e., both groups may be attached to adjacent ring carbon atoms, e.g., $-(\text{CH}_2)_n\text{OFG}^1$ may be attached to C-2 and OFG^2 may be attached to C-3; $-(\text{CH}_2)_n\text{OFG}^1$ may be attached to C-3 and OFG^2 may be attached to C-2; $-(\text{CH}_2)_n\text{OFG}^1$ may be attached to C-3 and OFG^2 may be attached to C-4; or $-(\text{CH}_2)_n\text{OFG}^1$ may be attached to C-4 and

OFG² may be attached to C-3. The piperidine-based monomers may therefore contain linkages (e.g., carbon-carbon bonds) wherein bond rotation is restricted about that particular linkage, e.g. restriction resulting from the presence of a ring. Thus, - (CH₂)_nOFG¹ and OFG² may be *cis* or *trans* with respect to one another in any of the pairings delineated above. Accordingly, all *cis/trans* isomers are expressly included. The monomers may also contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of the monomers are expressly included. The tethering attachment point is preferably nitrogen.

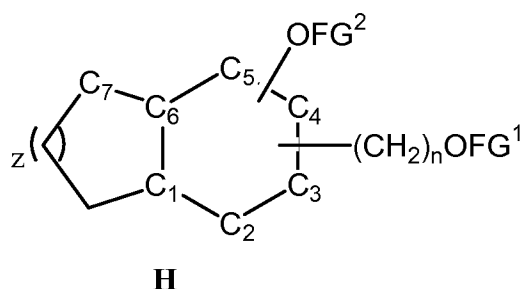
In certain embodiments, the carrier may be based on the piperazine ring system (**F**), e.g., X is N(CO)R⁷ or NR⁷, Y is NR⁸, and Z is CR¹¹R¹², or the morpholine ring system (**G**), e.g., X is N(CO)R⁷ or NR⁷, Y is O, and Z is CR¹¹R¹². OFG¹ is preferably



attached to a primary carbon, e.g., an exocyclic alkylene group, e.g., a methylene group, connected to one of the carbons in the six-membered ring (-CH₂OFG¹ in **F** or **G**). OFG² is preferably attached directly to one of the carbons in the six-membered rings (-OFG² in **F** or **G**). For both **F** and **G**, -CH₂OFG¹ may be attached to C-2 and OFG² may be attached to C-3; or *vice versa*. In certain embodiments, CH₂OFG¹ and OFG² may be geminally

substituted to one of the above-referenced carbons. The piperazine- and morpholine-based monomers may therefore contain linkages (e.g., carbon-carbon bonds) wherein bond rotation is restricted about that particular linkage, e.g. restriction resulting from the presence of a ring. Thus, CH_2OFG^1 and OFG^2 may be *cis* or *trans* with respect to one another in any of the pairings delineated above. Accordingly, all *cis/trans* isomers are expressly included. The monomers may also contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of the monomers are expressly included. R''' can be, e.g., $\text{C}_1\text{-C}_6$ alkyl, preferably CH_3 . The tethering attachment point is preferably nitrogen in both **F** and **G**.

In certain embodiments, the carrier may be based on the decalin ring system, e.g., X is CH_2 ; Y is CR^9R^{10} ; Z is $\text{CR}^{11}\text{R}^{12}$, and R^5 and R^{11} together form C_6 cycloalkyl (**H**, $z = 2$), or the indane ring system, e.g., X is CH_2 ; Y is CR^9R^{10} ; Z is $\text{CR}^{11}\text{R}^{12}$, and R^5 and R^{11} together form C_5 cycloalkyl (**H**, $z = 1$). OFG^1 is preferably attached to a primary carbon,



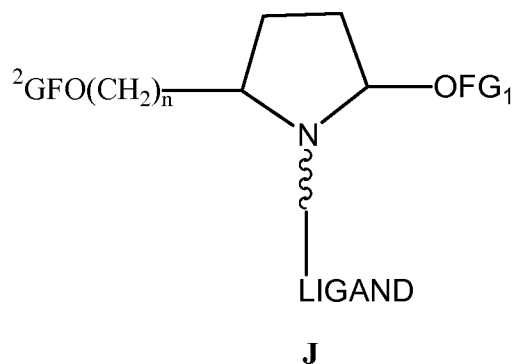
e.g., an exocyclic methylene group ($n=1$) or ethylene group ($n=2$) connected to one of C-2, C-3, C-4, or C-5 [$-(\text{CH}_2)_n\text{OFG}^1$ in **H**]. OFG^2 is preferably attached directly to one of C-2, C-3, C-4, or C-5 ($-\text{OFG}^2$ in **H**). $-(\text{CH}_2)_n\text{OFG}^1$ and OFG^2 may be disposed in a geminal manner on the ring, i.e., both groups may be attached to the same carbon, e.g., at C-2, C-3, C-4, or C-5. Alternatively, $-(\text{CH}_2)_n\text{OFG}^1$ and OFG^2 may be disposed in a vicinal manner on the ring, i.e., both groups may be attached to adjacent ring carbon atoms, e.g., $-(\text{CH}_2)_n\text{OFG}^1$ may be attached to C-2 and OFG^2 may be attached to C-3; $-(\text{CH}_2)_n\text{OFG}^1$ may be attached to C-3 and OFG^2 may be attached to C-2; $-(\text{CH}_2)_n\text{OFG}^1$

may be attached to C-3 and OFG² may be attached to C-4; or -(CH₂)_nOFG¹ may be attached to C-4 and OFG² may be attached to C-3; -(CH₂)_nOFG¹ may be attached to C-4 and OFG² may be attached to C-5; or -(CH₂)_nOFG¹ may be attached to C-5 and OFG² may be attached to C-4. The decalin or indane-based monomers may therefore contain

5 linkages (e.g., carbon-carbon bonds) wherein bond rotation is restricted about that particular linkage, e.g. restriction resulting from the presence of a ring. Thus, -(CH₂)_nOFG¹ and OFG² may be *cis* or *trans* with respect to one another in any of the pairings delineated above. Accordingly, all *cis/trans* isomers are expressly included. The monomers may also contain one or more asymmetric centers and thus occur as

10 racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of the monomers are expressly included. In a preferred embodiment, the substituents at C-1 and C-6 are *trans* with respect to one another. The tethering attachment point is preferably C-6 or C-7.

Other carriers may include those based on 3-hydroxyproline (**J**). Thus, -(CH₂)_nOFG¹ and OFG² may be *cis* or *trans* with respect to one another. Accordingly, all *cis/trans* isomers are expressly included. The monomers may also contain one or more asymmetric centers



20 and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of the monomers are expressly included. The tethering attachment point is preferably nitrogen.

In certain embodiments, a moiety, e.g., a ligand may be connected indirectly to the carrier *via* the intermediacy of an intervening tether. Tethers are connected to the carrier at the tethering attachment point (TAP) and may include any C₁-C₁₀₀ carbon-containing moiety, (e.g. C₁-C₇₅, C₁-C₅₀, C₁-C₂₀, C₁-C₁₀, C₁-C₆), preferably having at least one nitrogen atom. In preferred embodiments, the nitrogen atom forms part of a terminal amino group on the tether, which may serve as a connection point for the ligand.

Preferred tethers (underlined) include TAP-(CH₂)_nNH₂; TAP-C(O)(CH₂)_nNH₂; or TAP-NR^{'''}(CH₂)_nNH₂, in which n is 1-6 and R^{'''} is C₁-C₆ alkyl. and R^d is hydrogen or a ligand. In other embodiments, the nitrogen may form part of a terminal oxyamino group,

e.g., -ONH₂, or hydrazino group, -NHNH₂. The tether may optionally be substituted, e.g., with hydroxy, alkoxy, perhaloalkyl, and/or optionally inserted with one or more additional heteroatoms, e.g., N, O, or S. Preferred tethered ligands may include, e.g., TAP-(CH₂)_nNH(LIGAND),

TAP-C(O)(CH₂)_nNH(LIGAND), or TAP-NR^{'''}(CH₂)_nNH(LIGAND);

TAP-(CH₂)_nONH(LIGAND), TAP-C(O)(CH₂)_nONH(LIGAND), or

TAP-NR^{'''}(CH₂)_nONH(LIGAND); TAP-(CH₂)_nNHNH₂(LIGAND),

TAP-C(O)(CH₂)_nNHNH₂(LIGAND), or TAP-NR^{'''}(CH₂)_nNHNH₂(LIGAND).

In other embodiments the tether may include an electrophilic moiety, preferably at the terminal position of the tether. Preferred electrophilic moieties include, e.g., an aldehyde, alkyl halide, mesylate, tosylate, nosylate, or brosylate, or an activated carboxylic acid ester, e.g. an NHS ester, or a pentafluorophenyl ester. Preferred tethers (underlined) include TAP-(CH₂)_nCHO; TAP-C(O)(CH₂)_nCHO; or TAP-NR^{'''}(CH₂)_nCHO, in which n is 1-6 and R^{'''} is C₁-C₆ alkyl; or TAP-(CH₂)_nC(O)ONHS; TAP-C(O)(CH₂)_nC(O)ONHS; or

TAP-NR^{'''}(CH₂)_nC(O)ONHS, in which n is 1-6 and R^{'''} is C₁-C₆ alkyl;

TAP-(CH₂)_nC(O)OC₆F₅; TAP-C(O)(CH₂)_nC(O)OC₆F₅; or TAP-NR^{'''}(CH₂)_nC(O)

OC₆F₅, in which n is 1-6 and R^{'''} is C₁-C₆ alkyl; or -(CH₂)_nCH₂LG; TAP-

C(O)(CH₂)_nCH₂LG; or TAP-NR^{'''}(CH₂)_nCH₂LG, in which n is 1-6 and R^{'''} is C₁-C₆ alkyl (LG can be a leaving group, e.g., halide, mesylate, tosylate, nosylate, brosylate).

Tethering can be carried out by coupling a nucleophilic group of a ligand, e.g., a thiol or amino group with an electrophilic group on the tether.

Tethered Entities

5 A wide variety of entities can be tethered to an iRNA agent, e.g., to the carrier of an RRMS. Examples are described below in the context of an RRMS but that is only preferred, entities can be coupled at other points to an iRNA agent.

Preferred moieties are ligands, which are coupled, preferably covalently, either directly or indirectly via an intervening tether, to the RRMS carrier. In preferred embodiments, the ligand is attached to the carrier *via* an intervening tether. As discussed
10 above, the ligand or tethered ligand may be present on the RRMS monomer when the RRMS monomer is incorporated into the growing strand. In some embodiments, the ligand may be incorporated into a “precursor” RRMS after a “precursor” RRMS monomer has been incorporated into the growing strand. For example, an RRMS monomer having, e.g., an amino-terminated tether (i.e., having no associated ligand), e.g.,
15 TAP-(CH₂)_n NH₂ may be incorporated into a growing sense or antisense strand. In a subsequent operation, i.e., after incorporation of the precursor monomer into the strand, a ligand having an electrophilic group, e.g., a pentafluorophenyl ester or aldehyde group, can subsequently be attached to the precursor RRMS by coupling the electrophilic group of the ligand with the terminal nucleophilic group of the precursor RRMS tether.

20 In preferred embodiments, a ligand alters the distribution, targeting or lifetime of an iRNA agent into which it is incorporated. In preferred embodiments a ligand provides an enhanced affinity for a selected target, e.g, molecule, cell or cell type, compartment, e.g., a cellular or organ compartment, tissue, organ or region of the body, as, e.g., compared to a species absent such a ligand. Preferred ligands will not take part in duplex
25 pairing in a duplexed nucleic acid.

Preferred ligands can improve transport, hybridization, and specificity properties and may also improve nuclease resistance of the resultant natural or modified oligoribonucleotide, or a polymeric molecule comprising any combination of monomers described herein and/or natural or modified ribonucleotides.

Ligands in general can include therapeutic modifiers, e.g., for enhancing uptake; diagnostic compounds or reporter groups e.g., for monitoring distribution; cross-linking agents; and nuclease-resistance conferring moieties. General examples include lipids, steroids, vitamins, sugars, proteins, peptides, polyamines, and peptide mimics.

- 5 Ligands can include a naturally occurring substance, such as a protein (e.g., human serum albumin (HSA), low-density lipoprotein (LDL), or globulin); carbohydrate (e.g., a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid); or a lipid. The ligand may also be a recombinant or synthetic molecule, such as a synthetic polymer, e.g., a synthetic polyamino acid. Examples of polyamino acids include
- 10 polyamino acid is a polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolid) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacrylic acid), N-isopropylacrylamide polymers, or polyphosphazine.
- 15 Example of polyamines include: polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an alpha helical peptide.

- Ligands can also include targeting groups, e.g., a cell or tissue targeting agent,
- 20 e.g., a lectin, glycoprotein, lipid or protein, e.g., an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, bone cell. A targeting group can be a thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, Mucin carbohydrate, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids,
- 25 multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, biotin, or an RGD peptide or RGD peptide mimetic.

- Other examples of ligands include dyes, intercalating agents (*e.g.* acridines), cross-linkers (*e.g.* psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, Sapphyrin),
- 30 polycyclic aromatic hydrocarbons (*e.g.*, phenazine, dihydrophenazine), artificial

endonucleases (*e.g.* EDTA), lipophilic molecules, *e.g.* cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis-O(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid, O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine) and peptide conjugates (*e.g.*, antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (*e.g.*, PEG-40K), MPEG, [MPEG]₂, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (*e.g.* biotin), transport/absorption facilitators (*e.g.*, aspirin, vitamin E, folic acid), synthetic ribonucleases (*e.g.*, imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu³⁺ complexes of tetraazamacrocycles), dinitrophenyl, HRP, or AP.

Ligands can be proteins, *e.g.*, glycoproteins, or peptides, *e.g.*, molecules having a specific affinity for a co-ligand, or antibodies *e.g.*, an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell. Ligands may also include hormones and hormone receptors. They can also include non-peptidic species, such as lipids, lectins, carbohydrates, vitamins, cofactors, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, or multivalent fucose. The ligand can be, for example, a lipopolysaccharide, an activator of p38 MAP kinase, or an activator of NF- κ B.

The ligand can be a substance, *e.g.* a drug, which can increase the uptake of the iRNA agent into the cell, for example, by disrupting the cell's cytoskeleton, *e.g.*, by disrupting the cell's microtubules, microfilaments, and/or intermediate filaments. The drug can be, for example, taxon, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholide A, indanocine, or myoservin.

The ligand can increase the uptake of the iRNA agent into the cell by activating an inflammatory response, for example. Exemplary ligands that would have such an effect include tumor necrosis factor alpha (TNFalpha), interleukin-1 beta, or gamma interferon.

In one aspect, the ligand is a lipid or lipid-based molecule. Such a lipid or lipid-based molecule preferably binds a serum protein, *e.g.*, human serum albumin (HSA). An

HSA binding ligand allows for distribution of the conjugate to a target tissue, e.g., a non-kidney target tissue of the body. Preferably, the target tissue is the liver, preferably parenchymal cells of the liver. Other molecules that can bind HSA can also be used as ligands. For example, neproxin or aspirin can be used. A lipid or lipid-based ligand can
5 (a) increase resistance to degradation of the conjugate, (b) increase targeting or transport into a target cell or cell membrane, and/or (c) can be used to adjust binding to a serum protein, e.g., HSA.

A lipid based ligand can be used to modulate, e.g., control the binding of the conjugate to a target tissue. For example, a lipid or lipid-based ligand that binds to HSA
10 more strongly will be less likely to be targeted to the kidney and therefore less likely to be cleared from the body. A lipid or lipid-based ligand that binds to HSA less strongly can be used to target the conjugate to the kidney.

In a preferred embodiment, the lipid based ligand binds HSA. Preferably, it binds HSA with a sufficient affinity such that the conjugate will be preferably distributed to a
15 non-kidney tissue. However, it is preferred that the affinity not be so strong that the HSA-ligand binding cannot be reversed.

In another preferred embodiment, the lipid based ligand binds HSA weakly or not at all, such that the conjugate will be preferably distributed to the kidney. Other moieties that target to kidney cells can also be used in place of or in addition to the lipid based
20 ligand.

In another aspect, the ligand is a moiety, e.g., a vitamin, which is taken up by a target cell, e.g., a proliferating cell. These are particularly useful for treating disorders characterized by unwanted cell proliferation, e.g., of the malignant or non-malignant type, e.g., cancer cells. Exemplary vitamins include vitamin A, E, and K. Other
25 exemplary vitamins include are B vitamin, e.g., folic acid, B12, riboflavin, biotin, pyridoxal or other vitamins or nutrients taken up by cancer cells. Also included are HSA and low density lipoprotein (LDL).

In another aspect, the ligand is a cell-permeation agent, preferably a helical cell-permeation agent. Preferably, the agent is amphipathic. An exemplary agent is a peptide
30 such as tat or antennopodia. If the agent is a peptide, it can be modified, including a

peptidylmimetic, invertomers, non-peptide or pseudo-peptide linkages, and use of D-amino acids. The helical agent is preferably an alpha-helical agent, which preferably has a lipophilic and a lipophobic phase.

5 The ligand can be a peptide or peptidomimetic. A peptidomimetic (also referred to herein as an oligopeptidomimetic) is a molecule capable of folding into a defined three-dimensional structure similar to a natural peptide. The attachment of peptide and peptidomimetics to iRNA agents can affect pharmacokinetic distribution of the iRNA, such as by enhancing cellular recognition and absorption. The peptide or peptidomimetic moiety can be about 5-50 amino acids long, *e.g.*, about 5, 10, 15, 20, 25, 30, 35, 40, 45, or
10 50 amino acids long.

A peptide or peptidomimetic can be, for example, a cell permeation peptide, cationic peptide, amphipathic peptide, or hydrophobic peptide (*e.g.*, consisting primarily of Tyr, Trp or Phe). The peptide moiety can be a dendrimer peptide, constrained peptide or crosslinked peptide. In another alternative, the peptide moiety can include a

5 hydrophobic membrane translocation sequence (MTS). An exemplary hydrophobic MTS-containing peptide is RFGF. An RFGF analogue containing a hydrophobic MTS can also be a targeting moiety. The peptide moiety can be a “delivery” peptide, which can carry large polar molecules including peptides, oligonucleotides, and protein across cell membranes. For example, sequences from the HIV Tat protein and the *Drosophila*

10 Antennapedia protein have been found to be capable of functioning as delivery peptides. A peptide or peptidomimetic can be encoded by a random sequence of DNA, such as a peptide identified from a phage-display library, or one-bead-one-compound (OBOC) combinatorial library (Lam *et al.*, *Nature*, 354:82-84, 1991). Preferably the peptide or peptidomimetic tethered to an iRNA agent via an incorporated monomer unit is a cell

15 targeting peptide such as an arginine-glycine-aspartic acid (RGD)-peptide, or RGD mimic. A peptide moiety can range in length from about 5 amino acids to about 50 amino acids. The peptide moieties can have a structural modification, such as to increase stability or direct conformational properties. Any of the structural modifications described below can be utilized.

20 An RGD peptide moiety can be used to target a tumor cell, such as an endothelial tumor cell or a breast cancer tumor cell (Zitzmann *et al.*, *Cancer Res.*, 62:5139-43, 2002). An RGD peptide can facilitate targeting of an iRNA agent to tumors of a variety of other tissues, including the lung, kidney, spleen, or liver (Aoki *et al.*, *Cancer Gene Therapy* 8:783-787, 2001). The RGD peptide can be linear or cyclic, and can be modified, *e.g.*,

25 glycosylated or methylated to facilitate targeting to specific tissues. For example, a glycosylated RGD peptide can deliver an iRNA agent to a tumor cell expressing $\alpha_v\beta_3$ (Haubner *et al.*, *Jour. Nucl. Med.*, 42:326-336, 2001).

Peptides that target markers enriched in proliferating cells can be used. *E.g.*, RGD containing peptides and peptidomimetics can target cancer cells, in particular cells

that exhibit an $I_V\beta_3$ integrin. Thus, one could use RGD peptides, cyclic peptides containing RGD, RGD peptides that include D-amino acids, as well as synthetic RGD mimics. In addition to RGD, one can use other moieties that target the $I_V\beta_3$ integrin ligand. Generally, such ligands can be used to control proliferating cells and angiogenesis. Preferred conjugates of this type include an iRNA agent that targets PECAM-1, VEGF, or other cancer gene, e.g., a cancer gene described herein.

A “cell permeation peptide” is capable of permeating a cell, e.g., a microbial cell, such as a bacterial or fungal cell, or a mammalian cell, such as a human cell. A microbial cell-permeating peptide can be, for example, an α -helical linear peptide (e.g., LL-37 or Ceropin P1), a disulfide bond-containing peptide (e.g., α -defensin, β -defensin or batenecin), or a peptide containing only one or two dominating amino acids (e.g., PR-39 or indolicidin). A cell permeation peptide can also include a nuclear localization signal (NLS). For example, a cell permeation peptide can be a bipartite amphipathic peptide, such as MPG, which is derived from the fusion peptide domain of HIV-1 gp41 and the NLS of SV40 large T antigen (Simeoni *et al.*, Nucl. Acids Res. 31:2717-2724, 2003).

In one embodiment, a targeting peptide tethered to an RRMS can be an amphipathic α -helical peptide. Exemplary amphipathic α -helical peptides include, but are not limited to, cecropins, lycotoxins, paradaxins, buforin, CPF, bombinin-like peptide (BLP), cathelicidins, ceratotoxins, *S. clava* peptides, hagfish intestinal antimicrobial peptides (HFIAPs), magainines, brevinins-2, dermaseptins, melittins, pleurocidin, H₂A peptides, *Xenopus* peptides, esculentinis-1, and caerins. A number of factors will preferably be considered to maintain the integrity of helix stability. For example, a maximum number of helix stabilization residues will be utilized (*e.g.*, leu, ala, or lys), and a minimum number helix destabilization residues will be utilized (*e.g.*, proline, or cyclic monomeric units). The capping residue will be considered (for example Gly is an exemplary N-capping residue and/or C-terminal amidation can be used to provide an extra H-bond to stabilize the helix. Formation of salt bridges between residues with opposite charges, separated by $i \pm 3$, or $i \pm 4$ positions can provide stability. For example, cationic residues such as lysine, arginine, homo-arginine, ornithine or histidine can form salt bridges with the anionic residues glutamate or aspartate.

Peptide and peptidomimetic ligands include those having naturally occurring or modified peptides, *e.g.*, D or L peptides; α , β , or γ peptides; N-methyl peptides; azapeptides; peptides having one or more amide, *i.e.*, peptide, linkages replaced with one or more urea, thiourea, carbamate, or sulfonyl urea linkages; or cyclic peptides.

Methods for making iRNA agents

iRNA agents can include modified or non-naturally occurring bases, *e.g.*, bases described in copending and coowned United States Provisional Application Serial No. 60/463,772, filed on April 17, 2003, which is hereby incorporated by reference and/or in copending and coowned United States Provisional Application Serial No. 60/465,802, filed on April 25, 2003, which is hereby incorporated by reference. Monomers and iRNA agents which include such bases can be made by the methods found in United States Provisional Application Serial No. 60/463,772, filed on April 17, 2003, and/or in United States Provisional Application Serial No. 60/465,802, filed on April 25, 2003.

In addition, the invention includes iRNA agents having a modified or non-naturally occurring base and another element described herein. *E.g.*, the invention

includes an iRNA agent described herein, e.g., a palindromic iRNA agent, an iRNA agent having a non canonical pairing, an iRNA agent which targets a gene described herein, e.g., a gene active in the liver, an iRNA agent having an architecture or structure described herein, an iRNA associated with an amphipathic delivery agent described
5 herein, an iRNA associated with a drug delivery module described herein, an iRNA agent administered as described herein, or an iRNA agent formulated as described herein, which also incorporates a modified or non-naturally occurring base.

The synthesis and purification of oligonucleotide peptide conjugates can be performed by established methods. See, for example, Trufert *et al.*, Tetrahedron,
10 52:3005, 1996; and Manoharan, "Oligonucleotide Conjugates in Antisense Technology," in Antisense Drug Technology, ed. S.T. Crooke, Marcel Dekker, Inc., 2001.

In one embodiment of the invention, a peptidomimetic can be modified to create a constrained peptide that adopts a distinct and specific preferred conformation, which can increase the potency and selectivity of the peptide. For example, the constrained peptide
15 can be an azapeptide (Gante, Synthesis, 405-413, 1989). An azapeptide is synthesized by replacing the α -carbon of an amino acid with a nitrogen atom without changing the structure of the amino acid side chain. For example, the azapeptide can be synthesized by using hydrazine in traditional peptide synthesis coupling methods, such as by reacting hydrazine with a "carbonyl donor," e.g., phenylchloroformate.

In one embodiment of the invention, a peptide or peptidomimetic (e.g., a peptide or peptidomimetic tethered to an RRMS) can be an N-methyl peptide. N-methyl peptides are composed of N-methyl amino acids, which provide an additional methyl group in the peptide backbone, thereby potentially providing additional means of resistance to
20 proteolytic cleavage. N-methyl peptides can be synthesized by methods known in the art (see, for example, Lindgren *et al.*, Trends Pharmacol. Sci. 21:99, 2000; Cell Penetrating Peptides: Processes and Applications, Langel, ed., CRC Press, Boca Raton, FL, 2002; Fische *et al.*, Bioconjugate. Chem. 12: 825, 2001; Wander *et al.*, J. Am. Chem. Soc., 124:13382, 2002). For example, an Ant or Tat peptide can be an N-methyl peptide.

In one embodiment of the invention, a peptide or peptidomimetic (e.g., a peptide
30 or peptidomimetic tethered to an RRMS) can be a β -peptide. β -peptides form stable

secondary structures such as helices, pleated sheets, turns and hairpins in solutions. Their cyclic derivatives can fold into nanotubes in the solid state. β -peptides are resistant to degradation by proteolytic enzymes. β -peptides can be synthesized by methods known in the art. For example, an Ant or Tat peptide can be a β -peptide.

5 In one embodiment of the invention, a peptide or peptidomimetic (*e.g.*, a peptide or peptidomimetic tethered to an RRMS) can be an oligocarbamate. Oligocarbamate peptides are internalized into a cell by a transport pathway facilitated by carbamate transporters. For example, an Ant or Tat peptide can be an oligocarbamate.

10 In one embodiment of the invention, a peptide or peptidomimetic (*e.g.*, a peptide or peptidomimetic tethered to an RRMS) can be an oligourea conjugate (or an oligothiourea conjugate), in which the amide bond of a peptidomimetic is replaced with a urea moiety. Replacement of the amide bond provides increased resistance to degradation by proteolytic enzymes, *e.g.*, proteolytic enzymes in the gastrointestinal tract. In one embodiment, an oligourea conjugate is tethered to an iRNA agent for use in oral
15 delivery. The backbone in each repeating unit of an oligourea peptidomimetic can be extended by one carbon atom in comparison with the natural amino acid. The single carbon atom extension can increase peptide stability and lipophilicity, for example. An oligourea peptide can therefore be advantageous when an iRNA agent is directed for passage through a bacterial cell wall, or when an iRNA agent must traverse the blood-
20 brain barrier, such as for the treatment of a neurological disorder. In one embodiment, a hydrogen bonding unit is conjugated to the oligourea peptide, such as to create an increased affinity with a receptor. For example, an Ant or Tat peptide can be an oligourea conjugate (or an oligothiourea conjugate).

25 The siRNA peptide conjugates of the invention can be affiliated with, *e.g.*, tethered to, RRMSs occurring at various positions on an iRNA agent. For example, a peptide can be terminally conjugated, on either the sense or the antisense strand, or a peptide can be bisconjugated (one peptide tethered to each end, one conjugated to the sense strand, and one conjugated to the antisense strand). In another option, the peptide can be internally conjugated, such as in the loop of a short hairpin iRNA agent. In yet

another option, the peptide can be affiliated with a complex, such as a peptide-carrier complex.

A peptide-carrier complex consists of at least a carrier molecule, which can encapsulate one or more iRNA agents (such as for delivery to a biological system and/or a cell), and a peptide moiety tethered to the outside of the carrier molecule, such as for targeting the carrier complex to a particular tissue or cell type. A carrier complex can carry additional targeting molecules on the exterior of the complex, or fusogenic agents to aid in cell delivery. The one or more iRNA agents encapsulated within the carrier can be conjugated to lipophilic molecules, which can aid in the delivery of the agents to the interior of the carrier.

A carrier molecule or structure can be, for example, a micelle, a liposome (*e.g.*, a cationic liposome), a nanoparticle, a microsphere, or a biodegradable polymer. A peptide moiety can be tethered to the carrier molecule by a variety of linkages, such as a disulfide linkage, an acid labile linkage, a peptide-based linkage, an oxyamino linkage or a hydrazine linkage. For example, a peptide-based linkage can be a GFLG peptide. Certain linkages will have particular advantages, and the advantages (or disadvantages) can be considered depending on the tissue target or intended use. For example, peptide based linkages are stable in the blood stream but are susceptible to enzymatic cleavage in the lysosomes.

Asymmetric Modifications

In one aspect, the invention features an iRNA agent which can be asymmetrically modified as described herein.

In addition, the invention includes iRNA agents having asymmetrical modifications and another element described herein. *E.g.*, the invention includes an iRNA agent described herein, *e.g.*, a palindromic iRNA agent, an iRNA agent having a non canonical pairing, an iRNA agent which targets a gene described herein, *e.g.*, a gene active in the liver, an iRNA agent having an architecture or structure described herein, an iRNA associated with an amphipathic delivery agent described herein, an iRNA associated with a drug delivery module described herein, an iRNA agent administered as

described herein, or an iRNA agent formulated as described herein, which also incorporates an asymmetrical modification.

iRNA agents of the invention can be asymmetrically modified. An asymmetrical modification is one in which a strand has a modification which is not present on the other strand. An asymmetrical modification is a modification found on one strand but not on the other strand. Any modification, e.g., any modification described herein, can be present as an asymmetrical modification. An asymmetrical modification can confer any of the desired properties associated with a modification, e.g., those properties discussed herein. *E.g.*, an asymmetrical modification can: confer resistance to degradation, an alteration in half life; target the iRNA agent to a particular target, e.g., to a particular tissue; modulate, e.g., increase or decrease, the affinity of a strand for its complement or target sequence; or hinder or promote modification of a terminal moiety, e.g., modification by a kinase or other enzymes involved in the RISC mechanism pathway. The designation of a modification as having one property does not mean that it has no other property, e.g., a modification referred to as one which promotes stabilization might also enhance targeting.

While not wishing to be bound by theory or any particular mechanistic model, it is believed that asymmetrical modification allows an iRNA agent to be optimized in view of the different or “asymmetrical” functions of the sense and antisense strands. For example, both strands can be modified to increase nuclease resistance, however, since some changes can inhibit RISC activity, these changes can be chosen for the sense strand. In addition, since some modifications, e.g., targeting moieties, can add large bulky groups that, e.g., can interfere with the cleavage activity of the RISC complex, such modifications are preferably placed on the sense strand. Thus, targeting moieties, especially bulky ones (e.g. cholesterol), are preferentially added to the sense strand. In one embodiment, an asymmetrical modification in which a phosphate of the backbone is substituted with S, e.g., a phosphorothioate modification, is present in the antisense strand, and a 2' modification, e.g., 2' OMe is present in the sense strand. A targeting moiety can be present at either (or both) the 5' or 3' end of the sense strand of the iRNA agent. In a preferred example, a P of the backbone is replaced with S in the antisense

strand, 2'OMe is present in the sense strand, and a targeting moiety is added to either the 5' or 3' end of the sense strand of the iRNA agent.

In a preferred embodiment an asymmetrically modified iRNA agent has a modification on the sense strand which modification is not found on the antisense strand and the antisense strand has a modification which is not found on the sense strand.

Each strand can include one or more asymmetrical modifications. By way of example: one strand can include a first asymmetrical modification which confers a first property on the iRNA agent and the other strand can have a second asymmetrical modification which confers a second property on the iRNA. E.g., one strand, e.g., the sense strand can have a modification which targets the iRNA agent to a tissue, and the other strand, e.g., the antisense strand, has a modification which promotes hybridization with the target gene sequence.

In some embodiments both strands can be modified to optimize the same property, e.g., to increase resistance to nucleolytic degradation, but different modifications are chosen for the sense and the antisense strands, e.g., because the modifications affect other properties as well. E.g., since some changes can affect RISC activity these modifications are chosen for the sense strand.

In an embodiment one strand has an asymmetrical 2' modification, e.g., a 2' OMe modification, and the other strand has an asymmetrical modification of the phosphate backbone, e.g., a phosphorothioate modification. So, in one embodiment the antisense strand has an asymmetric 2' OMe modification and the sense strand has an asymmetrical phosphorothioate modification (or vice versa). In a particularly preferred embodiment the RNAi agent will have asymmetrical 2'-O alkyl, preferably, 2'-OMe modifications on the sense strand and asymmetrical backbone P modification, preferably a phosphorothioate modification in the antisense strand. There can be one or multiple 2'-OMe modifications, e.g., at least 2, 3, 4, 5, or 6, of the subunits of the sense strand can be so modified. There can be one or multiple phosphorothioate modifications, e.g., at least 2, 3, 4, 5, or 6, of the subunits of the antisense strand can be so modified. It is preferable to have an iRNA agent wherein there are multiple 2'-OMe modifications on the sense strand and multiple phosphorothioate modifications on the antisense strand. All of the subunits on one or both

strands can be so modified. A particularly preferred embodiment of multiple asymmetric modification on both strands has a duplex region about 20-21, and preferably 19, subunits in length and one or two 3' overhangs of about 2 subunits in length.

Asymmetrical modifications are useful for promoting resistance to degradation by nucleases, e.g., endonucleases. iRNA agents can include one or more asymmetrical modifications which promote resistance to degradation. In preferred embodiments the modification on the antisense strand is one which will not interfere with silencing of the target, e.g., one which will not interfere with cleavage of the target. Most if not all sites on a strand are vulnerable, to some degree, to degradation by endonucleases. One can determine sites which are relatively vulnerable and insert asymmetrical modifications which inhibit degradation. It is often desirable to provide asymmetrical modification of a UA site in an iRNA agent, and in some cases it is desirable to provide the UA sequence on both strands with asymmetrical modification. Examples of modifications which inhibit endonucleolytic degradation can be found herein. Particularly favored modifications include: 2' modification, e.g., provision of a 2' OMe moiety on the U, especially on a sense strand; modification of the backbone, e.g., with the replacement of an O with an S, in the phosphate backbone, e.g., the provision of a phosphorothioate modification, on the U or the A or both, especially on an antisense strand; replacement of the U with a C5 amino linker; replacement of the A with a G (sequence changes are preferred to be located on the sense strand and not the antisense strand); and modification of the at the 2', 6', 7', or 8' position. Preferred embodiments are those in which one or more of these modifications are present on the sense but not the antisense strand, or embodiments where the antisense strand has fewer of such modifications.

Asymmetrical modification can be used to inhibit degradation by exonucleases. Asymmetrical modifications can include those in which only one strand is modified as well as those in which both are modified. In preferred embodiments the modification on the antisense strand is one which will not interfere with silencing of the target, e.g., one which will not interfere with cleavage of the target. Some embodiments will have an asymmetrical modification on the sense strand, e.g., in a 3' overhang, e.g., at the 3' terminus, and on the antisense strand, e.g., in a 3' overhang, e.g., at the 3' terminus. If

the modifications introduce moieties of different size it is preferable that the larger be on the sense strand. If the modifications introduce moieties of different charge it is preferable that the one with greater charge be on the sense strand.

Examples of modifications which inhibit exonucleolytic degradation can be found
5 herein. Particularly favored modifications include: 2' modification, e.g., provision of a 2' OMe moiety in a 3' overhang, e.g., at the 3' terminus (3' terminus means at the 3' atom of the molecule or at the most 3' moiety, e.g., the most 3' P or 2' position, as indicated by the context); modification of the backbone, e.g., with the replacement of a P with an S, e.g., the provision of a phosphorothioate modification, or the use of a
10 methylated P in a 3' overhang, e.g., at the 3' terminus; combination of a 2' modification, e.g., provision of a 2' O Me moiety and modification of the backbone, e.g., with the replacement of a P with an S, e.g., the provision of a phosphorothioate modification, or the use of a methylated P, in a 3' overhang, e.g., at the 3' terminus; modification with a 3' alkyl; modification with an abasic pyrrolidine in a 3' overhang, e.g., at the 3' terminus;
15 modification with naproxene, ibuprofen, or other moieties which inhibit degradation at the 3' terminus.

Modifications, e.g., those described herein, which affect targeting can be provided as asymmetrical modifications. Targeting modifications which can inhibit silencing, e.g., by inhibiting cleavage of a target, can be provided as asymmetrical modifications of the
20 sense strand. A biodistribution altering moiety, e.g., cholesterol, can be provided in one or more, e.g., two, asymmetrical modifications of the sense strand. Targeting modifications which introduce moieties having a relatively large molecular weight, e.g., a molecular weight of more than 400, 500, or 1000 daltons, or which introduce a charged moiety (e.g., having more than one positive charge or one negative charge) can be placed
25 on the sense strand.

Modifications, e.g., those described herein, which modulate, e.g., increase or decrease, the affinity of a strand for its complement or target, can be provided as asymmetrical modifications. These include: 5 methyl U; 5 methyl C; pseudouridine, Locked nucleic acids, 2 thio U and 2-amino-A. In some embodiments one or more of
30 these is provided on the antisense strand.

Asymmetrical modification can be used to optimize the activity of an iRNA agent structure, e.g., by being placed selectively within the iRNA. In preferred embodiments asymmetrical modifications which result in one or more of the following are used: modifications of the 5' end of the sense strand which inhibit kinase activation of the sense strand, including, e.g., attachments of conjugates which target the molecule or the use modifications which protect against 5' exonucleolytic degradation; or modifications of either strand, but preferably the sense strand, which enhance binding between the sense and antisense strand and thereby promote a "tight" structure at this end of the molecule.

The end region of the iRNA agent defined by the 3' end of the sense strand and the 5' end of the antisense strand is also important for function. This region can include the terminal 2, 3, or 4 paired nucleotides and any 3' overhang. Preferred embodiments include asymmetrical modifications of either strand, but preferably the sense strand, which decrease binding between the sense and antisense strand and thereby promote an "open" structure at this end of the molecule. Such modifications include placing conjugates which target the molecule or modifications which promote nuclease resistance on the sense strand in this region.

Exemplary modifications for asymmetrical placement in the sense strand include the following:

(a) backbone modifications, e.g., modification of a backbone P, including replacement of P with S, or P substituted with alkyl or allyl, e.g., Me, and dithioates (S-P=S); these modifications can be used to promote nuclease resistance;

(b) 2'-O alkyl, e.g., 2'-OMe, 3'-O alkyl, e.g., 3'-OMe (at terminal and/or internal positions); these modifications can be used to promote nuclease resistance or to enhance binding of the sense to the antisense strand, the 3' modifications can be used at the 5' end of the sense strand to avoid sense strand activation by RISC;

(c) 2'-5' linkages (with 2'-H, 2'-OH and 2'-OMe and with P=O or P=S) these modifications can be used to promote nuclease resistance or to inhibit binding of the sense to the antisense strand, or can be used at the 5' end of the sense strand to avoid sense strand activation by RISC;

(d) L sugars (e.g., L ribose, L-arabinose with 2'-H, 2'-OH and 2'-OMe); these modifications can be used to promote nuclease resistance or to inhibit binding of the sense to the antisense strand, or can be used at the 5' end of the sense strand to avoid sense strand activation by RISC;

5 (e) modified sugars (e.g., locked nucleic acids (LNA's), hexose nucleic acids (HNA's) and cyclohexene nucleic acids (CeNA's)); these modifications can be used to promote nuclease resistance or to inhibit binding of the sense to the antisense strand, or can be used at the 5' end of the sense strand to avoid sense strand activation by RISC;

(f) nucleobase modifications (e.g., C-5 modified pyrimidines, N-2 modified purines, N-7 modified purines, N-6 modified purines), these modifications can be used to promote nuclease resistance or to enhance binding of the sense to the antisense strand;

(g) cationic groups and Zwitterionic groups (preferably at a terminus), these modifications can be used to promote nuclease resistance;

15 (h) conjugate groups (preferably at terminal positions), e.g., naproxen, biotin, cholesterol, ibuprofen, folic acid, peptides, and carbohydrates; these modifications can be used to promote nuclease resistance or to target the molecule, or can be used at the 5' end of the sense strand to avoid sense strand activation by RISC.

Exemplary modifications for asymmetrical placement in the antisense strand include the following:

20 (a) backbone modifications, e.g., modification of a backbone P, including replacement of P with S, or P substituted with alkyl or allyl, e.g., Me, and dithioates (S-P=S);

(b) 2'-O alkyl, e.g., 2'-OMe, (at terminal positions);

(c) 2'-5' linkages (with 2'-H, 2'-OH and 2'-OMe) e.g., terminal at the 3' end);
25 e.g., with P=O or P=S preferably at the 3'-end, these modifications are preferably excluded from the 5' end region as they may interfere with RISC enzyme activity such as kinase activity;

(d) L sugars (e.g., L ribose, L-arabinose with 2'-H, 2'-OH and 2'-OMe); e.g., terminal at the 3' end; e.g., with P=O or P=S preferably at the 3'-end, these modifications
30 are preferably excluded from the 5' end region as they may interfere with kinase activity;

(e) modified sugars (e.g., LNA's, HNA's and CeNA's); these modifications are preferably excluded from the 5' end region as they may contribute to unwanted enhancements of pairing between the sense and antisense strands, it is often preferred to have a "loose" structure in the 5' region, additionally, they may interfere with kinase
5 activity;

(f) nucleobase modifications (e.g., C-5 modified pyrimidines, N-2 modified purines, N-7 modified purines, N-6 modified purines);

(g) cationic groups and Zwitterionic groups (preferably at a terminus);
conjugate groups (preferably at terminal positions), e.g., naproxen, biotin,
10 cholesterol, ibuprofen, folic acid, peptides, and carbohydrates, but bulky groups or generally groups which inhibit RISC activity should be less preferred.

The 5'-OH of the antisense strand should be kept free to promote activity. In some preferred embodiments modifications that promote nuclease resistance should be included at the 3' end, particularly in the 3' overhang.

15 In another aspect, the invention features a method of optimizing, e.g., stabilizing, an iRNA agent. The method includes selecting a sequence having activity, introducing one or more asymmetric modifications into the sequence, wherein the introduction of the asymmetric modification optimizes a property of the iRNA agent but does not result in a decrease in activity.

20 The decrease in activity can be less than a preselected level of decrease. In preferred embodiments decrease in activity means a decrease of less than 5, 10, 20, 40, or 50 % activity, as compared with an otherwise similar iRNA lacking the introduced modification. Activity can, e.g., be measured in vivo, or in vitro, with a result in either being sufficient to demonstrate the required maintenance of activity.

25 The optimized property can be any property described herein and in particular the properties discussed in the section on asymmetrical modifications provided herein. The modification can be any asymmetrical modification, e.g., an asymmetric modification described in the section on asymmetrical modifications described herein. Particularly preferred asymmetric modifications are 2'-O alkyl modifications, e.g., 2'-OMe

modifications, particularly in the sense sequence, and modifications of a backbone O, particularly phosphorothioate modifications, in the antisense sequence.

In a preferred embodiment a sense sequence is selected and provided with an asymmetrical modification, while in other embodiments an antisense sequence is selected and provided with an asymmetrical modification. In some embodiments both sense and antisense sequences are selected and each provided with one or more asymmetrical modifications.

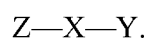
Multiple asymmetric modifications can be introduced into either or both of the sense and antisense sequence. A sequence can have at least 2, 4, 6, 8, or more modifications and all or substantially all of the monomers of a sequence can be modified.

Z-X-Y Architecture

In one aspect, the invention features an iRNA agent which can have a Z-X-Y architecture or structure such as those described herein and those described in copending, co-owned United States Provisional Application Serial No. 60/510,246, filed on October 9, 2003, which is hereby incorporated by reference, and in copending, co-owned United States Provisional Application Serial No. 60/510,318, filed on October 10, 2003, which is hereby incorporated by reference.

In addition, the invention includes iRNA agents having a Z-X-Y structure and another element described herein. E.g., the invention includes an iRNA agent described herein, e.g., a palindromic iRNA agent, an iRNA agent having a non canonical pairing, an iRNA agent which targets a gene described herein, e.g., a gene active in the liver, an iRNA associated with an amphipathic delivery agent described herein, an iRNA associated with a drug delivery module described herein, an iRNA agent administered as described herein, or an iRNA agent formulated as described herein, which also incorporates a Z-X-Y architecture.

The invention provides an iRNA agent having a first segment, the Z region, a second segment, the X region, and optionally a third region, the Y region:



It may be desirable to modify subunits in one or both of Z and/or Y on one hand and X on the other hand. In some cases they will have the same modification or the same class of modification but it will more often be the case that the modifications made in Z
5 and/or Y will differ from those made in X.

The Z region typically includes a terminus of an iRNA agent. The length of the Z region can vary, but will typically be from 2-14, more preferably 2-10, subunits in length. It typically is single stranded, i.e., it will not base pair with bases of another strand, though it may in some embodiments self associate, e.g., to form a loop structure. Such
10 structures can be formed by the end of a strand looping back and forming an intrastrand duplex. E.g., 2, 3, 4, 5 or more intra-strand bases pairs can form, having a looped out or connecting region, typically of 2 or more subunits which do not pair. This can occur at one or both ends of a strand. A typical embodiment of a Z region is a single strand overhang, e.g., an overhang of the length described elsewhere herein. The Z region can
15 thus be or include a 3' or 5' terminal single strand. It can be sense or antisense strand but if it is antisense it is preferred that it is a 3'-overhang. Typical inter-subunit bonds in the Z region include: P=O; P=S; S-P=S; P-NR₂; and P-BR₂. Chiral P=X, where X is S, N, or B) inter-subunit bonds can also be present. (These inter-subunit bonds are discussed in more detail elsewhere herein.) Other preferred Z region subunit modifications (also
20 discussed elsewhere herein) can include: 3'-OR, 3'SR, 2'-OMe, 3'-OMe, and 2'OH modifications and moieties; alpha configuration bases; and 2' arabino modifications.

The X region will in most cases be duplexed, in the case of a single strand iRNA agent, with a corresponding region of the single strand, or in the case of a double stranded iRNA agent, with the corresponding region of the other strand. The length of the X
25 region can vary but will typically be between 10-45 and more preferably between 15 and 35 subunits. Particularly preferred region X's will include 17, 18, 19, 29, 21, 22, 23, 24, or 25 nucleotide pairs, though other suitable lengths are described elsewhere herein and can be used. Typical X region subunits include 2'-OH subunits. In typical embodiments phosphate inter-subunit bonds are preferred while phosphorothioate or non-phosphate
30 bonds are absent. Other modifications preferred in the X region include: modifications

to improve binding, e.g., nucleobase modifications; cationic nucleobase modifications; and C-5 modified pyrimidines, e.g., allylamines. Some embodiments have 4 or more consecutive 2'OH subunits. While the use of phosphorothioate is sometimes non preferred they can be used if they connect less than 4 consecutive 2'OH subunits.

5 The Y region will generally conform to the the parameters set out for the Z regions. However, the X and Z regions need not be the same, different types and numbers of modifications can be present, and infact, one will usually be a 3' overhang and one will usually be a 5' overhang.

10 In a preferred embodiment the iRNA agent will have a Y and/or Z region each having ribonucleosides in which the 2'-OH is substituted, e.g., with 2'-OMe or other alkyl; and an X region that includes at least four consecutive ribonucleoside subunits in which the 2'-OH remains unsubstituted.

15 The subunit linkages (the linkages between subunits) of an iRNA agent can be modified, e.g., to promote resistance to degradation. Numerous examples of such modifications are disclosed herein, one example of which is the phosphorothioate linkage. These modifications can be provided bewteen the subunits of any of the regions, Y, X, and Z. However, it is preferred that their occureceis minimized and in particular it is preferred that consecutive modified linkages be avoided.

20 In a preferred embodiment the iRNA agent will have a Y and Z region each having ribonucleosides in which the 2'-OH is substituted, e.g., with 2'-OMe; and an X region that includes at least four consecutive subunits, e.g., ribonucleoside subunits in which the 2'-OH remains unsubstituted.

25 As mentioned above, the subunit linkages of an iRNA agent can be modified, e.g., to promote resistance to degradation. These modifications can be provided between the subunits of any of the regions, Y, X, and Z. However, it is preferred that they are minimized and in particular it is preferred that consecutive modified linkages be avoided.

30 Thus, in a preferred embodiment, not all of the subunit linkages of the iRNA agent are modified and more preferably the maximum number of consecutive subunits linked by other than a phosphodiester bond will be 2, 3, or 4. Particulary preferred iRNA agents will not have four or more consecutive subunits, e.g., 2'-hydroxyl ribonucleoside

subunits, in which each subunits is joined by modified linkages – i.e. linkages that have been modified to stabilize them from degradation as compared to the phosphodiester linkages that naturally occur in RNA and DNA.

It is particularly preferred to minimize the occurrence in region X. Thus, in preferred embodiments each of the nucleoside subunit linkages in X will be phosphodiester linkages, or if subunit linkages in region X are modified, such modifications will be minimized. E.g., although the Y and/or Z regions can include inter subunit linkages which have been stabilized against degradation, such modifications will be minimized in the X region, and in particular consecutive modifications will be minimized. Thus, in preferred embodiments the maximum number of consecutive subunits linked by other than a phosphodiester bond will be 2, 3, or 4. Particular preferred X regions will not have four or more consecutive subunits, e.g., 2'-hydroxyl ribonucleoside subunits, in which each subunits is joined by modified linkages – i.e. linkages that have been modified to stabilize them from degradation as compared to the phosphodiester linkages that naturally occur in RNA and DNA.

In a preferred embodiment Y and /or Z will be free of phosphorothioate linkages, though either or both may contain other modifications, e.g., other modifications of the subunit linkages.

In a preferred embodiment region X, or in some cases, the entire iRNA agent, has no more than 3 or no more than 4 subunits having identical 2' moieties.

In a preferred embodiment region X, or in some cases, the entire iRNA agent, has no more than 3 or no more than 4 subunits having identical subunit linkages.

In a preferred embodiment one or more phosphorothioate linkages (or other modifications of the subunit linkage) are present in Y and/or Z, but such modified linkages do not connect two adjacent subunits, e.g., nucleosides, having a 2' modification, e.g., a 2'-O-alkyl moiety. E.g., any adjacent 2'-O-alkyl moieties in the Y and/or Z, are connected by a linkage other than a phosphorothioate linkage.

In a preferred embodiment each of Y and/or Z independently has only one phosphorothioate linkage between adjacent subunits, e.g., nucleosides, having a 2' modification, e.g., 2'-O-alkyl nucleosides. If there is a second set of adjacent subunits,

e.g., nucleosides, having a 2' modification, e.g., 2'-O-alkyl nucleosides, in Y and/or Z that second set is connected by a linkage other than a phosphorothioate linkage, e.g., a modified linkage other than a phosphorothioate linkage.

5 In a preferred embodiment each of Y and/or Z independently has more than one phosphorothioate linkage connecting adjacent pairs of subunits, e.g., nucleosides, having a 2' modification, e.g., 2'-O-alkyl nucleosides, but at least one pair of adjacent subunits, e.g., nucleosides, having a 2' modification, e.g., 2'-O-alkyl nucleosides, are be connected by a linkage other than a phosphorothioate linkage, e.g., a modified linkage other than a phosphorothioate linkage.

10 In a preferred embodiment one of the above recited limitation on adjacent subunits in Y and or Z is combined with a limitation on the subunits in X. E.g., one or more phosphorothioate linkages (or other modifications of the subunit linkage) are present in Y and/or Z, but such modified linkages do not connect two adjacent subunits, e.g., nucleosides, having a 2' modification, e.g., a 2'-O-alkyl moiety. E.g., any adjacent 2'-O-alkyl moieties in the Y and/or Z, are connected by a linkage other than a phosphorothioate linkage. In addition, the X region has no more than 3 or no more than 4 identical subunits, e.g., subunits having identical 2' moieties or the X region has no more than 3 or no more than 4 subunits having identical subunit linkages.

15 A Y and/or Z region can include at least one, and preferably 2, 3 or 4 of a modification disclosed herein. Such modifications can be chosen, independently, from any modification described herein, e.g., from nuclease resistant subunits, subunits with modified bases, subunits with modified intersubunit linkages, subunits with modified sugars, and subunits linked to another moiety, e.g., a targeting moiety. In a preferred embodiment more than 1 of such subunits can be present but in some embodiments it is preferred that no more than 1, 2, 3, or 4 of such modifications occur, or occur consecutively. In a preferred embodiment the frequency of the modification will differ between Y and /or Z and X, e.g., the modification will be present one of Y and/or Z or X and absent in the other.

20 An X region can include at least one, and preferably 2, 3 or 4 of a modification disclosed herein. Such modifications can be chosen, independently, from any

30

modification described herein, e.g., from nuclease resistant subunits, subunits with modified bases, subunits with modified intersubunit linkages, subunits with modified sugars, and subunits linked to another moiety, e.g., a targeting moiety. In a preferred embodiment more than 1 of such subunits can be present but in some embodiments it is preferred that no more than 1, 2, 3, or 4 of such modifications occur, or occur consecutively.

An RRMS (described elsewhere herein) can be introduced at one or more points in one or both strands of a double-stranded iRNA agent. An RRMS can be placed in a Y and/or Z region, at or near (within 1, 2, or 3 positions) of the 3' or 5' end of the sense strand or at near (within 2 or 3 positions of) the 3' end of the antisense strand. In some embodiments it is preferred to not have an RRMS at or near (within 1, 2, or 3 positions of) the 5' end of the antisense strand. An RRMS can be positioned in the X region, and will preferably be positioned in the sense strand or in an area of the antisense strand not critical for antisense binding to the target.

Differential Modification of Terminal Duplex Stability (DMTDS)

In one aspect, the invention features an iRNA agent which can have differential modification of terminal duplex stability (DMTDS).

In addition, the invention includes iRNA agents having DMTDS and another element described herein. E.g., the invention includes an iRNA agent described herein, e.g., a palindromic iRNA agent, an iRNA agent having a non canonical pairing, an iRNA agent which targets a gene described herein, e.g., a gene active in the liver, an iRNA agent having an architecture or structure described herein, an iRNA associated with an amphipathic delivery agent described herein, an iRNA associated with a drug delivery module described herein, an iRNA agent administered as described herein, or an iRNA agent formulated as described herein, which also incorporates DMTDS.

iRNA agents can be optimized by increasing the propensity of the duplex to disassociate or melt (decreasing the free energy of duplex association), in the region of the 5' end of the antisense strand duplex. This can be accomplished, e.g., by the inclusion of subunits which increase the propensity of the duplex to disassociate or melt in the region of the 5' end of the antisense strand. It can also be accomplished by the

attachment of a ligand that increases the propensity of the duplex to disassociate or melt in the region of the 5' end. While not wishing to be bound by theory, the effect may be due to promoting the effect of an enzyme such as helicase, for example, promoting the effect of the enzyme in the proximity of the 5' end of the antisense strand.

5 The inventors have also discovered that iRNA agents can be optimized by decreasing the propensity of the duplex to disassociate or melt (increasing the free energy of duplex association), in the region of the 3' end of the antisense strand duplex. This can be accomplished, e.g., by the inclusion of subunits which decrease the propensity of the duplex to disassociate or melt in the region of the 3' end of the antisense strand. It
10 can also be accomplished by the attachment of ligand that decreases the propensity of the duplex to disassociate or melt in the region of the 5' end.

Modifications which increase the tendency of the 5' end of the duplex to dissociate can be used alone or in combination with other modifications described herein, e.g., with modifications which decrease the tendency of the 3' end of the duplex to
15 dissociate. Likewise, modifications which decrease the tendency of the 3' end of the duplex to dissociate can be used alone or in combination with other modifications described herein, e.g., with modifications which increase the tendency of the 5' end of the duplex to dissociate.

Decreasing the stability of the AS 5' end of the duplex

20 Subunit pairs can be ranked on the basis of their propensity to promote dissociation or melting (e.g., on the free energy of association or dissociation of a particular pairing, the simplest approach is to examine the pairs on an individual pair basis, though next neighbor or similar analysis can also be used). In terms of promoting dissociation:

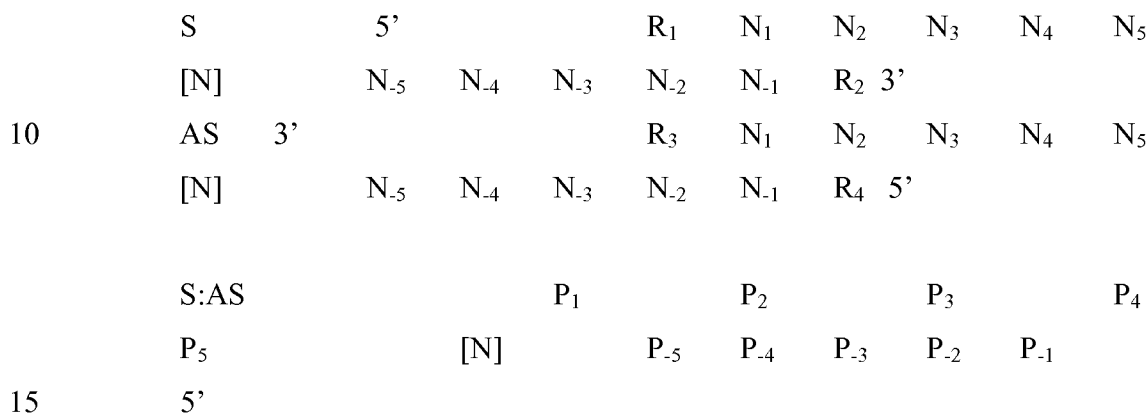
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A:U	is preferred over	G:C;
G:U	is preferred over	G:C;
I:C	is preferred over	G:C (I=inosine);

mismatches, e.g., non-canonical or other than canonical pairings (as described elsewhere herein) are preferred over canonical (A:T, A:U, G:C) pairings; pairings which include a universal base are preferred over canonical pairings.

5

A typical ds iRNA agent can be diagrammed as follows:



S indicates the sense strand; AS indicates antisense strand; R₁ indicates an optional (and nonpreferred) 5' sense strand overhang; R₂ indicates an optional (though preferred) 3' sense overhang; R₃ indicates an optional (though preferred) 3' antisense sense overhang; R₄ indicates an optional (and nonpreferred) 5' antisense overhang; N indicates subunits; [N] indicates that additional subunit pairs may be present; and P_x, indicates a pairing of sense N_x and antisense N_x. Overhangs are not shown in the P diagram. In some embodiments a 3' AS overhang corresponds to region Z, the duplex region corresponds to region X, and the 3' S strand overhang corresponds to region Y, as described elsewhere herein. (The diagram is not meant to imply maximum or minimum lengths, on which guidance is provided elsewhere herein.)

It is preferred that pairings which decrease the propensity to form a duplex are used at 1 or more of the positions in the duplex at the 5' end of the AS strand. The terminal pair (the most 5' pair in terms of the AS strand) is designated as P₋₁, and the subsequent pairing positions (going in the 3' direction in terms of the AS strand) in the

duplex are designated, P₋₂, P₋₃, P₋₄, P₋₅, and so on. The preferred region in which to modify to modulate duplex formation is at P₋₅ through P₋₁, more preferably P₋₄ through P₋₁, more preferably P₋₃ through P₋₁. Modification at P₋₁, is particularly preferred, alone or with modification(s) other position(s), e.g., any of the positions just identified. It is
 5 preferred that at least 1, and more preferably 2, 3, 4, or 5 of the pairs of one of the recited regions be chosen independently from the group of: (A:U), (G:U), (I:C), mismatched pairs, e.g., non-canonical or other than canonical pairings or pairings which include a universal base.

In preferred embodiments the change in subunit needed to achieve a pairing
 10 which promotes dissociation will be made in the sense strand, though in some embodiments the change will be made in the antisense strand.

In a preferred embodiment the at least 2, or 3, of the pairs in P₋₁, through P₋₄, are pairs which promote dissociation.

In a preferred embodiment the at least 2, or 3, of the pairs in P₋₁, through P₋₄, are
 15 A:U.

In a preferred embodiment the at least 2, or 3, of the pairs in P₋₁, through P₋₄, are G:U.

In a preferred embodiment the at least 2, or 3, of the pairs in P₋₁, through P₋₄, are I:C.

In a preferred embodiment the at least 2, or 3, of the pairs in P₋₁, through P₋₄, are
 20 mismatched pairs, e.g., non-canonical or other than canonical pairings pairings.

In a preferred embodiment the at least 2, or 3, of the pairs in P₋₁, through P₋₄, are pairings which include a universal base.

Increasing the stability of the AS 3' end of the duplex

25 Subunit pairs can be ranked on the basis of their propensity to promote stability and inhibit dissociation or melting (e.g., on the free energy of association or dissociation of a particular pairing, the simplest approach is to examine the pairs on an individual pair basis, though next neighbor or similar analysis can also be used). In terms of promoting duplex stability: G:C is preferred over A:U. Watson-Crick matches (A:T, A:U, G:C) are

preferred over non-canonical or other than canonical pairings. Analogs that increase stability are preferred over Watson-Crick matches (A:T, A:U, G:C). 2-amino-A:U is preferred over A:U. 2-thio U or 5 Me-thio-U:A are preferred over U:A. G-clamp (an analog of C having 4 hydrogen bonds):G is preferred over C:G. Guanadinium-G-clamp:G is preferred over C:G. Psuedo uridine:A is preferred over U:A. Sugar modifications, e.g., 2' modifications, e.g., 2'F, ENA, or LNA, which enhance binding are preferred over non-modified moieties and can be present on one or both strands to enhance stability of the duplex. It is preferred that pairings which increase the propensity to form a duplex are used at 1 or more of the positions in the duplex at the 3' end of the AS strand. The terminal pair (the most 3' pair in terms of the AS strand) is designated as P₁, and the subsequent pairing positions (going in the 5' direction in terms of the AS strand) in the duplex are designated, P₂, P₃, P₄, P₅, and so on. The preferred region in which to modify to modulate duplex formation is at P₅ through P₁, more preferably P₄ through P₁, more preferably P₃ through P₁. Modification at P₁, is particularly preferred, alone or with mdification(s) at other position(s), e.g., any of the positions just identified. It is preferred that at least 1, and more preferably 2, 3, 4, or 5 of the pairs of the recited regions be chosen independently from the group of: G:C, a pair having an analog that increases stability over Watson-Crick matches (A:T, A:U, G:C), 2-amino-A:U, 2-thio U or 5 Me-thio-U:A, G-clamp (an analog of C having 4 hydrogen bonds):G, guanadinium-G-clamp:G, psuedo uridine:A, a pair in which one or both subunits has a sugar modification, e.g., a 2' modification, e.g., 2'F, ENA, or LNA, which enhance binding.

In a preferred embodiment the at least 2, or 3, of the pairs in P₁, through P₄, are pairs which promote duplex stability.

In a preferred embodiment the at least 2, or 3, of the pairs in P₁, through P₄, are G:C.

In a preferred embodiment the at least 2, or 3, of the pairs in P₁, through P₄, are a pair having an analog that increases stability over Watson-Crick matches.

In a preferred embodiment the at least 2, or 3, of the pairs in P₁, through P₄, are 2-amino-A:U.

In a preferred embodiment the at least 2, or 3, of the pairs in P₁, through P₄, are 2-thio U or 5 Me-thio-U:A.

In a preferred embodiment the at least 2, or 3, of the pairs in P₁, through P₄, are G-clamp:G.

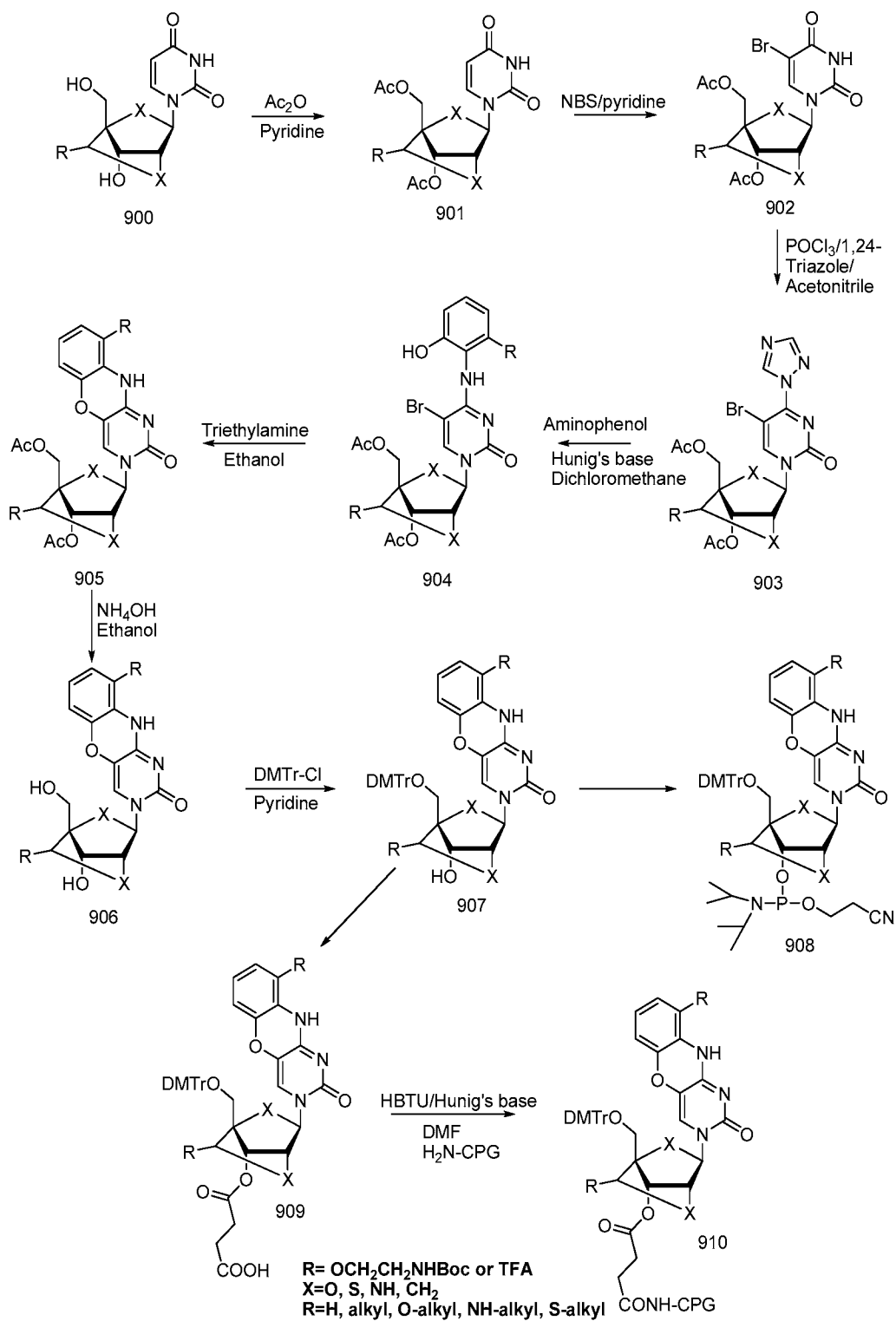
5 In a preferred embodiment the at least 2, or 3, of the pairs in P₁, through P₄, are guanidinium-G-clamp:G.

In a preferred embodiment the at least 2, or 3, of the pairs in P₁, through P₄, are psuedo uridine:A.

10 In a preferred embodiment the at least 2, or 3, of the pairs in P₁, through P₄, are a pair in which one or both subunits has a sugar modification, e.g., a 2' modification, e.g., 2'F, ENA, or LNA, which enhances binding.

G-clamps and guanidinium G-clamps are discussed in the following references:
Holmes and Gait, "The Synthesis of 2'-O-Methyl G-Clamp Containing Oligonucleotides and Their Inhibition of the HIV-1 Tat-TAR Interaction," *Nucleosides, Nucleotides & Nucleic Acids*, 22:1259-1262, 2003; Holmes *et al.*, "Steric inhibition of human immunodeficiency virus type-1 Tat-dependent trans-activation in vitro and in cells by oligonucleotides containing 2'-O-methyl G-clamp ribonucleoside analogues," *Nucleic Acids Research*, 31:2759-2768, 2003; Wilds, *et al.*, "Structural basis for recognition of guanosine by a synthetic tricyclic cytosine analogue: Guanidinium G-clamp," *Helvetica Chimica Acta*, 86:966-978, 2003; Rajeev, *et al.*, "High-Affinity Peptide Nucleic Acid Oligomers Containing Tricyclic Cytosine Analogues," *Organic Letters*, 4:4395-4398, 2002; Ausin, *et al.*, "Synthesis of Amino- and Guanidino-G-Clamp PNA Monomers," *Organic Letters*, 4:4073-4075, 2002; Maier *et al.*, "Nuclease resistance of oligonucleotides containing the tricyclic cytosine analogues phenoxazine and 9-(2-aminoethoxy)-phenoxazine ("G-clamp") and origins of their nuclease resistance properties," *Biochemistry*, 41:1323-7, 2002; Flanagan, *et al.*, "A cytosine analog that confers enhanced potency to antisense oligonucleotides," *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 96:3513-8, 1999. Gclamps may also comprise a locked nucleic acid. Methods of synthesizing such LNA-Gclamps are shown in the following schematics.

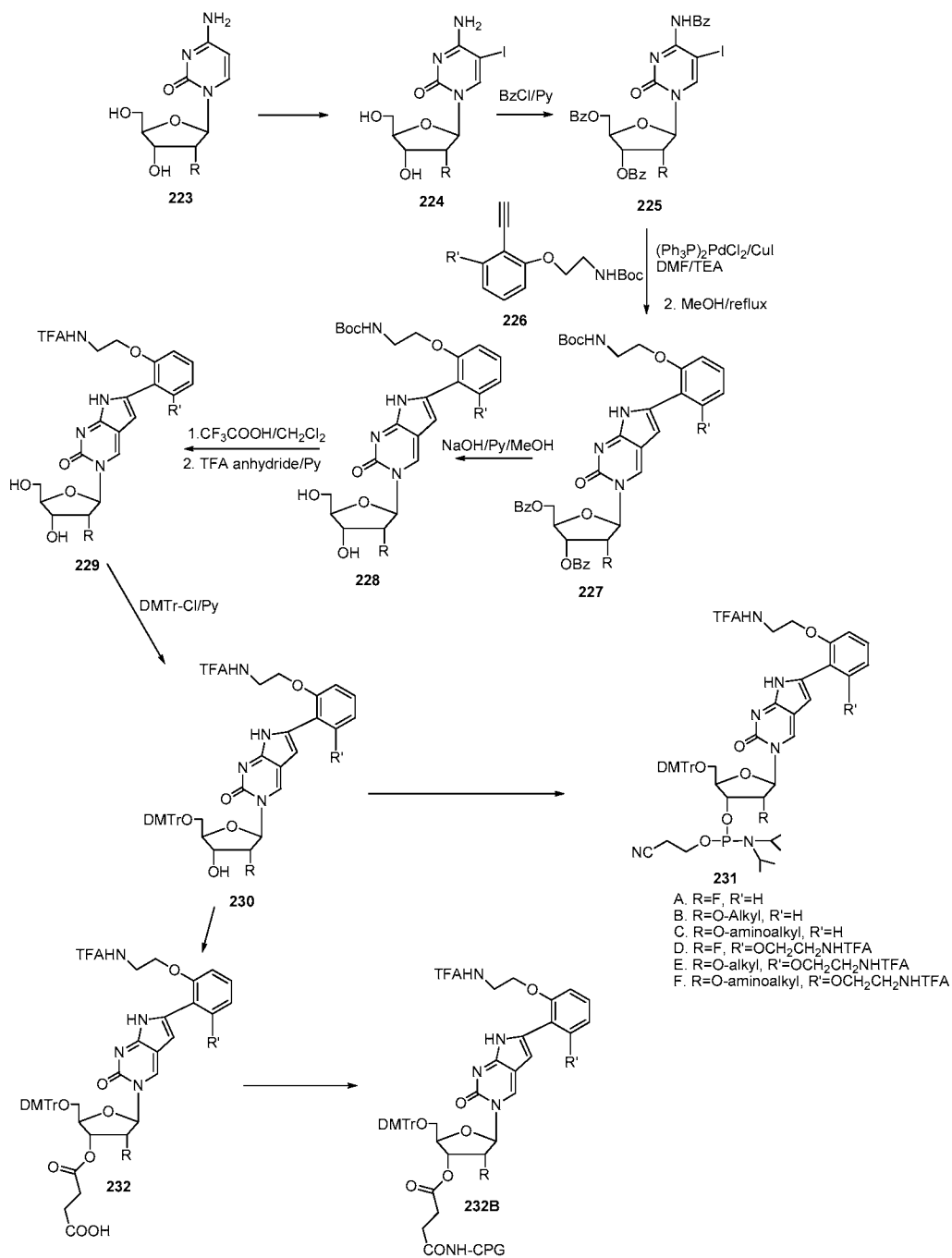
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Compound 900 is synthesized utilizing the literature procedure (Tetrahedron Lett., 49, 7168, 2008). Treatment of 900 with acetic anhydride in pyridine followed by reaction with *N*-bromosuccinimide gives the protected 5-bromo LNA 902. This on reaction with phosphorus oxychloride and 1,2,4-triazole in acetonitrile gives the triazolyl derivative 903 which on treatment with aminophenol is converted to the corresponding N-substituted cytidine derivative 904. Cyclization of 904 is accomplished by treating with triethylamine in ethanol to give the protected LNA- Phenoxazine or G-Clamp. Removal of the protecting groups is accomplished by treating with ammonium hydroxide at room temperature to give 906. This on reaction with DMTr-Cl gives the 5'-DMTr derivative 907 which on phosphitylation gives the target phosphoramidite 908.

The dimethoxytrityl derivative 907 is converted to the corresponding succinate 909 by the treatment with succinic anhydride in pyridine in the presence of DMAP. This is activated using HBTU in DMF in the presence of Hunig's base and the activated ester is coupled with long chain alkyl amine-CPG to give 910.

Pyrrolopyrimidine Gclamp.



Compound 225A: To a suspension of 223A (10g) in dry methanol (20 ml) was
 5 added ICl (10g) and the mixture was heated under reflux for 20 h. The solvent was

evaporated and the crude was purified by silica gel column chromatography using a gradient 0-25% methanol in dichloromethane to give 11.2 g of 224A. To a cold solution of 224A (1 g, 2.69 mmol) in dry pyridine (20 ml) was added benzoyl chloride (1.26 ml, 10.8 mmol) and the mixture was stirred at room temperature overnight. Reaction was
5 quenched by the addition of water. The mixture was diluted with dichloromethane and washed with sat. sodium bicarbonate solution. Organic layer was evaporated and coevaporated with toluene. The residue was purified by silica gel column chromatography using a gradient 0-5% methanol in dichloromethane to give 1.6 g of 225A.

10 Compound 227A: A solution of 225A (4.2g, 6.1 4mmol) and 226 (2.3 g, 8.8 mmol) in anhydrous DMF (60 ml) and triethylamine (30 ml) was degassed by bubbling argon. To this solution Dichloro bis (triphenylphosphine) palladium (0.42 g) and copper iodide (0.23 g) were added and the mixture was heated at 48°C for 18h. The solvent was evaporated and the residue was dissolved in methanol (300 ml). The reaction mixture was
15 heated under reflux for 18 h. The solvent was evaporated and the residue was purified by silica gel column chromatography. The product was eluted using a gradient of 0-5% methanol in dichloromethane. Evaporation of the appropriate fractions containing the product gave 3.8 g of 227A.

Compound 228A: To a cold (ice bath) solution of 227A (3.8 g) in a mixture of
20 pyridine (60 ml) and methanol (60 ml) was added 1 N NaOH (11 ml). After stirring the mixture at 0-5°C for 1h, the reaction was quenched by the addition of dilute HCl (1N, 11 ml). Methanol was evaporated and the solution was diluted with dichloromethane (250 ml) and washed with water (50 ml). Organic layer was dried over sodium sulfate and evaporated. The residue was co-evaporated with toluene and purified by silica gel column
25 chromatography to give 1.8 g of 228A.

¹H NMR (400 MHz, DMSO) δ 11.47 (s, 1H), 8.71 (s, 1H), 7.78 – 7.66 (m, 1H), 7.38 – 7.25 (m, 1H), 7.11 (t, *J* = 6.6, 2H), 7.00 (t, *J* = 7.5, 1H), 6.76 (s, 1H), 6.04 (d, *J* = 17.5, 1H), 5.57 (d, *J* = 6.5, 1H), 5.29 (t, *J* = 5.0, 1H), 4.93 (dd, *J* = 52.9, 4.0, 1H), 4.22 – 4.05 (m, 3H), 3.97 (d, *J* = 8.7, 1H), 3.94 – 3.82 (m, 1H), 3.75 – 3.62 (m, 1H), 3.38 (dd, *J* = 11.1, 5.5, 2H), 1.34 (s, 9H).
30

^{19}F NMR (376 MHz, DMSO) δ -203.50 (m).

MS: Calcd: 505 Found: 504 (M-1)-

Compound 229A: A solution of compound 228A (0.8g) in 50% TFA-dichloromethane (20 ml) was stirred at 0°C for 2h. After this time the reaction mixture was evaporated. The residue was co-evaporated with toluene (25 ml) followed by anhydrous pyridine (20 ml). This was dissolved in dry pyridine (15 ml), the solution was cooled in an ice bath and trifluoroacetic anhydride (2 ml) was added. The reaction mixture was stirred at 0-5°C for 2h. Reaction was quenched by the addition of methanol, diluted with dichloromethane (150 ml) and washed with water (50 ml). Organic layer was dried over sodium sulfate and evaporated. The residue was co-evaporated with toluene (20 ml) and purified by silica gel column chromatography. The product was eluted using a gradient of 0-10% methanol in dichloromethane. Appropriate fractions containing the product were evaporated to give 0.4 g of pure 229A.

Compound 230A: To a solution of 229A (0.4 g) in anhydrous pyridine was added 4,4'-DMT-Cl (0.4 g) and the mixture was stirred at room temperature for 5h. The reaction mixture was diluted with dichloromethane (100 ml) and washed with water (50 ml). Organic layer was evaporated and the residue was co-evaporated with toluene. The product was purified using silica gel column chromatography using a gradient of 0-5% methanol in dichloromethane to give 0.36 g of 230A.

^1H NMR (400 MHz, DMSO) δ 11.39 (s, 1H), 9.57 (t, J = 5.3, 1H), 8.54 (s, 1H), 7.64 – 7.54 (m, 1H), 7.43 (d, J = 7.5, 3H), 7.36 – 7.26 (m, 9H), 7.21 (t, J = 7.2, 1H), 7.13 (d, J = 8.3, 1H), 7.02 (t, J = 7.6, 1H), 6.86 (dd, J = 8.9, 7.4, 5H), 6.02 (d, J = 18.6, 1H), 5.77 – 5.68 (m, 1H), 5.01 (dd, J = 52.9, 3.9, 1H), 4.55 – 4.37 (m, 1H), 4.14 (dd, J = 13.3, 7.4, 4H), 4.00 (q, J = 7.1, 1H), 3.70 – 3.60 (m, 8H), 3.57 – 3.42 (m, 4H), 3.34 (d, J = 11.1, 2H). ^{19}F NMR (376 MHz, DMSO) δ -77.22 (s), -202.65 (m).

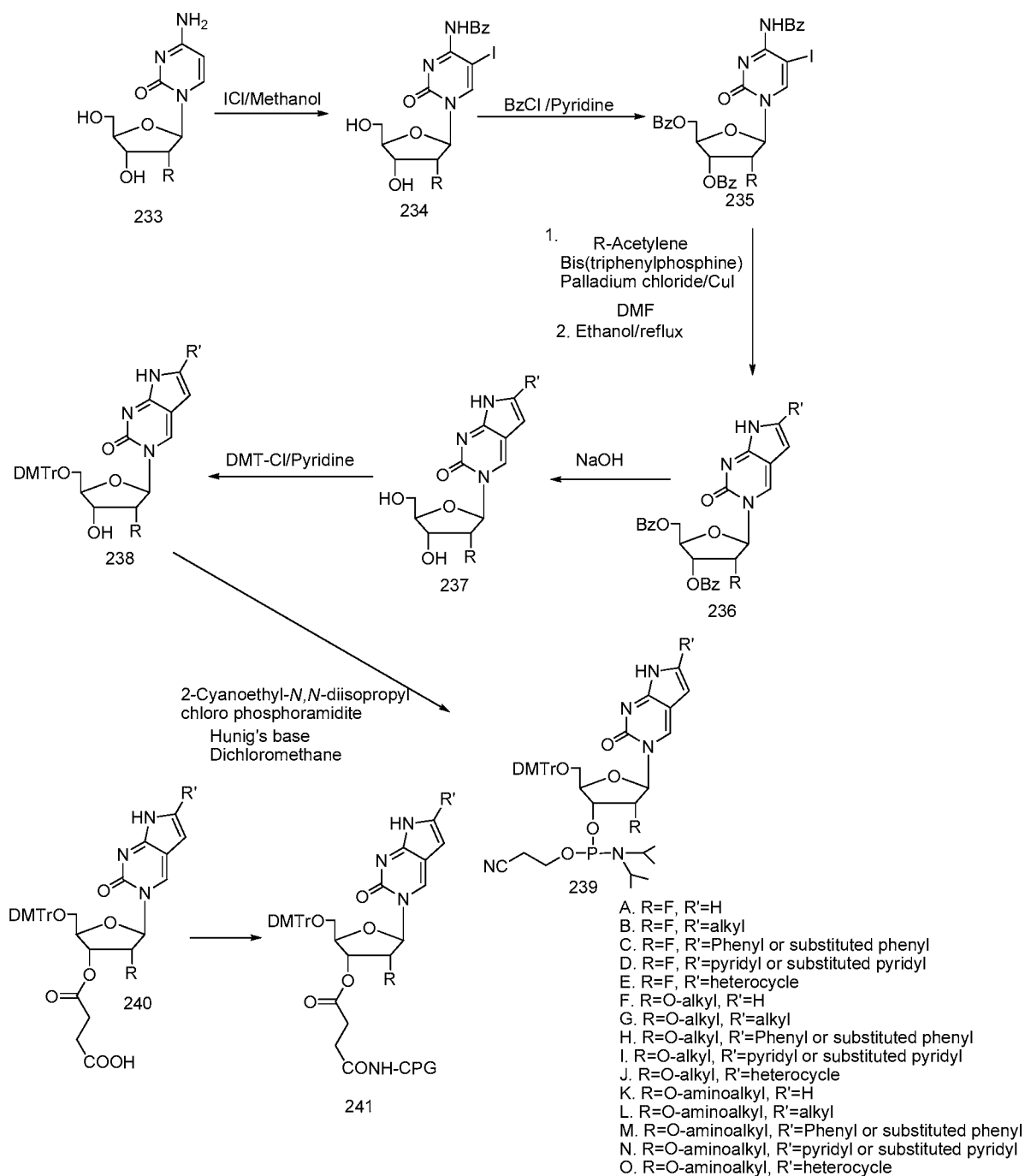
Compound 230 B (2'-OMe PC-GClamp): In a similar manner as described for 230A, compound 230B was synthesized starting from Tri-benzoyl-2'-OMe-5-iodocytidine and 226.

^1H NMR (400 MHz, DMSO) δ 11.35 (s, 1H), 9.56 (t, J = 5.4, 1H), 8.57 (s, 1H), 7.64 – 7.54 (m, 1H), 7.43 (d, J = 7.5, 2H), 7.38 – 7.26 (m, 8H), 7.21 (t, J = 7.2, 1H), 7.13

(d, $J = 8.3$, 1H), 7.02 (t, $J = 7.5$, 1H), 6.86 (t, $J = 8.2$, 4H), 5.94 (s, 1H), 5.87 (s, 1H), 5.22 (d, $J = 7.5$, 1H), 4.32 (td, $J = 8.1$, 5.1, 1H), 4.14 (t, $J = 5.5$, 2H), 4.06 (d, $J = 7.5$, 1H), 3.73 (d, $J = 4.8$, 1H), 3.66 (s, 3H), 3.63 (s, 3H), 3.53 (s, 3H). ^{19}F NMR (376 MHz, DMSO) δ -77.15 (s).

5 Compound 231: To a solution of 230 (1mmol) in dichloromethane (10ml) is added 2-cyanoethyl-tetraisopropylphosphoramidite (1.3 mmol) and dicyanoimidazole (0.9 mmol). The mixture is stirred at room temperature for 6 h, diluted with dichloromethane and washed with sodium bicarbonate solution. Organic layer is dried over sodium sulfate and evaporated. The residue is subjected to column chromatography
10 to give compound 231.

 Solid support derivatization: The dimethoxytrityl derivative 230 is converted to the corresponding succinate 232 by the treatment with succinic anhydride in pyridine in the presence of DMAP. This is activated using HBTU in DMF in the presence of Hunig's base and the activated ester is coupled with long chain alkyl amine-CPG to give
15 232B.



5 Tribenzoyl-5-iodocytidine 235 on reaction with 3-ethynylpyridine (or 4-ethynylpyridine) in DMF in the presence of bis(triphenylphosphine)palladium(II)

dichloride and CuI gave 236. Deprotection of 236 with sodium hydroxide in a mixture of methanol and pyridine gave 237 which on reaction with DMTr-Cl in pyridine gives the corresponding 5'-dimethoxytrityl derivative 238. This on phosphitylation with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite in dichloromethane in the presence of Hunig's base gives the target monomer 239.

Derivatization of the solid support: The dimethoxytrityl derivative 238 is converted to the corresponding succinate 240 by the treatment with succinic anhydride in pyridine in the presence of DMAP. This is activated using HBTU in DMF in the presence of Hunig's base and the activated ester is coupled with long chain alkyl amine-CPG to give 241.

Simultaneously decreasing the stability of the AS 5' end of the duplex and increasing the stability of the AS 3' end of the duplex

As is discussed above, an iRNA agent can be modified to both decrease the stability of the AS 5' end of the duplex and increase the stability of the AS 3' end of the duplex. This can be effected by combining one or more of the stability decreasing modifications in the AS 5' end of the duplex with one or more of the stability increasing modifications in the AS 3' end of the duplex. Accordingly a preferred embodiment includes modification in P₋₅ through P₋₁, more preferably P₋₄ through P₋₁ and more preferably P₋₃ through P₋₁. Modification at P₋₁, is particularly preferred, alone or with other position, e.g., the positions just identified. It is preferred that at least 1, and more preferably 2, 3, 4, or 5 of the pairs of one of the recited regions of the AS 5' end of the duplex region be chosen independently from the group of: (A:U), (G:U), (I:C), mismatched pairs, e.g., non-canonical or other than canonical pairings which include a universal base; and a modification in P₅ through P₁, more preferably P₄ through P₁ and more preferably P₃ through P₁. Modification at P₁, is particularly preferred, alone or with other position, e.g., the positions just identified. It is preferred that at least 1, and more preferably 2, 3, 4, or 5 of the pairs of one of the recited regions of the AS 3' end of the duplex region be chosen independently from the group of: G:C, a pair having an analog that increases stability over Watson-Crick matches (A:T, A:U, G:C), 2-amino-A:U, 2-thio U or 5 Me-thio-U:A, G-clamp (an analog of C having 4 hydrogen bonds):G,

guanadinium-G-clamp:G, pseudo uridine:A, a pair in which one or both subunits has a sugar modification, e.g., a 2' modification, e.g., 2'F, ENA, or LNA, which enhance binding.

The invention also includes methods of selecting and making iRNA agents having
5 DMTDS, e.g., when screening a target sequence for candidate sequences for use as iRNA agents one can select sequences having a DMTDS property described herein or one which can be modified, preferably with as few changes as possible, especially to the AS strand, to provide a desired level of DMTDS.

The invention also includes, providing a candidate iRNA agent sequence, and
10 modifying at least one P in P₋₅ through P₋₁ and/or at least one P in P₅ through P₁ to provide a DMTDS iRNA agent.

DMTDS iRNA agents can be used in any method described herein, e.g., to silence any gene disclosed herein, to treat any disorder described herein, in any formulation described herein, and generally in and/or with the methods and compositions described
15 elsewhere herein. DMTDS iRNA agents can incorporate other modifications described herein, e.g., the attachment of targeting agents or the inclusion of modifications which enhance stability, e.g., the inclusion of nuclease resistant monomers or the inclusion of single strand overhangs (e.g., 3' AS overhangs and/or 3' S strand overhangs) which self associate to form intrastrand duplex structure.

20

III. Delivery of Targeting Constructs

A targeting construct (such as an iRNA agent) can be linked, e.g., noncovalently linked to a polymer for the efficient delivery of the targeting construct to an avian cell or cell culture. The targeting construct can, for example, be complexed with cyclodextrin.

25 Cyclodextrins have been used as delivery vehicles of therapeutic compounds.

Cyclodextrins can form inclusion complexes with drugs that are able to fit into the hydrophobic cavity of the cyclodextrin. In other examples, cyclodextrins form non-covalent associations with other biologically active molecules such as oligonucleotides and derivatives thereof. The use of cyclodextrins creates a water-soluble drug delivery
30 complex that can be modified with targeting or other functional groups. Cyclodextrin

cellular delivery system for oligonucleotides described in U.S. Pat. No. 5,691,316, which is hereby incorporated by reference, are suitable for use in methods of the invention. In this system, an oligonucleotide is noncovalently complexed with a cyclodextrin, or the oligonucleotide is covalently bound to adamantine which in turn is non-covalently associated with a cyclodextrin.

The delivery molecule can include a linear cyclodextrin copolymer or a linear oxidized cyclodextrin copolymer having at least one ligand bound to the cyclodextrin copolymer. Delivery systems, as described in U.S. Patent No. 6,509,323, herein incorporated by reference, are suitable for use in methods of the invention. A targeting construct can be bound to the linear cyclodextrin copolymer and/or a linear oxidized cyclodextrin copolymer. Either or both of the cyclodextrin or oxidized cyclodextrin copolymers can be crosslinked to another polymer and/or bound to a ligand.

A composition for iRNA delivery can employ an "inclusion complex," a molecular compound having the characteristic structure of an adduct. In this structure, the "host molecule" spatially encloses at least part of another compound in the delivery vehicle. The enclosed compound (the "guest molecule") is situated in the cavity of the host molecule without affecting the framework structure of the host. A "host" is preferably cyclodextrin, but can be any of the molecules suggested in U.S. Patent Publ. 2003/0008818, herein incorporated by reference in its entirety.

Cyclodextrins can interact with a variety of ionic and molecular species, and the resulting inclusion compounds belong to the class of "host-guest" complexes. Within the host-guest relationship, the binding sites of the host and guest molecules should be complementary in the stereoelectronic sense. A composition of the invention can contain at least one polymer and at least one therapeutic agent, generally in the form of a particulate composite of the polymer and therapeutic agent, *e.g.*, the targeting construct. The targeting construct can contain one or more complexing agents. At least one polymer of the particulate composite can interact with the complexing agent in a host-guest or a guest-host interaction to form an inclusion complex between the polymer and the complexing agent. The polymer and, more particularly, the complexing agent can be

used to introduce functionality into the composition. For example, at least one polymer of the particulate composite has host functionality and forms an inclusion complex with a complexing agent having guest functionality. Alternatively, at least one polymer of the particulate composite has guest functionality and forms an inclusion complex with a complexing agent having host functionality. A polymer of the particulate composite can also contain both host and guest functionalities and form inclusion complexes with guest complexing agents and host complexing agents. A polymer with functionality can, for example, facilitate cell targeting and/or cell contact (*e.g.*, targeting or contact to a liver cell), intercellular trafficking, and/or cell entry and release.

Upon forming the particulate composite, the targeting construct may or may not retain its biological or therapeutic activity. Upon release from the composition, specifically, from the polymer of the particulate composite, the activity of the targeting construct is restored. Accordingly, the particulate composite advantageously affords the targeting construct protection against loss of activity due to, for example, degradation and offers enhanced bioavailability. Thus, a composition may be used to provide stability, particularly storage or solution stability, to a targeting construct or any active chemical compound. The targeting construct may be further modified with a ligand prior to or after particulate composite or therapeutic composition formation. The ligand can provide further functionality. For example, the ligand can be a targeting moiety.

Physiological Effects

The targeting constructs described herein can be designed such that determining therapeutic toxicity is made easier by the complementarity of the targeting construct with both a human and a non-human animal sequence. By these methods, a nucleic acid based targeting construct can consist of a sequence that is fully complementary to a nucleic acid sequence from a human and a nucleic acid sequence from at least one non-human animal, *e.g.*, a non-human mammal, such as a rodent, ruminant or primate or avian species. By determining the toxicity of the targeting construct in the non-human mammal, one can extrapolate the toxicity of the targeting construct in a human. For a more strenuous toxicity test, the targeting construct can be complementary to a human and more than one, *e.g.*, two or three or more, non-human animals.

The methods described herein can be used to correlate any physiological effect of a targeting construct on a human, *e.g.*, any unwanted effect, such as a toxic effect, or any positive, or desired effect.

5 In one aspect, the invention features a delivery conjugate or module, such as those described herein and those described in copending, co-owned United States Provisional Application Serial No. 60/454,265, filed on March 12, 2003, which is hereby incorporated by reference.

10 In addition, the invention includes targeting constructs which are iRNA agents and are described herein, *e.g.*, a palindromic iRNA agent, an iRNA agent having a non canonical pairing, an iRNA agent which targets a gene described herein, an iRNA agent having a chemical modification described herein, *e.g.*, a modification which enhances resistance to degradation, an iRNA agent having an architecture or structure described herein, an iRNA agent administered as described herein, or an iRNA agent formulated as described herein, combined with, associated with, and delivered by such a drug delivery
15 conjugate or module.

The iRNA agents can be complexed to a delivery agent that features a modular complex. The complex can include a carrier agent linked to one or more of (preferably two or more, more preferably all three of): (a) a condensing agent (*e.g.*, an agent capable of attracting, *e.g.*, binding, a nucleic acid, *e.g.*, through ionic or electrostatic interactions);
20 (b) a fusogenic agent (*e.g.*, an agent capable of fusing and/or being transported through a cell membrane, *e.g.*, an endosome membrane); and (c) a targeting group, *e.g.*, a cell or tissue targeting agent, *e.g.*, a lectin, glycoprotein, lipid or protein, *e.g.*, an antibody, that binds to a specified cell type.

A targeting construct, *e.g.*, iRNA agent or siRNA agent described herein, can be
25 linked, *e.g.*, coupled or bound, to the modular complex. The iRNA agent can interact with the condensing agent of the complex, and the complex can be used to deliver an iRNA agent to a cell, *e.g.*, *in vitro* or *in vivo*.

The fusogenic agent and the condensing agent can be different agents or the one and the same agent. For example, a polyamino chain, *e.g.*, polyethyleneimine (PEI), can
30 be the fusogenic and/or the condensing agent.

The delivery agent can be a modular complex. For example, the complex can include a carrier agent linked to one or more of (preferably two or more, more preferably all three of):

(a) a condensing agent (*e.g.*, an agent capable of attracting, *e.g.*, binding, a nucleic acid, *e.g.*, through ionic interaction),

(b) a fusogenic agent (*e.g.*, an agent capable of fusing and/or being transported through an avian cell membrane, *e.g.*, an endosome membrane), and

(c) a targeting group, *e.g.*, an avian cell or tissue targeting agent, *e.g.*, a lectin, glycoprotein, lipid or protein, *e.g.*, an antibody, that binds to a specified cell type.

10 A targeting group can be a thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, Mucin carbohydrate, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12,
15 biotin, Neproxin, or an RGD peptide or RGD peptide mimetic.

The carrier agent of a modular complex described herein can be a substrate for attachment of one or more of: a condensing agent, a fusogenic agent, and a targeting group. The carrier agent would preferably lack an endogenous enzymatic activity. The agent would preferably be a biological molecule, preferably a macromolecule.

20 Polymeric biological carriers are preferred. It would also be preferred that the carrier molecule be biodegradable.

The carrier agent can be a naturally occurring substance, such as a protein (*e.g.*, human serum albumin (HSA), low-density lipoprotein (LDL), or globulin); carbohydrate (*e.g.*, a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid); or
25 lipid. The carrier molecule can also be a recombinant or synthetic molecule, such as a synthetic polymer, *e.g.*, a synthetic polyamino acid. Examples of polyamino acids include polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolid) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA),
30 polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacrylic

acid), N-isopropylacrylamide polymers, or polyphosphazine. Other useful carrier molecules can be identified by routine methods.

A carrier agent can be characterized by one or more of: (a) is at least 1 Da in size; (b) has at least 5 charged groups, preferably between 5 and 5000 charged groups; (c) is present in the complex at a ratio of at least 1:1 carrier agent to fusogenic agent; (d) is present in the complex at a ratio of at least 1:1 carrier agent to condensing agent; (e) is present in the complex at a ratio of at least 1:1 carrier agent to targeting agent.

A fusogenic agent of a modular complex described herein can be an agent that is responsive to, *e.g.*, changes charge depending on, the pH environment. Upon encountering the pH of an endosome, it can cause a physical change, *e.g.*, a change in osmotic properties which disrupts or increases the permeability of the endosome membrane. Preferably, the fusogenic agent changes charge, *e.g.*, becomes protonated, at pH lower than physiological range. For example, the fusogenic agent can become protonated at pH 4.5-6.5. The fusogenic agent can serve to release the iRNA agent into the cytoplasm of an avian cell after the complex is taken up, *e.g.*, via endocytosis, by the cell, thereby increasing the cellular concentration of the iRNA agent in the cell.

In one embodiment, the fusogenic agent can have a moiety, *e.g.*, an amino group, which, when exposed to a specified pH range, will undergo a change, *e.g.*, in charge, *e.g.*, protonation. The change in charge of the fusogenic agent can trigger a change, *e.g.*, an osmotic change, in a vesicle, *e.g.*, an endocytic vesicle, *e.g.*, an endosome. For example, the fusogenic agent, upon being exposed to the pH environment of an endosome, will cause a solubility or osmotic change substantial enough to increase the porosity of (preferably, to rupture) the endosomal membrane.

The fusogenic agent can be a polymer, preferably a polyamino chain, *e.g.*, polyethyleneimine (PEI). The PEI can be linear, branched, synthetic or natural. The PEI can be, *e.g.*, alkyl substituted PEI, or lipid substituted PEI.

In other embodiments, the fusogenic agent can be polyhistidine, polyimidazole, polypyridine, polypropyleneimine, mellitin, or a polyacetal substance, *e.g.*, a cationic polyacetal. In some embodiment, the fusogenic agent can have an alpha helical structure. The fusogenic agent can be a membrane disruptive agent, *e.g.*, mellittin.

A fusogenic agent can have one or more of the following characteristics: (a) is at least 1Da in size; (b) has at least 10 charged groups, preferably between 10 and 5000 charged groups, more preferably between 50 and 1000 charged groups; (c) is present in the complex at a ratio of at least 1:1 fusogenic agent to carrier agent; (d) is present in the
5 complex at a ratio of at least 1:1 fusogenic agent to condensing agent; (e) is present in the complex at a ratio of at least 1:1 fusogenic agent to targeting agent.

Other suitable fusogenic agents can be tested and identified by a skilled artisan. The ability of a compound to respond to, *e.g.*, change charge depending on, the pH environment can be tested by routine methods, *e.g.*, in a cellular assay. For example, a
10 test compound is combined or contacted with a cell, and the cell is allowed to take up the test compound, *e.g.*, by endocytosis. An endosome preparation can then be made from the contacted cells and the endosome preparation compared to an endosome preparation from control cells. A change, *e.g.*, a decrease, in the endosome fraction from the contacted cell vs. the control cell indicates that the test compound can function as a
15 fusogenic agent. Alternatively, the contacted cell and control cell can be evaluated, *e.g.*, by microscopy, *e.g.*, by light or electron microscopy, to determine a difference in endosome population in the cells. The test compound can be labeled. In another type of assay, a modular complex described herein is constructed using one or more test or putative fusogenic agents. The modular complex can be constructed using a labeled
20 nucleic acid instead of the targeting construct. The ability of the fusogenic agent to respond to, *e.g.*, change charge depending on, the pH environment, once the modular complex is taken up by the cell, can be evaluated, *e.g.*, by preparation of an endosome preparation, or by microscopy techniques, as described above. A two-step assay can also be performed, wherein a first assay evaluates the ability of a test compound alone to
25 respond to, *e.g.*, change charge depending on, the pH environment; and a second assay evaluates the ability of a modular complex that includes the test compound to respond to, *e.g.*, change charge depending on, the pH environment.

The condensing agent of a modular complex described herein can interact with (*e.g.*, attracts, holds, or binds to) a targeting construct and act to (a) condense, *e.g.*, reduce
30 the size or charge of the iRNA agent and/or (b) protect the targeting construct, *e.g.*,

protect the iRNA agent against degradation. The condensing agent can include a moiety, *e.g.*, a charged moiety, that can interact with a nucleic acid, *e.g.*, an iRNA agent, *e.g.*, by ionic interactions. The condensing agent would preferably be a charged polymer, *e.g.*, a polycationic chain. The condensing agent can be a polylysine (PLL), spermine, 5 spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, quarternary salt of a polyamine, or an alpha helical peptide.

A condensing agent can have the following characteristics: (a) at least 1Da in size; (b) has at least 2 charged groups, preferably between 2 and 100 charged groups; (c) 10 is present in the complex at a ratio of at least 1:1 condensing agent to carrier agent; (d) is present in the complex at a ratio of at least 1:1 condensing agent to fusogenic agent; (e) is present in the complex at a ratio of at least 1:1 condensing agent to targeting agent.

Other suitable condensing agents can be tested and identified by a skilled artisan, *e.g.*, by evaluating the ability of a test agent to interact with a targeting construct, *e.g.*, an 15 iRNA agent. The ability of a test agent to interact with a targeting construct, *e.g.*, an iRNA agent, *e.g.*, to condense or protect the iRNA agent, can be evaluated by routine techniques. In one assay, a test agent is contacted with a nucleic acid, and the size and/or charge of the contacted nucleic acid is evaluated by a technique suitable to detect changes in molecular mass and/or charge. Such techniques include non-denaturing gel 20 electrophoresis, immunological methods, *e.g.*, immunoprecipitation, gel filtration, ionic interaction chromatography, and the like. A test agent is identified as a condensing agent if it changes the mass and/or charge (preferably both) of the contacted nucleic acid, compared to a control. A two-step assay can also be performed, wherein a first assay evaluates the ability of a test compound alone to interact with, *e.g.*, bind to, *e.g.*, 25 condense the charge and/or mass of, a nucleic acid; and a second assay evaluates the ability of a modular complex that includes the test compound to interact with, *e.g.*, bind to, *e.g.*, condense the charge and/or mass of, a targeting construct.

Amphipathic Delivery Agents

In one aspect, the invention features an amphipathic delivery conjugate or module, such as those described herein and those described in copending, co-owned United States Provisional Application Serial No. 60/455,050, filed on March 13, 2003, which is hereby incorporated by reference.

In addition, the invention include a targeting construct described herein, e.g., a palindromic iRNA agent, an iRNA agent hving a non canonical pairing, an iRNA agent which targets a gene described herein, an iRNA agent having a chemical modification described herein, e.g., a modification which enhances resistance to degradation, an iRNA agent having an architecture or structure described herein, an iRNA agent administered as described herein, or an iRNA agent formulated as described herein, combined with, associated with, and delivered by such an amphipathic delivery conjugate.

An amphipathic molecule is a molecule having a hydrophobic and a hydrophilic region. Such molecules can interact with (e.g., penetrate or disrupt) lipids, e.g., a lipid bilayer of a cell. As such, they can serve as delivery agent for an associated (e.g., bound) iRNA (e.g., an iRNA or sRNA described herein). A preferred amphipathic molecule to be used in the compositions described herein (e.g., the amphipathic iRNA constructs descriebd herein) is a polymer. The polymer may have a secondary structure, e.g., a repeating secondary structure.

One example of an amphipathic polymer is an amphipathic polypeptide, e.g., a polypeptide having a secondary structure such that the polypeptide has a hydrophilic and a hybrophobic face. The design of amphipathic peptide structures (e.g., alpha-helical polypeptides) is routine to one of skill in the art.

Another example of an amphipathic polymer is a polymer made up of two or more amphipathic subunits, e.g., two or more subunits containing cyclic moieties (e.g., a cyclic moiety having one or more hydrophilic groups and one or more hydrophobic groups). For example, the subunit may contain a steroid, e.g., cholic acid; or an aromatic moiety. Such moieties preferably can exhibit atropisomerism, such that they can form opposing hydrophobic and hydrophilic faces when in a polymer structure.

The ability of a putative amphipathic molecule to interact with a lipid membrane, e.g., a cell membrane, can be tested by routine methods, e.g., in a cell free or cellular assay. For example, a test compound is combined or contacted with a synthetic lipid bilayer, a cellular membrane fraction, or a cell, and the test compound is evaluated for its ability to interact with, penetrate or disrupt the lipid bilayer, cell membrane or cell. The test compound can be labeled in order to detect the interaction with the lipid bilayer, cell membrane or cell. In another type of assay, the test compound is linked to a reporter molecule or a targeting construct (e.g., an iRNA or siRNA described herein) and the ability of the reporter molecule or targeting construct to penetrate the lipid bilayer, cell membrane or cell is evaluated. A two-step assay can also be performed, wherein a first assay evaluates the ability of a test compound alone to interact with a lipid bilayer, cell membrane or cell; and a second assay evaluates the ability of a construct (e.g., a construct described herein) that includes the test compound and a reporter or targeting construct to interact with a lipid bilayer, cell membrane or cell.

An amphipathic polymer useful in the compositions described herein has at least 2, preferably at least 5, more preferably at least 10, 25, 50, 100, 200, 500, 1000, 2000, 50000 or more subunits (e.g., amino acids or cyclic subunits). A single amphipathic polymer can be linked to one or more, e.g., 2, 3, 5, 10 or more targeting constructs (e.g., iRNA or siRNA agents described herein). In some embodiments, an amphipathic polymer can contain both amino acid and cyclic subunits, e.g., aromatic subunits.

The invention features a composition that includes a targeting construct (e.g., an iRNA or siRNA described herein) in association with an amphipathic molecule. Such compositions may be referred to herein as "amphipathic iRNA constructs." Such compositions and constructs are useful in the delivery or targeting of targeting constructs, e.g., delivery or targeting of iRNA agents to an avian cell. While not wanting to be bound by theory, such compositions and constructs can increase the porosity of, e.g., can penetrate or disrupt, a lipid (e.g., a lipid bilayer of a cell), e.g., to allow entry of the targeting construct into a cell.

In one aspect, the invention relates to a composition comprising an targeting construct (e.g., an iRNA or siRNA agent described herein) linked to an amphipathic

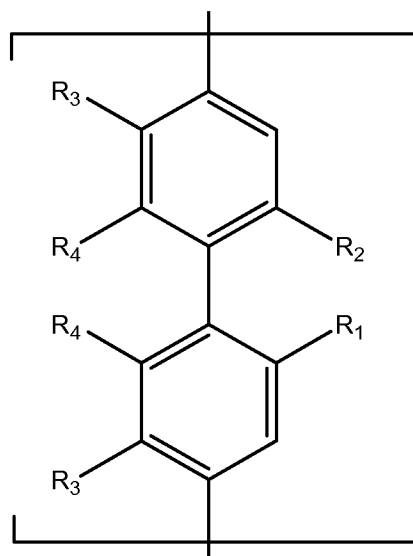
molecule. The targeting construct and the amphipathic molecule may be held in continuous contact with one another by either covalent or noncovalent linkages.

The amphipathic molecule of the composition or construct is preferably other than a phospholipid, e.g., other than a micelle, membrane or membrane fragment.

5 The amphipathic molecule of the composition or construct is preferably a polymer. The polymer may include two or more amphipathic subunits. One or more hydrophilic groups and one or more hydrophobic groups may be present on the polymer. The polymer may have a repeating secondary structure as well as a first face and a second face. The distribution of the hydrophilic groups and the hydrophobic groups along the
10 repeating secondary structure can be such that one face of the polymer is a hydrophilic face and the other face of the polymer is a hydrophobic face.

The amphipathic molecule can be a polypeptide, e.g., a polypeptide comprising an α -helical conformation as its secondary structure.

In one embodiment, the amphipathic polymer includes one or more subunits
15 containing one or more cyclic moiety (e.g., a cyclic moiety having one or more hydrophilic groups and/or one or more hydrophobic groups). In one embodiment, the polymer is a polymer of cyclic moieties such that the moieties have alternating hydrophobic and hydrophilic groups. For example, the subunit may contain a steroid, e.g., cholic acid. In another example, the subunit may contain an aromatic moiety. The
20 aromatic moiety may be one that can exhibit atropisomerism, e.g., a 2,2'-bis(substituted)-1-1'-binaphthyl or a 2,2'-bis(substituted) biphenyl. A subunit may include an aromatic moiety of Formula (M):



(M)

5 Referring to Formula M, R_1 is C_1 - C_{100} alkyl optionally substituted with aryl, alkenyl, alkynyl, alkoxy or halo and/or optionally inserted with O, S, alkenyl or alkynyl; C_1 - C_{100} perfluoroalkyl; or OR_5 .

R_2 is hydroxy; nitro; sulfate; phosphate; phosphate ester; sulfonic acid; OR_6 ; or C_1 - C_{100} alkyl optionally substituted with hydroxy, halo, nitro, aryl or alkyl sulfinyl, aryl
 10 or alkyl sulfonyl, sulfate, sulfonic acid, phosphate, phosphate ester, substituted or unsubstituted aryl, carboxyl, carboxylate, amino carbonyl, or alkoxycarbonyl, and/or optionally inserted with O, NH, S, S(O), SO_2 , alkenyl, or alkynyl.

R_3 is hydrogen, or when taken together with R_4 forms a fused phenyl ring.

R_4 is hydrogen, or when taken together with R_3 forms a fused phenyl ring.

15 R_5 is C_1 - C_{100} alkyl optionally substituted with aryl, alkenyl, alkynyl, alkoxy or halo and/or optionally inserted with O, S, alkenyl or alkynyl; or C_1 - C_{100} perfluoroalkyl; and R_6 is C_1 - C_{100} alkyl optionally substituted with hydroxy, halo, nitro, aryl or alkyl sulfinyl, aryl or alkyl sulfonyl, sulfate, sulfonic acid, phosphate, phosphate ester,

substituted or unsubstituted aryl, carboxyl, carboxylate, amino carbonyl, or alkoxy carbonyl, and/or optionally inserted with O, NH, S, S(O), SO₂, alkenyl, or alkynyl.

Increasing cellular uptake of dsRNAs

5 A method of the invention that can include the administration of a targeting construct and a drug that affects the uptake of the targeting construct into the cell. The drug can be administered before, after, or at the same time that the targeting construct is administered. The drug can be covalently linked to the targeting construct. The drug can have a transient effect on the cell.

10 The drug can increase the uptake of the targeting construct into the cell, for example, by disrupting the cell's cytoskeleton, *e.g.*, by disrupting the cell's microtubules, microfilaments, and/or intermediate filaments. The drug can be, for example, taxon, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholid A, indanocine, or myoservin.

15 The drug can also increase the uptake of the targeting construct into the cell by activating an inflammatory response, for example. Exemplary drug's that would have such an effect include tumor necrosis factor alpha (TNFalpha), interleukin-1 beta, or gamma interferon.

IV. Formulations

20 **Nucleic acid lipid particles**

In one embodiment, an avian targeting construct, *e.g.*, a dsRNA, featured in the invention is fully encapsulated in a lipid formulation, *e.g.*, to form a SPLP, pSPLP, SNALP, or other nucleic acid-lipid particle. As used herein, the term "SNALP" refers to a stable nucleic acid-lipid particle, including SPLP. A SNALP represents a vesicle of 25 lipids coating a reduced aqueous interior comprising a nucleic acid such as an iRNA agent or a plasmid from which a iRNA agent is transcribed. SNALPs are described, *e.g.*, in U.S. Patent Application Publication Nos. 20060240093, 20070135372, and in International Application No. WO 2009082817. These applications are incorporated herein by reference in their entirety.

As used herein, the term "SPLP" refers to a nucleic acid-lipid particle comprising plasmid DNA encapsulated within a lipid vesicle. SNALPs and SPLPs typically contain a cationic lipid, a non-cationic lipid, and a lipid that prevents aggregation of the particle (*e.g.*, a PEG-lipid conjugate). SNALPs and SPLPs are extremely useful for systemic applications, as they exhibit extended circulation lifetimes following intravenous (i.v.) injection and accumulate at distal sites (*e.g.*, sites physically separated from the administration site). SPLPs include "pSPLP," which include an encapsulated condensing agent-nucleic acid complex as set forth in PCT Publication No. WO 00/03683. The particles of the present invention typically have a mean diameter of about 50 nm to about 150 nm, more typically about 60 nm to about 130 nm, more typically about 70 nm to about 110 nm, most typically about 70 nm to about 90 nm, and are substantially nontoxic. In addition, the nucleic acids when present in the nucleic acid-lipid particles of the present invention are resistant in aqueous solution to degradation with a nuclease. Nucleic acid-lipid particles and their method of preparation are disclosed in, *e.g.*, U.S. Patent Nos. 5,976,567; 5,981,501; 6,534,484; 6,586,410; 6,815,432; and PCT Publication No. WO 96/40964, each of which is incorporated herein by reference in its entirety.

In one embodiment, the lipid to drug ratio (mass/mass ratio) (*e.g.*, lipid to targeting construct, *e.g.*, dsRNA ratio) will be in the range of from about 1:1 to about 50:1, from about 1:1 to about 25:1, from about 3:1 to about 15:1, from about 4:1 to about 10:1, from about 5:1 to about 9:1, or about 6:1 to about 9:1.

The cationic lipid may be, for example, N,N-dioleoyl-N,N-dimethylammonium chloride (DODAC), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-dimethyl-2,3-dioleoyloxypropylamine (DODMA), 1,2-DiLinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-Dilinenyloxy-N,N-dimethylaminopropane (DLenDMA), 1,2-Dilinoylelcarbamoxyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-Dilinoxyloxy-3-(dimethylamino)acetoxyp propane (DLin-DAC), 1,2-Dilinoxyloxy-3-morpholinopropane (DLin-MA), 1,2-Dilinoylel-3-dimethylaminopropane (DLinDAP), 1,2-Dilinoylelthio-3-dimethylaminopropane (DLin-S-DMA), 1-Linoylel-2-linoxyloxy-3-

dimethylaminopropane (DLin-2-DMAP), 1,2-Dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-Dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-Dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), or 3-(N,N-Dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-Dioleoylamino)-1,2-propanedio (DOAP), 1,2-Dilinoleyloxy-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), 1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA), 2,2-Dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA) or analogs thereof, (3aR,5s,6aS)-N,N-dimethyl-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)tetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-amine (ALN100), (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (MC3), 1,1'-(2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethylazanediy)l)didodecan-2-ol (Tech G1), or a mixture thereof. The cationic lipid may comprise from about 20 mol % to about 50 mol % or about 40 mol % of the total lipid present in the particle.

In another embodiment, the compound 2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane can be used to prepare lipid-siRNA nanoparticles. Synthesis of 2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane is described in United States provisional patent application number 61/107,998 filed on October 23, 2008, which is herein incorporated by reference.

In one embodiment, the lipid-siRNA particle includes 40% 2, 2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane: 10% DSPC: 40% Cholesterol: 10% PEG-C-DOMG (mole percent) with a particle size of 63.0 ± 20 nm and a 0.027 siRNA/Lipid Ratio.

The non-cationic lipid may be an anionic lipid or a neutral lipid including, but not limited to, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyl-oleoylphosphatidylcholine (POPC), palmitoyl-oleoylphosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidyl-ethanolamine

(DSPE), 16-O-monomethyl PE, 16-O-dimethyl PE, 18-1 -trans PE, 1 -stearoyl-2-oleoyl-phosphatidylethanolamine (SOPE), cholesterol, or a mixture thereof. The non-cationic lipid may be from about 5 mol % to about 90 mol %, about 10 mol %, or about 58 mol % if cholesterol is included, of the total lipid present in the particle.

5 The conjugated lipid that inhibits aggregation of particles may be, for example, a polyethyleneglycol (PEG)-lipid including, without limitation, a PEG-diacylglycerol (DAG), a PEG-dialkylxypropyl (DAA), a PEG-phospholipid, a PEG-ceramide (Cer), or a mixture thereof. The PEG-DAA conjugate may be, for example, a PEG-dilauryloxypropyl (Ci₂), a PEG-dimyristyloxypropyl (Ci₄), a PEG-dipalmitoyloxypropyl
10 (Ci₆), or a PEG- distearyloxypropyl (C₁₈). The conjugated lipid that prevents aggregation of particles may be from 0 mol % to about 20 mol % or about 2 mol % of the total lipid present in the particle.

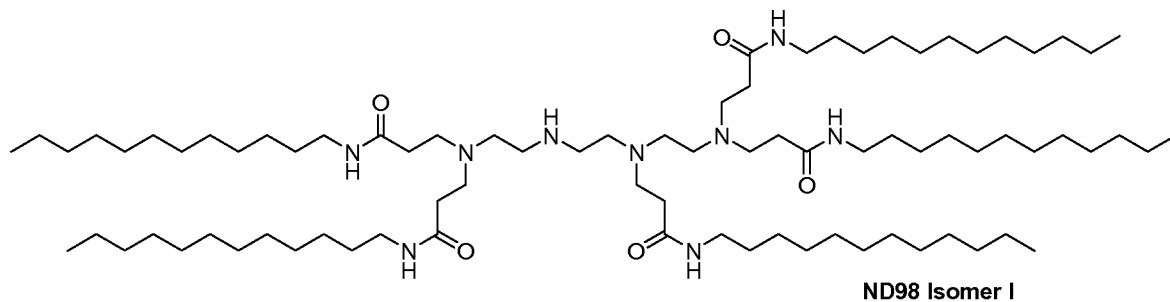
 In some embodiments, the nucleic acid-lipid particle further includes cholesterol at, *e.g.*, about 10 mol % to about 60 mol % or about 48 mol % of the total lipid present in
15 the particle.

LNP01

 In one embodiment, the lipidoid ND98·4HCl (MW 1487) (see U.S. Patent Application No. 12/056,230, filed 3/26/2008, which is herein incorporated by reference), Cholesterol (Sigma-Aldrich), and PEG-Ceramide C16 (Avanti Polar Lipids) can be used
20 to prepare lipid-dsRNA nanoparticles (*i.e.*, LNP01 particles). Stock solutions of each in ethanol can be prepared as follows: ND98, 133 mg/ml; Cholesterol, 25 mg/ml, PEG-Ceramide C16, 100 mg/ml. The ND98, Cholesterol, and PEG-Ceramide C16 stock solutions can then be combined in a, *e.g.*, 42:48:10 molar ratio. The combined lipid solution can be mixed with aqueous dsRNA (*e.g.*, in sodium acetate pH 5) such that the
25 final ethanol concentration is about 35-45% and the final sodium acetate concentration is about 100-300 mM. Lipid-dsRNA nanoparticles typically form spontaneously upon mixing. Depending on the desired particle size distribution, the resultant nanoparticle mixture can be extruded through a polycarbonate membrane (*e.g.*, 100 nm cut-off) using, for example, a thermobarrel extruder, such as Lipex Extruder (Northern Lipids, Inc). In
30 some cases, the extrusion step can be omitted. Ethanol removal and simultaneous buffer

exchange can be accomplished by, for example, dialysis or tangential flow filtration.

Buffer can be exchanged with, for example, phosphate buffered saline (PBS) at about pH 7, *e.g.*, about pH 6.9, about pH 7.0, about pH 7.1, about pH 7.2, about pH 7.3, or about pH 7.4.



Formula 1

LNP01 formulations are described, *e.g.*, in International Application Publication No. WO 2008/042973, which is hereby incorporated by reference.

Additional exemplary lipid-dsRNA formulations are as follows:

10

Table 2 Lipid Nanoparticle formulations

	Cationic Lipid	cationic lipid/non-cationic lipid/cholesterol/PEG-lipid conjugate Lipid:dsRNA (e.g., siRNA) ratio
SNALP	1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA)	DLinDMA/DPPC/Cholesterol/PEG-cDMA (57.1/7.1/34.4/1.4) lipid:siRNA ~ 7:1
S-XTC	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DPPC/Cholesterol/PEG-cDMA 57.1/7.1/34.4/1.4 lipid:siRNA ~ 7:1
LNP05	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid:siRNA ~ 6:1
LNP06	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid:siRNA ~ 11:1
LNP07	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid:siRNA ~ 6:1

LNP08	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid:siRNA ~ 11:1
LNP09	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA 10:1
LNP10	(3aR,5s,6aS)-N,N-dimethyl-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)tetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-amine (ALN100)	ALN100/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA 10:1
LNP11	(6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (MC3)	MC-3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA 10:1
LNP12	1,1'-(2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl)pipe razin-1-yl)ethylazanediyldidodecan-2-ol (C12-200)	C12-200/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA 10:1
LNP13	XTC	XTC/DSPC/Chol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA: 33:1
LNP14	MC3	MC3/DSPC/Chol/PEG-DMG 40/15/40/5 Lipid:siRNA: 11:1
LNP15	MC3	MC3/DSPC/Chol/PEG-DSG/GalNAc-PEG-DSG 50/10/35/4.5/0.5 Lipid:siRNA: 11:1
LNP16	MC3	MC3/DSPC/Chol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA: 7:1
LNP17	MC3	MC3/DSPC/Chol/PEG-DSG 50/10/38.5/1.5 Lipid:siRNA: 10:1
LNP18	MC3	MC3/DSPC/Chol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA: 12:1
LNP19	MC3	MC3/DSPC/Chol/PEG-DMG 50/10/35/5 Lipid:siRNA: 8:1
LNP20	MC3	MC3/DSPC/Chol/PEG-DPG 50/10/38.5/1.5 Lipid:siRNA: 10:1

LNP21	C12-200	C12-200/DSPC/Chol/PEG-DSG 50/10/38.5/1.5 Lipid:siRNA: 7:1
LNP22	XTC	XTC/DSPC/Chol/PEG-DSG 50/10/38.5/1.5 Lipid:siRNA: 10:1

DSPC: distearoylphosphatidylcholine

DPPC: dipalmitoylphosphatidylcholine

PEG-DMG: PEG-didimyrystoyl glycerol (C14-PEG, or PEG-C14) (PEG with avg mol
5 wt of 2000)

PEG-DSG: PEG-distyryl glycerol (C18-PEG, or PEG-C18) (PEG with avg mol wt of
2000)

PEG-cDMA: PEG-carbamoyl-1,2-dimyristyloxypropylamine (PEG with avg mol wt of
2000)

10

SNALP (1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA))

comprising formulations are described in International Publication No. WO2009/127060,
filed April 15, 2009, which is hereby incorporated by reference in its entirety.

XTC comprising formulations are described, e.g., in U.S. Provisional Serial
15 No. 61/239,686, filed September 3, 2009 as well as PCT/US10/22614 filed January 29,
2010 each of which is hereby incorporated by reference in its entirety. Further XTC
formulations useful in the present invention are disclosed in PCT/US08/088588 filed 31-
Dec-2008 and PCT/US08/88587 filed 31-Dec-2008 and PCT/US09/041442 filed 22-Apr-
2009 and PCT/US09/061897 filed 23-Oct-2009 and PCT/US10/38224 filed June 10,
20 2010, each of which is hereby incorporated by reference in its entirety.

MC3 comprising formulations are described, e.g., in U.S. Provisional Serial
No. 61/244,834, filed September 22, 2009, and U.S. Provisional Serial No. 61/185,800,
filed June 10, 2009, and PCT/US09/63933 filed November 10, 2009 and
PCT/US09/63927 filed 10-Nov-2009 and PCT/US09/63931 filed 10-Nov-2009 and
25 PCT/US09/63897 filed 10-Nov-2009, each of which are hereby incorporated by reference
in its entirety.

ALNY-100 comprising formulations are described, e.g., International patent
application number PCT/US09/63933, filed on November 10, 2009, which is hereby
incorporated by reference in its entirety. The synthesis is outlined in Example 8.

C12-200 comprising formulations are described in U.S. Provisional Serial No. 61/175,770, filed May 5, 2009, as well as PCT/US10/33777 which are hereby incorporated by reference in its entirety. The synthesis is outlined in Example 8.

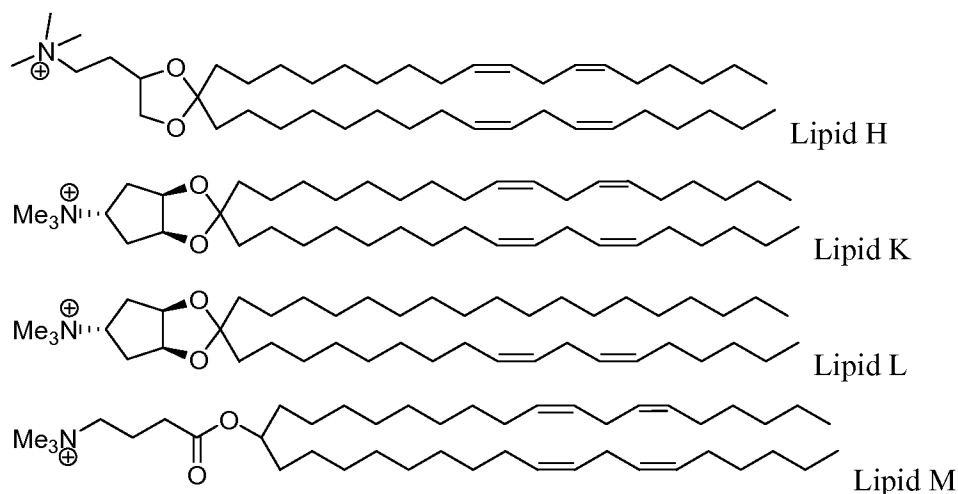
Transfection reagents useful in the present invention are disclosed in US
5 provisional 61/267,419 filed December 7, 2009, which is hereby incorporated by reference in its entirety.

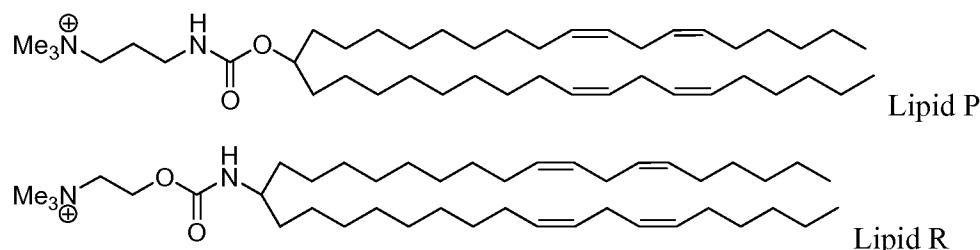
Formulations for targeting immune cells useful in the present invention are disclosed in PCT/US10/033747 filed May 5, 2010, which is hereby incorporated by reference in its entirety.

10 Pyrrolidine cationic lipids useful in the formulations of the present invention are disclosed in USSN 12/123,922 filed May 20, 2008 which is hereby incorporated by reference in its entirety.

In one embodiment, the reagent that facilitates targeting construct uptake used herein comprises a cationic lipid as described in e.g., U.S. Application Ser.
15 No. 61/267,419, filed 7 December 2009, and U.S. Application Ser. No. 61/334,398, filed 13 May 2010. In various embodiments, the targeting construct composition described herein comprisescomprises a cationic lipid selected from the group consisting of: "Lipid H", "Lipid K"; "Lipid L", "Lipid M"; "Lipid P"; or "Lipid R", whose formulas are indicated as follows:

20





Also contemplated herein are various formulations of the lipids described above,
 5 such as, e.g., K8, P8 and L8 which refer to formulations comprising Lipid K, P, and L,
 respectively. Some exemplary lipid formulations for use with the methods and
 compositions described herein are found in e.g., Table 3:

Table 3. Example lipid formulations

Formulation Number	Cationic Lipid Number	Cationic Lipid Mol %	DOPE %	Cholesterol %
1	200 (Lipid H)	48.08	51.92	-
2	200 (Lipid H)	47.94	47.06	5
3	201 (Lipid K)	45.56	54.44	-
4 (K8)	201 (Lipid K)	47.94	47.06	5
5 (L8)	202 (Lipid L)	47.94	47.06	5
6	203 (Lipid M)	53.01	44.49	2.5
7	203 (Lipid M)	47.94	47.06	5
8 (P8)	204 (Lipid P)	47.94	47.06	5
9	205 (Lipid R)	47.94	47.06	5

10

In another embodiment, the targeting construct composition described herein
 further comprises a lipid formulation comprising a lipid selected from the group
 consisting of Lipid H, Lipid K, Lipid L, Lipid M, Lipid P, and Lipid R, and further
 comprises a neutral lipid and a sterol. In particular embodiments, the lipid formulation
 15 comprises between approximately 25 mol % - 100 mol% of the lipid. In another
 embodiment, the lipid formulation comprises between 0 mol% - 50 mol% cholesterol. In
 still another embodiment, the lipid formulation comprises between 30 mol% - 65 mol%
 of a neutral lipid. In particular embodiments, the lipid formulation comprises the relative
 mol% of the components as listed in Table 4 as follows:

Table 4. Example lipid formulae

Series	Lipid (Mol%)	DOPE	Chol
1	45.56	54.44	0
2	48.08	51.92	0
3	50.60	49.40	0
4	53.10	46.90	0
5	52.73	37.27	10
6	52.92	42.08	5
7	53.01	44.49	2.5
8	47.94	47.06	5

Other particles

In vivo delivery can also be by a beta-glucan delivery system, such as those described in U.S. Patent Nos. 5,032,401 and 5,607,677, and U.S. Publication No. 2005/0281781, which are hereby incorporated by reference in their entirety. *In vitro* introduction into a cell includes methods known in the art such as electroporation and lipofection.

Chitosan and minicell nanoparticles may also be utilized.

10 Liposomal formulations

There are many organized surfactant structures that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up *in vivo*.

It is desirable to use a liposome which is highly deformable and able to pass through fine pores with a diameter less than 50 nm, under the influence of a suitable gradient.

Further advantages of liposomes include; liposomes obtained from natural
5 phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Important considerations in the preparation of liposome
10 formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with the
15 cellular membranes and as the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over other formulations. Such
20 advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

Liposomes fall into two broad classes. Cationic liposomes are positively charged
25 liposomes which interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang *et al.*, *Biochem. Biophys. Res. Commun.*, 1987, 147, 980-985).

Liposomes which are pH-sensitive or negatively charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver
5 DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou *et al.*, Journal of Controlled Release, 1992, 19, 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can
10 be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC,
15 and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Liposomes also include “sterically stabilized” liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes
20 lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G_{M1}, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for
25 sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen *et al.*, FEBS Letters, 1987, 223, 42; Wu *et al.*, Cancer Research, 1993, 53, 3765).

Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos *et al.* (Ann. N.Y. Acad. Sci., 1987, 507, 64) reported the ability of monosialoganglioside G_{M1}, galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon *et al.* (Proc. Natl. Acad. Sci. U.S.A., 1988, 85, 6949). U.S. Pat. No. 4,837,028 and WO 88/04924, both to Allen *et al.*, disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G_{M1} or a galactocerebroside sulfate ester. U.S. Pat. No. 5,543,152 (Webb *et al.*) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-sn-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim *et al.*).

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto *et al.* (Bull. Chem. Soc. Jpn., 1980, 53, 2778) described liposomes comprising a nonionic detergent, 2C₁₂15G, that contains a PEG moiety. Illum *et al.* (FEBS Lett., 1984, 167, 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (*e.g.*, PEG) are described by Sears (U.S. Pat. Nos. 4,426,330 and 4,534,899). Klivanov *et al.* (FEBS Lett., 1990, 268, 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume *et al.* (Biochimica et Biophysica Acta, 1990, 1029, 91) extended such observations to other PEG-derivatized phospholipids, *e.g.*, DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle *et al.* (U.S. Pat. Nos. 5,013,556 and 5,356,633) and Martin *et al.* (U.S. Pat. No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Pat. No. 5,225,212 (both to Martin *et al.*) and in WO 94/20073 (Zalipsky *et al.*) Liposomes comprising PEG-modified

ceramide lipids are described in WO 96/10391 (Choi *et al.*). U.S. Pat. No. 5,540,935 (Miyazaki *et al.*) and U.S. Pat. No. 5,556,948 (Tagawa *et al.*) describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

A number of liposomes comprising nucleic acids are known in the art. WO 96/40062 to Thierry *et al.* discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Pat. No. 5,264,221 to Tagawa *et al.* discloses protein-bonded liposomes and asserts that the contents of such liposomes may include a dsRNA. U.S. Pat. No. 5,665,710 to Rahman *et al.* describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love *et al.* discloses liposomes comprising dsRNAs targeted to the raf gene.

Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes may be described as lipid droplets which are so highly deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, *e.g.*, they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated delivery of serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, N.Y., 1988, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and

are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers
5 such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include
10 carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

15 If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative
20 charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, N.Y., 1988, p. 285).

25 Compositions and formulations for parenteral, intraparenchymal (into the brain), intrathecal, intraventricular or intrahepatic administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations of targeting constructs. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids. Particularly preferred are formulations that target the liver when treating hepatic disorders such as hepatic carcinoma.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Emulsions

The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogeneous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter (see e.g., Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York,

N.Y., Volume 1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi *et al.*, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, Pa., 1985, p. 301). Other means of stabilizing emulsions entail the use of emulsifiers that may be
5 incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (see e.g., *Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems*, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Idson, in *Pharmaceutical Dosage Forms*,
10 Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (see e.g., *Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems*, Allen, LV.,
15 Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988, volume 1, p. 199). Surfactants may be classified into
20 different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (see e.g., *Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems*, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p.
25 285).

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman,
30 Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335;

Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid.

Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (see e.g., *Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems*, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of ease of formulation, as well as efficacy from an absorption and bioavailability standpoint (see e.g., *Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems*, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rosoff, in

Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat
5 nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of targeting constructs are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and
10 thermodynamically stable liquid solution (see e.g., Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Microemulsions commonly are prepared via a combination of three to
15 five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., 1985, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (see e.g., Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rosoff, in Pharmaceutical Dosage Forms,
20 Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed
25 spontaneously.
30

Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (see e.g., U.S. Patent Nos. 6,191,105; 7,063,860; 7,070,802; 7,157,099; Constantinides *et al.*, Pharmaceutical Research, 1994, 11, 1385-1390; Ritschel, Meth. Find. Exp. Clin. Pharmacol., 1993, 13, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (see e.g., U.S. Patent Nos. 6,191,105; 7,063,860; 7,070,802; 7,157,099; Constantinides *et al.*, Pharmaceutical Research, 1994, 11, 1385; Ho *et al.*, J. Pharm. Sci., 1996, 85, 138-143). Often

microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile targeting construct drugs, peptides or iRNAs. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of nucleic acid based targeting constructs, e.g., iRNAs and nucleic acids, from the gastrointestinal tract, as well as improve the local cellular uptake.

Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the targeting constructs and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories--surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 92). Each of these classes has been discussed above.

Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of targeting constructs, particularly iRNAs to avian cells. It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (see e.g., Malmsten, M. Surfactants and polymers in drug delivery, Informa Health Care, New York, NY, 2002; Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of targeting constructs through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (see e.g., Malmsten, M. Surfactants and polymers in drug delivery, Informa Health Care, New York, NY, 2002; Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92); and perfluorochemical emulsions, such as FC-43. Takahashi *et al.*, J. Pharm. Pharmacol., 1988, 40, 252).

Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C₁₋₂₀ alkyl esters thereof (e.g., methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (*i.e.*, oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, *etc.*) (see e.g., Tuitou, E., et al. Enhancement in Drug Delivery, CRC Press, Danvers, MA, 2006; Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; El Hariri *et al.*, J. Pharm. Pharmacol., 1992, 44, 651-654).

Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (see e.g., Malmsten, M. Surfactants and polymers in drug delivery, Informa Health Care, New York, NY, 2002; Brunton, Chapter 38 in: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th Ed., Hardman *et al.* Eds., McGraw-Hill, New York, 1996, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. Suitable bile salts include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium

dehydrocholate), deoxycholic acid (sodium deoxycholate), glucolic acid (sodium glucolate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate),
5 ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (see e.g., Malmsten, M. Surfactants and polymers in drug delivery, Informa Health Care, New York, NY, 2002; Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Swinyard, Chapter 39 In: Remington's Pharmaceutical Sciences, 18th Ed., Gennaro, ed.,
10 Mack Publishing Co., Easton, Pa., 1990, pages 782-783; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Yamamoto *et al.*, J. Pharm. Exp. Ther., 1992, 263, 25; Yamashita *et al.*, J. Pharm. Sci., 1990, 79, 579-583).

Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by
15 forming complexes therewith, with the result that absorption of targeting constructs through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, J. Chromatogr., 1993, 618, 315-339).

20 Suitable chelating agents include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (*e.g.*, sodium salicylate, 5-methoxysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of beta-diketones (enamines)(see e.g., Katdare, A. et al., Excipient development for pharmaceutical, biotechnology, and drug delivery, CRC Press, Danvers,
25 MA, 2006; Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Buur *et al.*, J. Control Rel., 1990, 14, 43-51).

Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate
30 insignificant activity as chelating agents or as surfactants but that nonetheless enhance

absorption of targeting constructs through the alimentary mucosa (see e.g., Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33). This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita *et al.*, J. Pharm. Pharmacol., 1987, 39, 621-626).

Agents that enhance uptake of targeting constructs at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi *et al.*, U.S. Pat. No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo *et al.*, PCT Application WO 97/30731), are also known to enhance the cellular uptake of dsRNAs. Examples of commercially available transfection reagents include, for example Lipofectamine™ (Invitrogen; Carlsbad, CA), Lipofectamine 2000™ (Invitrogen; Carlsbad, CA), 293fectin™ (Invitrogen; Carlsbad, CA), Cellfectin™ (Invitrogen; Carlsbad, CA), DMRIE-C™ (Invitrogen; Carlsbad, CA), FreeStyle™ MAX (Invitrogen; Carlsbad, CA), Lipofectamine™ 2000 CD (Invitrogen; Carlsbad, CA), Lipofectamine™ (Invitrogen; Carlsbad, CA), RNAiMAX (Invitrogen; Carlsbad, CA), Oligofectamine™ (Invitrogen; Carlsbad, CA), Optifect™ (Invitrogen; Carlsbad, CA), X-tremeGENE Q2 Transfection Reagent (Roche; Grenzachstrasse, Switzerland), DOTAP Liposomal Transfection Reagent (Grenzacherstrasse, Switzerland), DOSPER Liposomal Transfection Reagent (Grenzacherstrasse, Switzerland), or Eugene (Grenzacherstrasse, Switzerland), Transfectam® Reagent (Promega; Madison, WI), TransFast™ Transfection Reagent (Promega; Madison, WI), Tfx™-20 Reagent (Promega; Madison, WI), Tfx™-50 Reagent (Promega; Madison, WI), DreamFect™ (OZ Biosciences; Marseille, France), EcoTransfect (OZ Biosciences; Marseille, France), TransPass^a D1 Transfection Reagent (New England Biolabs; Ipswich, MA, USA), LyoVec™/LipoGen™ (Invivogen; San Diego, CA, USA), PerFectin Transfection Reagent (Genlantis; San Diego, CA, USA), NeuroPORTER Transfection Reagent (Genlantis; San Diego, CA, USA), GenePORTER Transfection reagent (Genlantis; San Diego, CA, USA), GenePORTER 2 Transfection

reagent (Genlantis; San Diego, CA, USA), Cytofectin Transfection Reagent (Genlantis; San Diego, CA, USA), BaculoPORTER Transfection Reagent (Genlantis; San Diego, CA, USA), TroganPORTER™ transfection Reagent (Genlantis; San Diego, CA, USA), RiboFect (Bioline; Taunton, MA, USA), PlasFect (Bioline; Taunton, MA, USA),
5 UniFECTOR (B-Bridge International; Mountain View, CA, USA), SureFECTOR (B-Bridge International; Mountain View, CA, USA), or HiFect™ (B-Bridge International, Mountain View, CA, USA), among others.

Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols
10 such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

Carriers

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, “carrier compound” or “carrier” can refer to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity per se) but
15 is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation.

Excipients

In contrast to a carrier compound, a “pharmaceutical carrier” or “excipient” is a
20 pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, *etc.*, when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical
25 carriers include, but are not limited to, binding agents (*e.g.*, pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, *etc.*); fillers (*e.g.*, lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, *etc.*); lubricants (*e.g.*, magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated
30 vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, *etc.*);

disintegrants (*e.g.*, starch, sodium starch glycolate, *etc.*); and wetting agents (*e.g.*, sodium lauryl sulphate, *etc.*).

Pharmaceutically acceptable organic or inorganic excipients which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations of nucleic acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Other Components

The compositions of the present invention may additionally contain other adjunct components. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, *e.g.*, lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances that increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

In addition to their administration, as discussed above, the targeting constructs
5 featured in the invention can be administered in combination with other known agents. Further, toxicity and therapeutic efficacy of compounds of the invention can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio
10 between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds that exhibit high therapeutic indices are preferred.

V. Uses for biological products and biomolecules

The invention relates in particular to the use of a targeting construct to modulate
15 the expression of one or more avian transcripts in the bioprocessing or bioproduction of biological products and/or biomolecules. The resultant biological products and/or biomolecules produced by these methods have utility in the treatment of human pathological conditions, diseases, symptoms or disorders.

As used herein, in the context of the use of the biological products or
20 biomolecules insofar as it relates to any of the other conditions recited herein below, the terms “treat,” “treatment,” and the like mean to relieve or alleviate at least one symptom associated with a condition, or to slow or reverse the progression or anticipated progression of a condition, such as slowing the progression of a malignancy or cancer, or increasing the clearance of an infectious organism to alleviate/reduce the symptoms
25 caused by the infection, *e.g.*, hepatitis caused by infection with a hepatitis virus.

By “lower” in the context of a disease marker or symptom is meant a statistically significant decrease in such level. The decrease can be, for example, at least 10%, at least 20%, at least 30%, at least 40% or more, and is preferably down to a level accepted as within the range of normal for an individual without such disorder.

As used herein, the phrases “therapeutically effective amount” and “prophylactically effective amount” refer to an amount that provides a therapeutic benefit in the treatment, prevention, or management of pathological processes or an overt symptom of pathological processes. The specific amount that is therapeutically effective
5 can be readily determined by an ordinary medical practitioner, and may vary depending on factors known in the art, such as, for example, the type of pathological processes mediated by avian expression, the patient’s history and age, the stage of pathological processes mediated by avian expression, and the administration of other agents that inhibit pathological processes mediated by avian expression.

10 As used herein, a “pharmaceutical composition” comprises a pharmacologically effective amount of a targeting construct and a pharmaceutically acceptable carrier. As used herein, “pharmacologically effective amount,” “therapeutically effective amount” or simply “effective amount” refers to that amount of a biological product or biomolecule to produce the intended pharmacological, therapeutic or preventive result. For example, if a
15 given clinical treatment is considered effective when there is at least a 10% reduction in a measurable parameter associated with a disease or disorder, a therapeutically effective amount of a biological product or biomolecule for the treatment of that disease or disorder is the amount necessary to effect at least a 10% reduction in that parameter.

The term “pharmaceutically acceptable carrier” refers to a carrier for
20 administration of a biological product or biomolecule. Such carriers include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The term specifically excludes cell culture medium. For biological product or biomolecules administered orally, pharmaceutically acceptable carriers include, but are not limited to pharmaceutically acceptable excipients such as inert diluents, disintegrating
25 agents, binding agents, lubricating agents, sweetening agents, flavoring agents, coloring agents and preservatives. Suitable inert diluents include sodium and calcium carbonate, sodium and calcium phosphate, and lactose, while corn starch and alginic acid are suitable disintegrating agents. Binding agents may include starch and gelatin, while the lubricating agent, if present, will generally be magnesium stearate, stearic acid or talc. If
30 desired, the tablets may be coated with a material such as glyceryl monostearate or

glyceryl distearate, to delay absorption in the gastrointestinal tract. Agents included in biological product or biomolecule formulations are described further herein below.

Biological therapies use the body's immune system, either directly or indirectly, to fight cancer or to lessen the side effects that may be caused by some cancer treatments.

5 In one sense, biological product or biomolecules can be considered in this group of therapies in that it can stimulate immune system action against a tumor, for example. However, this approach can also be considered with other such biological approaches, e.g., immune response modifying therapies such as the administration of interferons, interleukins, colony-stimulating factors, monoclonal antibodies, vaccines, gene therapy,
10 and nonspecific immunomodulating agents are also envisioned as anti-cancer therapies to be combined with the biological product or biomolecule.

Efficacy of treatment or amelioration of disease can be assessed, for example by measuring disease progression, disease remission, symptom severity, reduction in pain, quality of life, dose of a medication required to sustain a treatment effect, level of a
15 disease marker or any other measurable parameter appropriate for a given disease being treated or targeted for prevention. It is well within the ability of one skilled in the art to monitor efficacy of treatment or prevention by measuring any one of such parameters, or any combination of parameters. In connection with the administration of a biological product or biomolecule or pharmaceutical composition thereof, "effective against" a
20 cancer indicates that administration in a clinically appropriate manner results in a beneficial effect for at least a statistically significant fraction of patients, such as a improvement of symptoms, a cure, a reduction in disease load, reduction in tumor mass or cell numbers, extension of life, improvement in quality of life, or other effect generally recognized as positive by medical doctors familiar with treating the particular type of
25 cancer.

A treatment or preventive effect is evident when there is a statistically significant improvement in one or more parameters of disease status, or by a failure to worsen or to develop symptoms where they would otherwise be anticipated. As an example, a favorable change of at least 10% in a measurable parameter of disease, and preferably at
30 least 20%, 30%, 40%, 50% or more can be indicative of effective treatment. Efficacy for

a given biological product or biomolecule drug or formulation of that drug can also be judged using an experimental animal model for the given disease as known in the art. When using an experimental animal model, efficacy of treatment is evidenced when a statistically significant reduction in a marker or symptom is observed.

5 Infections are broadly classified as bacterial, viral, fungal, or parasitic based on the category of infectious organism or agent involved. Other less common types of infection are also known in the art, including, e.g., infections involving rickettsiae, mycoplasmas, and agents causing scrapie, bovine spongiform encephalopathy (BSE), and prion diseases (e.g., kuru and Creutzfeldt-Jacob disease). Examples of bacteria, viruses,
10 fungi, and parasites which cause infection are well known in the art. An infection can be acute, subacute, chronic, or latent, and it can be localized or systemic. As defined herein, a “chronic infection” refers to those infections that are not cleared by the normal actions of the innate or adaptive immune responses and persist in the subject for a long duration of time, on the order of weeks, months, and years. A chronic infection may reflect latency
15 of the infectious agent, and may include periods in which no infectious symptoms are present, i.e., asymptomatic periods. Examples of chronic infections include, but are not limited to, HIV infection and herpesvirus infections. Furthermore, an infection can be predominantly intracellular or extracellular during at least one phase of the infectious organism's or agent's life cycle in the host.

20 Exemplary viruses include, but are not limited to: *Retroviridae* (e.g., human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III), HIV-2, LAV or HTLV-III/LAV, or HIV-III, and other isolates, such as HIV-LP; *Picornaviridae* (e.g., polio viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); *Calciviridae* (e.g., strains that cause gastroenteritis); *Togaviridae* (e.g.,
25 equine encephalitis viruses, rubella viruses); *Flaviviridae* (e.g., dengue viruses, encephalitis viruses, yellow fever viruses); *Coronaviridae* (e.g., coronaviruses); *Rhabdoviridae* (e.g., vesicular stomatitis viruses, rabies viruses); *Filoviridae* (e.g., ebola viruses); *Paramyxoviridae* (e.g., parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); adenovirus; *Orthomyxoviridae* (e.g., influenza viruses);
30 *Bunyaviridae* (e.g., Hantaan viruses, bunya viruses, phleboviruses and Nairo viruses);

Arena viridae (hemorrhagic fever viruses); *Reoviridae* (e.g., reoviruses, orbiviruses and rotaviruses, i.e., Rotavirus A, Rotavirus B, Rotavirus C); *Birnaviridae*; *Hepadnaviridae* (Hepatitis A and B viruses); *Parvoviridae* (parvoviruses); *Papovaviridae* (papilloma viruses, polyoma viruses); *Adenoviridae* (most adenoviruses); *Herpesviridae* (herpes simplex virus (HSV) 1 and 2, Human herpes virus 6, Human herpes virus 7, Human herpes virus 8, varicella zoster virus, cytomegalovirus (CMV), herpes virus; Epstein-Barr virus; Rous sarcoma virus; West Nile virus; Japanese equine encephalitis, Norwalk, papilloma virus, parvovirus B19; *Poxviridae* (variola viruses, vaccinia viruses, pox viruses); and *Iridoviridae* (e.g., African swine fever virus); Hepatitis D virus, Hepatitis E virus, and unclassified viruses (e.g., the etiological agents of Spongiform encephalopathies, the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1=enterally transmitted; class 2=parenterally transmitted (i.e., Hepatitis C); Norwalk and related viruses, and astroviruses).

Bacteria include both Gram negative and Gram positive bacteria. Examples of Gram positive bacteria include, but are not limited to *Pasteurella* species, *Staphylococci* species, and *Streptococcus* species. Examples of Gram negative bacteria include, but are not limited to, *Escherichia coli* (including enterohaemorrhagic E. coli (EHEC) strain 0104), *Pseudomonas* species, and *Salmonella* species. Specific examples of infectious bacteria include but are not limited to: *Helicobacter pylori*, *Borrelia burgdorferi*, *Legionella pneumophila*, *Mycobacteria* spp. (e.g., *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansasii*, *M. gordonae*, *M. leprae*), *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Listeria monocytogenes*, *Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus* (viridans group), *Streptococcus faecalis*, *Streptococcus bovis*, *Streptococcus* (anaerobic spp.), *Streptococcus pneumoniae*, pathogenic *Campylobacter* spp., *Enterococcus* spp., *Haemophilus influenzae* (*Hemophilus influenza B*, and *Hemophilus influenza non-typable*), *Bacillus anthracis*, *Corynebacterium diphtheriae*, *Corynebacterium* spp., *Erysipelothrix rhusiopathiae*, *Clostridium perfringens*, *Clostridium tetani*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pasteurella*

multocida, *Bacteroides* spp., *Fusobacterium nucleatum*, *Streptobacillus moniliformis*,
Treponema pallidum, *Treponema pertenue*, *Leptospira*, *Rickettsia*, *Actinomyces israelii*,
meningococcus, *pertussis*, *pneumococcus*, *shigella*, *tetanus*, *Vibrio cholerae*, *yersinia*,
Pseudomonas species, *Clostridia* species, *Salmonella typhi*, *Shigella dysenteriae*,
5 *Yersinia pestis*, *Brucella* species, *Legionella pneumophila*, *Rickettsiae*, *Chlamydia*,
Clostridium perfringens, *Clostridium botulinum*, *Staphylococcus aureus*, *Pseudomonas*
aeruginosa, *Cryptosporidium parvum*, *Streptococcus pneumoniae*, and *Bordetella*
pertussis.

Exemplary fungi and yeast include, but are not limited to, *Cryptococcus*
10 *neoformans*, *Candida albicans*, *Candida tropicalis*, *Candida stellatoidea*, *Candida*
glabrata, *Candida krusei*, *Candida parapsilosis*, *Candida guilliermondii*, *Candida*
viswanathii, *Candida lusitanae*, *Rhodotorula mucilaginosa*, *Aspergillus fumigatus*,
Aspergillus flavus, *Blastomyces dermatitidis*, *Aspergillus clavatus*, *Cryptococcus*
neoformans, *Chlamydia trachomatis*, *Coccidioides immitis*, *Cryptococcus laurentii*,
15 *Cryptococcus albidus*, *Cryptococcus gattii*, *Nocardia* spp, *Histoplasma capsulatum*,
Pneumocystis jirovecii (or *Pneumocystis carinii*), *Stachybotrys chartarum*, and any
combination thereof.

Exemplary parasites include, but are not limited to: *Entamoeba histolytica*;
Plasmodium species (*Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*,
20 *Plasmodium vivax*), *Leishmania* species (*Leishmania tropica*, *Leishmania braziliensis*,
Leishmania donovani), *Toxoplasmosis* (*Toxoplasma gondii*), *Trypanosoma gambiense*,
Trypanosoma rhodesiense (African sleeping sickness), *Trypanosoma cruzi* (Chagas'
disease), Helminths (flat worms, round worms), *Babesia microti*, *Babesia divergens*,
Giardia lamblia, and any combination thereof.

25 The invention further relates to the use of a biological product or biomolecule for
the treatment of an infectious disease, such as hepatitis B or a chronic bacterial infection,
in combination with other pharmaceuticals and/or other therapeutic methods, e.g., with
known pharmaceuticals and/or known therapeutic methods, such as, for example, those
which are currently employed for treating such infectious diseases or disorders (e.g.,
30 antibiotics, anti-viral agents).

In other embodiments, administration of a biological product or biomolecule is performed in combination with an anti-viral medicament or agent.

In other embodiments, administration of a biological product or biomolecule is performed in combination with an anti-fungal medicament or agent. An "antifungal medicament" is an agent that kills or inhibits the growth or function of infective fungi. Anti-fungal medicaments are sometimes classified by their mechanism of action. Some anti-fungal agents function as cell wall inhibitors by inhibiting glucose synthase, other antifungal agents function by destabilizing membrane integrity, and other antifungal agents function by breaking down chitin (e.g., chitinase) or immunosuppression (501 cream).

In further embodiments, administration of a biological product or biomolecule is administered in combination with an anti-parasitic medicament or agent. An "antiparasitic medicament" refers to an agent that kills or inhibits the growth or function of infective parasites.

Delivery of Biological Products

The delivery of a biological product or biomolecule to a subject in need thereof can be achieved in a number of different ways. In vivo delivery can be performed directly by administering a composition comprising a biological product or biomolecule, e.g, antibody, etc to a subject. Alternatively, delivery can be performed indirectly by administering one or more vectors that encode and direct the expression of the biological product or biomolecule. These alternatives are discussed further below.

"Introducing into a cell," when referring to a biological product or biomolecule, means facilitating or effecting uptake or absorption into the cell, as is understood by those skilled in the art. Absorption or uptake of a biological product or biomolecule can occur through unaided diffusive or active cellular processes, or by auxiliary agents or devices. The meaning of this term is not limited to cells *in vitro*; a biological product or biomolecule may also be "introduced into a cell," wherein the cell is part of a living organism. In such an instance, introduction into the cell will include the delivery to the organism. For example, for *in vivo* delivery, biological product or biomolecules can be injected into a tissue site or administered systemically. It is also contemplated by the

inventors that introduction into cells or tissues may effected *ex vivo*, *in situ* and *in ovo*. In the case of transplants or within the field of stem cell technologies, it is contemplated that “introduction into a cell” will embrace the introduction to cells of any lineage or state, whether presently stem cells or which are intended to produce stem cells or progenitors or precursors thereof, as well as tissues, explants, organs and even organ systems.

When the organism to be treated is a mammal such as a human, the biological product of the methods described here may be administered by any means known in the art including, but not limited to oral, intraperitoneal, or parenteral routes, including intracranial (*e.g.*, intraventricular, intraparenchymal and intrathecal), intravenous, intramuscular, subcutaneous, transdermal, airway (aerosol), nasal, rectal, and topical (including buccal and sublingual) administration. In certain embodiments, the compositions are administered by intravenous infusion or injection.

Direct delivery

In general, any method of delivering a nucleic acid molecule can be adapted for use with a biological product or biomolecule (see *e.g.*, Akhtar S. and Julian RL. (1992) Trends Cell. Biol. 2(5):139-144 and WO94/02595, which are incorporated herein by reference in their entireties). However, there are three factors that are important to consider in order to successfully deliver a biological product or biomolecule *in vivo*: (a) biological stability of the delivered molecule, (2) preventing non-specific effects, and (3) accumulation of the delivered molecule in the target tissue. The non-specific effects of a targeting construct can be minimized by local administration, for example by direct injection or implantation into a tissue (as a non-limiting example, a tumor) or topically administering the preparation.

Local administration to a treatment site maximizes local concentration of the agent, limits the exposure of the agent to systemic tissues that may otherwise be harmed by the agent or that may degrade the agent, and permits a lower total dose of the biological product or biomolecule to be administered. Several studies have shown successful knockdown of gene products when an iRNA agent, biological product or biomolecule, is administered locally.

For administering a biological product or biomolecule systemically for the treatment of a disease, the biological product or biomolecule can be modified or alternatively delivered using a drug delivery system; both methods act to prevent the rapid degradation of the molecule by endo- and exo-nucleases (in the case of nucleic acid based targeting constructs) in vivo. Modification of a biological product or biomolecule or the pharmaceutical carrier can also permit targeting of the biological product or biomolecule composition to the target tissue and avoid undesirable off-target effects. Biological product or biomolecule molecules can be modified by chemical conjugation to lipophilic groups such as cholesterol to enhance cellular uptake and prevent degradation. In like fashion, the biological product or biomolecule of the present invention may be conjugated to one or more aptamers.

In an alternative embodiment, the biological product or biomolecule can be delivered using drug delivery systems such as a nanoparticle, a dendrimer, a polymer, liposomes, or a cationic delivery system. Positively charged cationic delivery systems facilitate binding of a targeting construct molecule (when negatively charged) and also enhance interactions at the negatively charged cell membrane to permit efficient uptake of a biological product or biomolecule by the cell. Cationic lipids, dendrimers, or polymers can either be bound to a biological product or biomolecule, or induced to form a vesicle or micelle (see e.g., Kim SH., et al (2008) *Journal of Controlled Release* 129(2):107-116) that encases a biological product or biomolecule. The formation of vesicles or micelles further prevents degradation of the biological product or biomolecule when administered systemically.

Methods for making and administering cationic biological product or biomolecule complexes are well within the abilities of one skilled in the art (see e.g., Sorensen, DR., et al (2003) *J. Mol. Biol* 327:761-766; Verma, UN., et al (2003) *Clin. Cancer Res.* 9:1291-1300; Arnold, AS et al (2007) *J. Hypertens.* 25:197-205, which are incorporated herein by reference in their entirety). Some non-limiting examples of drug delivery systems useful for systemic delivery of biological product or biomolecule include DOTAP (Sorensen, DR., et al (2003), *supra*; Verma, UN., et al (2003), *supra*), Oligofectamine, "solid nucleic acid lipid particles" (Zimmermann, TS., et al (2006) *Nature* 441:111-114),

cardiolipin (Chien, PY., et al (2005) Cancer Gene Ther. 12:321-328; Pal, A., et al (2005) Int J. Oncol. 26:1087-1091), polyethyleneimine (Bonnet ME., et al (2008) Pharm. Res. Aug 16 Epub ahead of print; Aigner, A. (2006) J. Biomed. Biotechnol. 71659), Arg-Gly-Asp (RGD) peptides (Liu, S. (2006) Mol. Pharm. 3:472-487), and polyamidoamines (Tomalia, DA., et al (2007) Biochem. Soc. Trans. 35:61-67; Yoo, H., et al (1999) Pharm. Res. 16:1799-1804). In some embodiments, a biological product or biomolecule forms a complex with cyclodextrin for systemic administration. Methods for administration and pharmaceutical compositions of a biological product or biomolecule and cyclodextrins can be found in U.S. Patent No. 7, 427, 605, which is herein incorporated by reference in its entirety.

Methods of direct delivery are disclosed in PCT/US2007/079203 filed September 21, 2007 (Applicant docket number ALE-027) incorporated by reference herein in its entirety. These methods are useful in the present invention. Disclosed in PCT/US09/38437 filed 26-Mar-2009 (Applicant docket number ALN-062) and USSN 12/591,629 25-Nov-2009 (Applicant docket number ALN-080) are further methods of delivery useful in the present invention. Each of these documents is incorporated by reference herein in its entirety.

VI. Kits and Assays

Any of the compositions described herein may be comprised in a kit. In a non-limiting example, reagents for generating targeting constructs, including iRNA agents and specifically siRNA molecules are included in a kit. The kit may further include reagents or instructions for creating or synthesizing the targeting construct. It may also include one or more buffers, such as a nuclease buffer, transcription buffer, or a hybridization buffer, compounds for preparing the DNA template or a dsRNA, and components for isolating the resultant template, dsRNA, or siRNA. Other kits of the invention may include components for making a nucleic acid array comprising iRNA agents, e.g., siRNA, and thus, may include, for example, a solid support.

The components of the kits may be packaged either in aqueous media or in lyophilized form. The container means of the kits will generally include at least one vial,

test tube, flask, bottle, syringe or other container means, into which a component may be placed, and preferably, suitably aliquoted. Where there are more than one component in the kit (labeling reagent and label may be packaged together), the kit also will generally contain a second, third or other additional container into which the additional components
5 may be separately placed. However, various combinations of components may be comprised in a vial. The kits of the present invention also will typically include a means for containing the targeting constructs, e.g., nucleic acids, and any other reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained.

10 When the components of the kit are provided in one and/or more liquid solutions, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred. However, the components of the kit may be provided as dried powder(s). When reagents and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent
15 may also be provided in another container means. In some embodiments, labeling dyes are provided as a dried power. It is contemplated that 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 120, 130, 140, 150, 160, 170, 180, 190, 200, 300, 400, 500, 600, 700, 800, 900, 1000 micrograms or at least or at most those amounts of dried dye are provided in kits of the invention. The dye may then be resuspended in any suitable solvent, such as DMSO.

20 The container means will generally include at least one vial, test tube, flask, bottle, syringe and/or other container means, into which the targeting construct, e.g., nucleic acid formulations are placed, preferably, suitably allocated. The kits may also comprise a second container means for containing a sterile, pharmaceutically acceptable buffer and/or other diluent.

25 The kits of the present invention may also typically include a means for containing the vials in close confinement for commercial sale, such as, e.g., injection and/or blow-molded plastic containers into which the desired vials are retained.

Kits may also include components that facilitate isolation of a DNA template, long dsRNA, or iRNA agent, e.g., siRNA. It may also include components that preserve
30 or maintain the nucleic acids or that protect against their degradation. Such components

may be RNase-free or protect against RNases, such as RNase inhibitors. Such kits generally will comprise, in suitable means, distinct containers for each individual reagent or solution.

5 A kit can include instructions for employing the kit components as well the use of any other reagent not included in the kit. Instructions may include variations that can be implemented.

10 In some embodiments, kits are provided for testing the effect of a targeting construct or a series of targeting constructs on the production of a biological product by the cell, where the kits comprise a substrate having one or more assay surfaces suitable for culturing cells under conditions that allow production of a biological product. In some
10 embodiments, the exterior of the substrate comprises wells, indentations, demarcations, or the like at positions corresponding to the assay surfaces. In some embodiments, the wells, indentations, demarcations, or the like retain fluid, such as cell culture media, over the assay surfaces.

15 In some embodiments, the assay surfaces on the substrate are sterile and are suitable for culturing host cells (e.g., avian host cells) under conditions representative of the culture conditions during large-scale (e.g., industrial scale) production of the biological product. Advantageously, kits provided herein offer a rapid, cost-effective means for testing a wide-range of agents and/or conditions on the production of a
20 biological product, allowing the cell culture conditions to be established prior to full-scale production of the biological product.

25 In some embodiments, one or more assay surfaces of the substrate comprise a concentrated test agent, such as a targeting construct, such that the addition of suitable media to the assay surfaces results in a desired concentration of the targeting construct surrounding the assay surface. In some embodiments, the targeting construct may be printed or ingrained onto the assay surface, or provided in a lyophilized form, e.g., within wells, such that the effector molecules can be reconstituted upon addition of an appropriate amount of media. In some embodiments, the targeting constructs are reconstituted by plating cells onto assay surfaces of the substrate.

In some embodiments, kits provided herein further comprise cell culture media suitable for culturing a cell under conditions allowing for the production of a biological product and/or biomolecule of interest. The media can be in a ready to use form or can be concentrated (e.g., as a stock solution), lyophilized, or provided in another reconstitutable form.

In further embodiments, kits provided herein further comprise one or more reagents suitable for detecting production of the biological product or biomolecule by the cell, cell culture, or tissue culture. In further embodiments, the reagent(s) are suitable for detecting a property of the cell, such as maximum cell density, cell viability, or the like, which is indicative of production of the desired biological product. In some embodiments, the reagent(s) are suitable for detecting the biological product or a property thereof, such as the *in vitro* or *in vivo* biological activity, homogeneity, or structure of the biological product or biomolecule.

In some embodiments, one or more assay surfaces of the substrate further comprise a carrier for which facilitates uptake of targeting constructs by cells. Carriers for targeting constructs are known in the art and are described herein. For example, in some embodiments, the carrier is a lipid formulation such as LIPOFECTAMINETM transfection reagent (Invitrogen; Carlsbad, CA) or a related formulation. Examples of such carrier formulations are described herein. In some embodiments, the reagent that facilitates targeting construct uptake comprises a charged lipid, an emulsion, a liposome, a cationic or non-cationic lipid, an anionic lipid, a transfection reagent or a penetration enhancer as described throughout the application herein. In particular embodiments, the reagent that facilitates targeting construct uptake comprises a charged lipid as described in U.S. Application Ser. No. 61/267,419, filed on December 7, 2009.

In some embodiments, one or more assay surfaces of the substrate comprise a targeting construct or series of targeting constructs and a carrier, each in concentrated form, such that plating test cells onto the assay surface(s) results in a concentration the targeting construct(s) and the carrier effective for facilitating uptake of the targeting construct (s) by the cells and modulation of the expression of one or more genes targeted by the targeting constructs.

In some embodiments, the substrate further comprises a matrix which facilitates 3-dimensional cell growth and/or production of the biological product by the cells. In further embodiments, the matrix facilitates anchorage-dependent growth of cells. Non-limiting examples of matrix materials suitable for use with various kits described
5 herein include agar, agarose, methylcellulose, alginate hydrogel (e.g., 5% alginate + 5% collagen type I), chitosan, hydroactive hydrocolloid polymer gels, polyvinyl alcohol-hydrogel (PVA-H), polylactide-co-glycolide (PLGA), collagen vitrigel, PHEMA (poly(2-hydroxylmethacrylate)) hydrogels, PVP/PEO hydrogels, BD PURAMATRIX™ hydrogels, and copolymers of 2-methacryloyloxyethyl phosphorylcholine (MPC).

10 In some embodiments, the substrate comprises a microarray plate, a biochip, or the like which allows for the high-throughput, automated testing of a range of test agents, conditions, and/or combinations thereof on the production of a biological product or biomolecule by cultured cells. For example, the substrate may comprise a 2-dimensional microarray plate or biochip having m columns and n rows of assay surfaces (e.g., residing
15 within wells) which allow for the testing of m x n combinations of test agents and/or conditions (e.g., on a 24-, 96- or 384-well microarray plate). The microarray substrates are preferably designed such that all necessary positive and negative controls can be carried out in parallel with testing of the agents and/or conditions.

In further embodiments, kits are provided comprising one or more microarray
20 substrates seeded with a set of targeting constructs designed to modulate a particular pathway, function, or property of a cell which affects the production of the biological product or biomolecule. For example, in some embodiments, the targeting constructs are directed against target genes comprising a pathway involved in the expression, folding, secretion, or post-translational modification of a recombinant protein product by the cell.

25 In further embodiments, kits are provided herein comprising one or more microarray substrates seeded with a set of targeting constructs designed to address a particular problem or class of problems associated with the production of an immunogenic agent in cell-based systems. For example, in some embodiments, the targeting constructs are directed against target genes expressed by latent or endogenous
30 viruses; or involved in cell processes, such as cell cycle progression, cell metabolism or

apoptosis which inhibit or interfere production or purification of the biological product.

In further embodiments, the targeting constructs are directed against target genes that mediate enzymatic degradation, aggregation, misfolding, or other processes that reduce the activity, homogeneity, stability, and/or other qualities of the biological product. In yet

5 further embodiments, the targeting constructs are directed against target genes that affect the infectivity of exogenous or adventitious contaminating microbes. In one embodiment, the biological product or biomolecule includes a glycoprotein, and the targeting constructs are directed against target genes involved in glycosylation (e.g., fucosylation) and/or proteolytic processing of glycoproteins by the host cell. In another embodiment, 10 the biological product is a multi-subunit recombinant protein and the targeting constructs are directed against target genes involved in the folding and/or secretion of the protein by the host cell. In another embodiment, the targeting constructs are directed against target genes involved in post-translation modification of the biological product in the cells, such as methionine oxidation, glycosylation, disulfide bond formation, pyroglutamation and/or 15 protein deamidation. In another embodiment, the targeting constructs are directed against avian transcripts involved in any one or more of the functions outlined in Table 5.

In some embodiments, kits provided herein allow for the selection or optimization of at least one factor for enhancing production of the biological product or biomolecule.

For example, the kits may allow for the selection of a targeting construct from among a series of candidate targeting constructs, or for the selection of a concentration or 20 concentration range from a wider range of concentrations of a given targeting construct.

In some embodiments, the kits allow for selection of one or more targeting constructs from a series of candidate targeting constructs directed against a common target gene. In further embodiments, the kits allow for selection of one or more targeting constructs from 25 a series of candidate targeting constructs directed against two or more functionally related target genes or two or more target genes of a common host cell pathway.

In some embodiments, kits provided herein allow for the selection or optimization of a combination of two or more factors in the production of a biological product or biomolecule. For example, the kits may allow for the selection of a suitable targeting 30 construct from among a series of candidate targeting constructs as well as a concentration

of the targeting construct. In further embodiments, kits provided herein allow for the selection of a first targeting construct from a first series of candidate targeting constructs and a second targeting construct from a second series of candidate targeting constructs. In some embodiments, the first and/or second series of candidate targeting constructs are directed against a common target gene. In further embodiments, the first and/or second series of targeting constructs are directed against two or more functionally related target genes or two or more target genes of a common host cell pathway.

In another embodiment, a kit for enhancing production of a biological product in a cell, comprising at least a first targeting construct, a portion of which is complementary to at least a first target gene of a latent or endogenous virus; a second targeting construct, a portion of which is complementary to at least a second target gene of the cellular immune response; and, optionally, a third targeting construct, a portion of which is complementary to at least a third target gene of a cellular process. The kit can further comprise at least additional targeting construct that targets a cellular process including, but not limited to, carbon metabolism and transport, apoptosis, RNAi uptake and/or efficiency, reactive oxygen species production, cell cycle control, protein folding, pyroglutamation protein modification, deamidase, glycosylation, disulfide bond formation, protein secretion, gene amplification, viral replication, viral infection, viral particle release, control of cellular pH, and protein production.

VII. Bioprocessing

In one embodiment of the invention are methods for producing a biological product or biomolecule in an avian host cell by contacting the cell with a targeting construct (e.g., iRNA agent) capable of modulating expression of an avian transcript, wherein the modulation enhances production of the biological product or biomolecule. According to the present invention, bioprocessing methods may be improved by targeting (using one or more of the targeting constructs of the present invention) avian genes or transcripts expressed endogenously or expressed as a result of engineering the host cells to express said targets. They may also be improved by supplementing, replacing or adding one or more avian transcripts or transcript variants or avian products.

Bioprocessing methods of using targeting constructs (e.g., siRNA, miRNA, dsRNA, saRNA, shRNA, piRNA, tkRNAi, eiRNA, pdRNA, a gapmer, an antagomir, or a ribozyme, etc.) are disclosed in co-owned applications 61/223,370, filed July 6, 2009, entitled COMPOSITIONS AND METHODS FOR ENHANCING PRODUCTION OF A BIOLOGICAL PRODUCT by Maraganore et al.; U.S. Provisional Patent Application No. 61/244,868 filed September 22, 2009, entitled COMPOSITIONS AND METHODS FOR ENHANCING PRODUCTION OF A BIOLOGICAL PRODUCT, by Maraganore et al.; U.S. Provisional Patent Application No. 61/293,980, filed January 11, 2010, entitled COMPOSITIONS AND METHODS FOR ENHANCING PRODUCTION OF A BIOLOGICAL PRODUCT, by Rossomando et al.; U.S. Provisional Patent Application No. 61/319,589, filed March 31, 2010, entitled CELL-BASED BIOPROCESSING by Rossomando et al; each of which is incorporated fully herein by reference. These methods may be employed in the process of targeting avian transcripts to improve bioprocessing.

As such, methods of practicing bioprocessing including enhancing bioprocessing by controlling host cell immune responses, improving host cell viability, improving post-translational processing taught in the aforementioned commonly owned applications are also applicable to the avian systems described herein. Likewise, the methods for reducing bicontamination by endogenous, exogenous, latent and adventitious viruses are also applicable here.

The phrase “genome information” as used herein and throughout the claims and specification is meant to refer to sequence information from partial or entire genome of an organism, including protein coding and non-coding regions. These sequences are present every cell originating from the same organisms. As opposed to the transcriptome sequence information, genome information comprises not only coding regions, but also, for example, intronic sequences, promoter sequences, silencer sequences and enhancer sequences. Thus, the “genome information” can refer to, for example an avian genome, a human genome, a mouse genome, a rat genome. One can use complete genome information or partial genome information to add an additional dimension to the database sequences to increase the potential targets to modify with a targeting construct.

The phrase “play a role” refers to any activity of a transcript or a protein in a molecular pathway known to a skilled artisan or identified elsewhere in this specification. Such pathways and cellular activities include, but are not limited to apoptosis, cell division, glycosylation, growth rate, a cellular productivity, a peak cell density, a sustained cell viability, a rate of ammonia production or consumption, or a rate of lactate production.

A “bioreactor”, as used herein, refers generally to any reaction vessel suitable for growing and maintaining host cells such that the host cells produce a biological product, and for recovering such biological product. Bioreactors described herein include cell culture systems of varying sizes, such as small culture flasks, Nunc multilayer cell factories, small high yield bioreactors (e.g., MiniPerm, INTEGRA-CELLine), spinner flasks, hollow fiber-WAVE bags (Wave Biotech, Tagelswangen, Switzerland), and industrial scale bioreactors. In some embodiments, the biological product is produced in a “large scale culture” bioreactor having a 1 L capacity or more, suitable for pharmaceutical or industrial scale production of biological products (e.g., a volume of at least 1 L, at least 2 L, at least 5 L, at least 10 L, at least 25 L, at least 50 L, at least 100 L, or more, inclusive), often including means of monitoring pH, glucose, lactate, temperature, and/or other bioprocess parameters. In one embodiment, a large scale culture is at least 1 L in volume.

In one embodiment, a large scale culture is at least 2 L in volume. In one embodiment, a large scale culture is at least 5 L in volume. In one embodiment, a large scale culture is at least 25 L in volume. In one embodiment, a large scale culture is at least 40 L in volume. In one embodiment, a large scale culture is at least 50 L in volume. In one embodiment, a large scale culture is at least 100 L in volume.

A “host cell”, as used herein, is any avian cell, cell culture, cellular biomass or avian tissue, capable of being grown and maintained in cell culture under conditions allowing for production and recovery of useful quantities of a biological product or biomolecule, as defined herein. A host cell can be a hybridoma derived from the combination of any avian cell with a yeast, insect, amphibian, fish, reptile, bird, mammal or human cell. Host cells can be unmodified cells or cell lines, or cell lines which have

been genetically modified (e.g., to facilitate production of a biological product). In some embodiments, the host cell is a cell line that has been modified to allow for growth under desired conditions, such as in serum-free media, in cell suspension culture, or in adherent cell culture.

5 In some embodiments, the host cells are suitable for growth in suspension cultures.

In some embodiments, the host cell is an attachment dependent cell which is grown and maintained in adherent culture. Examples of avian host cells useful in methods provided herein include avian cells from the superorders including both the

10 Palaeognathae and Neognathae. Orders of birds within the Palaeognathae include the Struthioniformes (ostriches, emus, kiwis) and Tinamiformes (tinamous) any of whose cells may encode avian transcripts as targets of the invention. The superorder Neognathae includes the orders *Anseriformes* (waterfowl), *Galliformes* (fowl), *Charadriiformes* (gulls, button-quails, plover), *Gaviiformes* (loons), *Podicipediformes*

15 (grebes), *Procellariiformes* (albatrosses, petrels), *Sphenisciformes* (penguins), *Pelecaniformes* (pelicans), *Phaethontiformes* (tropicbirds), *Ciconiiformes* (storks), *Cathartiformes* (New World vultures), *Phoenicopteriformes* (flamingos), *Falconiformes* (falcons, eagles, hawks), *Gruiformes* (cranes), *Pteroclidiformes* (sandgrouse), *Columbiformes* (doves and pigeons), *Psittaciformes* (parrots), *Cuculiformes* (cuckoos and

20 turacos), *Opisthocomiformes* (hoatzin), *Strigiformes* (owls), *Caprimulgiformes* (nightjars), *Apodiformes* (swifts and hummingbirds), *Coraciiformes* (kingfishers), *Piciformes* (woodpeckers), *Trogoniformes* (trogons), *Coliiformes* (mousebirds) and *Passeriformes* (passerines).

In one embodiment, the avian host cells are those of the Order *Anseriformes*

25 (waterfowl) and in particular those of ducks. Cells of ducks which may be used in the present invention include those from Abacot Ranger (also known as Streicher), Allier Duck (Blanc d'Allier), Ancona duck, Aylesbury Duck, Bali Duck, Black East Indian, Blue Swedish duck, Buff Orpington Duck, Call Duck, Challans, Cayuga Duck, Chara Chemballi Duck, Crested Duck, Danish Duck, Duclair, Dutch Hookbill

30 (kromsnaveleend), East Indie Duck, Forest Duck (Eend van Vorst), Gimbsheimer,

Golden Cascade, Gressingham Duck (Wild Mallard crossed with Pekin), Huttengem Duck, Indian Runner Duck, Magpie Duck, Majorcan Duck, Muscovy duck, Orpington duck, Pekin Duck (also known as Long Island duck), Rouen Duck, Saxony Duck, Semois, Silver Appleyard Duck, Silver Bantam Duck, Termonde Duck, Venetian Duck (Germanata Veneta), Welsh Harlequin Duck, Wood Duck and the Swedish Duck.

In one embodiment the cells are from the Pekin duck. Cells of the Pekin duck can be obtained from ATCC (Mannassas, VA; number CCL-141; *Anas platyrhynchos domesticus*). Other duck cells which may be used in the methods of the present invention include those disclosed in US Publications 20090081251, 20100062489 and 20100226912, each of which is incorporated herein in its entirety.

Host cells can be unmodified or genetically modified (e.g., a cell from a transgenic animal). For example, CEFs from transgenic chicken eggs can have one or more genes essential for the IFN pathway, e.g., interferon receptor, STAT1, etc., has been disrupted, i.e., is a “knockout.” See, e.g., Sang, 12 Trends Biotech. 415 (1994); Perry et al., 2 Transgenic Res. 125 (1993); Stern, 212 Curr Top Micro. Immunol. 195-206 (1996); Shuman, 47 Experientia 897 (1991). Also, the cell can be modified to allow for growth under desired conditions, e.g., incubation at 30°C.

In some embodiments, the host cell is an attachment dependent cell which is grown and maintained in adherent culture. In some embodiments, the host cell is contained in an egg, such as a fish, amphibian, or avian egg.

“Isolating biological product from the host cell” means at least one step in separating the biological product away from host cellular material, e.g., the host cell, host cell culture medium, host cellular biomass, or host tissue. Thus, isolating biological products that are secreted into, and ultimately harvested from, the host cell culture media are encompassed in the phrase “isolated from the host cell.” A useful quantity includes an amount, including an aliquot or sample, used to screen for or monitor production, including monitoring modulation of target gene expression.

The present invention provides for the production of biological product or biomolecules such as a polypeptide, a metabolite, a nutraceutical, a chemical intermediate, a biofuel, a food additive, an antibiotic (antiviral, antibacterial, antifungal,

etc), or an immunogenic agent. More specifically, a “biological product” can include any substance capable of being produced by a host cell and recovered in useful quantities, including but not limited to, polypeptides (e.g., glycoproteins, antibodies, peptide-based growth factors), carbohydrates, lipids, fatty acids, metabolites (e.g., polyketides, macrolides), and chemical intermediates. This also includes the term “biologics”, a preparation, such as a drug, a vaccine, or an antitoxin, that is synthesized from living organisms or their products, and used as a diagnostic, preventive, or therapeutic agent. Thus, biological products can be used for a wide range of applications, including as biotherapeutic agents, vaccines, research or diagnostic reagents, fermented foods, food additives, nutraceuticals, biofuels, industrial enzymes (e.g., glucoamylase, lipase), industrial chemicals (e.g., lactate, fumarate, glycerol, ethanol), in the fields of biodefense, biowarfare, environmental applications and the like.

In some embodiments, the biological product is a polypeptide. The polypeptide can be a recombinant polypeptide or a polypeptide endogenous to the host cell. In some embodiments, the polypeptide is a glycoprotein and the host cell is a mammalian cell. Non-limiting examples of polypeptides that can be produced according to methods provided herein include receptors, membrane proteins, cytokines, chemokines, hormones, enzymes, growth factors, growth factor receptors, antibodies, antibody derivatives and other immune effectors, interleukins, interferons, erythropoietin, integrins, soluble major histocompatibility complex antigens, binding proteins, transcription factors, translation factors, oncoproteins or proto-oncoproteins, muscle proteins, myeloproteins, neuroactive proteins, tumor growth suppressors, structural proteins, and blood proteins (e.g., thrombin, serum albumin, Factor VII, Factor VIII, Factor IX, Factor X, Protein C, von Willebrand factor, etc.). As used herein, a polypeptide encompasses glycoproteins or other polypeptides which has undergone post-translational modification, such as deamidation, glycation, and the like. In some embodiments, the biological product is an antibody (e.g., a monoclonal antibody). Monoclonal antibodies produced in mammalian host cells contain an N-linked glycosylation site on each heavy chain. The heavy chain glycans are typically complex structures with high levels of core fucosylation. The fucose residues attached via an α 1,6 linkage to the innermost N-acetylglucosamine (GlcNAc)

residues of the Fc region N-linked oligosaccharides are the most important carbohydrate structures for antibody activity. For example, non-fucosylated antibodies are associated with dramatically increased antibody-dependent cellular cytotoxicity (ADCC) activity. ADCC activity can also be assessed in vivo, e.g., in an animal model such as that disclosed in Clynes et al., 95 PNAS 652 56 (1998).

In other embodiments, the biological product or biomolecule is an immunogenic viral, bacterial, protozoan, or recombinant protein derived from an expression vector. An example approach for producing viral-based vaccines involves the use of attenuated live virus vaccines, which are capable of replication but are not pathogenic, and, therefore, provide lasting immunity and afford greater protection against disease. The conventional methods for producing attenuated viruses involve the chance isolation of host range mutants, many of which are temperature sensitive, e.g., the virus is passaged through unnatural hosts, and progeny viruses which are immunogenic, yet not pathogenic, are selected. Efficient vaccine production requires the growth of large quantities of virus produced in high yields from a host system. Different types of virus require different growth conditions in order to obtain acceptable yields. The host in which the virus is grown is therefore of great significance. As a function of the virus type, a virus can be grown in embryonated eggs, primary tissue culture cells, or in established cell lines.

Thus, in some embodiments of the present invention, the biological product or biomolecule is a viral product, for example, naturally occurring viral strains, variants or mutants; mutagenized viruses (e.g., generated by exposure to mutagens, repeated passages and/or passage in non-permissive hosts), reassortants (in the case of segmented viral genomes), and/or genetically engineered viruses (e.g., using the “reverse genetics” techniques) having the desired phenotype. The viruses of these embodiments can be attenuated; i.e., they are infectious and can replicate in vivo, but generate low titers resulting in subclinical levels of infection that are generally non pathogenic.

Additionally, the biological product or biomolecule of the present invention can be derived from an intracellular parasite for which a biological product can be enhanced using the compositions, cells, and/or methods of the present invention, e.g., using a targeting construct. For example, alternative embodiments of the present invention

provide for production of a bacterial immunogen in a eukaryotic avian cell. These bacteria include *Shigella flexneri*, *Listeria monocytogenes*, *Rickettsia tsutsugamushi*, *Rickettsia rickettsiae*, *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Legionella pneumophila*, *Chlamydia* ssp. Additional embodiments of the present invention provide
5 for production of a protozoan immunogen, in a eukaryotic cell. These protozoa include *Plasmodium falciparum*, *Trypanosoma cruzi*, and *Leishmania donovani*.

In some embodiments, the enhancement of production of a biological product is achieved by improving viability of the cells in culture. As used herein, the term “improving cell viability” refers to an increase in cell density (e.g., as assessed by a
10 Trypan Blue exclusion assay) or a decrease in apoptosis (e.g., as assessed using a TUNEL assay) of at least 10% in the presence of a targeting construct(s) compared to the cell density or apoptosis levels in the absence of such a treatment. In some embodiments, the increase in cell density or decrease in apoptosis in response to treatment with a targeting construct(s) is at least 20%, at least 30%, at least 40%, at least 50%, at least
15 60%, at least 70%, at least 80%, at least 90%, at least 95%, or even 100% compared to untreated cells. In some embodiments, the increase in cell density in response to treatment with a targeting construct(s) is at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, at least 50-fold, at least 100-fold, at least 1000-fold or higher than the cell density in the absence of the targeting construct(s).

20 As used herein, “immunogenic agent” refers to an agent used to stimulate the immune system of a subject, so that one or more functions of the immune system are increased and directed towards the immunogenic agent. An antigen or immunogen is intended to mean a molecule containing one or more epitopes that can stimulate a host immune system to make a secretory, humoral and/or cellular immune response specific to
25 that antigen. Immunogenic agents can be used in the production of antibodies, both isolated polyclonal antibodies and monoclonal antibodies, using techniques known in the art. Immunogenic agents include vaccines.

As used herein, “vaccine” refers to an agent used to stimulate the immune system of a subject so that protection is provided against an antigen not recognized as a self-
30 antigen by the subject’s immune system. Immunization refers to the process of inducing a

high level of antibody and/or cellular immune response in a subject, that is directed against a pathogen or antigen to which the organism has been exposed. Vaccines and immunogenic agents as used herein, refer to a subject's immune system: the anatomical features and mechanisms by which a subject produces antibodies and/or cellular immune responses against an antigenic material that invades the subject's cells or extra-cellular fluids. In the case of antibody production, the antibody so produced can belong to any of the immunological classes, such as immunoglobulins, A, D, E, G, or M. Vaccines that stimulate production of immunoglobulin A (IgA) are of interest, because IgA is the principal immunoglobulin of the secretory system in warm-blooded animals. Vaccines are likely to produce a broad range of other immune responses in addition to IgA formation, for example cellular and humoral immunity. Immune responses to antigens are well-studied and reported widely. See, e.g., Elgert, IMMUNOL. (Wiley Liss, Inc., 1996); Stites et al., BASIC & CLIN. IMMUNOL., (7th Ed., Appleton & Lange, 1991). By contrast, the phrase "immune response of the host cell" refers to the responses of unicellular host organisms to the presence of foreign bodies.

"Bioprocessing" as used herein is an exemplary process for the industrial-scale production of a biological product or biomolecule (e.g., a heterologous polypeptide) in cell culture (e.g., in a host cell), that typically includes the following steps: (a) inoculating host cells containing a transgene encoding the heterologous protein into a seed culture vessel containing cell culture medium and propagating the cells to reach a minimum threshold cross-seeding density; (b) transferring the propagated seed culture cells, or a portion thereof, to a large-scale bioreactor; (c) propagating the large-scale culture under conditions allowing for rapid growth and cell division until the cells reach a predetermined density; (d) maintaining the culture under conditions that disfavor continued cell growth and/or host cell division and facilitate expression of the heterologous protein.

Steps (a) to (c) of the above method generally comprise a "growth" phase, whereas step (d) generally comprises a "production" phase. In some embodiments, fed batch culture or continuous cell culture conditions are tailored to enhance growth and division of the host cells in the growth phase and to disfavor cell growth and/or division

and facilitate expression of the heterologous protein during the production phase. For example, in some embodiments, a heterologous protein is expressed at levels of about 1 mg/L, or about 2.5 mg/L, or about 5 mg/L, or about 1 g/L, or about 5 g/L, or about 15 g/L, or higher. The rate of cell growth and/or division can be modulated by varying culture conditions, such as temperature, pH, dissolved oxygen (dO₂) and the like. For example, suitable conditions for the growth phase can include a pH of between about pH 6.5 and pH 7.5, a temperature between about 30°C to 38°C, and a CO₂ between about 5% to 90% saturation. In some embodiments, the expression of a heterologous protein can be enhanced in the production phase by inducing a temperature shift to a lower culture temperature (e.g., from about 37°C to about 30°C), increasing the concentration of solutes in the cell culture medium, or adding a toxin (e.g., sodium butyrate) to the cell culture medium. In some embodiments, the expression of a heterologous protein can be enhanced in the production phase by inducing a temperature shift to about 28°C, e.g., to increase protein expression in the absence of cell division. A variety of additional protocols and conditions for enhancing growth and/or protein expression during the production phase are known in the art.

The host cells can be cultured in a stirred tank bioreactor system in a fed batch culture process in which the host cells and culture medium are supplied to the bioreactor initially and additional culture nutrients are fed, continuously or in discrete increments, throughout the cell culture process. The fed batch culture process can be semi-continuous, wherein periodically whole culture (including cells and medium) is removed and replaced by fresh medium. Alternatively, a simple batch culture process can be used in which all components for cell culturing (including the cells and culture medium) are supplied to the culturing vessel at the start of the process. A continuous perfusion process can also be used, in which the cells are immobilized in the culture, e.g., by filtration, encapsulation, anchoring to microcarriers, or the like, and the supernatant is continuously removed from the culturing vessel and replaced with fresh medium during the process.

In one embodiment, after the production phase the biological product or biomolecule is recovered from the cell culture medium using various methods known in the art. For example, recovering a secreted heterologous protein typically involves

removal of host cells and debris from the medium, for example, by centrifugation or filtration. In some cases, particularly if the biological product is a protein is not secreted, protein recovery can also be performed by lysing the cultured host cells, e.g., by mechanical shear, osmotic shock, or enzymatic treatment, to release the contents of the cells into the homogenate. The protein can then be separated from subcellular fragments, insoluble materials, and the like by differential centrifugation, filtration, affinity chromatography, hydrophobic interaction chromatography, ion-exchange chromatography, size exclusion chromatography, electrophoretic procedures (e.g., preparative isoelectric focusing (IEF)), ammonium sulfate precipitation, and the like.

Procedures for recovering and purifying particular types of proteins are known in the art.

In some embodiments, it is desirable to adapt cells to serum free media and adapt adherent cells to cell growth in suspension. In some embodiments, cells are adapted to grow in serum-free medium. In one aspect of the invention, adaptation of cells is facilitated by increasing cell plasticity by using a targeting construct that targets genes involved in control of plasticity.

Stem Cells

In one embodiment of the invention, avian directed agents (targeting constructs) find utility in the study, research, manipulation, production and application of stem cell-based technologies. According to the present invention, the differentiation status, survival, proliferation or regeneration of a stem cell or population of cells may be altered by the administration or introduction into a cell of a targeting construct. It is also contemplated that cellular populations may be affected or altered by the administration of a transcript which comprises an avian transcript or gene. It is also contemplated that cells may be contacted with a combination of one or more targeting constructs or avian transcripts to modify the differentiation status, survival, proliferation or regeneration of the cells.

Several protein coding genes have been identified in the art which affect the differentiation status, survival, proliferation or regeneration of stem cells or their precursors (See US Publication 20100166714 and 20060246446 which discloses genes

affecting cardiac stem cells each of which is incorporated herein by reference in its entirety).

In one embodiment of the invention are provided compositions comprising targeting constructs that function to regulate or modulate the expression of avian transcripts which in turn alter or modulate the protein coding genes which have been associated with differentiation status, survival, proliferation or regeneration of stem cells. For example, targeting constructs of the present invention may be used to target transcription factors known to alter the status of stem cell lineages. These targeting constructs may target the transcripton factors at the DNA, RNA or protein level.

Systems and methods for selecting targeting constructs

Based on the known Duck transcriptome, we have developed methods and systems for selecting targeting constructs to affect the cells through manipulating cellular processes, for example, to improve production of biomolecules in the cells.

Accordingly, the invention provides databases and system comprising and using the Duck transcriptome sequences and optionally also an organized compilation of the Duck transcriptome outlining at least one functional aspect of each of the transcript, such as the transcripts role in a particular cellular process or pathway, and the corresponding siRNAs to allow design and selection of targets and targeting constructs for optimization of biological processes, particularly in the Duck cells.

Functional aspects of transcripts relate to their role in, for example apoptosis, cell cycle, DNA amplification (DHFR), virus gene production, e.g., in the case of viral promoters that are used to drive biomolecule production in the cells, glycosylation, carbon metabolism, prooxidant enzymes, protein folding, methionine oxidation, protein pyroglutamation, disulfide bond formation, protein secretion, cell viability, specific productivity of cell, nutrient requirements, internal cell pH. Other cellular processes are known to a skilled artisan, and can be found, for example, at Gene Ontology database available through world wide web at geneontology.org.

Analogous systems useful for exploitation of the duck transcriptome are taught in for example U.S. Provisional Patent Application No. 61/354,932, filed June 15, 2010, entitled CHINESE HAMSTER OVARY (CHO) CELL TRANSCRIPTOME,

CORRESPONDING SIRNAS AND USES THEREOF, by Rossomando et al.; which is incorporated fully herein by reference. These methods may be employed in the process of targeting avian transcripts to improve bioprocessing.

The terms “system”, “computing device”, and “computer-based system” refer to the computer hardware, associated software, and data storage devices used to analyze the information of the present invention. In one embodiment, the computer-based systems of the present invention comprises one or more central processing units (e.g., CPU, PAL, PLA, PGA), input means (e.g., keyboard, cursor control device, touch screen), output means (e.g., computer display, printer) and data storage devices (e.g., RAM, ROM, volatile and non-volatile memory devices, hard disk drives, network attached storage, optical storage devices, magnetic storage devices, solid state storage devices). As such, any convenient computer-based system can be employed in the present invention. Further, the computing device can included an embedded system based on a combination computing hardware and associated software or firmware.

A “processor” includes any hardware and/or software combination which can perform the functions under program control. For example, any processor herein can be a programmable digital microprocessor such as available in the form of an embedded system, a programmable controller, mainframe, server or personal computer (desktop or portable). Where the processor is selectively programmable, suitable programs, software or firmware can be communicated from a remote location to the processor, or previously saved in a computer program product (such as a portable or fixed computer readable storage medium, whether magnetic, optical or solid state device based). For example, a magnetic medium or optical disk can store the program or operating instructions and can be read and transferred to each processor at its corresponding station.

“Computer readable medium” as used herein refers to any storage or transmission medium that participates in providing instructions and/or data to a computer for execution and/or processing. Examples of storage media include floppy disks, magnetic media (tape, disk), UBS, optical media (CD-ROM, DVD, Blu-Ray), solid state media, a hard disk drive, a RAM, a ROM or integrated circuit, a magneto-optical disk, or a computer readable card such as a PCMCIA card and the like, whether or not such devices are

internal or external to the computer. A file containing information can be “stored” on computer readable medium, where “storing” means recording information such that it is accessible and retrievable at a later date by a computer.

With respect to computer readable media, “permanent memory” or “non-volatile memory” refers to memory that is permanently stored on a data storage medium. Permanent memory is not erased by termination of the electrical supply to a computer or processor. A computer hard-drive, ROM, CD-ROM, floppy disk and DVD are all examples of permanent memory. Random Access Memory (RAM) is an example of non-permanent or volatile memory.

To “record” or “store” data, programming or other information on a computer readable medium refers to a process for storing information, using any convenient method. Any convenient data storage structure can be chosen, based on the means used to access the stored information.

A “memory” or “memory unit” refers to any device which can store information for subsequent retrieval by a processor, and can include magnetic or optical devices (such as a hard disk, floppy disk, CD, or DVD), or solid state memory devices (such as volatile or non-volatile RAM). A memory or memory unit can have more than one physical memory device of the same or different types (for example, a memory can have multiple memory devices such as multiple hard drives or multiple solid state memory devices or some combination of hard drives and solid state memory devices)

Accordingly, the present invention provides a system for selecting a sequence of at least one targeting construct suitable for modulating protein expression in a cell, the system comprising: a computing device, having a processor and associated memory, and a database comprising at least one cell transcriptome information, the information comprising, a sequence for each transcript of the transcriptome, and optionally, a name of the transcript, and a pathway the transcript plays a role; and at least one targeting construct information, the information comprising at least the sequence of the targeting construct and optionally target specificity of the targeting construct, wherein each targeting construct is designed to match at least one or more sequences in the at least one cell transcriptome; a computer program, stored in memory, executed by the computing

device and configured to receive from a user via a user input device, parameters comprising a cell type selection, a target organism selection, a cellular pathway selection, a cross-reactivity selection, a target gene name and/or sequence selection, and optionally a method of delivery selection comprising either in vivo or in vitro delivery options; and
5 further optionally user address information; a first module configured to check the parameters against the sequences in the database for a matching combination of the parameters and transcriptome transcript sequences; and a second module to display a selected sequence of at least one targeting construct suitable for modulating protein expression in the cell.

10 The computing device and associated programs stored in memory can be adapted and configured to provide a user interface, such as a graphical user interface which allows the user to input search target parameters, for example, using one or more drop down menus or structured or free form text input, and selects the appropriate parameters for finding an appropriate target in the desired cell. For example, if a user wishes to find a target for
15 modulating carbon metabolism in an Avian cell, the user identifies the target cell as “Avian” e.g, chicken, duck, etc, and pathway as “carbon metabolism”, and the server performs a search through the database that would identify, e.g., transcripts for Gluts, PTEN and LDH genes and matches them with the appropriate siRNA molecules from the siRNA database part. This output information can be presented to the user on a computer
20 display or other output device, such as a printer.

The system can be a stand alone system or an internet-based system, wherein the computations and selection of targeting constructs is performed in same or different locations. The transcriptome information can be stored in database and accessed by computing device. As used herein, the term database includes any organization of data
25 regardless of whether it is structured or unstructured that allows retrieval of the information requested. The database can be a flat file or set of flat files stored in memory, one or more tables stored in memory, a set of discrete data elements stored in memory. The database can also include any well known database program that allows a user to directly or indirectly (through another program) access the data. Examples of
30 these include Microsoft Access, Oracle database, and MySQL.

In an alternative embodiment the system can be a network based system.

One can also provide a system by selling software to be run by a computer, wherein the databases and algorithms matching the parameters with sequence information and other information are provided to the user. The user may then either synthesize the
5 targeting constructs or separately order them from a third party provider.

In some embodiments, the system further comprises a storage module for storing the at least one targeting construct in a container, wherein if there are two or more targeting constructs, each targeting construct is stored in a separate container, and a robotic handling module, which upon selection of the matching combination, selects a
10 matching container, and optionally adds to the container additives based on a user selection for in vivo or in vitro delivery, and optionally further packages the container comprising the matching targeting construct to be sent to the user address.

The storage module can be a refrigerated module linked to the system components. The system may also be linked to a nucleic acid or other biomolecule
15 synthesizer.

The robotic handling module can be any system that can retrieve, and optionally mix components from the storage module, and or the biomolecule synthesizer, and optionally package the container(s). The robotic handling module can comprise one or more parts functioning based upon the commands from the system. The robotic handling
20 module may be in the same or different location as where the computations are performed.

In some embodiments, the system further comprises genome information of the cell, wherein by a user selection, the targeting constructs can be matched to target genomic sequences, comprising promoters, enhancers, introns and exons present in the genome.
25

VIII. Business Methods

Given the broad application of improved bioprocessing methods in avian cells, the present invention may also be used in commerce in other fields. Provided herein are improved methods of protein production (including enzymes and antibodies) which can
30 be exploited in the marketplace to alter current distribution, storage and chemical

processing methods. These methods provide alternatives to current economic paradigms around manufacture, transport and distribution chains in the areas of not only biological processes (including food production) but those of crop science, electronics and textiles.

- In one embodiment is a a method of doing a business for the production of a
- 5 biological product, comprising providing a service for the rapid production of a biological product from an avian host cell using one or more iRNA targeting constructs of the present invention and assessing the efficacy of the biological product. In this embodiment, the biological product may be useful in the treatment of one or more infections and assessment of efficacy may be against one or more infective agents
- 10 suspected of causing the infection. To be clear, the infection being targeted may be that of a human subject or may be the infection of a separate cell culture or batch or lot of cells. The resultant effective biological product may then be provided to a consumer or customer where the end result may be the treatment of an animal subject or the treatment of a culture of cells.
- 15 Further, the biological products made by the processes of the present invention or via the utilization of the targeting constructs of the present invention may be useful in the manufacture of textiles, in the chemicals industry and/or in the manufacture, cleaning or production of electronic devices or components. To this end the biological products may be enzymes that find utility in the above-mentioned processes or structural proteins
- 20 likewise useful.

EXAMPLES**Example 1. Identification of Avian Transcripts (ATs)**

Duck transcriptome

5 The duck (*Anas platyrhynchos*) transcriptome was obtained from Ensembl
(www.ensembl.org) and was supplemented with novel transcripts and miRNAs from
internal sequencing of the CCL-141 cell line. The Avian Transcripts (SEQ ID NOs 1-
18039) are listed in Lengthy Table 1 which is incorporated herein.

10 Certain avian genes of the present invention were identified from the Ensembl
database (www.ensembl.org) and their respective RNA transcripts were extracted (SEQ
ID 1-17169, having transcript names ENSAPLT000000000001 through
ENSAPLT00000017169). Other microRNA avian transcripts were identified via internal
discovery efforts and these are also listed in Table 1 (SEQ ID NOs: 17170 through
18039). To be clear, Table 1 lists the Ensembl gene identifier of each avian transcript
15 with the prefix, ENSAPLT while internal identifiers have been given to transcripts
identified internally.

Example 2. Function of Avian Transcripts

20 Evaluation of the avian transcripts to determine function of each was conducted
by comparative analysis to publicly available databases using the transcript sequence
information. Table 5 lists the functional annotation for a subset of the transcripts listed in
Table 1. The avian transcripts are identified here by SEQ ID number.

Table 5. Molecular Function

Transcript SEQ ID	Predicted Molecular Function
11327	1-phosphatidylinositol-3-kinase activity;phosphotransferase activity, alcohol group as acceptor;inositol or phosphatidylinositol kinase activity;protein binding;phosphatidylinositol binding;binding
615	2 iron, 2 sulfur cluster binding;protein homodimerization activity;protein binding
6882	2-polyprenyl-6-methoxy-1,4-benzoquinone methyltransferase activity;methyltransferase activity
1135, 9920, 11273, 11279, 13394	3',5'-cyclic-nucleotide phosphodiesterase activity;catalytic activity
7953, 8928	3',5'-cyclic-nucleotide phosphodiesterase activity;catalytic activity;3',5'-

	cyclic-AMP phosphodiesterase activity
7672	3',5'-cyclic-nucleotide phosphodiesterase activity;catalytic activity;protein binding;3',5'-cyclic-GMP phosphodiesterase activity
2982	3'-5'-exoribonuclease activity;RNA binding;polyribonucleotide nucleotidyltransferase activity
14147	4-alpha-glucanotransferase activity;amylase activity;amylo-alpha-1,6-glucosidase activity;catalytic activity;protein binding;polyubiquitin binding;polysaccharide binding;glycogen debranching enzyme activity
12859	4-aminobutyrate transaminase activity;transaminase activity;pyridoxal phosphate binding;protein homodimerization activity;protein binding
7182	6-pyruvoyltetrahydropterin synthase activity;protein binding;identical protein binding;protein homodimerization activity;metal ion binding
2649	7S RNA binding
14151	acetylglucosaminyltransferase activity
11300	acetylglucosaminyltransferase activity;transferase activity;transferase activity, transferring glycosyl groups;protein xylosyltransferase activity
3388, 4395, 10637, 10640	acid phosphatase activity
11289	acid phosphatase activity;inositol hexakisphosphate 5-kinase activity;inositol 1,3,4,5,6-pentakisphosphate kinase activity;diphosphoinositol-pentakisphosphate kinase activity
8615, 13494	acid phosphatase activity;protein binding
2046, 2904, 11065	acid-amino acid ligase activity
16725	acid-amino acid ligase activity;binding
8429	acid-amino acid ligase activity;binding;protein binding;zinc ion binding;thyroid hormone receptor binding
2992	acid-amino acid ligase activity;phosphopantetheine binding
4165, 16094	acid-amino acid ligase activity;protein binding
13953	acid-amino acid ligase activity;protein binding;ligase activity
4472	acid-amino acid ligase activity;protein binding;ubiquitin-protein ligase activity;proline-rich region binding;protein domain specific binding;beta-2 adrenergic receptor binding;sodium channel inhibitor activity;RNA polymerase binding;ubiquitin binding;phosphoserine binding;phosphothreonine binding
10945	acid-amino acid ligase activity;protein binding;ubiquitin-protein ligase activity;R-SMAD binding;I-SMAD binding;activin binding
10322	acid-amino acid ligase activity;protein binding;ubiquitin-protein ligase activity;SMAD binding;identical protein binding
11093	acid-amino acid ligase activity;protein binding;ubiquitin-protein ligase activity;syntaxin binding
2489	acid-amino acid ligase activity;ubiquitin-protein ligase activity;metal ion binding;binding
15267	acid-amino acid ligase activity;ubiquitin-protein ligase activity;protein binding;transcription coactivator activity
11268, 16025	actin binding
6656	actin binding;binding

7700, 10122	actin binding;calcium ion binding;hydrolase activity, hydrolyzing O-glycosyl compounds;protein binding
1919	actin binding;oxidoreductase activity;transition metal ion binding
8712	actin binding;phospholipid binding;phosphatidylinositol binding;protein binding;clathrin binding
3709, 9624	actin binding;profilin binding
12597, 14230	actin binding;Rho GTPase binding;binding
4212	actin binding;sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;protein binding
13434	actin filament binding
5772	acyl-CoA binding
12754	acyl-CoA dehydrogenase activity;oxidoreductase activity, acting on the CH-CH group of donors
11099	acyl-CoA oxidase activity;oxidoreductase activity, acting on the CH-CH group of donors;acyl-CoA dehydrogenase activity
13545	acyl-CoA thioesterase activity
13401	acyl-CoA thioesterase activity;protein binding
5734	acylglycerol lipase activity;protein homodimerization activity
4643, 7450	acyltransferase activity
5746	acyltransferase activity;1-acylglycerol-3-phosphate O-acyltransferase activity
10248	acyltransferase activity;ATP binding;ATPase activity, coupled to transmembrane movement of substances
14940	acyltransferase activity;carnitine O-octanoyltransferase activity
6399	acyltransferase activity;DNA binding;catalytic activity;ATP binding;glycerol-3-phosphate O-acyltransferase activity
2280	acyltransferase activity;glycerol-3-phosphate O-acyltransferase activity
5656	acyltransferase activity;nucleotide binding;aminoacyl-tRNA ligase activity;ATP binding;protein binding;dihydrolipoyllysine-residue acetyltransferase activity
12923	acyltransferase activity;protein binding
9535	acyltransferase activity;transferase activity
16614	adenosylhomocysteinase activity
8290	adenosylhomocysteinase activity;protein binding
12422	adenyl-nucleotide exchange factor activity;protein homodimerization activity;chaperone binding;protein binding;unfolded protein binding
8271	alpha-1,3-mannosylglycoprotein 2-beta-N-acetylglucosaminyltransferase activity;beta-1,3-galactosyl-O-glycosyl-glycoprotein beta-1,3-N-acetylglucosaminyltransferase activity
598	alpha2-adrenergic receptor activity;protein homodimerization activity;protein heterodimerization activity;protein binding;alpha-2A adrenergic receptor binding;epinephrine binding;G-protein coupled receptor activity
8718	alpha-L-fucosidase activity
5646	alpha-mannosidase activity;catalytic activity;carbohydrate

	binding;mannosidase activity;heme binding
13425	alpha-tubulin binding;gamma-tubulin binding;protein binding
9514	amidophosphoribosyltransferase activity;transferase activity;transferase activity, transferring glycosyl groups;metal ion binding;iron-sulfur cluster binding;4 iron, 4 sulfur cluster binding
10911	aminoacylase activity;catalase activity;oxidoreductase activity, acting on peroxide as acceptor;heme binding;protein homodimerization activity;NADP+ or NADPH binding;peroxidase activity;iron ion binding;oxidoreductase activity;metal ion binding;protein binding
14316	aminoacyl-tRNA ligase activity;ATP binding
2999	aminoacyl-tRNA ligase activity;ATP binding;nucleotide binding;tryptophan-tRNA ligase activity;protein binding
1793	aminopeptidase activity
10689	aminopeptidase activity;manganese ion binding
14008	aminopeptidase activity;manganese ion binding;protein homodimerization activity;metalloaminopeptidase activity
2575	aminopeptidase activity;metalloexopeptidase activity
13584	antioxidant activity;oxidoreductase activity
8103	antioxidant activity;oxidoreductase activity;identical protein binding;protein kinase binding;caspase inhibitor activity;protein C-terminus binding;protein binding;kinase binding
8174	ARF GTPase activator activity;zinc ion binding
6100, 15849	ARF GTPase activator activity;zinc ion binding;GTP binding
7121	ARF GTPase activator activity;zinc ion binding;phosphatidylinositol-3,4,5-trisphosphate binding;protein binding;inositol 1,3,4,5 tetrakisphosphate binding
4105, 5937, 6919, 10164	ARF GTPase activator activity;zinc ion binding;protein binding
2885, 13887, 14713, 15655	ARF guanyl-nucleotide exchange factor activity
1894, 2771	ARF guanyl-nucleotide exchange factor activity;binding
10669	ARF guanyl-nucleotide exchange factor activity;binding;guanyl-nucleotide exchange factor activity
12766	ARF guanyl-nucleotide exchange factor activity;protein binding
3715	ARF guanyl-nucleotide exchange factor activity;protein binding;phosphatidylinositol-3,4,5-trisphosphate binding
7192	arginine-tRNA ligase activity;ATP binding;nucleotide binding;aminoacyl-tRNA ligase activity
1875, 6120, 14210	aspartic-type endopeptidase activity
14965	aspartic-type endopeptidase activity;endopeptidase activity;protein binding
12348	aspartic-type endopeptidase activity;protein binding;ATP binding;chromatin binding
4865	aspartic-type endopeptidase activity;zinc ion binding
8700, 11725, 15101	ATP binding
11162	ATP binding;5-formyltetrahydrofolate cyclo-ligase activity
12818	ATP binding;ATPase activity

4608, 13992	ATP binding;ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism;catalytic activity;ATPase activity;hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances
6379	ATP binding;ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism;catalytic activity;calcium-transporting ATPase activity;calcium ion transmembrane transporter activity;hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances;calmodulin binding;calcium ion binding;PDZ domain binding;protein C-terminus binding;protein binding;calcium-dependent ATPase activity
4952, 11254	ATP binding;ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism;catalytic activity;calcium-transporting ATPase activity;hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances;signal transducer activity;manganese-transporting ATPase activity;manganese ion binding;calcium ion binding
6909, 10171, 11797, 13669	ATP binding;ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism;catalytic activity;magnesium ion binding;phospholipid-translocating ATPase activity
8812	ATP binding;ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism;catalytic activity;magnesium ion binding;phospholipid-translocating ATPase activity;hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances
2472	ATP binding;ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism;catalytic activity;magnesium ion binding;phospholipid-translocating ATPase activity;phospholipid transporter activity
7640	ATP binding;ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism;catalytic activity;metal ion binding;copper ion binding;copper-exporting ATPase activity;metal ion transmembrane transporter activity;hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances
5291, 9238, 15455	ATP binding;ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism;magnesium ion binding;phospholipid-translocating ATPase activity
9982	ATP binding;ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism;magnesium ion binding;phospholipid-translocating ATPase activity;hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances
13459	ATP binding;ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism;queuine tRNA-ribosyltransferase activity;catalytic activity;calcium-transporting ATPase activity;calcium ion transmembrane transporter activity;hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances
8784, 11941	ATP binding;ATPase activity, coupled to transmembrane movement of substances
4044, 9186	ATP binding;ATPase activity, coupled to transmembrane movement of substances;ATPase activity;nucleotide binding;nucleoside-triphosphatase activity

15752	ATP binding;ATPase activity, coupled to transmembrane movement of substances;ATPase activity;nucleotide binding;nucleoside-triphosphatase activity;oxidoreductase activity
3667	ATP binding;ATPase activity, coupled to transmembrane movement of substances;ATPase activity;nucleotide binding;nucleoside-triphosphatase activity;phosphopantetheine binding
3541	ATP binding;ATPase activity, coupled to transmembrane movement of substances;ATPase activity;nucleotide binding;nucleoside-triphosphatase activity;protein homodimerization activity
2409	ATP binding;ATPase activity, coupled to transmembrane movement of substances;ATPase activity;nucleotide binding;nucleoside-triphosphatase activity;protein homodimerization activity;peptide-transporting ATPase activity;substrate-specific transmembrane transporter activity
14397	ATP binding;ATPase activity, coupled to transmembrane movement of substances;ATPase activity;nucleotide binding;nucleoside-triphosphatase activity;transporter activity;protein kinase activity
11575	ATP binding;ATPase activity, coupled to transmembrane movement of substances;efflux transmembrane transporter activity;heme-transporting ATPase activity;heme binding
3006, 6428, 7316, 7435	ATP binding;ATPase activity;nucleotide binding;nucleoside-triphosphatase activity
9239	ATP binding;ATPase activity;nucleotide binding;nucleoside-triphosphatase activity;phospholipid transporter activity;protein homodimerization activity;protein heterodimerization activity;glycoprotein transporter activity;sterol-transporting ATPase activity;toxin transporter activity;cholesterol transporter activity;ADP binding
14716	ATP binding;ATPase activity;nucleotide binding;nucleoside-triphosphatase activity;protein homodimerization activity;protein heterodimerization activity
4888	ATP binding;inositol pentakisphosphate 2-kinase activity
16040	ATP binding;kinase activity
4244	ATP binding;ligase activity, forming aminoacyl-tRNA and related compounds;nucleotide binding;protein binding;aminoacyl-tRNA editing activity
16711	ATP binding;mismatched DNA binding
11928	ATP binding;nucleic acid binding;zinc ion binding
7041, 7315, 15147	ATP binding;nucleobase, nucleoside, nucleotide kinase activity;adenylate kinase activity
8474	ATP binding;nucleobase, nucleoside, nucleotide kinase activity;cAMP-dependent protein kinase regulator activity;nucleotide binding;nucleoside-triphosphatase activity
9309	ATP binding;nucleotide binding;nucleoside-triphosphatase activity
16082	ATP binding;nucleotide binding;nucleoside-triphosphatase activity;4 iron, 4 sulfur cluster binding
7424	ATP binding;nucleotide binding;nucleoside-triphosphatase activity;binding;ATPase activity
3483	ATP binding;nucleotide binding;nucleoside-triphosphatase activity;binding;protein binding;syntaxin binding;ATPase activity, coupled

15946	ATP binding;phosphotransferase activity, alcohol group as acceptor;nucleotide binding;nucleoside-triphosphatase activity;kinase activity;protein binding
13917	ATP binding;receptor activity;transcription cofactor activity;transcription factor binding;transcription activator activity;transcription coactivator activity;protein binding;RNA polymerase II transcription mediator activity;ligand-dependent nuclear receptor binding;estrogen receptor binding;thyroid hormone receptor binding;peroxisome proliferator activated receptor binding;retinoic acid receptor binding;vitamin D receptor binding;DNA binding;nuclear hormone receptor binding;chromatin binding
13841	ATP binding;small protein activating enzyme activity;catalytic activity
5519, 10032	ATP binding;small protein activating enzyme activity;catalytic activity;ISG15 activating enzyme activity
9508	ATP binding;small protein activating enzyme activity;transporter activity;lipid binding;catalytic activity;protein binding;FAT10 activating enzyme activity
15271	ATP binding;zinc ion binding
15739	ATPase activator activity;protein binding;chaperone binding
6638, 7279	ATPase activity
15968	ATPase activity, coupled to transmembrane movement of substances;proton-transporting ATPase activity, rotational mechanism;protein binding
13387	ATPase binding
11190	ATP-dependent peptidase activity;nucleotide binding;nucleoside-triphosphatase activity;ATP binding;serine-type endopeptidase activity;ATPase activity;single-stranded RNA binding;sequence-specific DNA binding;protein binding;mitochondrial heavy strand promoter anti-sense binding;mitochondrial light strand promoter anti-sense binding;DNA polymerase binding;G-quadruplex DNA binding;ADP binding;single-stranded DNA binding
4017	ATP-dependent peptidase activity;protein binding
5595	beta-amyloid binding;protein binding;ATP binding
10961	beta-catenin binding;DNA binding;transcription factor binding;transcription coactivator activity;histone acetyltransferase activity;protein binding;acetyltransferase activity;acyltransferase activity
14897	beta-catenin binding;identical protein binding;protein binding
7602	beta-catenin binding;protein binding;armadillo repeat domain binding
9368	beta-tubulin binding;protein binding;single-stranded DNA binding
11267	beta-tubulin binding;Ran GTPase binding;Ran guanyl-nucleotide exchange factor activity
1033, 2724, 2884, 3338, 4061, 4890, 5763, 6284, 6719, 6769, 6785, 7419, 7653, 8085, 8199, 8332, 8453, 8869, 8892, 8966, 10000, 10143, 10603, 11082, 11122, 11124, 11234, 11792, 12039, 12931, 12978, 13282, 13978, 14398, 14817, 15469,	binding

15689	
2986, 5484, 8791, 8794, 8820, 10708	binding;DNA binding
14604	binding;methylated histone residue binding;protein binding
16600	binding;peptidase inhibitor activity
2764	binding;phosphotransferase activity, alcohol group as acceptor
3828, 4476, 4485, 6983, 7205, 7400, 7441, 8233, 8865, 10866, 11863, 13552, 14162, 14511, 15677, 17003, 17114	binding;protein binding
4014	binding;protein binding;acetylglucosaminyltransferase activity;protein N-acetylglucosaminyltransferase activity;enzyme activator activity
4118	binding;protein binding;chromatin binding
3036	binding;protein binding;enzyme binding
12698	binding;protein binding;kinesin binding
14854	binding;protein binding;phospholipase A2 activator activity
6448	binding;protein binding;protein domain specific binding;cation channel activity
7165	binding;protein binding;protein domain specific binding;RPTP-like protein binding;cadherin binding;receptor binding;protein phosphatase binding
5186	binding;protein binding;receptor activity
7790	binding;protein binding;sphingomyelin phosphodiesterase D activity
1277	binding;protein binding;structural molecule activity
12356	binding;protein complex binding;protein binding
4184	binding;protein transporter activity
13351	binding;structural constituent of ribosome;protein binding
4041	binding;TBP-class protein binding
16100	binding;transcription coactivator activity;protein binding;estrogen receptor binding
15777	binding;transforming growth factor beta receptor binding;SMAD binding;protein binding
5332	binding;translation repressor activity
13330	binding;ubiquitin-protein ligase activity;protein binding
3014	binding;zinc ion binding;hormone activity
2674, 2680, 2681	biotin binding;catalytic activity;ATP binding;ligase activity;enzyme binding;propionyl-CoA carboxylase activity
15781	calcium channel activity;ion channel activity;binding;phosphatidylinositol binding;inositol-1,4,5-trisphosphate receptor activity;calcium-release channel activity
16873	calcium channel activity;receptor binding;leak channel activity;gap junction hemi-channel activity

655, 2029, 2557, 2816, 3137, 4164, 4258, 4388, 4493, 5680, 5918, 6173, 6371, 7540, 7632, 7664, 7823, 7841, 8341, 8855, 9098, 9213, 9404, 10133, 11138, 11681, 12336, 12414, 13561, 14996, 15636, 16449, 16920, 17081, 17138	calcium ion binding
3688	calcium ion binding;acyltransferase activity;l-acylglycerophosphocholine O-acyltransferase activity
14655	calcium ion binding;ATP binding
7979	calcium ion binding;binding;enzyme inhibitor activity;extracellular matrix structural constituent
6009	calcium ion binding;binding;extracellular matrix structural constituent;protein binding
5850	calcium ion binding;calcium-dependent phospholipid binding
4653	calcium ion binding;calcium-dependent phospholipid binding;protein binding;calcium-dependent protein binding
10165, 10407	calcium ion binding;collagen binding;heparin binding;protein binding;heparan sulfate proteoglycan binding
1836	calcium ion binding;epidermal growth factor receptor binding;epidermal growth factor receptor activity;protein binding
6040	calcium ion binding;G-protein coupled receptor activity
7052	calcium ion binding;histone-lysine N-methyltransferase activity;protein binding
11486	calcium ion binding;integrin binding;phosphatidylethanolamine binding;phosphatidylserine binding
2912	calcium ion binding;L-glutamate transmembrane transporter activity;protein binding;L-aspartate transmembrane transporter activity
12751	calcium ion binding;low-density lipoprotein receptor activity;receptor binding;protein homodimerization activity;protein binding;coreceptor activity;toxin transporter activity;kinase inhibitor activity;Wnt-protein binding;apolipoprotein binding
5336	calcium ion binding;oxidoreductase activity;protein binding
1127, 1130, 1139, 2506, 5360, 6341, 7973, 10282, 11044, 11435, 11798, 13579, 14974, 15001, 16270	calcium ion binding;protein binding
6500	calcium ion binding;protein binding;actin binding
7588	calcium ion binding;protein binding;extracellular matrix binding
10710	calcium ion binding;protein binding;glycosaminoglycan binding
15816	calcium ion binding;protein binding;G-protein coupled receptor activity;secondary active sulfate transmembrane transporter activity;transporter activity;transmembrane receptor activity
9259	calcium ion binding;protein binding;Notch binding
9916	calcium ion binding;protein binding;nucleotide binding;aminoacyl-tRNA ligase activity;ATP binding
9806	calcium ion binding;protein binding;peptidase inhibitor activity;Notch binding

14011	calcium ion binding;protein binding;protein dimerization activity;protein homodimerization activity;calcium-dependent protein binding;binding, bridging
5490, 13409	calcium ion binding;protein C-terminus binding
9713	calcium ion binding;protein dimerization activity;protein heterodimerization activity;protein binding
13895	calcium ion binding;protein heterodimerization activity;identical protein binding
11844	calcium ion binding;protein homodimerization activity;identical protein binding;protein binding;RAGE receptor binding;calcium-dependent protein binding;S100 beta binding
14318	calcium ion binding;receptor agonist activity
16947	calcium ion binding;transcription factor binding
13593	calcium, potassium:sodium antiporter activity
16237	calcium:cation antiporter activity;calcium:sodium antiporter activity
13986	calcium-activated potassium channel activity
2011	calcium-dependent cysteine-type endopeptidase activity
11184	calcium-dependent protein binding
2349, 2919	calmodulin binding
13637	calmodulin binding;calcium-activated potassium channel activity
10200	calmodulin binding;calcium-activated potassium channel activity;DNA binding
15439, 16827	calmodulin binding;transcription regulator activity
7239, 8186	cAMP-dependent protein kinase regulator activity
6536	cAMP-dependent protein kinase regulator activity;protein binding
5789	carbonate dehydratase activity;zinc ion binding;protein binding
5799	carbon-carbon lyase activity;catalytic activity;citrate (pro-3S)-lyase activity
8231	carbon-nitrogen ligase activity, with glutamine as amido-N-donor;fatty acid amide hydrolase activity
7730	carbon-sulfur lyase activity
5043, 11428	carboxylesterase activity
5464	carboxy-lyase activity;pyridoxal phosphate binding;protein binding
15670	caspase inhibitor activity;protein binding
15652	catalase activity;protein binding
2411, 2779, 3257, 3910, 5180, 5242, 6275, 6887, 6888, 7120, 7329, 8381, 8798, 8814, 9230, 11239, 11454, 11862, 13573, 14825, 15784, 15825, 15867, 16118, 16775	catalytic activity
6034	catalytic activity;4 iron, 4 sulfur cluster binding;iron-sulfur cluster binding
8867	catalytic activity;4 iron, 4 sulfur cluster binding;transferase activity;iron-sulfur cluster binding
16414, 16579	catalytic activity;6-phosphofructo-2-kinase activity;ATP binding

4648	catalytic activity;6-phosphofructo-2-kinase activity;ATP binding;identical protein binding;protein binding
3357, 16650	catalytic activity;acetate-CoA ligase activity;AMP binding;protein binding
9292	catalytic activity;acyl carrier activity
10647	catalytic activity;ATP binding;protein binding
6403	catalytic activity;axon guidance receptor activity
14362	catalytic activity;biotin-[acetyl-CoA-carboxylase] ligase activity;biotin-[propionyl-CoA-carboxylase (ATP-hydrolyzing)] ligase activity;biotin binding;enzyme binding;biotin-protein ligase activity
3340	catalytic activity;branched-chain-amino-acid transaminase activity
13641, 13957	catalytic activity;coenzyme binding;3-beta-hydroxy-delta5-steroid dehydrogenase activity;dTDP-4-dehydrorhamnose reductase activity
14346	catalytic activity;coenzyme binding;3-beta-hydroxy-delta5-steroid dehydrogenase activity;dTDP-4-dehydrorhamnose reductase activity;protein binding
13195	catalytic activity;coenzyme binding;3-beta-hydroxy-delta5-steroid dehydrogenase activity;dTDP-4-dehydrorhamnose reductase activity;protein binding;methionine adenosyltransferase regulator activity
13900	catalytic activity;coenzyme binding;3-beta-hydroxy-delta5-steroid dehydrogenase activity;UDP-glucose 4-epimerase activity;dTDP-4-dehydrorhamnose reductase activity;protein homodimerization activity
8584	catalytic activity;coenzyme binding;binding
14446	catalytic activity;coenzyme binding;GDP-mannose 4,6-dehydratase activity;oxidoreductase activity
16759	catalytic activity;CTP synthase activity;protein binding
3992	catalytic activity;ferric-chelate reductase activity
10738	catalytic activity;flavin adenine dinucleotide binding;oxidoreductase activity;ATP binding;alkylglycerone-phosphate synthase activity
14846	catalytic activity;glutathione transferase activity;protein homodimerization activity;protein binding
8749	catalytic activity;glycerophosphodiester phosphodiesterase activity;carbohydrate binding;phosphoric diester hydrolase activity
5137	catalytic activity;hydrolase activity
12942	catalytic activity;long-chain fatty acid-CoA ligase activity
13353	catalytic activity;metal ion binding;4 iron, 4 sulfur cluster binding;iron-sulfur cluster binding
1974, 2236	catalytic activity;metalloendopeptidase activity;ubiquinol-cytochrome-c reductase activity;zinc ion binding;metal ion binding;protein binding
6581	catalytic activity;molybdenum ion binding;pyridoxal phosphate binding
3693	catalytic activity;phosphatidylinositol-5-phosphate binding;phosphatidylinositol-3-phosphate binding;phosphatidylinositol-4-phosphate binding
12242	catalytic activity;phosphoglycolate phosphatase activity;acireductone synthase activity;metal ion binding

6583	catalytic activity;phosphoprotein phosphatase activity;protein tyrosine phosphatase activity;protein binding;hydrolase activity
8599, 10318, 12700, 14551	catalytic activity;protein binding
13646	catalytic activity;protein binding;glycerone-phosphate O-acyltransferase activity;transferase activity;acyltransferase activity
8530, 9095	catalytic activity;protein binding;lipid binding;oxidoreductase activity;transferase activity;transferase activity, transferring acyl groups other than amino-acyl groups;sterol binding;propanoyl-CoA C-acyltransferase activity
3125	catalytic activity;protein binding;long-chain fatty acid-CoA ligase activity
8987	catalytic activity;protein binding;very long-chain fatty acid-CoA ligase activity;long-chain fatty acid-CoA ligase activity
8361	catalytic activity;protein kinase activity;ATP binding;3-oxoacyl-[acyl-carrier-protein] synthase activity
1226	catalytic activity;protein serine/threonine phosphatase activity
714	catalytic activity;pyridoxal phosphate binding
14150	catalytic activity;sphingosine-1-phosphate phosphatase activity
7775	catalytic activity;thiosulfate sulfurtransferase activity;protein binding;nucleotidyltransferase activity;URM1 activating enzyme activity
2549, 13243	catalytic activity;transcription factor binding
12187	catalytic activity;transcription factor binding;oxidoreductase activity;protein binding
15815	catalytic activity;transketolase activity;protein binding
7876	catalytic activity;transporter activity
12683	catalytic activity;UDP-glucose 6-dehydrogenase activity;binding;oxidoreductase activity;oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor;NAD ⁺ or NADH binding
10388	catalytic activity;zinc ion binding;protein binding
3027, 4577, 5414, 7961, 12562	cation transmembrane transporter activity
9618	cation transmembrane transporter activity;cytochrome-c oxidase activity;copper ion binding;heme binding;protein binding
8340	cation transmembrane transporter activity;nucleotide binding
4336	cation:chloride symporter activity;transporter activity
2711	ceramidase activity
2562	chloride channel activity
5171	chloride channel activity;protein binding
10207	cholesterol binding
10672	chondroitin-glucuronate 5-epimerase activity
6147	chromatin binding;catalytic activity
9701	chromatin binding;catalytic activity;protein binding
9440	chromatin binding;DNA binding;methylated histone residue binding

15718	chromatin binding;histone demethylase activity (H3-K36 specific)
8112	chromatin binding;methylated histone residue binding;ubiquitin-protein ligase activity;protein binding;single-stranded RNA binding
7833	chromatin binding;protein tyrosine kinase activity;protein binding;histone kinase activity;histone binding;histone acetyl-lysine binding;zinc ion binding;nucleotide binding;vitamin D receptor activator activity;protein complex scaffold;vitamin D receptor binding
4882	chromatin binding;sequence-specific DNA binding transcription factor activity;calcium ion binding;protein binding;chromatin DNA binding;sequence-specific DNA binding;receptor activity
15855	cobalamin binding
4251	copper ion binding
11542	copper ion binding;copper chaperone activity;enzyme activator activity
13915	copper ion binding;electron carrier activity;low-density lipoprotein binding
4862	copper ion binding;electron carrier activity;protein binding;enzyme binding
5585	copper ion binding;oxidoreductase activity
6534	copper ion transmembrane transporter activity
16478	cyanate hydratase activity;guanyl-nucleotide exchange factor activity;GTP binding;GTPase binding
13317	cyanate hydratase activity;guanyl-nucleotide exchange factor activity;GTP binding;GTPase binding;binding
13733	cyclin-dependent protein kinase regulator activity
3582	cyclin-dependent protein kinase regulator activity;protein binding;zinc ion binding
10386	cyclin-dependent protein kinase regulator activity;zinc ion binding;ubiquitin-protein ligase activity
11211, 13932, 14532	cysteine-type endopeptidase activity
8782, 8807	cysteine-type endopeptidase activity;calcium ion binding;protein binding;low-density lipoprotein receptor activity
3405, 3626, 3628, 3632, 9554, 10805	cysteine-type endopeptidase activity;calcium-dependent cysteine-type endopeptidase activity
9776, 15370	cysteine-type endopeptidase activity;cysteine-type peptidase activity
13972	cysteine-type endopeptidase activity;cysteine-type peptidase activity;peptidase activity;protein binding
6982	cysteine-type endopeptidase activity;cysteine-type peptidase activity;protein binding
905, 1146, 1151	cysteine-type endopeptidase activity;nucleotide binding;nucleoside-triphosphatase activity;phosphopantetheine binding;ATP binding;ATPase activity
8300, 15973	cysteine-type endopeptidase activity;peptidase activity
1020	cysteine-type endopeptidase activity;protein binding
9216	cysteine-type endopeptidase activity;protein binding;peptidase activity;cysteine-type peptidase activity;hydrolase activity
11229	cysteine-type endopeptidase activity;protein binding;zinc ion binding
3608	cysteine-type endopeptidase activity;receptor activity;binding

13870	cysteine-type endopeptidase activity;Rho guanyl-nucleotide exchange factor activity
4589	cysteine-type endopeptidase activity;sequence-specific DNA binding transcription factor activity;protein binding;DNA binding;chromatin binding
6117, 14988	cysteine-type peptidase activity
11148	cysteine-type peptidase activity;SUMO-specific protease activity
7552, 15599	cytochrome-c oxidase activity
2171	cytochrome-c oxidase activity;copper ion binding;heme binding;protein binding;structural constituent of nuclear pore
1795	cytokine activity;chemoattractant activity;protein binding
13021	cytokine activity;frizzled binding;protein homodimerization activity;cell surface binding
12637	cytokine receptor activity
8613	cytokine receptor activity;oncostatin-M receptor activity;growth factor binding
6414, 13079	cytoskeletal adaptor activity;SH3 domain binding;protein binding
10914	cytoskeletal protein binding
6818	damaged DNA binding;exodeoxyribonuclease III activity;protein binding;3'-5' exonuclease activity
14778	damaged DNA binding;single-stranded DNA binding;endodeoxyribonuclease activity;protein binding;protein C-terminus binding;structure-specific DNA binding;protein N-terminus binding;DNA binding;nuclease activity;nucleic acid binding;endonuclease activity
9300	delta-catenin binding;protein binding
4901	diacylglycerol kinase activity;electron carrier activity;iron-sulfur cluster binding;protein binding
11621	diacylglycerol kinase activity;protein binding;zinc ion binding
7622	diacylglycerol kinase activity;protein binding;zinc ion binding;calcium ion binding
10533	diacylglycerol kinase activity;protein binding;zinc ion binding;calcium ion binding;catalytic activity;flavin adenine dinucleotide binding
12608	diacylglycerol kinase activity;protein homodimerization activity;protein heterodimerization activity
8382	dipeptidase activity
6693, 7551	diphosphomevalonate decarboxylase activity;ATP binding;kinase activity;Hsp70 protein binding;protein homodimerization activity
15775	disulfide oxidoreductase activity
2316, 2335, 2404, 2802, 3307, 3364, 4363, 5072, 5542, 5778, 6894, 8408, 8492, 9147, 9409, 9975, 10097, 10488, 11308, 12121, 12438, 13111, 13800, 14719, 14790, 15877	DNA binding
11667	DNA binding;ATP binding;chromatin binding;nucleic acid binding;helicase activity;hydrolase activity

592, 2906	DNA binding;ATP binding;chromatin binding;nucleic acid binding;helicase activity;protein binding
8533	DNA binding;ATP binding;hydrolase activity
4820	DNA binding;ATP binding;nucleic acid binding;helicase activity
9672	DNA binding;ATP binding;nucleic acid binding;helicase activity;ATPase activity;protein binding;histone binding;transcription activator activity
10457	DNA binding;ATP binding;nucleic acid binding;helicase activity;ATP-dependent helicase activity;hydrolase activity;nucleotide binding;ATPase activity;protein binding
6373	DNA binding;ATP binding;nucleic acid binding;helicase activity;binding
2725	DNA binding;ATP binding;nucleic acid binding;helicase activity;chromatin binding
13756	DNA binding;ATP binding;nucleic acid binding;helicase activity;protein binding;DNA-dependent ATPase activity;transcription regulator activity
6696	DNA binding;ATP binding;nucleic acid binding;helicase activity;protein binding;zinc ion binding;catalytic activity;chromo shadow domain binding;chromatin binding
2302	DNA binding;ATP binding;nucleic acid binding;helicase activity;protein kinase binding;transcription cofactor activity
17049	DNA binding;ATP binding;nucleic acid binding;helicase activity;transcription elongation regulator activity;chromatin binding;protein complex binding;protein C-terminus binding;protein N-terminus binding;protein binding;DNA-dependent ATPase activity
10782	DNA binding;ATP binding;nucleic acid binding;helicase activity;zinc ion binding;protein binding
3586	DNA binding;ATP binding;nucleotide binding;nucleoside-triphosphatase activity;ATP-dependent DNA helicase activity;DNA helicase activity;protein binding
14869	DNA binding;ATP binding;protein binding;nucleic acid binding;helicase activity;zinc ion binding
13354	DNA binding;ATP-dependent DNA helicase activity;double-stranded telomeric DNA binding;telomeric DNA binding;protein C-terminus binding;promoter binding;protein binding;5'-deoxyribose-5-phosphate lyase activity
14867	DNA binding;beta-catenin binding;protein kinase binding;RNA polymerase II transcription factor activity;gamma-catenin binding;sequence-specific DNA binding transcription factor activity;transcription factor binding;sequence-specific DNA binding;protein binding;specific transcriptional repressor activity;nuclear hormone receptor binding
3001, 11266	DNA binding;binding
2773, 5013	DNA binding;catalytic activity;protein binding
16529	DNA binding;catalytic activity;protein binding;protein phosphatase 2A binding
16305	DNA binding;catalytic activity;zinc ion binding;hydrolase activity;DNA 5'-adenosine monophosphate hydrolase activity;metal ion binding

7930	DNA binding;chromatin binding;transcription coactivator activity;signal transducer activity;receptor binding;protein binding;transcription repressor activity;ligand-dependent nuclear receptor transcription coactivator activity;thyroid hormone receptor coactivator activity;transcription regulator activity
12879	DNA binding;DNA topoisomerase type I activity
5617	DNA binding;DNA topoisomerase type I activity;nucleotide binding;protein binding
8925	DNA binding;DNA-directed DNA polymerase activity;binding;protein binding;transcription repressor activity
10641	DNA binding;DNA-directed DNA polymerase activity;catalytic activity
6803	DNA binding;DNA-directed DNA polymerase activity;catalytic activity;microtubule binding;protein binding;lyase activity;enzyme binding
5527, 14548, 16059	DNA binding;DNA-directed RNA polymerase activity
4271	DNA binding;DNA-directed RNA polymerase activity;protein binding
2963	DNA binding;DNA-directed RNA polymerase activity;protein dimerization activity;protein binding
5624	DNA binding;DNA-directed RNA polymerase activity;protein dimerization activity;protein kinase activity;protein binding
8088	DNA binding;DNA-directed RNA polymerase activity;protein kinase activity
16800	DNA binding;DNA-directed RNA polymerase activity;transferase activity, transferring phosphorus-containing groups
14565	DNA binding;DNA-directed RNA polymerase activity;zinc ion binding;protein kinase activity
7712	DNA binding;double-stranded telomeric DNA binding;protein binding;protein C-terminus binding;telomeric DNA binding;protein homodimerization activity
7478	DNA binding;endonuclease activity;4 iron, 4 sulfur cluster binding;catalytic activity;hydrolase activity
15613	DNA binding;extracellular matrix binding
16977	DNA binding;heat shock protein binding;protein binding;chaperone binding
3652	DNA binding;nuclease activity;binding
12480	DNA binding;nucleic acid binding
5215	DNA binding;nucleic acid binding;protein binding
9100	DNA binding;nucleotide binding;protein binding;sequence-specific DNA binding;sequence-specific DNA binding transcription factor activity;protein heterodimerization activity;promoter binding
1613, 2117, 2166, 2235, 2267, 2314, 2323, 2339, 2340, 3315, 4005, 5688, 7488, 9902, 12726, 15823, 16277	DNA binding;protein binding
9080	DNA binding;protein binding;protein kinase binding
13651	DNA binding;protein binding;single-stranded DNA binding
11299	DNA binding;protein binding;zinc ion binding

3882	DNA binding;protein binding;zinc ion binding;calcium ion binding;histone-lysine N-methyltransferase activity;chromatin binding
3825	DNA binding;protein binding;zinc ion binding;histone methyltransferase activity (H3-K4 specific)
13648	DNA binding;protein binding;zinc ion binding;histone-lysine N-methyltransferase activity
1099	DNA binding;protein binding;zinc ion binding;transcription activator activity;chromatin binding
17158	DNA binding;sequence-specific DNA binding transcription factor activity;ATP binding
7822	DNA binding;sequence-specific DNA binding transcription factor activity;protein binding
3287	DNA binding;sequence-specific DNA binding transcription factor activity;protein binding;protein C-terminus binding;transcription regulator activity
11755	DNA binding;sequence-specific DNA binding transcription factor activity;protein binding;transcription activator activity;transcription regulator activity;sequence-specific DNA binding
9458	DNA binding;sequence-specific DNA binding transcription factor activity;protein binding;transcription activator activity;transcription repressor activity;sequence-specific DNA binding
8692	DNA binding;sequence-specific DNA binding transcription factor activity;protein binding;ubiquitin protein ligase binding
7845	DNA binding;sequence-specific DNA binding transcription factor activity;receptor activity;protein binding;zinc ion binding;sequence-specific DNA binding;metal ion binding;steroid hormone receptor activity
5314	DNA binding;sequence-specific DNA binding transcription factor activity;RNA polymerase II transcription factor activity;sequence-specific DNA binding
2787	DNA binding;sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding
7584	DNA binding;sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;transcription activator activity;protein binding
8546	DNA binding;sequence-specific DNA binding transcription factor activity;transcription regulator activity;sequence-specific DNA binding
11107	DNA binding;sequence-specific DNA binding transcription factor activity;transcription repressor activity;general transcriptional repressor activity;sequence-specific DNA binding
2384	DNA binding;sequence-specific DNA binding transcription factor activity;zinc ion binding;transcription repressor activity;enzyme binding;sequence-specific DNA binding;nucleic acid binding;transcription regulator activity;promoter binding
14466	DNA binding;signal transducer activity;endonuclease activity;zinc ion binding;protein binding;histone methyltransferase activity (H3-K27 specific)
11041	DNA binding;signal transducer activity;protein binding;histone methyltransferase activity;chromatin binding
11182	DNA binding;signal transducer activity;protein binding;transcription corepressor activity

6086	DNA binding;site-specific DNA-methyltransferase (cytosine-N4-specific) activity
7855	DNA binding;transcription corepressor activity;histone deacetylase binding;protein binding;sequence-specific DNA binding;sequence-specific DNA binding transcription factor activity;protein domain specific binding;transcription repressor activity;promoter binding;retinoic acid receptor binding;retinoid X receptor binding;thyroid hormone receptor binding;histone deacetylase regulator activity
6533	DNA binding;transcription factor binding;transcription activator activity;zinc ion binding;histone acetyltransferase activity;protein binding;acetyltransferase activity
4524	DNA binding;transcription regulator activity
2292	DNA binding;transcription regulator activity;nucleic acid binding
5714	DNA binding;transporter activity
15577	DNA binding;transporter activity;zinc ion binding
2442, 5214, 7382, 15420, 15700	DNA binding;zinc ion binding
11043	DNA binding;zinc ion binding;protein binding
12968	DNA binding;zinc ion binding;protein dimerization activity
6483, 6510	DNA binding;zinc ion binding;sequence-specific DNA binding transcription factor activity
10980	DNA binding;zinc ion binding;sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;protein binding;transcription activator activity
15521	DNA binding;zinc ion binding;sequence-specific DNA binding transcription factor activity;transcription factor binding
15369	DNA binding;zinc ion binding;signal transducer activity
4576	DNA-directed DNA polymerase activity;protease binding;protein binding;DNA binding;transferase activity;nucleotidyltransferase activity
5219	DNA-directed RNA polymerase activity
10220	DNA-directed RNA polymerase activity;nucleotide binding;catalytic activity;protein kinase activity
4542	dolichyl-diphosphooligosaccharide-protein glycotransferase activity;protein binding
10324	dopamine beta-monooxygenase activity
14722	dopamine receptor binding
4423	double-stranded RNA binding;protein binding
3500	double-stranded RNA binding;protein kinase inhibitor activity;protein binding;protein kinase binding
10096	double-stranded RNA binding;RNA binding;adenosine deaminase activity;double-stranded RNA adenosine deaminase activity
6027	electron carrier activity
12599	electron carrier activity;iron-sulfur cluster binding;dihydroorotate oxidase activity;dihydroorotate dehydrogenase activity
1743	electron carrier activity;iron-sulfur cluster binding;oxidoreductase activity;oxidoreductase activity, acting on NADH or NADPH;4 iron, 4 sulfur cluster binding;NADH dehydrogenase activity;NADH

	dehydrogenase (ubiquinone) activity
6168, 9638	electron carrier activity;protein disulfide oxidoreductase activity
13343	electron carrier activity;protein disulfide oxidoreductase activity;protein binding
17156	electron carrier activity;protein disulfide oxidoreductase activity;protein binding;DNA binding;transcription activator activity
7161	endodeoxyribonuclease activity, producing 5'-phosphomonoesters
15651	endopeptidase activity;serine-type endopeptidase activity;peptide binding;transmembrane receptor protein tyrosine kinase activity;ATP binding
15649	endopeptidase activity;serine-type endopeptidase activity;protein binding
11378	endopeptidase activity;threonine-type endopeptidase activity;RNA binding;NF-kappaB binding;protein binding
8257	endopeptidase inhibitor activity;protein binding
15153	endopeptidase inhibitor activity;protein binding;semaphorin receptor binding
9805	endopeptidase inhibitor activity;semaphorin receptor binding
14977	enzyme binding
16647	enzyme inhibitor activity;histone deacetylase binding;histone binding
17047	epoxide hydrolase activity
3146	ethanolamine kinase activity
9283	ethanolamine kinase activity;choline kinase activity;drug binding;protein homodimerization activity;cholinesterase activity
16526	eukaryotic initiation factor 4E binding;protein binding
3402	eukaryotic translation initiation factor 2alpha kinase activity
12460	exonuclease activity;nucleic acid binding
5222, 5338	exonuclease activity;nucleic acid binding;3'-5' exonuclease activity
2610, 2712, 16340	extracellular ligand-gated ion channel activity
16063	extracellular ligand-gated ion channel activity;benzodiazepine receptor activity
10558	extracellular ligand-gated ion channel activity;protein binding
14639	extracellular matrix binding
13437	extracellular matrix structural constituent;binding
13821	extracellular matrix structural constituent;binding;protein binding
10969	extracellular matrix structural constituent;protein binding;enzyme binding;glycosphingolipid binding;calcium ion binding;unfolded protein binding
3443	extracellular matrix structural constituent;protein binding;glycosphingolipid binding;calcium ion binding;signal transducer activity;unfolded protein binding
8658	extracellular matrix structural constituent;receptor binding;peptidase inhibitor activity;SMAD binding
13547	extracellular matrix structural constituent;receptor binding;structural molecule activity

6024	farnesyltransferase activity;geranyltransferase activity
9104	ferrous iron binding;DNA-N1-methyladenine dioxygenase activity
12183	fibronectin binding;receptor activity;protein binding;identical protein binding
14955	folic acid transporter activity;heme transporter activity
10984	formate-tetrahydrofolate ligase activity;ATP binding;catalytic activity;protein homodimerization activity;protein binding
12148	fructosamine-3-kinase activity
16265	GABA-A receptor activity;ion channel activity;extracellular ligand-gated ion channel activity
13916	galactokinase activity;ATP binding;kinase activity;galactose binding
3925	galactosylceramidase activity
15435	galactosylceramide sulfotransferase activity
1227	galactosyltransferase activity
747	galactosyltransferase activity;UDP-galactose:beta-N-acetylglucosamine beta-1,3-galactosyltransferase activity
3533	gamma-glutamylcyclotransferase activity;protein homodimerization activity
13980	gamma-glutamyltransferase activity
12633	general RNA polymerase II transcription factor activity
15361	general RNA polymerase II transcription factor activity;protein kinase activity;protein N-terminus binding;RNA polymerase II carboxy-terminal domain kinase activity;DNA-dependent ATPase activity
10272	glucosamine-6-phosphate deaminase activity
16438	glucuronyl-galactosyl-proteoglycan 4-alpha-N-acetylglucosaminyltransferase activity;alpha-1,4-N-acetylglucosaminyltransferase activity
14832	glutamate-ammonia ligase activity
3193	glutamate-cysteine ligase activity;hydrolase activity, hydrolyzing O-glycosyl compounds;magnesium ion binding;glutamate binding;coenzyme binding;ADP binding;protein heterodimerization activity
9462	glutathione peroxidase activity
16256	glutathione synthase activity;ATP binding;magnesium ion binding;protein homodimerization activity;glutathione binding
3288	glycerol kinase activity;phosphotransferase activity, alcohol group as acceptor
11123	glycerol-3-phosphate dehydrogenase activity;calcium ion binding;oxidoreductase activity
14417	glycerophosphodiester phosphodiesterase activity;phosphoric diester hydrolase activity
4025	glycine hydroxymethyltransferase activity;pyridoxal phosphate binding;protein homodimerization activity
16019	glycine hydroxymethyltransferase activity;transferase activity;transferase activity, transferring nitrogenous groups;pyridoxal phosphate binding;glycine C-acetyltransferase activity
16992	glycogen (starch) synthase activity

13718	glycosaminoglycan binding
10772, 14688	glycosaminoglycan binding;heparin binding
10320, 14452	glycylpeptide N-tetradecanoyltransferase activity
4012, 4800	GPI-anchor transamidase activity;protein binding
3124, 15499	G-protein coupled receptor activity
3462	G-protein coupled receptor activity;beta-amyloid binding
10562, 15074	G-protein coupled receptor activity;calcium channel regulator activity;group II metabotropic glutamate receptor activity
9316	G-protein coupled receptor activity;electron carrier activity;transmembrane receptor activity
14497	G-protein coupled receptor activity;group III metabotropic glutamate receptor activity
2592	G-protein coupled receptor activity;group III metabotropic glutamate receptor activity;calcium ion binding;adenylate cyclase inhibitor activity;glutamate binding;glutamate receptor activity;serine binding;calcium channel regulator activity
4659, 7586, 8978	G-protein coupled receptor activity;protein binding
9486	G-protein coupled receptor activity;protein binding;transmembrane receptor activity
2713, 3276, 17089	G-protein coupled receptor activity;transmembrane receptor activity
5264, 6751, 12982	granulocyte macrophage colony-stimulating factor receptor binding;growth factor activity;actin binding;sugar:hydrogen symporter activity;lamin binding;protein binding
1899, 9890	growth factor activity
12270	growth factor activity;heparin binding
3733	growth factor activity;protein heterodimerization activity;BMP receptor binding;protein binding
2847, 5805, 6539, 6903, 7294, 9752, 9835, 10019, 11632, 13559, 14730, 14998, 15167	GTP binding
16371	GTP binding;alpha-tubulin binding;protein binding
1454, 7543	GTP binding;GTPase activity
14970	GTP binding;GTPase activity;protein binding;identical protein binding
5220	GTP binding;GTPase activity;translation elongation factor activity
16714	GTP binding;microtubule binding;protein binding;GDP binding;GTPase activity
10605	GTP binding;nucleotide binding;nucleoside-triphosphatase activity;7S RNA binding;protein binding;GTPase activity;drug binding;endoplasmic reticulum signal peptide binding;GDP binding;ribonucleoprotein binding
2887, 13632	GTP binding;nucleotide binding;nucleoside-triphosphatase activity;serine-type peptidase activity;heparin binding
1396, 5381, 5648, 6804, 10871, 11764	GTP binding;protein binding
12287	GTP binding;protein binding;protein complex scaffold;enzyme regulator activity
16738	GTP binding;protein heterodimerization activity;protein binding
3831, 8137, 14143, 15237	GTPase activator activity

7292	GTPase activator activity;alpha-tubulin binding;beta-tubulin binding;protein C-terminus binding;protein binding;gamma-tubulin binding
11696	GTPase activator activity;binding
15363	GTPase activator activity;binding;Ras GTPase activator activity;protein binding
14192	GTPase activator activity;identical protein binding;protein binding
14091, 15722	GTPase activator activity;protein binding
8157, 11634, 14827, 15207, 17136	GTPase activity;GTP binding
3451	GTPase activity;GTP binding;identical protein binding;protein binding
8124, 10750	GTPase activity;GTP binding;protein binding
1107, 1944, 2160, 2678, 2755, 2902, 4102, 4250, 5901, 6015, 6964, 7028, 7148, 8213, 8682, 10497, 10543, 11379, 12164, 12218, 12948, 13348, 13619, 14299, 14610, 14614, 15297, 15642, 16644, 16822	GTPase activity;protein binding;GTP binding
3673, 7483	GTPase activity;protein binding;GTP binding;ATP binding;transcription factor binding
4447	GTPase activity;protein binding;GTP binding;ATP binding;transcription factor binding;GDP binding
5107	GTPase activity;protein binding;GTP binding;Edg-2 lysophosphatidic acid receptor binding
15710	GTPase activity;protein binding;GTP binding;GDP binding
9308	GTPase activity;protein binding;GTP binding;guanyl nucleotide binding
13223	GTPase activity;protein binding;GTP binding;nucleotide binding;nucleoside-triphosphatase activity
2636	GTPase activity;protein binding;GTP binding;oxidoreductase activity;GDP binding
9690	GTPase activity;protein binding;GTP binding;protein kinase activity;ATP binding
4499	GTPase activity;protein binding;GTP binding;protein transporter activity;GDP binding
14276	GTPase activity;protein binding;GTP binding;Ras GTPase binding
10190, 10276	GTPase inhibitor activity;calcium ion binding;protein binding;heparin binding;protein homodimerization activity;laminin-1 binding;proteoglycan binding;chemorepellent activity;Roundabout binding
5386, 14632, 15907, 16200	guanyl-nucleotide exchange factor activity
7462	guanyl-nucleotide exchange factor activity;GTP binding;GTPase binding;ATP binding
10625, 10919, 11140	guanyl-nucleotide exchange factor activity;GTP binding;GTPase binding;binding
3004	guanyl-nucleotide exchange factor activity;GTP binding;GTPase binding;binding;Rho guanyl-nucleotide exchange factor activity;Rho GTPase binding
2356, 15616	guanyl-nucleotide exchange factor activity;GTP binding;GTPase

	binding;transporter activity;protein binding
1208	guanyl-nucleotide exchange factor activity;protein binding
5681	guanyl-nucleotide exchange factor activity;translation initiation factor activity;protein binding
3669, 5492, 8706, 10366, 16534	heat shock protein binding
15687	heat shock protein binding;electron carrier activity;iron-sulfur cluster binding
13140	heat shock protein binding;hormone activity;Hsp70 protein binding;proteasome binding;chaperone binding
7018	heat shock protein binding;nucleic acid binding;zinc ion binding
10671	heat shock protein binding;protein binding
14014	heat shock protein binding;protein binding;misfolded protein binding;ATPase binding;chaperone binding;ATPase activator activity;disulfide oxidoreductase activity
1647, 6142	heat shock protein binding;unfolded protein binding
2106, 5491, 10240	heat shock protein binding;unfolded protein binding;protein binding
14828	heat shock protein binding;unfolded protein binding;protein binding;small GTPase regulator activity;interferon-gamma receptor binding;protein kinase binding;NF-kappaB binding;transcription factor binding
12475, 13050	hedgehog receptor activity
8095	hedgehog receptor activity;protein binding
14103	helicase activity;protein binding;ATPase activity;nucleotide binding;nucleoside-triphosphatase activity;ATP binding;DNA binding
16190	heme binding;growth factor activity
16812	heme binding;iron ion binding;oxidoreductase activity
12346	heme binding;oxidoreductase activity;cytochrome-b5 reductase activity;NAD(P)H oxidase activity;oxidoreductase activity, acting on NADH or NADPH, heme protein as acceptor
8713	heme binding;oxidoreductase activity;iron ion binding;oxidoreductase activity, acting on paired donors, with oxidation of a pair of donors resulting in the reduction of molecular oxygen to two molecules of water;protein binding
2492	heme oxygenase (decyclizing) activity;protein binding;electron carrier activity
6582, 7951, 14690	heparan sulfate proteoglycan binding
15537	heparan sulfate proteoglycan binding;protein binding;peptidyl-dipeptidase inhibitor activity
7299	heparan sulfate proteoglycan binding;sequence-specific DNA binding transcription factor activity;signal transducer activity
7550	heparin binding
1646	heparin binding;receptor binding;frizzled binding
11966	hexokinase activity;ATP binding;phosphotransferase activity, alcohol group as acceptor
4889	histone acetyltransferase activity;protein binding;phosphorylase kinase regulator activity

8722	homocysteine S-methyltransferase activity
12845	hormone activity
15142	hormone activity;endothelin B receptor binding
15307	hormone activity;protein kinase activity;ATP binding
1060	hormone binding;transcription corepressor activity;protein homodimerization activity;thyroid hormone binding;NADP+ or NADPH binding
8506, 13937	hydrogen ion transmembrane transporter activity
9990	hydrogen ion transmembrane transporter activity;ATPase activity
14001	hydrogen ion transmembrane transporter activity;ATPase activity;protein binding
7814	hydrogen ion transmembrane transporter activity;protein binding;hydrogen-exporting ATPase activity, phosphorylative mechanism
7375	hydrogen ion transporting ATP synthase activity, rotational mechanism
15262	hydrogen ion transporting ATP synthase activity, rotational mechanism;proton-transporting ATPase activity, rotational mechanism
8325	hydrogen ion transporting ATP synthase activity, rotational mechanism;proton-transporting ATPase activity, rotational mechanism;ATPase activity
12014	hydrogen ion transporting ATP synthase activity, rotational mechanism;proton-transporting ATPase activity, rotational mechanism;hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances;ATP binding
2876, 3648, 8482, 9157, 15121	hydrolase activity
16117	hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances;ATPase binding
12141	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds
3985	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in cyclic amides;hydrolase activity;hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds
10057	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides
4539, 7710, 9046, 14399	hydrolase activity, acting on ester bonds
2731, 2822, 3160, 4734, 16226, 16914	hydrolase activity, hydrolyzing O-glycosyl compounds
11598	hydrolase activity, hydrolyzing O-glycosyl compounds;alpha-galactosidase activity;catalytic activity;receptor binding;protein homodimerization activity;protein binding
15353	hydrolase activity, hydrolyzing O-glycosyl compounds;beta-mannosidase activity
17142	hydrolase activity, hydrolyzing O-glycosyl compounds;catalytic activity;cation binding
17131	hydrolase activity, hydrolyzing O-glycosyl compounds;chitinase activity
7752	hydrolase activity, hydrolyzing O-glycosyl compounds;protein binding
3371	hydrolase activity;adenosine-diphosphatase activity
15513	hydrolase activity;diphosphoinositol-polyphosphate diphosphatase activity

10883	hydrolase activity;DNA binding;DNA-directed RNA polymerase activity
11319	hydrolase activity;gamma-glutamyl-peptidase activity
14717	hydrolase activity;guanosine-diphosphatase activity;uridine-diphosphatase activity
16989	hydrolase activity;hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances;ATP binding
14184	hydrolase activity;isopentenyl-diphosphate delta-isomerase activity
9107	hydrolase activity;magnesium ion binding;diphosphoinositol-polyphosphate diphosphatase activity
4171	hydrolase activity;peptidase activity
15939, 16382	hydrolase activity;protein binding
12109	hydrolase activity;serine hydrolase activity;phosphate binding
15483	hydrolase activity;uridine-diphosphatase activity
16395	hydroxymethyl-, formyl- and related transferase activity;methionyl-tRNA formyltransferase activity;catalytic activity
8800	hydroxymethylbilane synthase activity
16900	hydroxymethylglutaryl-CoA synthase activity;catalytic activity
11613, 14403	identical protein binding
2791, 3261, 3727, 9735, 12606, 13305, 13867	identical protein binding;protein binding
11918, 14615	inorganic anion exchanger activity;anion transmembrane transporter activity;transporter activity
4504	inorganic anion exchanger activity;anion transmembrane transporter activity;transporter activity;protein binding
14793, 16262	inositol or phosphatidylinositol phosphatase activity
7849	inositol or phosphatidylinositol phosphatase activity;3'-nucleotidase activity
12358	inositol or phosphatidylinositol phosphatase activity;nucleic acid binding;protein binding;polyphosphatidylinositol phosphatase activity;phosphatidylinositol phosphate 5-phosphatase activity;phosphatidylinositol-4,5-bisphosphate 5-phosphatase activity
13012	inositol or phosphatidylinositol phosphatase activity;phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase activity
9273	inositol or phosphatidylinositol phosphatase activity;protein binding;inositol bisphosphate phosphatase activity;inositol trisphosphate phosphatase activity
6002	inositol or phosphatidylinositol phosphatase activity;protein homodimerization activity;identical protein binding;inositol-1(or 4)-monophosphatase activity
12546	inositol or phosphatidylinositol phosphatase activity;protein homodimerization activity;inositol-1(or 4)-monophosphatase activity
14640, 14647	inositol trisphosphate 3-kinase activity
11464	inositol trisphosphate 3-kinase activity;inositol tetrakisphosphate 3-kinase activity
9126	insulin receptor binding

9454	insulin receptor binding;fibroblast growth factor receptor binding
3647	insulin receptor binding;protein binding;protein kinase binding
5041	insulin receptor binding;receptor signaling protein activity
10115	insulin-like growth factor binding;protein binding
11468	insulin-like growth factor binding;serine-type endopeptidase activity;protein binding;catalytic activity
6449	integrin binding;calcium ion binding;heparin binding;structural molecule activity
4965	intramolecular transferase activity, phosphotransferases
3194, 5819, 6317, 6477, 8610, 10284, 12181, 12866, 14352	ion channel activity
16385	ion channel activity;extracellular ligand-gated ion channel activity
8292	ion channel activity;hydrolase activity
17157	ion channel activity;inward rectifier potassium channel activity;voltage-gated ion channel activity
2131	ion channel activity;inward rectifier potassium channel activity;voltage-gated ion channel activity;ATP binding;ATPase activity, coupled to transmembrane movement of substances
15137	ion channel activity;inward rectifier potassium channel activity;voltage-gated ion channel activity;PDZ domain binding;protein binding
3946	ion channel activity;inward rectifier potassium channel activity;voltage-gated ion channel activity;protein binding;G-protein activated inward rectifier potassium channel activity
975	ion channel activity;oxidoreductase activity;voltage-gated potassium channel activity
2972	ion channel activity;P-P-bond-hydrolysis-driven protein transmembrane transporter activity
4144	ion channel activity;protein binding;store-operated calcium channel activity
6029	ion channel activity;structural constituent of ribosome;protein binding;store-operated calcium channel activity
2961, 2971	ion channel activity;voltage-gated potassium channel activity;potassium channel activity;protein binding;potassium channel inhibitor activity
10095, 11507, 12448, 12544, 15276	ion channel activity;voltage-gated sodium channel activity
4177	ionotropic glutamate receptor activity;extracellular-glutamate-gated ion channel activity;kainate selective glutamate receptor activity
13531	ionotropic glutamate receptor activity;extracellular-glutamate-gated ion channel activity;protein binding;kainate selective glutamate receptor activity
5363, 11958, 15445, 15486	ionotropic glutamate receptor activity;extracellular-glutamate-gated ion channel activity;transporter activity
7633	ionotropic glutamate receptor activity;extracellular-glutamate-gated ion channel activity;transporter activity;adenylate cyclase inhibiting metabotropic glutamate receptor activity;kainate selective glutamate receptor activity;protein binding
14638	ionotropic glutamate receptor activity;extracellular-glutamate-gated ion channel activity;transporter activity;protein binding

14042	iron ion binding;cysteine dioxygenase activity
7005, 12748, 15453	iron ion binding;electron carrier activity;heme binding
970	iron ion binding;electron carrier activity;oxidoreductase activity;flavin adenine dinucleotide binding;ferric-chelate reductase activity
12802	iron ion binding;oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen;L-ascorbic acid binding
10599	iron ion binding;oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen
13507	iron ion binding;oxidoreductase activity;serine-type peptidase activity
15312	iron ion transmembrane transporter activity
16258	isomerase activity;bent DNA binding;double-stranded DNA binding
13166	isomerase activity;catalytic activity;carbohydrate binding
11347	isomerase activity;protein binding
15977	kinase activity;tRNA binding
6078	kinesin binding
3438	lactoylglutathione lyase activity;metal ion binding
10086	L-amino acid transmembrane transporter activity
13471	L-glutamate transmembrane transporter activity
13357	L-glutamate transmembrane transporter activity;protein binding
2483	ligase activity
4609	ligase activity;acid-amino acid ligase activity
5881	lipid binding;glycolipid transporter activity;glycolipid binding
12402	lipoate synthase activity;4 iron, 4 sulfur cluster binding;catalytic activity;iron-sulfur cluster binding;thiamine diphosphokinase activity;ATP binding
16810	magnesium ion binding;adenylosuccinate synthase activity;GTP binding;protein binding
16081	magnesium ion binding;four-way junction DNA binding;DNA binding;damaged DNA binding;double-stranded DNA binding;single-stranded DNA binding;protein binding;ATP binding;protein C-terminus binding;ATPase activity;centromeric DNA binding;mismatched DNA binding;guanine/thymine mispair binding;dinucleotide insertion or deletion binding;single guanine insertion binding;single thymine insertion binding;dinucleotide repeat insertion binding;oxidized purine DNA binding;MutLalpha complex binding;identical protein binding;protein homodimerization activity;ADP binding;protein kinase binding;enzyme binding
12364	magnesium ion binding;holo-[acyl-carrier-protein] synthase activity;protein binding
9414	magnesium ion binding;hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides;manganese ion binding;hydrolase activity
10617	magnesium ion binding;inorganic diphosphatase activity
7281	magnesium ion binding;inorganic diphosphatase activity;pyrophosphatase activity

2085	magnesium ion binding;oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor;NAD ⁺ or NADH binding;isocitrate dehydrogenase (NAD ⁺) activity
15457	magnesium ion binding;oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor;NAD ⁺ or NADH binding;isocitrate dehydrogenase (NADP ⁺) activity;protein homodimerization activity
1469, 13173	magnesium ion binding;protein kinase activity;protein tyrosine kinase activity;non-membrane spanning protein tyrosine kinase activity;protein binding;ATP binding;kinase activity;protein domain specific binding;manganese ion binding;protein serine/threonine kinase activity
11077	magnesium ion binding;ribose phosphate diphosphokinase activity
17023	magnesium ion binding;ribose phosphate diphosphokinase activity;identical protein binding;protein binding
6553, 12154	magnesium ion binding;ribulose-bisphosphate carboxylase activity
8021	magnesium ion binding;sequence-specific DNA binding
2698	malonyl-CoA decarboxylase activity
6394, 9648, 15692, 16824	mannosyl-oligosaccharide 1,2-alpha-mannosidase activity;calcium ion binding
8031	mannosyl-oligosaccharide 1,2-alpha-mannosidase activity;calcium ion binding;glycoprotein endo-alpha-1,2-mannosidase activity
3245, 6109, 10976, 14145, 14378	metal ion binding
6810	metal ion binding;copper ion binding;copper chaperone activity;copper-dependent protein binding
14883	metal ion binding;protein binding;zinc ion binding
14361	metal ion binding;zinc ion binding
12349, 14290, 14964	metal ion transmembrane transporter activity
9699	metal ion transmembrane transporter activity;zinc ion binding
10066	metallocarboxypeptidase activity;zinc ion binding
3210	metallocarboxypeptidase activity;zinc ion binding;binding
4802	metallocarboxypeptidase activity;zinc ion binding;carboxypeptidase activity;protein binding
10455	metallocarboxypeptidase activity;zinc ion binding;DNA-directed DNA polymerase activity;nucleotide binding;DNA binding;nucleic acid binding;chromatin binding;protein binding
1407, 6649, 8993, 10841, 10853, 12061, 12555, 17078	metalloendopeptidase activity
11680	metalloendopeptidase activity;ATP binding;nucleotide binding;nucleoside-triphosphatase activity;zinc ion binding
3449	metalloendopeptidase activity;endopeptidase activity
6594	metalloendopeptidase activity;enzyme regulator activity;zinc ion binding
13055	metalloendopeptidase activity;peptidase activity
6283	metalloendopeptidase activity;receptor binding;zinc ion binding;metallopeptidase activity
6314, 13690	metalloendopeptidase activity;zinc ion binding
14055	metalloendopeptidase activity;zinc ion binding;catalytic activity;metal ion binding;metallopeptidase activity

7080, 8068, 15126	metalloendopeptidase activity;zinc ion binding;metallopeptidase activity
1736	metalloendopeptidase activity;zinc ion binding;metallopeptidase activity;protein binding
2772, 9584, 10792	metalloendopeptidase activity;zinc ion binding;peptidase activity
9041	metalloendopeptidase activity;zinc ion binding;protein binding;heparin binding
4431	metalloexopeptidase activity;dipeptidyl-peptidase activity;dipeptidase activity
6765, 15136	metallopeptidase activity;metalloendopeptidase activity
4697, 10231	metallopeptidase activity;zinc ion binding
6832	metallopeptidase activity;zinc ion binding;aminopeptidase activity
3668, 10813	metallopeptidase activity;zinc ion binding;binding
12977	metallopeptidase activity;zinc ion binding;peptidase activity
14725	metallopeptidase activity;zinc ion binding;protein binding;aminopeptidase activity
13147	methylated-DNA-[protein]-cysteine S-methyltransferase activity;catalytic activity;methyltransferase activity
15097	methylmalonate-semialdehyde dehydrogenase (acylating) activity;oxidoreductase activity
7846	methylmalonyl-CoA epimerase activity
15767	methylmalonyl-CoA mutase activity;cobalamin binding;intramolecular transferase activity;metal ion binding;isomerase activity;catalytic activity
10154, 10944	methyltransferase activity
16511	methyltransferase activity;diphthine synthase activity
8318	methyltransferase activity;protein binding;protein methyltransferase activity
6947	methyltransferase activity;RNA binding
16695	microtubule motor activity;alpha-tubulin binding;beta-tubulin binding;protein binding;dynactin binding
4503, 15043, 15057	microtubule motor activity;ATP binding
7024	microtubule motor activity;ATP binding;ATPase activity
13432	microtubule motor activity;ATP binding;nucleotide binding
5610, 5668, 8521, 16245	microtubule motor activity;ATP binding;protein binding
11817, 15847	microtubule motor activity;protein binding
3053, 8435, 10985	molecular_function
14571	Mo-molybdopterin synthase activity
6077, 16649	monooxygenase activity;iron ion binding;electron carrier activity;heme binding
1397	monooxygenase activity;iron ion binding;electron carrier activity;heme binding;protein binding;oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, reduced flavin or flavoprotein as one donor, and incorporation of one atom of oxygen
7935	monooxygenase activity;iron ion binding;electron carrier activity;heme binding;retinoic acid binding

14441	monooxygenase activity;oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, NADH or NADPH as one donor, and incorporation of one atom of oxygen;flavin adenine dinucleotide binding
7971	motor activity
5349, 14366	motor activity;ATP binding;protein binding
7486	motor activity;ATP binding;signal transducer activity;protein binding
6081	N-acetylglucosamine-6-phosphate deacetylase activity;hydrolase activity;hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds
15063, 17162	NAD+ ADP-ribosyltransferase activity
10725	NAD+ ADP-ribosyltransferase activity;protein binding
16153	NADH dehydrogenase (ubiquinone) activity
15900	neurotransmitter:sodium symporter activity
4048, 12250	NF-kappaB binding;protein binding
14246	nitric-oxide synthase activity
4372	Notch binding;calcium ion binding;protein binding;growth factor activity;peptidase inhibitor activity
13514	nuclease activity
12392	nuclease activity;ATP binding;zinc ion binding
1202, 1907, 2476, 2835, 3649, 3940, 4466, 4992, 5224, 5281, 5382, 5389, 5667, 5697, 6039, 6129, 6607, 6854, 7669, 7906, 8315, 9542, 12137, 12503, 12776, 13441, 13526, 13588, 13817, 14851, 15035, 15122, 15485, 15902, 15922, 16307, 17096	nucleic acid binding
10401	nucleic acid binding;AF-2 domain binding;RNA polymerase II transcription mediator activity;receptor activator activity;ligand-dependent nuclear receptor transcription coactivator activity;estrogen receptor binding;protein binding
14309	nucleic acid binding;ATP binding;ATP-dependent helicase activity
13829	nucleic acid binding;ATP binding;ATP-dependent helicase activity;calcium ion binding
7214	nucleic acid binding;ATP binding;ATP-dependent helicase activity;calcium ion binding;G-protein beta/gamma-subunit complex binding
13790, 14978, 17116	nucleic acid binding;ATP binding;ATP-dependent helicase activity;helicase activity
5723	nucleic acid binding;ATP binding;ATP-dependent helicase activity;helicase activity;DNA binding;hydrolase activity
12215	nucleic acid binding;ATP binding;ATP-dependent helicase activity;helicase activity;nucleotide binding;nucleoside-triphosphatase activity
14420	nucleic acid binding;ATP binding;ATP-dependent helicase activity;helicase activity;protein binding;transcription repressor activity
7309	nucleic acid binding;ATP binding;ATP-dependent helicase

	activity;protein binding
13149	nucleic acid binding;binding
15621, 16095	nucleic acid binding;binding;protein binding
4545	nucleic acid binding;chromatin binding;sequence-specific DNA binding transcription factor activity;protein binding;beta-catenin binding;zinc ion binding;transcription repressor activity;histone acetyltransferase binding;histone deacetylase binding;sequence-specific DNA binding;repressing transcription factor binding
1905	nucleic acid binding;copper ion binding;primary amine oxidase activity;quinone binding;protein binding;eukaryotic cell surface binding
13234	nucleic acid binding;DNA binding;protein binding;transcription repressor activity;zinc ion binding
13439	nucleic acid binding;DNA binding;sequence-specific DNA binding transcription factor activity;zinc ion binding;transcription regulator activity;sequence-specific DNA binding;metal ion binding;hormone activity
13315	nucleic acid binding;DNA binding;zinc ion binding;metal ion binding
3864	nucleic acid binding;heat shock protein binding
9583, 13453	nucleic acid binding;helicase activity;ATP binding;ATP-dependent helicase activity
7794	nucleic acid binding;helicase activity;ATP binding;ATP-dependent helicase activity;ATP-dependent RNA helicase activity
9195, 13968	nucleic acid binding;helicase activity;ATP binding;ATP-dependent helicase activity;nucleotide binding;nucleoside-triphosphatase activity
13164	nucleic acid binding;helicase activity;ATP binding;ATP-dependent helicase activity;protein binding
13616	nucleic acid binding;helicase activity;ATP binding;ATP-dependent helicase activity;RNA binding;protein binding
14411	nucleic acid binding;helicase activity;ATP binding;nucleotide binding;nucleoside-triphosphatase activity
10012	nucleic acid binding;helicase activity;ATP binding;nucleotide binding;nucleoside-triphosphatase activity;protein binding
7481	nucleic acid binding;helicase activity;ATP binding;transcription repressor activity
9833, 16698	nucleic acid binding;helicase activity;ATP binding;zinc ion binding;ATP-dependent helicase activity;carboxy-lyase activity;protein binding
12712	nucleic acid binding;hydrolase activity;metal ion binding
6189, 12251, 15678	nucleic acid binding;methyltransferase activity
14795	nucleic acid binding;methyltransferase activity;endopeptidase activity;metallopeptidase activity
16472	nucleic acid binding;methyltransferase activity;metallopeptidase activity
3971	nucleic acid binding;methyltransferase activity;protein binding;protein methyltransferase activity
10864	nucleic acid binding;NAD ⁺ ADP-ribosyltransferase activity;protein binding
13101	nucleic acid binding;pancreatic ribonuclease activity;protein binding

8822	nucleic acid binding;peptidyl-prolyl cis-trans isomerase activity
10111	nucleic acid binding;peptidyl-prolyl cis-trans isomerase activity;protein binding
2642, 2669, 4309, 6452, 10053, 11384, 13445, 13461, 13688, 13704, 14407, 14530, 15365, 16267	nucleic acid binding;protein binding
6861	nucleic acid binding;protein binding;ligand-dependent nuclear receptor transcription coactivator activity
14915	nucleic acid binding;protein binding;methyltransferase activity;mRNA (nucleoside-2'-O-)-methyltransferase activity
3619	nucleic acid binding;protein binding;mRNA binding
7911	nucleic acid binding;protein binding;RNA binding;transcription corepressor activity;transcription factor binding
3909	nucleic acid binding;protein binding;structural molecule activity;protein binding, bridging
15060	nucleic acid binding;protein binding;transcription activator activity;actin monomer binding;transcription coactivator activity;actin binding
14702	nucleic acid binding;protein binding;transcription coactivator activity;actin binding
15585	nucleic acid binding;protein binding;zinc ion binding
10808	nucleic acid binding;protein dimerization activity
15438	nucleic acid binding;protein kinase activity;ATP binding;protein serine/threonine kinase activity;protein binding;ribonucleoprotein binding
13650	nucleic acid binding;pyridoxal phosphate binding;RNA binding
8772, 10160	nucleic acid binding;RNA binding
2812, 3602, 4436, 10861, 15133	nucleic acid binding;RNA binding;protein binding
4235	nucleic acid binding;RNA methyltransferase activity
6030	nucleic acid binding;RNA polymerase core enzyme binding
10277	nucleic acid binding;sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding
12742	nucleic acid binding;single-stranded DNA binding;RNA binding
5642	nucleic acid binding;two-component response regulator activity;DNA binding
1655, 1808, 2438, 2754, 3147, 4241, 4441, 5553, 6178, 6502, 7565, 7597, 11326, 11818, 12096, 12839, 13487, 13836, 14323, 14549, 15367, 16149, 16314	nucleic acid binding;zinc ion binding
5057	nucleic acid binding;zinc ion binding;DNA binding
14440	nucleic acid binding;zinc ion binding;DNA binding;sequence-specific DNA binding;promoter binding;protein binding;specific transcriptional repressor activity;repressing transcription factor binding
10937	nucleic acid binding;zinc ion binding;DNA binding;transcription activator activity
11546	nucleic acid binding;zinc ion binding;DNA binding;transcription regulator activity;metal ion binding

11838	nucleic acid binding;zinc ion binding;GTP binding;helicase activity;ATP binding;ATP-dependent helicase activity;protein binding
8866	nucleic acid binding;zinc ion binding;iron ion binding;electron carrier activity;heme binding
7241	nucleic acid binding;zinc ion binding;metal ion binding
7249, 11247	nucleic acid binding;zinc ion binding;NAD ⁺ ADP-ribosyltransferase activity;protein binding
8118	nucleic acid binding;zinc ion binding;nucleotide binding;nucleoside-triphosphatase activity
6642	nucleic acid binding;zinc ion binding;nucleotidyltransferase activity;protein binding;RNA uridylyltransferase activity
874, 15552, 16894	nucleic acid binding;zinc ion binding;protein binding
3571	nucleic acid binding;zinc ion binding;protein binding;DNA binding
14540	nucleic acid binding;zinc ion binding;protein binding;phosphopantetheine binding;DNA binding
16201	nucleic acid binding;zinc ion binding;RNA uridylyltransferase activity
15331	nucleic acid binding;zinc ion binding;sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;protein binding
12675	nucleic acid binding;zinc ion binding;SH3 domain binding;opioid peptide activity
8296	nucleic acid binding;zinc ion binding;single-stranded RNA binding;protein homodimerization activity;protein binding
3581, 3699	nucleic acid binding;zinc ion binding;transcription regulator activity
10668	nucleic acid binding;zinc ion binding;transcription regulator activity;RNA polymerase II transcription factor activity;translation elongation factor activity;protein binding;DNA binding;transcription elongation regulator activity
3697	nucleocytoplasmic transporter activity;protein binding
16158	nucleoside transmembrane transporter activity;protein binding
3376, 3403, 3867, 10470, 11562, 13706, 15418, 15723	nucleotide binding
4540	nucleotide binding;6-phosphofructokinase activity;ATP binding;kinase activity;transferase activity;identical protein binding;kinase binding;fructose-6-phosphate binding;fructose binding
4159, 13508, 15720	nucleotide binding;aminoacyl-tRNA ligase activity;ATP binding
5052	nucleotide binding;aminoacyl-tRNA ligase activity;ATP binding;heat shock protein binding;binding
14287	nucleotide binding;aminoacyl-tRNA ligase activity;ATP binding;histidine-tRNA ligase activity
6022	nucleotide binding;aminoacyl-tRNA ligase activity;ATP binding;isoleucine-tRNA ligase activity;protein binding
12489	nucleotide binding;aminoacyl-tRNA ligase activity;ATP binding;leucine-tRNA ligase activity
8579	nucleotide binding;aminoacyl-tRNA ligase activity;ATP binding;leucine-tRNA ligase activity;cysteine-tRNA ligase activity;protein binding
10381	nucleotide binding;aminoacyl-tRNA ligase activity;ATP binding;methionine-tRNA ligase activity

8893	nucleotide binding;aminoacyl-tRNA ligase activity;ATP binding;threonine-tRNA ligase activity;ligase activity, forming aminoacyl-tRNA and related compounds
5133	nucleotide binding;aminoacyl-tRNA ligase activity;ATP binding;tyrosine-tRNA ligase activity;RNA binding;protein binding
2539	nucleotide binding;binding;protein N-terminus binding;protein binding;chaperone binding
5507, 5510	nucleotide binding;calcitonin gene-related polypeptide receptor activity;catalytic activity;DNA-directed RNA polymerase activity
4899	nucleotide binding;catalytic activity;calcium-transporting ATPase activity;protein binding;ATP binding;ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism;hydrolase activity;hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances;metal ion binding;S100 alpha binding;protein C-terminus binding
14092	nucleotide binding;catalytic activity;glutamate dehydrogenase [NAD(P)+] activity;binding;protein binding;ATP binding;GTP binding;oxidoreductase activity;oxidoreductase activity, acting on the CH-NH2 group of donors, NAD or NADP as acceptor
7216	nucleotide binding;catalytic activity;metal ion binding
6124	nucleotide binding;damaged DNA binding;single-stranded DNA specific endodeoxyribonuclease activity;protein binding
9532	nucleotide binding;DNA binding;DNA-directed DNA polymerase activity;nucleic acid binding
7647, 9506, 12180	nucleotide binding;GTP binding
17001	nucleotide binding;GTP binding;GDP binding;alpha-tubulin binding;beta-tubulin binding
9998	nucleotide binding;GTP binding;GTPase activity;protein binding;ATP binding;transcription factor binding
9955	nucleotide binding;GTPase activity;GTP binding
7118, 7931, 8142	nucleotide binding;GTPase activity;structural molecule activity;GTP binding
8311	nucleotide binding;magnesium ion binding;5'-nucleotidase activity
10375	nucleotide binding;magnesium ion binding;calcium-transporting ATPase activity;calmodulin binding;ATP binding;ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism;hydrolase activity;hydrolase activity, acting on acid anhydrides;metal ion binding;catalytic activity;calcium ion transmembrane transporter activity;hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances
16357	nucleotide binding;motor activity;actin binding;ATP binding;protein binding
15841	nucleotide binding;motor activity;actin binding;calmodulin binding;ATP binding;protein binding
12235	nucleotide binding;mRNA binding;protein binding;nucleic acid binding;RNA binding
16013	nucleotide binding;nucleic acid binding
3739, 12930	nucleotide binding;nucleic acid binding;protein binding

10394	nucleotide binding;nucleic acid binding;protein binding;AU-rich element binding
9433	nucleotide binding;nucleoside binding;nucleic acid binding;DNA binding;chromatin binding;DNA-directed DNA polymerase activity;DNA primase activity;protein binding;transferase activity;nucleotidyltransferase activity;DNA polymerase processivity factor activity;protein complex binding;protein heterodimerization activity
12793	nucleotide binding;nucleoside-triphosphatase activity
1728, 1954, 3902, 7948, 8595, 13007	nucleotide binding;nucleoside-triphosphatase activity;ATP binding
15228	nucleotide binding;nucleoside-triphosphatase activity;ATP binding;ATPase activity;protein complex binding;protein C-terminus binding;protein binding;ATPase activity, coupled
8976	nucleotide binding;nucleoside-triphosphatase activity;ATP binding;binding;protein complex binding;protein C-terminus binding;protein binding;ATPase activity, coupled
10560	nucleotide binding;nucleoside-triphosphatase activity;ATP binding;hydrolase activity;ATPase activity;protein binding;receptor binding;transcription factor binding;thyrotropin-releasing hormone receptor binding
10503	nucleotide binding;nucleoside-triphosphatase activity;ATP binding;identical protein binding;protein binding
11659	nucleotide binding;nucleoside-triphosphatase activity;ATP binding;protein C-terminus binding
4329	nucleotide binding;nucleoside-triphosphatase activity;ATP binding;transporter activity;ATPase activity
2294, 4918	nucleotide binding;nucleoside-triphosphatase activity;DNA binding;ATP binding;DNA-dependent ATPase activity
15256	nucleotide binding;nucleoside-triphosphatase activity;DNA binding;ATP binding;DNA-dependent ATPase activity;crossover junction endodeoxyribonuclease activity
9581	nucleotide binding;nucleoside-triphosphatase activity;DNA binding;ATP binding;hydrolase activity
11165	nucleotide binding;nucleoside-triphosphatase activity;DNA replication origin binding;protein binding
4355	nucleotide binding;nucleoside-triphosphatase activity;guanylate kinase activity
8789	nucleotide binding;nucleoside-triphosphatase activity;microtubule motor activity;protein binding;ATP binding;ATPase activity
3637, 3855	nucleotide binding;nucleoside-triphosphatase activity;protein binding
3366, 4788, 4799, 5690, 14898, 15344, 16488, 16973	nucleotide binding;protein binding
5343	nucleotide binding;protein binding;GTP binding
10917	nucleotide binding;protein binding;LRR domain binding
5547	nucleotide binding;protein homodimerization activity;protein binding;ADP-ribosylation factor binding;Rab GTPase binding
9171	nucleotide binding;protein homodimerization activity;Rab GTPase binding;protein binding

2670	nucleotide binding;protein kinase activity;protein serine/threonine kinase activity;MAP kinase activity;protein binding;ATP binding;kinase activity;transferase activity
12040	nucleotide binding;protein kinase activity;protein serine/threonine kinase activity;protein binding;ATP binding;kinase activity;transferase activity
9057	nucleotide binding;translation elongation factor activity;GTPase activity;protein binding;GTP binding
6190	nucleotide-sugar transmembrane transporter activity;sugar:hydrogen symporter activity
5845	nucleotidyltransferase activity
3381, 10728	nucleotidyltransferase activity;catalytic activity
1122	nucleotidyltransferase activity;nicotinate-nucleotide adenylyltransferase activity
8554, 8635	nucleotidyltransferase activity;transferase activity
7733	odorant binding
10285	O-methyltransferase activity;methyltransferase activity;ATP binding
2828	O-methyltransferase activity;protein binding
2569, 2838, 4369, 5347, 5503, 6490, 6530, 7242, 8422, 8975, 10170, 12847, 13049, 13388, 13553, 13713, 14844, 14956, 15860, 17025	oxidoreductase activity
13635	oxidoreductase activity, acting on a sulfur group of donors, disulfide as acceptor;peptide-methionine-(S)-S-oxide reductase activity
12271	oxidoreductase activity, acting on a sulfur group of donors, oxygen as acceptor
10158	oxidoreductase activity, acting on CH-OH group of donors;glycerol-3-phosphate dehydrogenase [NAD ⁺] activity;oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor;NAD ⁺ or NADH binding
10592	oxidoreductase activity, acting on CH-OH group of donors;oxidoreductase activity;oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor;NAD ⁺ or NADH binding
14912	oxidoreductase activity, acting on NADH or NADPH
11213	oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor;oxidoreductase activity
4303	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor
15103, 16008	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor;NAD ⁺ or NADH binding;cofactor binding
6568	oxidoreductase activity;2,4-dienoyl-CoA reductase (NADPH) activity;NADPH binding
7695	oxidoreductase activity;3-hydroxyacyl-CoA dehydrogenase activity
8402	oxidoreductase activity;aldehyde dehydrogenase (NAD) activity;aminobutyraldehyde dehydrogenase activity;oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP

	as acceptor
2913, 7614	oxidoreductase activity;aminomethyltransferase activity
11622	oxidoreductase activity;chromatin binding;transcription factor binding;transcription repressor activity;protein binding;histone demethylase activity (H3-dimethyl-K4 specific);histone demethylase activity (H3-K9 specific);histone demethylase activity (H3-K4 specific);histone demethylase activity;ligand-dependent nuclear receptor transcription coactivator activity;MyoD binding;androgen receptor binding;flavin adenine dinucleotide binding;sequence-specific DNA binding transcription factor activity;promoter binding
8158	oxidoreductase activity;copper ion binding;protein binding;chaperone binding;ferroxidase activity
14968	oxidoreductase activity;D-amino-acid oxidase activity
10064	oxidoreductase activity;flavin adenine dinucleotide binding;2 iron, 2 sulfur cluster binding;caspase activator activity;protein binding
8404, 13911	oxidoreductase activity;FMN binding
3050, 6596, 13931	oxidoreductase activity;iron ion binding;oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen;L-ascorbic acid binding
15674	oxidoreductase activity;iron ion binding;oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen;L-ascorbic acid binding;protein binding
6992	oxidoreductase activity;iron-sulfur cluster binding;zinc ion binding
4740	oxidoreductase activity;malate dehydrogenase activity;oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor;L-malate dehydrogenase activity;catalytic activity
8407	oxidoreductase activity;NAD ⁺ or NADH binding;NADH dehydrogenase (ubiquinone) activity
10417	oxidoreductase activity;oxidoreductase activity, acting on a sulfur group of donors, oxygen as acceptor;prenylcysteine oxidase activity
3186	oxidoreductase activity;polyamine oxidase activity
16561	oxidoreductase activity;protein binding
4132, 15048	oxidoreductase activity;protein binding;kinase binding
13311	oxidoreductase activity;protein binding;nitric-oxide synthase activity;FMN binding;iron ion binding;calmodulin binding;heme binding;flavin adenine dinucleotide binding;NADP ⁺ or NADPH binding
9002	oxidoreductase activity;protein binding;signal transducer activity
15911	oxidoreductase activity;saccharopine dehydrogenase (NAD ⁺ , L-glutamate-forming) activity
13117	oxidoreductase activity;transporter activity
6731	oxidoreductase activity;zinc ion binding;flavin adenine dinucleotide binding;histone demethylase activity (H3-monomethyl-K4 specific);histone demethylase activity (H3-dimethyl-K4 specific)
5978	oxygen binding;heme binding
7272	p53 binding;protein binding;protein-lysine N-methyltransferase activity;histone-lysine N-methyltransferase activity

15924	palmitoyl-CoA hydrolase activity
8096	palmitoyltransferase activity
722, 4131	pantothenate kinase activity;ATP binding
3280	pantothenate kinase activity;ATP binding;protein binding
6729, 7130, 7397, 11664, 13005, 15458	peptidase activity
10820	peptidase activity;serine-type peptidase activity
2949	peptidase inhibitor activity
1449	peptidase inhibitor activity;endopeptidase inhibitor activity
7463	peptide transporter activity
13335	peptide-aspartate beta-dioxygenase activity
6653, 9475, 9654, 9655, 11983, 12886	peptidyl-prolyl cis-trans isomerase activity
15378	peptidyl-prolyl cis-trans isomerase activity;protein binding;heat shock protein binding
5436	peptidyl-prolyl cis-trans isomerase activity;protein binding;ribonucleoprotein binding
7523	peroxidase activity;antioxidant activity;oxidoreductase activity;peroxiredoxin activity;protein binding;thioredoxin peroxidase activity
6738	peroxidase activity;protein binding;electron carrier activity;protein-disulfide reductase activity
16949	peroxisome matrix targeting signal-2 binding
5509	PH domain binding
13664	phosphatase activity
9279	phosphatase activity;phosphotransferase activity, alcohol group as acceptor;5'-nucleotidase activity
2448	phosphatase activity;protein tyrosine phosphatase activity
12082	phosphatase activity;protein tyrosine phosphatase activity;calcium-activated potassium channel activity
922	phosphatase activity;protein tyrosine phosphatase activity;phosphatidylinositol-3-phosphatase activity
10713	phosphatase activity;protein tyrosine phosphatase activity;protein binding;inositol or phosphatidylinositol phosphatase activity
13550	phosphatase activity;zinc ion binding;protein tyrosine phosphatase activity
8234	phosphatase activity;zinc ion binding;protein tyrosine phosphatase activity;protein serine/threonine phosphatase activity
4837	phosphatidylcholine-sterol O-acyltransferase activity;calcium-independent phospholipase A2 activity
4646	phosphatidylinositol N-acetylglucosaminyltransferase activity
5275	phosphatidylinositol phosphate kinase activity;1-phosphatidylinositol-4-phosphate 5-kinase activity
4033	phosphatidylinositol phosphate kinase activity;protein binding;1-phosphatidylinositol-4-phosphate 5-kinase activity
7027	phosphatidylinositol-3,5-bisphosphate binding

16971	phosphatidylinositol-4,5-bisphosphate binding;phosphotyrosine binding;beta-amyloid binding;protein complex binding;protein binding;signaling adaptor activity;protein binding, bridging;clathrin binding;receptor signaling complex scaffold activity;low-density lipoprotein receptor binding
3026	phosphatidylinositol-4,5-bisphosphate binding;protein binding;phosphatidylinositol-5-phosphate binding;phosphatidylinositol-3-phosphate binding;phosphatidylinositol-3,5-bisphosphate binding;phosphatidic acid binding;phosphatidylinositol-4-phosphate binding;phosphatidylinositol-3,4-bisphosphate binding
10565	phospholipase A2 activity
11121	phospholipase A2 activity;calcium ion binding
8828	phospholipase activator activity;phospholipase binding;Rho GTPase activator activity
9201	phospholipase activity;protein binding;phospholipase A1 activity
7635	phospholipase C activity;phosphatidylinositol phospholipase C activity;calcium ion binding;phosphoric diester hydrolase activity
6774, 16321	phospholipase C activity;phosphatidylinositol phospholipase C activity;phosphoric diester hydrolase activity
13477	phospholipase C activity;phosphatidylinositol phospholipase C activity;phosphoric diester hydrolase activity;enzyme binding
12854	phospholipid binding
11019	phospholipid binding;protease binding;cell surface binding
4978	phosphomannomutase activity
12954, 13259	phosphopantetheine binding
9964	phosphopantetheine binding;catalytic activity
16349	phosphopantetheine binding;protein binding
5403	phosphoprotein phosphatase activity;actin binding;protein binding
15393	phosphoprotein phosphatase activity;protein binding;CTD phosphatase activity
4338, 13075	phosphoprotein phosphatase activity;protein tyrosine phosphatase activity;receptor activity;hydrolase activity;phosphatase activity
11000	phosphoribosylamine-glycine ligase activity;catalytic activity;hydroxymethyl-, formyl- and related transferase activity;phosphoribosylglycinamide formyltransferase activity;phosphoribosylformylglycinamide cyclo-ligase activity;ATP binding
16275	phosphoric diester hydrolase activity;single-stranded DNA binding;double-stranded DNA binding;protein binding;3'-tyrosyl-DNA phosphodiesterase activity
1092, 5692, 9050, 14536	phosphorus-oxygen lyase activity;adenylate cyclase activity
11430	phosphorus-oxygen lyase activity;guanylate cyclase activity
11675	phosphorus-oxygen lyase activity;guanylate cyclase activity;heme binding
16976	phosphorus-oxygen lyase activity;nucleotide binding;aminoacyl-tRNA ligase activity;ATP binding;guanylate cyclase activity

5806, 11169, 16635	phosphotransferase activity, alcohol group as acceptor
12334	phosphotransferase activity, alcohol group as acceptor;binding
2475	phosphotransferase activity, alcohol group as acceptor;binding;protein binding
7683	phosphotransferase activity, alcohol group as acceptor;binding;protein binding;kinase activity;phosphoprotein binding;protein serine/threonine kinase activity
3258	phosphotransferase activity, alcohol group as acceptor;inositol or phosphatidylinositol kinase activity;sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;oxidoreductase activity;transition metal ion binding;binding;protein binding
14523	phosphotransferase activity, alcohol group as acceptor;protein binding
7275	phosphotransferase activity, for other substituted phosphate groups
3502, 8263, 10988, 11496	polypeptide N-acetylgalactosaminyltransferase activity
2298	polypeptide N-acetylgalactosaminyltransferase activity;manganese ion binding
13069	polyubiquitin binding;protein binding
8423	potassium channel activity
9973	P-P-bond-hydrolysis-driven protein transmembrane transporter activity;protein binding
5922	P-P-bond-hydrolysis-driven protein transmembrane transporter activity;protein binding;ribosome binding
6192	P-P-bond-hydrolysis-driven protein transmembrane transporter activity;ribosome binding
11705	procollagen-lysine 5-dioxygenase activity;oxidoreductase activity;iron ion binding;oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen;L-ascorbic acid binding
3646	proline-rich region binding;protein binding
601, 843, 1096, 1131, 1169, 1339, 1438, 1459, 1473, 1632, 1730, 1806, 1856, 1864, 2026, 2032, 2042, 2137, 2146, 2238, 2287, 2396, 2401, 2422, 2455, 2631, 2635, 2699, 2721, 2723, 2757, 2763, 2830, 2845, 2860, 2889, 2952, 3086, 3087, 3159, 3251, 3282, 3329, 3367, 3387, 3421, 3434, 3463, 3514, 3532, 3540, 3549, 3618, 3653, 3684, 3710, 3713, 3728, 3736, 3813, 3874, 3887, 3900, 3916, 3958, 3981, 4009, 4030, 4077, 4113, 4157, 4200, 4263, 4300, 4374, 4442, 4464, 4489, 4510, 4513, 4534, 4601, 4642, 4665, 4728, 4761, 4804, 4924, 4928, 4945, 4973, 5032, 5037, 5165, 5194, 5283, 5483, 5518, 5568, 5579, 5582, 5620, 5660, 5674, 5700, 5720,	protein binding

5770, 5798, 5804, 5813, 5866, 5877, 5896, 5897, 5973, 5974, 5988, 5994, 5997, 6211, 6242, 6297, 6319, 6388, 6443, 6492, 6495, 6554, 6574, 6618, 6622, 6639, 6773, 6790, 6807, 6814, 6905, 6917, 6950, 6960, 6990, 7023, 7073, 7136, 7137, 7138, 7142, 7144, 7191, 7290, 7330, 7412, 7422, 7429, 7509, 7528, 7536, 7630, 7636, 7657, 7689, 7701, 7728, 7758, 7762, 7798, 7821, 7880, 7898, 7974, 7991, 7993, 8048, 8063, 8082, 8131, 8155, 8161, 8175, 8252, 8293, 8335, 8368, 8378, 8389, 8481, 8487, 8639, 8681, 8709, 8746, 8759, 8773, 8876, 8968, 9017, 9076, 9125, 9153, 9187, 9208, 9254, 9275, 9327, 9349, 9360, 9394, 9448, 9490, 9553, 9563, 9594, 9628, 9670, 9700, 9740, 9758, 9821, 9859, 9940, 9946, 9965, 10020, 10028, 10108, 10110, 10157, 10183, 10239, 10247, 10296, 10302, 10334, 10339, 10357, 10395, 10443, 10466, 10491, 10551, 10657, 10683, 10700, 10730, 10781, 10838, 10857, 10908, 10920, 10926, 10931, 10970, 11039, 11046, 11105, 11119, 11128, 11173, 11278, 11396, 11448, 11451, 11479, 11499, 11508, 11525, 11544, 11593, 11635, 11650, 11662, 11682, 11727, 11796, 11800, 11825, 11834, 11933, 11978, 12026, 12147, 12152, 12194, 12329, 12416, 12418, 12512, 12526, 12563, 12679, 12753, 12772, 12798, 12817, 12833, 12843, 12846, 12872, 12907, 12914, 12973, 12990, 13059, 13061, 13064, 13070, 13104, 13122, 13184, 13199, 13218, 13246, 13250, 13264, 13265, 13414, 13447, 13452, 13479, 13527, 13532, 13566, 13645, 13757, 13774, 13853, 13899, 13924, 13933, 13983, 13999, 14018, 14029, 14041, 14044, 14058, 14078, 14207, 14394, 14527, 14566,	

14586, 14595, 14627, 14661, 14675, 14692, 14751, 14779, 14866, 14904, 15030, 15091, 15107, 15135, 15160, 15229, 15231, 15292, 15337, 15431, 15441, 15464, 15479, 15482, 15501, 15532, 15603, 15691, 15707, 15708, 15721, 15725, 15728, 15735, 15773, 15786, 15790, 15935, 15963, 15965, 15978, 15991, 15993, 16000, 16006, 16047, 16147, 16173, 16284, 16292, 16315, 16320, 16392, 16406, 16410, 16424, 16425, 16457, 16484, 16509, 16603, 16737, 16796, 16802, 16870, 16881, 16895, 16915, 16918, 16922, 17020, 17034, 17035, 17037, 17074	
12240	protein binding;1-phosphatidylinositol-3-kinase activity
10151, 14887	protein binding;7S RNA binding;serine-type endopeptidase inhibitor activity
9384	protein binding;acid-amino acid ligase activity;sodium channel inhibitor activity;sodium channel regulator activity
9132, 9747	protein binding;actin binding
5252	protein binding;actin binding;GKAP/Homer scaffold activity
3294	protein binding;actin filament binding
10022	protein binding;angiotensin type II receptor activity
5618	protein binding;Atg12 ligase activity
6246	protein binding;Atg12 ligase activity;Atg8 ligase activity;enzyme binding;small conjugating protein ligase activity
13890	protein binding;ATP binding
14835	protein binding;ATP binding;phosphatidylinositol phosphate kinase activity;zinc ion binding
15909	protein binding;beta-catenin binding;cytoskeletal protein binding
7033	protein binding;beta-dystroglycan binding
3286, 5280, 6858, 7091, 8331	protein binding;binding
10758	protein binding;binding;identical protein binding
1333, 3480, 14264	protein binding;catalytic activity
10852	protein binding;catalytic activity;carbohydrate binding
3585	protein binding;catalytic activity;serine-type endopeptidase activity
13962	protein binding;chromatin binding
14557	protein binding;collagen binding
2673	protein binding;collagen binding;heparin binding;peptidase activator activity
15051	protein binding;connexin binding
14381	protein binding;cyclin-dependent protein kinase regulator activity

8273	protein binding;cysteine-type endopeptidase inhibitor activity
696	protein binding;cystic fibrosis transmembrane conductance regulator binding;small GTPase regulator activity
12439	protein binding;cytoskeletal adaptor activity;dynactin binding;Rab GTPase binding;dynein binding;proteinase activated receptor binding
8678	protein binding;cytoskeletal protein binding
3017	protein binding;delta-opioid receptor activity
8605, 9517, 11410	protein binding;DNA binding
3230	protein binding;DNA binding;sequence-specific DNA binding RNA polymerase II transcription factor activity
6679	protein binding;DNA binding;sequence-specific DNA binding transcription factor activity
2785, 3519, 7773, 8030	protein binding;DNA binding;zinc ion binding
744	protein binding;DNA binding;zinc ion binding;methyl-CpG binding
2370	protein binding;DNA binding;zinc ion binding;transcription repressor activity;sequence-specific DNA binding
11179	protein binding;drug transmembrane transporter activity
11711	protein binding;epidermal growth factor binding
14567	protein binding;GABA receptor binding
4586, 9818, 14106	protein binding;gamma-tubulin binding
10367	protein binding;glycoprotein binding
9169	protein binding;glycoprotein binding;carbohydrate binding;beta-amyloid binding
16159	protein binding;G-protein activated inward rectifier potassium channel activity
14251	protein binding;G-protein coupled photoreceptor activity
5380	protein binding;GTP binding;ATPase activity;ATP binding;Rab GTPase binding
12952	protein binding;GTPase inhibitor activity
4088	protein binding;guanyl-nucleotide exchange factor activity
2593	protein binding;heparin binding
12533	protein binding;histone deacetylase activity
5096	protein binding;histone-lysine N-methyltransferase activity
5157	protein binding;Hsp90 protein binding;histone binding
5966	protein binding;hydrogen ion transmembrane transporter activity;hydrogen ion transporting ATP synthase activity, rotational mechanism
2429	protein binding;hydrolase activity;catalytic activity
6532, 9850, 14558	protein binding;identical protein binding
3460	protein binding;identical protein binding;CARD domain binding
4972	protein binding;insulin receptor substrate binding;insulin-like growth factor receptor binding;ErbB-3 class receptor binding;insulin receptor binding;protein phosphatase binding;insulin binding
10953	protein binding;integrin binding;protein homodimerization activity;protein heterodimerization activity

6031	protein binding;iron ion binding
5900	protein binding;kinase activity
12885	protein binding;kinesin binding
13721	protein binding;kinetochore binding
4390	protein binding;laminin-1 binding
9410	protein binding;ligand-dependent nuclear receptor transcription coactivator activity;protein domain specific binding;transcription coactivator activity;histone acetyltransferase activity;ligand-dependent nuclear receptor binding
7887	protein binding;microtubule binding;tubulin binding;phospholipid binding;SH3 domain binding
6106	protein binding;microtubule motor activity;ATP binding
6545, 14568	protein binding;N-acetyltransferase activity;ribosome binding;acetyltransferase activity
12246	protein binding;neurexin binding
1242, 3861	protein binding;nitric-oxide synthase activity
16450	protein binding;Notch binding;transcription repressor activity
5183, 7516	protein binding;nucleic acid binding
5339, 9848	protein binding;nucleotide binding;ATP binding;identical protein binding
12913	protein binding;oxidoreductase activity
1569, 8859	protein binding;oxidoreductase activity, acting on a sulfur group of donors, disulfide as acceptor;flavin adenine dinucleotide binding
4409, 10251, 10788	protein binding;oxidoreductase activity;transition metal ion binding
13333	protein binding;oxidoreductase activity;transition metal ion binding;kinase binding;kinase activator activity
5297	protein binding;peptidase inhibitor activity;heparin binding;collagen binding;collagen V binding
16459	protein binding;peroxidase activity;heme binding;peptidase inhibitor activity;extracellular matrix structural constituent
16312	protein binding;phosphatidylinositol 3-kinase binding;kinesin binding;mitogen-activated protein kinase p38 binding;JUN kinase binding
4166, 5018, 6331, 7513, 9479, 11893, 13273, 13595, 13773, 16627	protein binding;phosphatidylinositol binding
16467	protein binding;phosphatidylinositol binding;1-phosphatidylinositol binding;protein homodimerization activity;ubiquitin protein ligase binding
13213	protein binding;phosphatidylinositol binding;microtubule motor activity;ATP binding
5794	protein binding;phosphatidylinositol binding;nucleic acid binding;zinc ion binding
6312	protein binding;phosphatidylinositol binding;protein C-terminus binding;low-density lipoprotein receptor binding
4143	protein binding;phosphatidylinositol binding;protein transporter activity

3849	protein binding;phosphatidylinositol binding;SH2 domain binding;phosphatidylinositol-3-phosphate binding;phosphatidylinositol-3,5-bisphosphate binding;phosphatidylinositol-5-phosphate binding;phosphatidylinositol-4-phosphate binding
7363	protein binding;phosphatidylinositol transporter activity
4579	protein binding;phosphatidylinositol-4,5-bisphosphate binding
1516, 12924	protein binding;phospholipase A2 activity
4148	protein binding;P-P-bond-hydrolysis-driven protein transmembrane transporter activity;unfolded protein binding
1291	protein binding;protein binding, bridging
15994	protein binding;protein complex binding;protein kinase regulator activity
5775, 7172	protein binding;protein complex scaffold
1877, 4086	protein binding;protein C-terminus binding
3822	protein binding;protein C-terminus binding;calcium ion binding
15805	protein binding;protein C-terminus binding;enzyme binding
8775	protein binding;protein C-terminus binding;receptor binding;phosphatase binding
6167	protein binding;protein C-terminus binding;transcription regulator activity;beta-catenin binding
12171	protein binding;protein disulfide isomerase activity
7195, 15065	protein binding;protein domain specific binding
8507	protein binding;protein domain specific binding;protein C-terminus binding
3018	protein binding;protein domain specific binding;protein kinase binding;NF-kappaB binding;heat shock protein binding
5091	protein binding;protein domain specific binding;receptor tyrosine kinase binding
6969, 10547	protein binding;protein heterodimerization activity
7504	protein binding;protein heterodimerization activity;hydrolase activity, hydrolyzing O-glycosyl compounds;identical protein binding
3879	protein binding;protein kinase activator activity
2832	protein binding;protein kinase activity;ATP binding;protein serine/threonine kinase activity;calmodulin binding
4671	protein binding;protein kinase B binding
1043, 14877	protein binding;protein kinase binding;kinesin binding;MAP-kinase scaffold activity
11048	protein binding;protein kinase binding;protein homodimerization activity;protein tyrosine kinase inhibitor activity
3318	protein binding;protein kinase binding;protein tyrosine kinase inhibitor activity
16331	protein binding;protein kinase C binding;Rho GTPase binding;GTP-dependent protein binding
11945	protein binding;protein N-terminal glutamine amidohydrolase activity
10739	protein binding;protein phosphatase 2A binding
4446	protein binding;protein self-association;PDZ domain binding

17038	protein binding;protein serine/threonine phosphatase activity
10356	protein binding;protein serine/threonine phosphatase inhibitor activity
5093, 11380, 16685	protein binding;protein transporter activity
15949	protein binding;Rab GTPase binding
11038	protein binding;Rab guanyl-nucleotide exchange factor activity
13596	protein binding;receptor activity
16994	protein binding;receptor activity;complement receptor activity
7514, 16077	protein binding;receptor binding
9691	protein binding;ribonucleoside-diphosphate reductase activity
3949	protein binding;RNA binding
1927	protein binding;RNA binding;hydrolase activity, acting on ester bonds
3215	protein binding;RNA binding;phenylalanine-tRNA ligase activity
6231	protein binding;semaphorin receptor activity
4544	protein binding;sequence-specific DNA binding transcription factor activity
13371	protein binding;sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding
4786, 13046	protein binding;sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;DNA binding;protein dimerization activity
8159	protein binding;sequence-specific DNA binding transcription factor activity;signal transducer activity
5581	protein binding;SH2 domain binding
998	protein binding;SH3/SH2 adaptor activity
4358	protein binding;SNARE binding;syntaxin binding;calmodulin binding;phospholipid binding
1000, 1002, 8904	protein binding;snoRNA binding
7107	protein binding;snRNA binding
9778	protein binding;stem cell factor receptor binding
10814	protein binding;structural molecule activity
3490	protein binding;structural molecule activity;binding
3530, 6923, 8630	protein binding;transcription coactivator activity
6229, 13995	protein binding;transcription coactivator activity;RNA polymerase II transcription factor binding;peroxisome proliferator activated receptor binding;histone deacetylase binding;phosphatidate phosphatase activity
4121	protein binding;transcription corepressor activity
12275	protein binding;transcription corepressor activity;chromatin binding
4997, 5002	protein binding;transcription corepressor activity;toxin receptor binding;protein domain specific binding
16883	protein binding;transcription elongation regulator activity
10807	protein binding;transcription regulator activity
2410	protein binding;transcription repressor activity

10214	protein binding;transcription repressor activity;transcription corepressor activity;repressing transcription factor binding
4531, 6941	protein binding;translation initiation factor activity
7471	protein binding;tRNA (guanine-N7-)-methyltransferase activity
14279	protein binding;U7 snRNA binding
9995, 16348	protein binding;ubiquitin protein ligase binding
12382	protein binding;unfolded protein binding
6499	protein binding;unfolded protein binding;enzyme inhibitor activity
1231, 1625, 3046, 4078, 5658, 6069, 7963, 9622, 9782, 15005	protein binding;voltage-gated potassium channel activity
1493, 1826, 2937, 5993, 7732, 13415, 16413	protein binding;voltage-gated potassium channel activity;ion channel activity
8504	protein binding;voltage-gated potassium channel activity;ion channel activity;delayed rectifier potassium channel activity;potassium channel regulator activity
2155	protein binding;voltage-gated potassium channel activity;nucleic acid binding;zinc ion binding;protein C-terminus binding
3119	protein binding;Wnt-protein binding;frizzled binding
15381	protein binding;WW domain binding;protein phosphatase type 1 regulator activity
850, 1298, 2089, 2494, 3080, 3178, 3293, 3468, 4049, 4126, 4175, 5213, 5345, 5869, 5959, 6197, 6232, 6421, 6472, 7625, 8334, 9236, 10428, 10494, 10757, 11045, 11142, 11767, 12098, 12380, 13148, 13233, 13729, 13879, 14456, 15379, 15392, 15941, 16210, 16251, 16342, 16381, 16386, 16794	protein binding;zinc ion binding
16829	protein binding;zinc ion binding;ATP-dependent peptidase activity;ubiquitin-protein ligase activity
3823, 4050, 8060, 13516	protein binding;zinc ion binding;binding
14336	protein binding;zinc ion binding;calcium ion binding;histone-lysine N-methyltransferase activity
3227	protein binding;zinc ion binding;calcium ion binding;histone-lysine N-methyltransferase activity;transcription cofactor activity;androgen receptor binding;histone methyltransferase activity;chromatin binding
6959	protein binding;zinc ion binding;catalytic activity;transcription activator activity
1858, 4694, 10234, 15412	protein binding;zinc ion binding;DNA binding
3008	protein binding;zinc ion binding;DNA binding;protein homodimerization activity;identical protein binding;specific transcriptional repressor activity
4029	protein binding;zinc ion binding;DNA binding;transcription activator activity;chromatin binding
6019	protein binding;zinc ion binding;GTP binding
6936, 15761	protein binding;zinc ion binding;hydrolase activity, hydrolyzing O-

	glycosyl compounds
10966	protein binding;zinc ion binding;hydrolase activity, hydrolyzing O-glycosyl compounds;ATP binding
6497	protein binding;zinc ion binding;MAP-kinase scaffold activity
1055, 7389	protein binding;zinc ion binding;metal ion binding
16282	protein binding;zinc ion binding;metal ion binding;adenosylmethionine decarboxylase activity
15294	protein binding;zinc ion binding;methyl-CpG binding;methylated histone residue binding
9919	protein binding;zinc ion binding;nucleic acid binding;transcription activator activity;transcription repressor activity
3570	protein binding;zinc ion binding;nucleic acid binding;transcription repressor activity;histone demethylase activity (H3-K36 specific)
14483	protein binding;zinc ion binding;oxidoreductase activity
5629	protein binding;zinc ion binding;PDZ domain binding
840	protein binding;zinc ion binding;peptidase inhibitor activity
8397	protein binding;zinc ion binding;protein kinase activity;protein N-terminus binding;RNA polymerase II carboxy-terminal domain kinase activity;DNA-dependent ATPase activity
15396	protein binding;zinc ion binding;Rab GTPase binding
4002, 9866	protein binding;zinc ion binding;RNA binding
12730	protein binding;zinc ion binding;sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding
9932	protein binding;zinc ion binding;sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;ubiquitin-protein ligase activity;transcription activator activity;androgen receptor binding;DNA binding
12399	protein binding;zinc ion binding;signal transducer activity
7853	protein binding;zinc ion binding;signal transducer activity;ubiquitin-protein ligase activity;identical protein binding
15990	protein binding;zinc ion binding;transcription repressor activity
1494, 5710, 8298, 9428, 11911, 12649, 14673, 16680	protein binding;zinc ion binding;ubiquitin-protein ligase activity
3484	protein binding;zinc ion binding;ubiquitin-protein ligase activity;PDZ domain binding
8522	protein binding;zinc ion binding;ubiquitin-protein ligase activity;RING-like zinc finger domain binding;transcription repressor activity;chromatin binding
11524	protein complex binding;protein binding;estrogen receptor binding;armadillo repeat domain binding;protein phosphatase 2A binding
9136	protein C-terminus binding;protein binding
4139	protein domain specific binding
13220	protein domain specific binding;protein binding
13013	protein heterodimerization activity;protein binding;positive transcription elongation factor activity;negative transcription elongation factor activity

3248, 11904	protein homodimerization activity
3554, 6509	protein homodimerization activity;protein binding
6169	protein homodimerization activity;protein heterodimerization activity;identical protein binding;protein binding
10361	protein homodimerization activity;protein heterodimerization activity;protein binding;glucuronosyltransferase activity;acetylglucosaminyltransferase activity;transferase activity, transferring glycosyl groups
10056	protein kinase activity;ATP binding;calcium ion binding;protein serine/threonine kinase activity;protein tyrosine kinase activity;protein binding
13433	protein kinase activity;ATP binding;calcium ion binding;protein serine/threonine kinase activity;protein tyrosine kinase activity;protein binding;receptor activity
10216	protein kinase activity;ATP binding;ephrin receptor activity;protein serine/threonine kinase activity;protein tyrosine kinase activity;axon guidance receptor activity
9893	protein kinase activity;ATP binding;heat shock protein binding;protein serine/threonine kinase activity;protein tyrosine phosphatase activity;phosphatase activity
6171	protein kinase activity;ATP binding;motor activity;protein serine/threonine kinase activity;microfilament motor activity;calmodulin binding;actin-dependent ATPase activity;plus-end directed microfilament motor activity;ADP binding
2133	protein kinase activity;ATP binding;protein adenyltransferase activity
13481, 13884	protein kinase activity;ATP binding;protein binding
11587	protein kinase activity;ATP binding;protein binding;phosphatidylinositol binding;protein serine/threonine kinase activity
6144	protein kinase activity;ATP binding;protein binding;phosphatidylinositol binding;protein serine/threonine kinase activity;protein C-terminus binding
8531, 14603	protein kinase activity;ATP binding;protein binding;protein serine/threonine kinase activity
3378, 5870, 10323, 11317	protein kinase activity;ATP binding;protein binding;protein serine/threonine kinase activity;protein tyrosine kinase activity
5954	protein kinase activity;ATP binding;protein binding;protein serine/threonine kinase activity;signal transducer activity
9828	protein kinase activity;ATP binding;protein binding;zinc ion binding;protein serine/threonine kinase activity;binding;protein kinase binding;JUN kinase kinase activity
14577	protein kinase activity;ATP binding;protein kinase inhibitor activity;transcription factor binding;protein binding;mitogen-activated protein kinase kinase binding;ubiquitin-protein ligase regulator activity;ubiquitin protein ligase binding
2502, 3359, 3455, 4104, 4327, 4937, 5117, 5970, 7749, 7925, 10013, 10202, 10329, 10809, 10846, 11002, 11311, 12820, 13513, 15085, 15477, 16399,	protein kinase activity;ATP binding;protein serine/threonine kinase activity

16728, 17050	
11272	protein kinase activity;ATP binding;protein serine/threonine kinase activity;calmodulin-dependent protein kinase activity
14989	protein kinase activity;ATP binding;protein serine/threonine kinase activity;catalytic activity;NF-kappaB-inducing kinase activity;protein binding;IkappaB kinase activity
15450	protein kinase activity;ATP binding;protein serine/threonine kinase activity;catalytic activity;protein binding;protein complex binding
12312	protein kinase activity;ATP binding;protein serine/threonine kinase activity;catalytic activity;protein C-terminus binding;protein binding;troponin I binding
3756	protein kinase activity;ATP binding;protein serine/threonine kinase activity;enzyme binding
16182	protein kinase activity;ATP binding;protein serine/threonine kinase activity;eukaryotic translation initiation factor 2alpha kinase activity;identical protein binding;protein binding
15347	protein kinase activity;ATP binding;protein serine/threonine kinase activity;GTP binding;protein binding
6746	protein kinase activity;ATP binding;protein serine/threonine kinase activity;heme binding
4730, 5968	protein kinase activity;ATP binding;protein serine/threonine kinase activity;magnesium ion binding
16948	protein kinase activity;ATP binding;protein serine/threonine kinase activity;magnesium ion binding;cAMP-dependent protein kinase activity
4054	protein kinase activity;ATP binding;protein serine/threonine kinase activity;magnesium ion binding;identical protein binding;protein binding
1395, 3474, 4167, 15983	protein kinase activity;ATP binding;protein serine/threonine kinase activity;magnesium ion binding;protein binding
14402	protein kinase activity;ATP binding;protein serine/threonine kinase activity;magnesium ion binding;protein binding;mitogen-activated protein kinase kinase kinase binding
17167	protein kinase activity;ATP binding;protein serine/threonine kinase activity;MAP kinase activity;JUN kinase activity;protein binding;kinase activity
2278, 6079	protein kinase activity;ATP binding;protein serine/threonine kinase activity;MAP kinase activity;protein binding
4282	protein kinase activity;ATP binding;protein serine/threonine kinase activity;MAP kinase activity;protein binding;magnesium ion binding
2958	protein kinase activity;ATP binding;protein serine/threonine kinase activity;MAP kinase activity;transcription factor binding;protein binding;ubiquitin protein ligase binding;magnesium ion binding;SH2 domain binding
8686, 14547	protein kinase activity;ATP binding;protein serine/threonine kinase activity;MAP kinase kinase kinase activity;magnesium ion binding;identical protein binding;protein binding
4746	protein kinase activity;ATP binding;protein serine/threonine kinase activity;MAP kinase kinase kinase activity;protein homodimerization activity;identical protein binding;protein binding

9777	protein kinase activity;ATP binding;protein serine/threonine kinase activity;oxidoreductase activity;magnesium ion binding;protein kinase binding;protein binding
14748	protein kinase activity;ATP binding;protein serine/threonine kinase activity;phosphatase regulator activity
2066, 4553, 4858, 5141, 8839, 9589, 9658, 10316, 10882, 11503, 11794, 11990, 12041, 12305, 13644, 13775, 13844, 13847, 13851, 15127, 15296, 16022, 16806, 16875	protein kinase activity;ATP binding;protein serine/threonine kinase activity;protein binding
8328	protein kinase activity;ATP binding;protein serine/threonine kinase activity;protein binding;beta-catenin binding;tau-protein kinase activity
11106, 14424	protein kinase activity;ATP binding;protein serine/threonine kinase activity;protein binding;kinase activity
2739	protein kinase activity;ATP binding;protein serine/threonine kinase activity;protein binding;MAP kinase kinase activity
6479	protein kinase activity;ATP binding;protein serine/threonine kinase activity;protein binding;phosphatase binding
11286	protein kinase activity;ATP binding;protein serine/threonine kinase activity;protein kinase binding;protein binding
4826	protein kinase activity;ATP binding;protein serine/threonine kinase activity;protein kinase binding;protein binding;ubiquitin protein ligase binding
6699, 6760	protein kinase activity;ATP binding;protein serine/threonine kinase activity;protein kinase C activity;protein binding;enzyme activator activity;enzyme binding;calcium-independent protein kinase C activity
7499, 14461, 16253	protein kinase activity;ATP binding;protein serine/threonine kinase activity;protein tyrosine kinase activity
632	protein kinase activity;ATP binding;protein serine/threonine kinase activity;protein tyrosine kinase activity;p53 binding;protein binding
5625, 5627	protein kinase activity;ATP binding;protein serine/threonine kinase activity;protein tyrosine kinase activity;protein phosphatase inhibitor activity;protein binding;myosin VI binding
13024	protein kinase activity;ATP binding;protein serine/threonine kinase activity;small GTPase regulator activity
4057, 11055	protein kinase activity;ATP binding;protein serine/threonine kinase activity;small GTPase regulator activity;protein binding
16155	protein kinase activity;ATP binding;protein serine/threonine kinase activity;small GTPase regulator activity;protein binding;protein tyrosine kinase activator activity;protein kinase binding;identical protein binding
11457	protein kinase activity;ATP binding;protein serine/threonine kinase activity;small GTPase regulator activity;protein binding;Rho GTPase binding
6299	protein kinase activity;ATP binding;protein serine/threonine kinase activity;transcription corepressor activity;SMAD binding;protein binding;virion binding
10479	protein kinase activity;ATP binding;protein serine/threonine kinase activity;zinc ion binding;protein binding

3379, 4112, 11003, 11753	protein kinase activity;ATP binding;small GTPase regulator activity;protein serine/threonine kinase activity;protein binding
13696	protein kinase activity;ATP binding;transmembrane receptor protein tyrosine kinase activity;protein serine/threonine kinase activity;protein tyrosine kinase activity;platelet-derived growth factor receptor binding;platelet-derived growth factor beta-receptor activity;growth factor binding;receptor binding;protein binding;platelet-derived growth factor binding
13578	protein kinase activity;ATP binding;transmembrane receptor protein tyrosine kinase activity;protein serine/threonine kinase activity;protein tyrosine kinase activity;protein binding
3991	protein kinase activity;protein binding;ATP binding;kinase activity;chemorepellent activity;ephrin receptor activity;protein serine/threonine kinase activity;protein tyrosine kinase activity
5938	protein kinase activity;protein binding;RNA polymerase II carboxy-terminal domain kinase activity;DNA-dependent ATPase activity
9493	protein kinase activity;protein serine/threonine kinase activity;protein tyrosine kinase activity;ATP binding
4416	protein kinase activity;protein tyrosine kinase activity;non-membrane spanning protein tyrosine kinase activity;protein binding;ATP binding;kinase activity;protein domain specific binding;nucleotide binding;transferase activity;protein serine/threonine kinase activity;receptor binding;SH2 domain binding
5662, 13560	protein kinase activity;protein tyrosine kinase activity;transmembrane receptor protein tyrosine kinase activity;protein binding;ATP binding;kinase activity;cytokine binding;protein homodimerization activity;protein serine/threonine kinase activity
14849	protein kinase activity;protein tyrosine kinase activity;transmembrane receptor protein tyrosine kinase activity;protein binding;ATP binding;kinase activity;protein serine/threonine kinase activity
14935	protein kinase activity;protein tyrosine kinase activity;transmembrane receptor protein tyrosine kinase activity;receptor signaling protein tyrosine kinase activity;insulin receptor activity;insulin-like growth factor receptor binding;protein binding;ATP binding;GTP binding;kinase activity;insulin-like growth factor I binding;insulin-like growth factor II binding;SH2 domain binding;phosphatidylinositol 3-kinase binding;insulin binding;insulin receptor substrate binding;PTB domain binding;nucleotide binding;receptor activity;transferase activity;insulin-like growth factor binding;protein serine/threonine kinase activity
2060	protein kinase activity;transmembrane receptor protein tyrosine kinase activity;protein binding;ATP binding;kinase activity;Wnt-protein binding;Wnt receptor activity;protein serine/threonine kinase activity;protein tyrosine kinase activity;frizzled binding;transmembrane receptor activity
11472	protein kinase binding;protein binding
9388	protein kinase C activity;calcium channel regulator activity;protein binding;kinase activity;metal ion binding;protein kinase activity;ATP binding;protein serine/threonine kinase activity
6840, 14556	protein methyltransferase activity

9196	protein methyltransferase activity;protein homodimerization activity;protein heterodimerization activity;identical protein binding;protein binding;S-adenosylmethionine-dependent methyltransferase activity;histone-arginine N-methyltransferase activity;protein-arginine omega-N asymmetric methyltransferase activity;protein-arginine omega-N monomethyltransferase activity
5064	protein N-terminal asparagine amidohydrolase activity
2891	protein N-terminus binding;protein binding
13299	protein phosphatase inhibitor activity
13113, 14292, 16430	protein phosphatase type 2A regulator activity;binding;protein binding
8765	protein phosphatase type 2A regulator activity;protein binding
9620	protein prenyltransferase activity
14242	protein prenyltransferase activity;acetylcholine receptor regulator activity
16308	protein serine/threonine kinase activity
3566	protein serine/threonine kinase activity;ATP binding
6728	protein serine/threonine kinase activity;ATP binding;protein binding
5225, 5308	protein serine/threonine kinase activity;ATP binding;protein binding;zinc ion binding
4356	protein serine/threonine kinase activity;ATP binding;protein tyrosine kinase activity;catalytic activity
586, 15162	protein serine/threonine phosphatase activity;catalytic activity
5294	protein serine/threonine phosphatase activity;catalytic activity;magnesium ion binding;phosphoprotein phosphatase activity;manganese ion binding;signal transducer activity;protein binding
7709, 15572	protein serine/threonine phosphatase activity;catalytic activity;protein binding
11979	protein serine/threonine/tyrosine kinase activity;peptidase activity;serine-type peptidase activity;hydrolase activity;metal ion binding
10077, 10686	protein transporter activity;binding
3511, 7799, 10311	protein transporter activity;binding;protein binding
9090	protein transporter activity;binding;protein binding;histone binding
2022	protein transporter activity;binding;protein binding;tRNA binding
10876	protein transporter activity;importin-alpha export receptor activity;binding;protein binding
13670	protein transporter activity;P-P-bond-hydrolysis-driven protein transmembrane transporter activity
9209	protein transporter activity;protein binding
12585	protein tyrosine kinase activator activity;SH3 domain binding;SH2 domain binding
2692, 10475, 14135	protein tyrosine phosphatase activity;phosphatase activity
2824	protein tyrosine phosphatase activity;phosphatase activity;beta-catenin binding;alpha-catenin binding;delta-catenin binding;gamma-catenin binding;protein binding;cadherin binding;alpha-actinin binding;thiolester hydrolase activity
6688	protein tyrosine phosphatase activity;phosphatase activity;phosphatidylinositol-3-phosphatase activity

10393	protein tyrosine phosphatase activity;phosphatase activity;phosphotyrosine binding;protein binding;ATPase binding
13787	protein tyrosine phosphatase activity;phosphatase activity;protein binding
14341	protein tyrosine phosphatase activity;phosphatase activity;protein binding;cytoskeletal protein binding;non-membrane spanning protein tyrosine phosphatase activity
10307	protein tyrosine phosphatase activity;phosphatase activity;protein binding;protein homodimerization activity
15425	protein tyrosine phosphatase activity;phosphatase activity;protein binding;protein kinase binding
8985	protein tyrosine phosphatase activity;phosphatase activity;protein tyrosine/serine/threonine phosphatase activity
6691	protein tyrosine phosphatase activity;phosphatase activity;protein tyrosine/serine/threonine phosphatase activity;protein binding
14019, 15573	protein tyrosine phosphatase activity;phosphatase activity;transferase activity;protein binding
9028	protein tyrosine phosphatase activity;phosphatase activity;transmembrane receptor protein tyrosine phosphatase activity;protein binding;cadherin binding
17019, 17022	protein tyrosine phosphatase activity;protein binding
14408	protein tyrosine phosphatase activity;protein tyrosine/serine/threonine phosphatase activity;phosphatase activity
5899	protein tyrosine phosphatase activity;protein tyrosine/serine/threonine phosphatase activity;phosphatase activity;identical protein binding;protein binding
2560	protein tyrosine/serine/threonine phosphatase activity;phosphatase activity
15675	protein tyrosine/serine/threonine phosphatase activity;phosphatase activity;mRNA guanylyltransferase activity;DNA ligase (ATP) activity;ATP binding;protein tyrosine phosphatase activity
12055	protein tyrosine/serine/threonine phosphatase activity;phosphatase activity;phosphoprotein phosphatase activity;actin binding
15760	protein tyrosine/serine/threonine phosphatase activity;phosphatase activity;protein binding
4385, 7112, 10312, 12110	protein tyrosine/serine/threonine phosphatase activity;phosphatase activity;protein tyrosine phosphatase activity
2760	protein tyrosine/serine/threonine phosphatase activity;phosphatase activity;protein tyrosine phosphatase activity;MAP kinase phosphatase activity;phosphoprotein phosphatase activity
3089, 13565	protein tyrosine/serine/threonine phosphatase activity;phosphatase activity;protein tyrosine phosphatase activity;protein binding
16365	protein tyrosine/serine/threonine phosphatase activity;phosphatase activity;protein tyrosine phosphatase activity;protein serine/threonine phosphatase activity;phosphatidylinositol-3-phosphatase activity;PDZ domain binding;protein binding;enzyme binding;phosphatidylinositol-3,4-bisphosphate 3-phosphatase activity;inositol-1,3,4,5-tetrakisphosphate 3-phosphatase activity;phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase activity

8035	protein tyrosine/serine/threonine phosphatase activity;phosphatase activity;protein tyrosine phosphatase activity;protein serine/threonine phosphatase activity;protein binding
5645	protein-glutamine gamma-glutamyltransferase activity;protein binding
8047	protein-L-isoaspartate (D-aspartate) O-methyltransferase activity
11370	pseudouridine synthase activity;snoRNA binding
11296	pyridoxal phosphate binding;cystathionine gamma-lyase activity
16005	pyrroline-5-carboxylate reductase activity;superoxide dismutase activity;metal ion binding
1210, 2170, 2213, 5926, 6640, 7282, 7314, 7878, 8059, 9963, 11482, 12634, 13675, 13942, 14781, 16152, 16512	Rab GTPase activator activity
8473, 8843, 15385	Rab GTPase activator activity;calcium ion binding
2788	Rab GTPase activator activity;nucleotide binding
4860, 12895, 15443, 16487	Rab GTPase activator activity;protein binding
15268	Rab GTPase activator activity;protein binding;Rab GTPase binding
14826	Rab GTPase activator activity;Rab GTPase binding
15040	Rab GTPase activator activity;transporter activity
11766	Ras GTPase activator activity;protein binding;GTPase activator activity;sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;protein phosphatase binding
2466, 3433, 16581	receptor activity
2043, 17166	receptor activity;adrenergic receptor activity;alpha2-adrenergic receptor activity;protein kinase binding;protein homodimerization activity;protein heterodimerization activity;protein binding;heterotrimeric G-protein binding;thioesterase binding;alpha-2C adrenergic receptor binding;norepinephrine binding;epinephrine binding
9220	receptor activity;angiostatin binding;protein binding
13222	receptor activity;binding;protein binding;glycine binding;extracellular-glycine-gated ion channel activity;extracellular-glycine-gated chloride channel activity;extracellular ligand-gated ion channel activity
5749	receptor activity;calcium channel activity;protein binding;ion channel activity
16938	receptor activity;collagen binding;transmembrane receptor activity;protein binding;actin filament binding
6245	receptor activity;G-protein coupled receptor activity;nucleic acid binding;pancreatic ribonuclease activity;nucleotide binding;nucleoside-triphosphatase activity
1306	receptor activity;insulin receptor binding;insulin-like growth factor receptor binding;protein binding;protein kinase binding;phosphatidylinositol 3-kinase binding
14991, 15280	receptor activity;protein binding
1880, 15916	receptor activity;protein binding;cytokine receptor activity
12291, 13571	receptor activity;protein binding;extracellular ligand-gated ion channel activity
3560	receptor activity;protein tyrosine phosphatase activity;phosphatase activity

13458	receptor activity;receptor binding
12925	receptor activity;steroid binding;protein binding;lipid binding;heme binding
8734	receptor activity;transcription cofactor activity;transcription activator activity;protein binding;RNA polymerase II transcription mediator activity;thyroid hormone receptor binding
15516	receptor activity;transcription cofactor activity;transcription activator activity;RNA polymerase II transcription mediator activity;thyroid hormone receptor binding;protein binding;identical protein binding
7105	receptor activity;transcription cofactor activity;transcription activator activity;thyroid hormone receptor coactivator activity;thyroid hormone receptor binding
16499	receptor activity;transcription cofactor activity;transcription activator activity;transcription coactivator activity;protein binding;RNA polymerase II transcription mediator activity;thyroid hormone receptor binding
7003, 9825, 10890	receptor binding
11642	receptor binding;enzyme inhibitor activity
12575	receptor binding;nucleotide binding;aminoacyl-tRNA ligase activity;ATP binding
9381	receptor binding;phosphatidylinositol-3-phosphate binding;estrogen receptor binding;phosphatidylinositol-3,5-bisphosphate binding;androgen receptor binding
8924	receptor binding;protein binding
5135	receptor binding;protein binding;protein homodimerization activity;protein C-terminus binding;PDZ domain binding
1014	receptor binding;protein binding;thrombin receptor activity
7522	receptor binding;protein binding;transcription regulator activity
8903	receptor binding;transcription factor binding;protein binding;nuclear hormone receptor binding;transcription regulator activity
3556	Rho GDP-dissociation inhibitor activity;magnesium ion binding;inorganic diphosphatase activity;protein binding
14741	Rho GTPase activator activity
15158	Rho GTPase activator activity;protein binding;Rac GTPase activator activity
10333, 10733	Rho GTPase activator activity;protein binding;SH2 domain binding
2882, 3237, 4685, 10514, 11318, 12994, 16437	Rho guanyl-nucleotide exchange factor activity
7524	Rho guanyl-nucleotide exchange factor activity;calcium ion binding;proline-rich region binding;protein binding;protein complex scaffold;kinase activator activity
13987	Rho guanyl-nucleotide exchange factor activity;calcium ion binding;protein binding
12362	Rho guanyl-nucleotide exchange factor activity;DNA binding
16963	Rho guanyl-nucleotide exchange factor activity;guanyl-nucleotide exchange factor activity;1-phosphatidylinositol binding

5524	Rho guanyl-nucleotide exchange factor activity;guanyl-nucleotide exchange factor activity;DNA binding;Ras guanyl-nucleotide exchange factor activity;protein binding
10521	Rho guanyl-nucleotide exchange factor activity;guanyl-nucleotide exchange factor activity;protein binding
3380	Rho guanyl-nucleotide exchange factor activity;guanyl-nucleotide exchange factor activity;protein binding;Rac guanyl-nucleotide exchange factor activity;Rac GTPase activator activity
6037	Rho guanyl-nucleotide exchange factor activity;guanyl-nucleotide exchange factor activity;protein binding;receptor signaling protein activity
3593	Rho guanyl-nucleotide exchange factor activity;guanyl-nucleotide exchange factor activity;protein binding;receptor signaling protein activity;phospholipid binding;ephrin receptor binding
7618	Rho guanyl-nucleotide exchange factor activity;guanyl-nucleotide exchange factor activity;protein domain specific binding;protein binding
9633	Rho guanyl-nucleotide exchange factor activity;guanyl-nucleotide exchange factor activity;protein homodimerization activity;Rab guanyl-nucleotide exchange factor activity;Rac guanyl-nucleotide exchange factor activity;Rab GTPase binding;protein serine/threonine kinase activator activity;protein binding
9520, 10259	Rho guanyl-nucleotide exchange factor activity;guanyl-nucleotide exchange factor activity;protein serine/threonine kinase activity;GTPase activator activity
10847	Rho guanyl-nucleotide exchange factor activity;guanyl-nucleotide exchange factor activity;signal transducer activity;protein binding;Rho GTPase binding
2659	Rho guanyl-nucleotide exchange factor activity;protein binding
15657	Rho guanyl-nucleotide exchange factor activity;protein binding;nucleic acid binding;methyltransferase activity;G-protein-coupled receptor binding
6708	Rho guanyl-nucleotide exchange factor activity;protein kinase activity;ATP binding;protein serine/threonine kinase activity
6735	Rho guanyl-nucleotide exchange factor activity;Rho GTPase binding;guanyl-nucleotide exchange factor activity;GTP binding;GTPase binding
11175	Rho guanyl-nucleotide exchange factor activity;signal transducer activity;protein binding
12228	Rho guanyl-nucleotide exchange factor activity;zinc ion binding;protein binding
16423	ribonuclease activity;ribonuclease P activity;protein binding
10374	ribonuclease activity;structural constituent of ribosome
5673	ribonucleoside-diphosphate reductase activity;oxidoreductase activity;transition metal ion binding
5942	ribulose-phosphate 3-epimerase activity;catalytic activity;protein binding
3068, 7746, 9362, 13674, 17102	RNA binding
16871	RNA binding;ATP binding;transferase activity;double-stranded RNA binding;DNA binding;protein binding;transcription regulator activity
6571	RNA binding;DNA binding;hydrolase activity, acting on ester bonds

7257	RNA binding;endoribonuclease activity;5'-3' exonuclease activity;protein binding
11302, 16060	RNA binding;neurotransmitter:sodium symporter activity
2428, 11255	RNA binding;nucleic acid binding
3187, 4116, 4581, 13656, 14435, 15391	RNA binding;protein binding
7072, 9630, 13828	RNA binding;pseudouridine synthase activity
4857	RNA binding;pseudouridine synthase activity;protein binding
16269, 16354	RNA binding;pseudouridine synthase activity;protein binding;DNA binding;ligand-dependent nuclear receptor transcription coactivator activity
13450	RNA binding;ribonuclease activity
4071, 13211	RNA binding;ribonuclease activity;guanyl-nucleotide exchange factor activity;protein binding
10833	RNA binding;ribonuclease activity;nucleotide binding;nucleoside-triphosphatase activity;nucleic acid binding;zinc ion binding
8140	RNA binding;ribonuclease III activity;double-stranded RNA binding;protein binding
6666	RNA binding;RNA methyltransferase activity;methyltransferase activity
14682	RNA binding;S-adenosylmethionine-dependent methyltransferase activity;methyltransferase activity
727, 3504, 6048	RNA binding;structural constituent of ribosome
16610, 16923	RNA binding;translation initiation factor activity;protein binding
1968	RNA polymerase binding
4676	RNA polymerase II transcription factor activity;ATP binding;catalytic activity;protein binding
4023	RNA polymerase II transcription factor activity;catalytic activity;beta-amyloid binding;protein binding
7810	RNA polymerase II transcription factor activity;DNA binding;protein N-terminus binding;transcription coactivator activity;protein binding;thyroid hormone receptor binding;vitamin D receptor binding
2541, 2715, 6725	RNA polymerase II transcription factor activity;protein binding
8475	RNA polymerase II transcription mediator activity
1238, 6690	RNA polymerase II transcription mediator activity;protein binding
10569	RNA polymerase II transcription mediator activity;receptor activity;transcription cofactor activity;transcription activator activity;transcription coactivator activity;thyroid hormone receptor binding
14255	Roundabout binding
6580	rRNA (adenine-N6,N6-)-dimethyltransferase activity;rRNA methyltransferase activity;protein binding
9891	rRNA (adenine-N6,N6-)-dimethyltransferase activity;rRNA methyltransferase activity;rRNA (adenine) methyltransferase activity
5873	rRNA N-glycosylase activity;protein binding
8683	scavenger receptor activity

4129	scavenger receptor activity;copper ion binding;oxidoreductase activity, acting on the CH-NH2 group of donors, oxygen as acceptor
9934	scavenger receptor activity;polysaccharide binding;catalytic activity;nucleic acid binding;hydrolase activity;metal ion binding
8384	scavenger receptor activity;protein binding;ferritin receptor activity
11248	scavenger receptor activity;serine-type endopeptidase activity;catalytic activity
1197	scavenger receptor activity;serine-type endopeptidase activity;catalytic activity;peptidase activity
2928, 2953	selenium binding
10346	semaphorin receptor activity
3657	sequence-specific DNA binding
6043, 7840, 9705, 15073	sequence-specific DNA binding transcription factor activity
5876	sequence-specific DNA binding transcription factor activity;DNA binding;protein domain specific binding;transcription factor binding;protein binding
13577	sequence-specific DNA binding transcription factor activity;DNA binding;sequence-specific DNA binding
16745	sequence-specific DNA binding transcription factor activity;iron ion binding;nucleotide binding;nucleoside-triphosphatase activity;microtubule motor activity;ATP binding;ATPase activity
2056	sequence-specific DNA binding transcription factor activity;nucleoside transmembrane transporter activity
5152, 5160, 8464	sequence-specific DNA binding transcription factor activity;protein binding
1256	sequence-specific DNA binding transcription factor activity;protein binding;beta-catenin binding;type I transforming growth factor beta receptor binding;promoter binding;ubiquitin protein ligase binding;transforming growth factor beta receptor, inhibitory cytoplasmic mediator activity;I-SMAD binding;activin binding;collagen binding
15058	sequence-specific DNA binding transcription factor activity;protein binding;DNA binding;transcription activator activity
13442	sequence-specific DNA binding transcription factor activity;protein binding;zinc ion binding;transcription activator activity;promoter binding;transcription repressor activity;protein self-association
600, 4878, 6968, 8741, 9427, 11116, 11671, 13036, 13379, 14628	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding
6249	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;binding;DNA binding;transcription repressor activity
5304	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;DNA binding
935, 13766	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;DNA binding;protein dimerization activity
13365	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;DNA binding;protein dimerization activity;promoter binding;cAMP response element binding

11840	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;DNA binding;protein dimerization activity;protein binding;transcription repressor activity
12806	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;DNA binding;protein dimerization activity;transcription repressor activity;identical protein binding;protein binding
15195	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;DNA binding;transcription factor binding;protein binding;enzyme binding;transcription activator activity
14258, 17069	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;DNA binding;transcription regulator activity
2695	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;DNA binding;transcription regulator activity;transcription repressor activity;chromatin binding
12102	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;nucleic acid binding;zinc ion binding;chromatin binding;transcription repressor activity;protein binding
15729	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;nucleic acid binding;zinc ion binding;protein binding
3970, 3975, 6747	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;nucleotide binding;protein binding
10810	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;promoter binding;transcription activator activity
10575, 14596, 16154	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;protein binding
9662	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;protein binding;DNA binding;protein heterodimerization activity;sequence-specific enhancer binding RNA polymerase II transcription factor activity
11774	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;protein kinase binding;transcription activator activity;protein binding;DNA binding
7088, 11782	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;sphingosine N-acyltransferase activity
14089	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;transcription activator activity
9019, 14169	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;transcription activator activity;transcription regulator activity;protein binding
11583	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;transcription regulator activity;DNA binding;transcription repressor activity
9081	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;transcription repressor activity;protein binding;DNA binding
7999, 12842	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;zinc ion binding

11402	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;zinc ion binding;DNA binding;protein binding
5119	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;zinc ion binding;DNA binding;protein dimerization activity;protein binding
1357, 12017	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;zinc ion binding;protein binding
8163	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;zinc ion binding;protein binding;chromatin binding
12996	sequence-specific DNA binding transcription factor activity;steroid hormone receptor activity;zinc ion binding;sequence-specific DNA binding
12593, 14704	sequence-specific DNA binding transcription factor activity;steroid hormone receptor activity;zinc ion binding;sequence-specific DNA binding;metal ion binding;protein binding;transcription regulator activity;DNA binding;transcription activator activity;protein heterodimerization activity
6076	sequence-specific DNA binding transcription factor activity;steroid hormone receptor activity;zinc ion binding;sequence-specific DNA binding;protein binding;DNA binding
11742	sequence-specific DNA binding transcription factor activity;steroid hormone receptor activity;zinc ion binding;sequence-specific DNA binding;transcription activator activity;RNA polymerase II core promoter proximal region sequence-specific DNA binding transcription factor activity involved in positive regulation of transcription
7546	sequence-specific DNA binding transcription factor activity;transcription activator activity;protein binding;DNA binding;sequence-specific DNA binding;promoter binding
3829	sequence-specific DNA binding transcription factor activity;transcription initiation factor activity;DNA binding;NF-kappaB binding
12306, 13533, 13684, 13919, 15206, 15893, 16255, 16746	sequence-specific DNA binding transcription factor activity;zinc ion binding
11686	sequence-specific DNA binding transcription factor activity;zinc ion binding;DNA binding;nucleotide binding;nucleoside-triphosphatase activity
5771	sequence-specific DNA binding transcription factor activity;zinc ion binding;nucleic acid binding;protein binding;ATP binding
9061, 11690	sequence-specific DNA binding transcription factor activity;zinc ion binding;palmitoyltransferase activity
7705, 17029	sequence-specific DNA binding transcription factor activity;zinc ion binding;protein binding
8695	sequence-specific DNA binding transcription factor activity;zinc ion binding;sequence-specific DNA binding
12655	sequence-specific DNA binding transcription factor activity;zinc ion binding;sequence-specific DNA binding;DNA binding
9997, 10319	sequence-specific DNA binding transcription factor activity;zinc ion binding;sequence-specific DNA binding;DNA binding;protein binding

10263	sequence-specific DNA binding transcription factor activity;zinc ion binding;sequence-specific DNA binding;protein binding
5174	sequence-specific DNA binding transcription factor activity;zinc ion binding;sequence-specific DNA binding;protein binding;phosphatidylinositol-3-phosphate binding
2552	sequence-specific DNA binding;DNA binding;sequence-specific DNA binding transcription factor activity
3472	sequence-specific DNA binding;hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances
3997	serine-type endopeptidase activity
5914, 11631	serine-type endopeptidase activity
6378, 13464	serine-type endopeptidase activity;calcium ion binding;catalytic activity
2219, 11930	serine-type endopeptidase activity;catalytic activity
6599	serine-type endopeptidase activity;catalytic activity;protein binding
5711	serine-type endopeptidase activity;catalytic activity;serine-type peptidase activity
5545	serine-type endopeptidase activity;peptidase activity;endopeptidase activity;heparin binding;nerve growth factor binding;eukaryotic cell surface binding
14805	serine-type endopeptidase activity;protein binding;catalytic activity
12696	serine-type endopeptidase inhibitor activity
13060	serine-type endopeptidase inhibitor activity;calcium ion binding
14436	serine-type endopeptidase inhibitor activity;mannosyltransferase activity
11330	serine-type endopeptidase inhibitor activity;peptidase inhibitor activity
9527, 14468	serine-type endopeptidase inhibitor activity;protein binding
8565, 14786	serine-type endopeptidase inhibitor activity;protein kinase activity;ATP binding;protein serine/threonine kinase activity
12467	serine-type endopeptidase inhibitor activity;structural molecule activity;receptor binding
11959	serine-type endopeptidase inhibitor activity;transcription regulator activity;sequence-specific DNA binding
4825, 8356, 12677, 13950, 14562	serine-type peptidase activity
6683	serine-type peptidase activity;protein binding
16014	SH2 domain binding
1854	shikimate kinase activity;ATP binding;pyridoxal phosphate binding;catalytic activity;nucleotide binding;nucleoside-triphosphatase activity;threonine synthase activity
14681	sialyltransferase activity;alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase activity
2553, 3384, 9121, 9336	sialyltransferase activity;transferase activity;transferase activity, transferring glycosyl groups
602, 1193, 3458, 3518, 3898, 4198, 4285, 6563, 7058, 9106, 16758, 17052	signal transducer activity
16066	signal transducer activity;3'-phosphoadenosine 5'-phosphosulfate transmembrane transporter activity

5182	signal transducer activity;actin binding;protein domain specific binding
9160	signal transducer activity;GTPase regulator activity;protein binding;GTPase activator activity;receptor signaling protein activity
4916, 9289	signal transducer activity;guanyl nucleotide binding
11491	signal transducer activity;guanyl nucleotide binding;GTP binding
9950	signal transducer activity;guanyl nucleotide binding;GTP binding;protein binding
3703	signal transducer activity;guanyl nucleotide binding;protein binding
9416	signal transducer activity;protein binding
6107, 6513	signal transducer activity;protein binding;frizzled binding;protease binding;beta-catenin binding;receptor binding;protein heterodimerization activity
16050	signal transducer activity;protein binding;guanyl nucleotide binding
9497, 12792	signal transducer activity;protein binding;identical protein binding
11461	signal transducer activity;protein binding;phosphatidylinositol binding
4286	signal transducer activity;protein binding;WW domain binding
12425, 15289	signal transducer activity;protein kinase activity;ATP binding;protein serine/threonine kinase activity
11022	signal transducer activity;protein kinase activity;ATP binding;protein serine/threonine kinase activity;G-protein coupled receptor kinase activity
8254	signal transducer activity;protein kinase activity;ATP binding;protein serine/threonine kinase activity;protein binding
9905	signal transducer activity;receptor activity;G-protein coupled receptor activity
933	signal transducer activity;receptor activity;G-protein coupled receptor activity;cannabinoid receptor activity;protein binding
3295	signal transducer activity;receptor activity;transmembrane receptor activity;G-protein coupled receptor activity;hydrogen ion transmembrane transporter activity;protein binding;Wnt receptor activity;Wnt-protein binding
15288	signal transducer activity;receptor activity;transmembrane receptor activity;G-protein coupled receptor activity;Wnt-protein binding;protein binding;PDZ domain binding
8948	signal transducer activity;receptor binding
11329	signal transducer activity;transcription regulator activity
10262	signal transducer activity;transcription regulator activity;transcription coactivator activity;protein N-terminus binding;histone acetyltransferase activity;protein binding;ligand-dependent nuclear receptor binding;nuclear hormone receptor binding;chromatin binding
4109	signal transducer activity;two-component response regulator activity;3',5'-cyclic-nucleotide phosphodiesterase activity;catalytic activity
14746	signal transducer activity;two-component sensor activity;ion channel activity
13692	signal transducer activity;two-component sensor activity;ion channel activity;nucleotide binding

7756	signal transducer activity;two-component sensor activity;ion channel activity;protein binding
13795	single-stranded DNA binding
3202, 5752, 9798, 12150, 12579, 13852, 15128, 15366, 16525	small conjugating protein ligase activity
1290	small conjugating protein ligase activity;DNA binding;protein binding;transcription regulator activity
6821	small conjugating protein ligase activity;oxidoreductase activity;catalytic activity
7407, 14881	small conjugating protein ligase activity;protein binding
5760	small conjugating protein ligase activity;ubiquitin-protein ligase activity;ISG15 ligase activity
9246, 11363	small conjugating protein ligase activity;ubiquitin-protein ligase activity;protein binding
15078, 15081	SNAP receptor activity
17014	SNAP receptor activity;protein binding
6280	SNAP receptor activity;protein binding;SNARE binding;calcium channel inhibitor activity
10859	sodium:dicarboxylate symporter activity;aminopeptidase activity;L-glutamate transmembrane transporter activity
7816	sodium:dicarboxylate symporter activity;protein binding;amino acid binding;glutamate binding;high-affinity glutamate transmembrane transporter activity
10030	sodium:dicarboxylate symporter activity;transporter activity;chloride channel activity;L-threonine transmembrane transporter activity;L-serine transmembrane transporter activity;L-proline transmembrane transporter activity;L-cystine transmembrane transporter activity;L-alanine transmembrane transporter activity;L-hydroxyproline transmembrane transporter activity;neutral amino acid transmembrane transporter activity
5965, 13497	sodium:hydrogen antiporter activity;solute:hydrogen antiporter activity
4428, 4849	sphingomyelin phosphodiesterase activity
9302, 13676	sphingosine N-acyltransferase activity
8354	sterol binding;DNA binding;binding
3477	sterol esterase activity;lipase activity
13239	structural constituent of cytoskeleton;protein C-terminus binding;identical protein binding;protein binding
8938	structural constituent of nuclear pore
2281, 4254, 4451, 4722, 5635, 5815, 5851, 6975, 8337, 8813, 8886, 11957, 12278, 13634, 14633, 14758, 15039, 15252, 15705, 16946, 16962, 16988, 17149	structural constituent of ribosome
3702	structural constituent of ribosome;caspase activator activity;translation activator activity
877, 2478, 3442, 3921, 3979, 5607, 8321, 9503, 9889, 11180, 12077, 12401, 15868, 17118	structural constituent of ribosome;protein binding

2430	structural constituent of ribosome;protein N-terminus binding
7339, 16595	structural constituent of ribosome;RNA binding;protein binding
7783, 12247	structural constituent of ribosome;rRNA binding
6918, 14327	structural molecule activity
9083	structural molecule activity;beta-catenin binding;gamma-catenin binding;protein binding;vinculin binding;cadherin binding;actin filament binding
16397	structural molecule activity;metal ion binding;iron-sulfur cluster binding
6101	structural molecule activity;protein binding
2977	structural molecule activity;protein binding;binding
7326	succinate dehydrogenase activity;protein binding;succinate dehydrogenase (ubiquinone) activity;electron carrier activity;oxidoreductase activity;oxidoreductase activity, acting on the CH-CH group of donors;flavin adenine dinucleotide binding
13529	sugar binding;glutamine-fructose-6-phosphate transaminase (isomerizing) activity;transporter activity
10279	sugar:hydrogen symporter activity
3539	sugar:hydrogen symporter activity;phosphatase activity;protein binding
4536, 14979	sugar:hydrogen symporter activity;protein binding
6460	sulfate adenylyltransferase (ATP) activity;ATP binding;kinase activity;transferase activity, transferring phosphorus-containing groups;adenylylsulfate kinase activity
643, 1371, 1876, 2510, 8309, 8669, 15449, 16866, 17092	sulfotransferase activity
10967	sulfotransferase activity;flavonol 3-sulfotransferase activity
15223	sulfotransferase activity;N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase activity;3'-phosphoadenosine 5'-phosphosulfate binding
7159	sulfotransferase activity;protein binding;[heparan sulfate]-glucosamine 3-sulfotransferase 1 activity
1073	sulfotransferase activity;protein binding;chondroitin 4-sulfotransferase activity
2714	sulfuric ester hydrolase activity;catalytic activity
10385	sulfuric ester hydrolase activity;catalytic activity;arylsulfatase activity
3795	syntaxin binding;neurotransmitter transporter activity
13691	telomeric DNA binding;protein binding
2899	thiamine diphosphokinase activity;ATP binding
8467	thiol oxidase activity
9206	thiol oxidase activity;protein binding
1719	thiosulfate sulfurtransferase activity
10439	threonine-type endopeptidase activity;endopeptidase activity
10252	threonine-type endopeptidase activity;endopeptidase activity;lipopolysaccharide binding
4357	threonine-type endopeptidase activity;endopeptidase activity;protein binding
6212	thymidylate kinase activity;ATP binding;cytidylate kinase activity;UMP

	kinase activity
16346	thymidylate synthase activity
9355, 13909	toxin binding
8497, 14876	transaminase activity;pyridoxal phosphate binding
4223	transcription activator activity
860	transcription activator activity;DNA binding;protein complex binding;protein binding
12604	transcription activator activity;protein binding
14448	transcription activator activity;transcription coactivator activity;chromatin binding;protein binding;ligand-dependent nuclear receptor transcription coactivator activity;enzyme binding;thyroid hormone receptor binding
13626	transcription coactivator activity;histone acetyltransferase activity
4962	transcription coactivator activity;ligand-dependent nuclear receptor transcription coactivator activity
10345	transcription coactivator activity;protein binding
15872	transcription coactivator activity;protein binding;androgen receptor binding;ribonucleoprotein binding
16762	transcription coactivator activity;protein binding;transcription regulator activity
9910	transcription coactivator activity;protein kinase binding;protein binding;peptide antigen binding
9429	transcription coactivator activity;TBP-class protein binding
16329	transcription cofactor activity;histone acetyltransferase activity;zinc ion binding;protein binding;transcription coactivator activity;transcription factor binding;p53 binding;acetyltransferase activity;MyoD binding;DNA binding;chromatin binding
12670	transcription cofactor activity;transcription coactivator activity;histone acetyltransferase activity;protein binding;N-acetyltransferase activity;transcription factor binding;acetyltransferase activity;histone deacetylase binding;protein kinase binding;cyclin-dependent protein kinase inhibitor activity;lysine N-acetyltransferase activity
16327	transcription corepressor activity;p53 binding;transcription repressor activity;protein binding;protein-lysine N-methyltransferase activity;histone-lysine N-methyltransferase activity
6446	transcription corepressor activity;protein N-terminus binding;protein binding
15028	transcription corepressor activity;sequence-specific DNA binding transcription factor activity;protein C-terminus binding;transcription activator activity;protein N-terminus binding;transcription coactivator activity;protein binding
13769	transcription factor binding
15493	transcription factor binding;protein binding
8255, 9844, 13068	transcription regulator activity
3341	transcription regulator activity;DNA binding
3243	transcription regulator activity;DNA binding;histone deacetylase binding;transcription repressor activity
7374	transcription regulator activity;DNA binding;protein binding

17021	transcription regulator activity;DNA binding;protein binding;transcription repressor activity
6730, 13188	transcription regulator activity;protein binding
11444	transcription regulator activity;protein binding;calcium ion binding
4020	transcription regulator activity;protein binding;DNA binding;sequence-specific DNA binding transcription factor activity;chromatin binding
8320	transcription regulator activity;protein binding;sequence-specific DNA binding;sequence-specific DNA binding transcription factor activity;protein dimerization activity
12204	transcription regulator activity;sequence-specific DNA binding transcription factor activity;protein binding
3331	transcription regulator activity;transcription coactivator activity;histone acetyltransferase activity
3336	transcription termination factor activity;transcription corepressor activity;protein binding
3536	transferase activity, transferring acyl groups other than amino-acyl groups;catalytic activity;acetyl-CoA C-acyltransferase activity
15196	transferase activity, transferring acyl groups other than amino-acyl groups;catalytic activity;protein binding
15562	transferase activity, transferring acyl groups, acyl groups converted into alkyl on transfer
9250, 16283	transferase activity, transferring alkyl or aryl (other than methyl) groups
13628, 13640	transferase activity, transferring alkyl or aryl (other than methyl) groups;poly(ADP-ribose) glycohydrolase activity
2995, 4411, 6268, 7155, 16493	transferase activity, transferring glycosyl groups
14526	transferase activity, transferring glycosyl groups;UDP-xylosyltransferase activity
2107, 4669, 9297	transferase activity, transferring hexosyl groups
5083	transferase activity, transferring hexosyl groups;acetylglactosaminyltransferase activity
5944, 8156	transferase activity, transferring hexosyl groups;alpha-1,3-mannosyltransferase activity
6201	transferase activity, transferring hexosyl groups;glucuronosyl-N-acetylglactosaminyl-proteoglycan 4-beta-N-acetylglactosaminyltransferase activity
14938	transferase activity, transferring hexosyl groups;glucuronosyltransferase activity;peptidoglycan glycosyltransferase activity;acetylglactosaminyltransferase activity;glucuronosyl-N-acetylglactosaminyl-proteoglycan 4-beta-N-acetylglactosaminyltransferase activity;glucuronylglactosylproteoglycan 4-beta-N-acetylglactosaminyltransferase activity
7711, 16017	transferase activity, transferring nitrogenous groups;pyridoxal phosphate binding
10249	transferase activity, transferring nitrogenous groups;pyridoxal phosphate binding;L-alanine:2-oxoglutarate aminotransferase activity
6230	transferase activity, transferring nitrogenous groups;pyridoxal phosphate binding;serine C-palmitoyltransferase activity;protein binding

3172	transferase activity, transferring phosphorus-containing groups
1466	transferase activity, transferring phosphorus-containing groups;dolichol kinase activity
2852	transferase activity;dynein binding
11600	transferase activity;transferase activity, transferring nitrogenous groups;pyridoxal phosphate binding;serine C-palmitoyltransferase activity
9949, 12115	trans-hexaprenyltranstransferase activity;protein heterodimerization activity;trans-octaprenyltranstransferase activity
8941	translation elongation factor activity;signal transducer activity;protein binding
10458, 14734	translation initiation factor activity;actin binding;Rho GTPase binding;binding
1201, 2671	translation initiation factor activity;protein binding
7312, 9722	translation release factor activity;translation release factor activity, codon specific
3174	transmembrane receptor activity
8014, 11762, 13430	transmembrane receptor activity
14943	transmembrane receptor activity;binding;protein binding
4013	transmembrane receptor activity;protein binding
1622, 14310	transmembrane receptor activity;protein kinase binding;protein binding;Wnt-protein binding
11078	transmembrane receptor protein serine/threonine kinase activity;transforming growth factor beta receptor activity;protein kinase activity;ATP binding;protein serine/threonine kinase activity
5730	transmembrane receptor protein serine/threonine kinase activity;transforming growth factor beta receptor activity;protein kinase activity;ATP binding;protein serine/threonine kinase activity;structural constituent of ribosome;activin receptor activity, type I
839, 1211, 3930, 4386, 4610, 4611, 6744, 6847, 7157, 9596, 12804, 13125, 14113, 15210, 16440	transporter activity
7715	transporter activity;G-protein coupled receptor activity;protein binding
4717	transporter activity;lipid binding;sugar:hydrogen symporter activity;calcium ion binding
12261	transporter activity;sugar binding;copper ion binding
6661	triglyceride lipase activity
15772	tRNA (guanine-N7-)-methyltransferase activity;methyltransferase activity
5837	tRNA binding;phenylalanine-tRNA ligase activity;ATP binding
6253	tRNA binding;protein homodimerization activity;cell surface binding
2078, 7933	tRNA dihydrouridine synthase activity;flavin adenine dinucleotide binding
8659	tropomyosin binding
10310	tubulin binding;protein binding
9611, 15664	tubulin-tyrosine ligase activity

7678	two-component response regulator activity;3',5'-cyclic-nucleotide phosphodiesterase activity;catalytic activity
11999	type 5 melanocortin receptor binding;type 4 melanocortin receptor binding;type 3 melanocortin receptor binding;adrenocorticotropin hormone receptor binding;type 1 melanocortin receptor binding
1372, 7293, 13262	ubiquinol-cytochrome-c reductase activity
5150	ubiquitin binding
1991, 10293	ubiquitin protein ligase binding
8732	ubiquitin protein ligase binding;cyclin binding;ubiquitin-protein ligase activity;protein binding
9698, 10964	ubiquitin protein ligase binding;protein binding
15248	ubiquitin protein ligase binding;protein kinase binding;protein homodimerization activity;protein binding
8768, 14755, 15726	ubiquitin thiolesterase activity
5844	ubiquitin thiolesterase activity;binding
8113	ubiquitin thiolesterase activity;calcium ion binding
15528	ubiquitin thiolesterase activity;calcium ion binding;calcium-dependent phospholipid binding;binding
7386	ubiquitin thiolesterase activity;cysteine-type endopeptidase activity;G-protein-coupled receptor binding;zinc ion binding;protein binding
1141	ubiquitin thiolesterase activity;endopeptidase inhibitor activity;proteasome binding;protein binding
6324	ubiquitin thiolesterase activity;protein binding
5546	ubiquitin thiolesterase activity;ubiquitin-specific protease activity;cysteine-type endopeptidase activity;protein binding
3058	ubiquitin thiolesterase activity;ubiquitin-specific protease activity;protein binding
2558	ubiquitin thiolesterase activity;zinc ion binding
9150	ubiquitin thiolesterase activity;zinc ion binding;cysteine-type endopeptidase activity;G-protein-coupled receptor binding
3444	ubiquitin thiolesterase activity;zinc ion binding;histone binding
3572, 11758	ubiquitin thiolesterase activity;zinc ion binding;protein binding
5009	ubiquitin thiolesterase activity;zinc ion binding;ubiquitin-specific protease activity;cysteine-type endopeptidase activity;transcription coactivator activity;ubiquitin binding;histone binding
9257, 13967	ubiquitin-protein ligase activity;protein binding
2315	ubiquitin-protein ligase activity;protein binding;protein C-terminus binding;zinc ion binding;metal ion binding
8717	ubiquitin-protein ligase activity;protein binding;specific transcriptional repressor activity;transcription activator activity;beta-catenin binding;protein phosphorylated amino acid binding
10121	ubiquitin-protein ligase activity;protein complex binding;protein binding
14084, 17009	ubiquitin-protein ligase activity;protein homodimerization activity
13797	ubiquitin-protein ligase activity;ubiquitin conjugating enzyme binding;enzyme binding
6137	ubiquitin-protein ligase activity;zinc ion binding

12507	ubiquitin-protein ligase activity;zinc ion binding;binding
4700	ubiquitin-specific protease activity;ubiquitin thiolesterase activity;protein binding
7476, 11839	UDP-glucose:glycoprotein glucosyltransferase activity;protein binding;unfolded protein binding
9488, 10577	UDP-N-acetylglucosamine-dolichyl-phosphate N-acetylglucosaminophosphotransferase activity
5290, 11627, 15141	unfolded protein binding
8185, 13753, 15854	unfolded protein binding;protein binding
13913	unfolded protein binding;transcription corepressor activity
7862	urease activity;nickel ion binding;heme binding
10665	uroporphyrinogen decarboxylase activity
13219	uroporphyrinogen-III synthase activity
8538	vinculin binding
6778	voltage-gated anion channel activity
2644	voltage-gated calcium channel activity
7295	voltage-gated chloride channel activity
5048, 10580, 12290	voltage-gated chloride channel activity;ion channel activity
1142, 14302	voltage-gated chloride channel activity;protein binding
2405, 10253	voltage-gated potassium channel activity
1212, 9099	voltage-gated potassium channel activity;ion channel activity;protein binding
11789	voltage-gated sodium channel activity
2102, 2196, 2274, 3636, 3680, 3964, 4743, 6236, 6434, 6496, 6795, 7053, 7086, 7323, 7420, 7753, 7779, 10029, 10278, 10707, 10712, 10784, 10956, 11010, 11011, 11013, 11097, 11540, 12116, 12959, 13022, 13581, 13700, 13928, 13930, 14000, 15711, 15746, 16273, 16502, 16535, 16941	zinc ion binding
15124	zinc ion binding;1-phosphatidylinositol binding;protein homodimerization activity;protein binding;GTP-dependent protein binding
3662	zinc ion binding;acid-amino acid ligase activity;heme binding;nucleic acid binding;ubiquitin-protein ligase activity;metal ion binding;protein kinase activity;ATP binding;protein binding
12686, 12694, 15364	zinc ion binding;actin binding
3476, 16928	zinc ion binding;binding;1-phosphatidylinositol binding;protein binding;beta-N-acetylglucosaminylglycopeptide beta-1,4-galactosyltransferase activity
8212	zinc ion binding;DNA binding
11183	zinc ion binding;DNA binding;protein binding
6925	zinc ion binding;DNA binding;RNA polymerase II transcription factor activity;protein binding

9224	zinc ion binding;DNA binding;sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding;transcription repressor activity;protein binding
9093	zinc ion binding;DNA binding;transcription corepressor activity
17105	zinc ion binding;histone demethylase activity (H3-K9 specific);histone demethylase activity (H3-K27 specific);iron ion binding;oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors;histone demethylase activity (H4-K20 specific);methylated histone residue binding;histone demethylase activity (H3-K36 specific)
1684	zinc ion binding;histone-lysine N-methyltransferase activity
10812	zinc ion binding;hydrogen ion transporting ATP synthase activity, rotational mechanism;proton-transporting ATPase activity, rotational mechanism
129, 533	zinc ion binding;hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides;NAD ⁺ binding;NAD ⁺ ADP-ribosyltransferase activity;protein binding
5306	zinc ion binding;hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides;NAD ⁺ binding;protein binding
9657	zinc ion binding;hydrolase activity;catalytic activity
14086	zinc ion binding;identical protein binding;protein binding;specific transcriptional repressor activity
3936, 4072	zinc ion binding;metal ion binding;protein binding
13981	zinc ion binding;methylated histone residue binding
8358	zinc ion binding;monooxygenase activity;electron carrier activity;oxidoreductase activity
4010	zinc ion binding;nucleic acid binding
12274	zinc ion binding;nucleic acid binding transcription factor activity;protein binding
10268	zinc ion binding;nucleic acid binding;DNA binding
10114	zinc ion binding;nucleic acid binding;methyltransferase activity
7699	zinc ion binding;nucleotide binding;protein binding
677	zinc ion binding;oxidoreductase activity
4169, 8999, 9439, 9544	zinc ion binding;oxidoreductase activity
5050	zinc ion binding;oxidoreductase activity;L-iditol 2-dehydrogenase activity;NAD ⁺ or NADH binding
11094	zinc ion binding;p53 binding;protein binding;protein-lysine N-methyltransferase activity;histone methyltransferase activity (H3-K36 specific);RNA polymerase II core binding
2018, 4249, 4763, 5212, 7662, 7670, 9912, 11033, 12446, 12713, 13438, 15491, 15492, 15634, 16713	zinc ion binding;protein binding
16945	zinc ion binding;protein binding;CD40 receptor binding;ubiquitin-protein ligase activity;sphingolipid binding
4437, 13498	zinc ion binding;protein binding;DNA binding;sequence-specific DNA binding transcription factor activity

14457	zinc ion binding;protein binding;DNA binding;transcription corepressor activity
9755	zinc ion binding;protein binding;DNA binding;transcription repressor activity;chromatin binding
14958	zinc ion binding;protein binding;electron carrier activity
9453	zinc ion binding;protein binding;GTP binding;ubiquitin-protein ligase activity;GTPase activity;GDP binding
8484	zinc ion binding;protein binding;K63-linked polyubiquitin binding
6441	zinc ion binding;protein binding;methylated histone residue binding;histone methyltransferase activity;chromatin binding
10950	zinc ion binding;protein binding;R-SMAD binding;co-SMAD binding
6506	zinc ion binding;protein binding;sequence-specific DNA binding;RNA polymerase II core promoter proximal region sequence-specific DNA binding;RNA polymerase II core promoter proximal region sequence-specific DNA binding transcription factor activity involved in positive regulation of transcription
1121	zinc ion binding;protein binding;signal transducer activity;ubiquitin-protein ligase activity
3015	zinc ion binding;protein binding;transcription corepressor activity
9992	zinc ion binding;protein binding;transcription regulator activity
12784	zinc ion binding;protein binding;ubiquitin-protein ligase activity
3858	zinc ion binding;protein binding;ubiquitin-protein ligase activity;chromatin binding;p53 binding;transcription coactivator activity;estrogen response element binding;histone acetyl-lysine binding;sequence-specific DNA binding;ligand-dependent nuclear receptor binding;transcription repressor activity;protein kinase activity
5397	zinc ion binding;protein binding;ubiquitin-protein ligase activity;metal ion binding
14047	zinc ion binding;protein binding;ubiquitin-protein ligase activity;protein kinase binding;histone deacetylase binding;protein N-terminus binding;transcription repressor activity;mitogen-activated protein kinase kinase binding;protein kinase B binding
9073	zinc ion binding;protein kinase C binding;protein kinase binding;protein binding;SH2 domain binding
8050	zinc ion binding;receptor activity;binding;oxidoreductase activity;electron carrier activity;iron-sulfur cluster binding
13759	zinc ion binding;retinoic acid-responsive element binding
11088	zinc ion binding;transcription activator activity;translation initiation factor activity;protein binding
5874	zinc ion binding;transcription coactivator activity
4789	zinc ion binding;transcription regulator activity;ligand-dependent nuclear receptor binding
15759	zinc ion binding;transcription regulator activity;translation initiation factor activity;protein binding;transcription factor binding;thyroid hormone receptor binding;DNA binding
889, 13830	zinc ion binding;transcription repressor activity;protein binding
16358	zinc ion binding;transcription repressor activity;transcription corepressor activity;transcription activator activity;transcription factor binding

15384	zinc ion binding;ubiquitin thiolesterase activity;protein binding
13337	zinc ion binding;ubiquitin thiolesterase activity;ubiquitin-specific protease activity;proline-rich region binding;protein kinase binding;protein binding
11313, 16942	zinc ion binding;ubiquitin-protein ligase activity
13160	zinc ion binding;ubiquitin-protein ligase activity;protein kinase binding;PDZ domain binding;protein binding;kinase binding;chaperone binding

From the table, it is clear that various cellular processes can be affected by targeting the transcripts listed. In one embodiment, targeting constructs are designed to modulate the expression of one or more of the avian transcripts listed in Table 5. Where similar functions have been annotated, it is expected that one or more targeting constructs, or a cocktail of targeting constructs may be designed to alter partial or entire signaling pathways associated with that particular function.

Example 3. Targeting Constructs: siRNA Design

Using the Avian transcripts identified in Example 1, oligonucleotide design was carried out to identify siRNAs targeting the transcripts of Table 1. All sequences were obtained as described above using both the ENSEMBL datasets as well as the internal microRNA sequencing results.

All siRNA duplexes were designed with 100% identity to their respective transcripts with a total of 1,744,220 strands (872,110 duplexes) having SEQ ID NO 18040 (the first sense strand) through SEQ ID NO 1762259 (the last antisense strand). These are presented in the sequence listing submitted with the present application and incorporated herein in its entirety.

siRNA Design and Specificity Prediction

Putative siRNA duplex designs were created in a sliding 19mer window across each avian transcript. The specificity of the 19mer oligonucleotide set was predicted from each sequence. The Avian Transcripts were used in a comprehensive search against the *Anas platyrhynchos* transcriptome (from the ENSEMBL dataset as well as novel microRNA transcripts identified via sequencing) using the BLASTN algorithm and a perl script was used to parse the alignment and generate a scorescore based on the position

and number of mismatches between the siRNA and any potential 'off-target' transcript. The off-target score is weighted to emphasize differences in the 'seed' region of siRNAs, in positions 2-9 from the 5' end of the molecule. The off-target score is calculated as follows: mismatches between the oligo and the transcript are given penalties. A

5 mismatch in the seed region in positions 2-9 of the oligo is given a penalty of 2.8; mismatches in the putative cleavage sites 10 and 11 are given a penalty of 1.2, and all other mismatches a penalty of 1. The off-target score for each oligo-transcript pair is then calculated by summing the mismatch penalties. The lowest off-target score from all the oligo-transcript pairs is then determined and used in subsequent sorting of oligos.

10 Both siRNAs strands were assigned to a category of specificity according to the calculated scores: a score above 3 qualifies as highly specific, equal to 3 as specific and between 2.2 and 2.8 as moderate specific. In picking which oligonucleotides to synthesize, these scores can be sorted from high to low by the off-target score of the antisense strand and the best (lowest off-target score) oligonucleotide pairs may be

15 synthesized for further study.

Example 4. Oligonucleotide synthesis

Source of reagents

Where the source of a reagent is not specifically given herein, such reagent may

20 be obtained from any supplier of reagents for molecular biology at a quality/purity standard for application in molecular biology.

Oligonucleotide Synthesis.

All oligonucleotides are synthesized on an AKTAoligopilot synthesizer. Commercially available controlled pore glass solid support (dT-CPG, 500Å, Prime

25 Synthesis) and RNA phosphoramidites with standard protecting groups, 5'-O-dimethoxytrityl N6-benzoyl-2'-*t*-butyldimethylsilyl-adenosine-3'-O-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-O-dimethoxytrityl-N4-acetyl-2'-*t*-butyldimethylsilyl-cytidine-3'-O-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-O-dimethoxytrityl-N2-isobutryl-2'-*t*-butyldimethylsilyl-guanosine-3'-O-N,N'-diisopropyl-2-

30 cyanoethylphosphoramidite, and 5'-O-dimethoxytrityl-2'-*t*-butyldimethylsilyl-uridine-3'-

O-N,N'-diisopropyl-2-cyanoethylphosphoramidite (Pierce Nucleic Acids Technologies) were used for the oligonucleotide synthesis. The 2'-F phosphoramidites, 5'-*O*-dimethoxytrityl-N4-acetyl-2'-fluoro-cytidine-3'-*O*-N,N'-diisopropyl-2-cyanoethyl-phosphoramidite and 5'-*O*-dimethoxytrityl-2'-fluoro-uridine-3'-*O*-N,N'-diisopropyl-2-cyanoethyl-phosphoramidite are purchased from (Promega). All phosphoramidites are used at a concentration of 0.2M in acetonitrile (CH₃CN) except for guanosine which is used at 0.2M concentration in 10% THF/ANC (v/v). Coupling/recycling time of 16 minutes is used. The activator is 5-ethyl thiotetrazole (0.75M, American International Chemicals); for the PO-oxidation iodine/water/pyridine is used and for the PS-oxidation PADS (2%) in 2,6-lutidine/ACN (1:1 v/v) is used.

3'-ligand conjugated strands are synthesized using solid support containing the corresponding ligand. For example, the introduction of cholesterol unit in the sequence is performed from a hydroxyprolinol-cholesterol phosphoramidite. Cholesterol is tethered to *trans*-4-hydroxyprolinol via a 6-aminohexanoate linkage to obtain a hydroxyprolinol-cholesterol moiety. 5'-end Cy-3 and Cy-5.5 (fluorophore) labeled iRNAs are synthesized from the corresponding Quasar-570 (Cy-3) phosphoramidite are purchased from Biosearch Technologies. Conjugation of ligands to 5'-end and or internal position is achieved by using appropriately protected ligand-phosphoramidite building block. An extended 15 min coupling of 0.1 M solution of phosphoramidite in anhydrous CH₃CN in the presence of 5-(ethylthio)-1*H*-tetrazole activator to a solid-support-bound oligonucleotide. Oxidation of the internucleotide phosphite to the phosphate is carried out using standard iodine-water as reported (1) or by treatment with *tert*-butyl hydroperoxide/acetonitrile/water (10: 87: 3) with 10 min oxidation wait time conjugated oligonucleotide. Phosphorothioate is introduced by the oxidation of phosphite to phosphorothioate by using a sulfur transfer reagent such as DDTT (purchased from AM Chemicals), PADS and or Beaucage reagent. The cholesterol phosphoramidite is synthesized in house and used at a concentration of 0.1 M in dichloromethane. Coupling time for the cholesterol phosphoramidite is 16 minutes.

Deprotection I (Nucleobase Deprotection)

After completion of synthesis, the support is transferred to a 100 mL glass bottle (VWR). The oligonucleotide is cleaved from the support with simultaneous deprotection of base and phosphate groups with 80 mL of a mixture of ethanolic ammonia [ammonia: ethanol (3:1)] for 6.5 h at 55°C. The bottle is cooled briefly on ice and then the ethanolic ammonia mixture is filtered into a new 250-mL bottle. The CPG is washed with 2 x 40 mL portions of ethanol/water (1:1 v/v). The volume of the mixture is then reduced to ~ 30 mL by roto-vap. The mixture is then frozen on dry ice and dried under vacuum on a speed vac.

Deprotection II (Removal of 2'-TBDMS group)

The dried residue is resuspended in 26 mL of triethylamine, triethylamine trihydrofluoride (TEA•3HF) or pyridine-HF and DMSO (3:4:6) and heated at 60°C for 90 minutes to remove the *tert*-butyldimethylsilyl (TBDMS) groups at the 2' position. The reaction is then quenched with 50 mL of 20 mM sodium acetate and the pH is adjusted to 6.5. Oligonucleotide is stored in a freezer until purification.

Analysis

The oligonucleotides are analyzed by high-performance liquid chromatography (HPLC) prior to purification and selection of buffer and column depends on nature of the sequence and or conjugated ligand.

HPLC Purification

The ligand-conjugated oligonucleotides are purified by reverse-phase preparative HPLC. The unconjugated oligonucleotides are purified by anion-exchange HPLC on a TSK gel column packed in house. The buffers are 20 mM sodium phosphate (pH 8.5) in 10% CH₃CN (buffer A) and 20 mM sodium phosphate (pH 8.5) in 10% CH₃CN, 1M NaBr (buffer B). Fractions containing full-length oligonucleotides are pooled, desalted, and lyophilized. Approximately 0.15 OD of desalted oligonucleotides are diluted in water to 150 µL and then pipetted into special vials for CGE and LC/MS analysis. Compounds are then analyzed by LC-ESMS and CGE.

iRNA preparation

For the general preparation of iRNA agents, specifically siRNAs, equimolar amounts of sense and antisense strand are heated in 1xPBS at 95°C for 5 min and slowly cooled to room temperature. Integrity of the duplex is confirmed by HPLC analysis.

Nucleic acid sequences are represented below using standard nomenclature, and specifically the abbreviations of Table 6. It will be understood that these monomers, when present in an oligonucleotide, are mutually linked by 5'-3'-phosphodiester bonds unless otherwise noted.

Table 6: Abbreviations of nucleotide monomers

Abbreviation	Nucleotide(s)
A	adenosine
C	cytidine
G	guanosine
T	thymidine
U	uridine
N	any nucleotide (G, A, C, T or U)
a	2'-O-methyladenosine
c	2'-O-methylcytidine
g	2'-O-methylguanosine
u	2'-O-methyluridine
dT	2'-deoxythymidine
s	phosphorothioate linkage

Example 5: Synthesis of iRNA Sequences

Sequences are synthesized on a MerMade 192 synthesizer at 1 μmol scale.

For all the sequences in the list, 'endolight' chemistry may be applied as detailed below.

- All pyrimidines (cytosine and uridine) in the sense strand contain 2'-O-Methyl bases (2' O-Methyl C and 2'-O-Methyl U)
- In the antisense strand, pyrimidines adjacent to (towards 5' position) ribo A nucleoside are replaced with their corresponding 2-O-Methyl nucleosides
- A two base dTsdT extension at 3' end of both sense and antisense sequences are introduced
- The sequence file is converted to a text file to make it compatible for loading in the MerMade 192 synthesis software

Synthesis, Cleavage and deprotection:

The synthesis of sequences uses solid supported oligonucleotide synthesis using phosphoramidite chemistry.

- 5 The synthesis of the above sequences are performed at 1um scale in 96 well plates. The amidite solutions are prepared at 0.1M concentration and ethyl thio tetrazole (0.6M in Acetonitrile) is used as activator.

- 10 The synthesized sequences are cleaved and deprotected in 96 well plates, using methylamine in the first step and fluoride reagent in the second step. The crude sequences are precipitated using acetone: ethanol (80:20) mix and the pellet re-suspended in 0.02M sodium acetate buffer. Samples from each sequence are analyzed by LC-MS to confirm the identity, UV for quantification and a selected set of samples by IEX chromatography to determine purity.

Purification and desalting:

- 15 Avian transcript siRNA sequences are purified on AKTA explorer purification system using Source 15Q column. A column temperature of 65C is maintained during purification. Sample injection and collection is performed in 96 well (1.8mL -deep well) plates. A single peak corresponding to the full length sequence is collected in the eluent. The purified sequences are desalted on a Sephadex G25 column using AKTA purifier.
- 20 The desalted sequences are analyzed for concentration (by UV measurement at A260) and purity (by ion exchange HPLC). The single strands are then submitted for annealing.

Example 6. Expression Analysis in Avian Cells

Cells and Growth Conditions:

- 25 Duck cells (ATCC, Mannassas, VA; ATCC number CCL-141; *Anas platyrhynchos domesticus*(duck, Pekin)) were plated into 24 well plates the night before infection at a cell density of 100k cells per well in Eagle's Minimum Essential Medium supplemented with fetal bovine serum to a final concentration of 10%. The following day the cells in half the wells were infected with B/Brisbane influenza virus at an MOI of 0.5 for 1 hr, and the media changed to DMEM supplemented with trypsin overnight (~16-

18hrs). The cells were expected to adhere, so any floating cells in the media were spun down.

RNA Preparation:

Qiagen RNeasy was used to isolate RNA from the infected and uninfected cells according to the manufacturer's directions. Each separate RNA sample for microRNA and transcriptome analysis was pooled from 4 wells of the 24well plate.

MicroRNA Sequencing:

The microRNA sequences from infected and uninfected CCL-141 cells were determined by LC Sciences, LLC (2575 West Bellfort St., Houston, TX 77054). In brief, total RNA was processed to generate a short sequence cDNA library that was then deep-sequenced with a next-gen Illumina IIG DNA sequencing machine. The resulting sequences were clustered in microRNA based on similarity to known microRNAs as well as by *ab-initio*, structure-based methods. Novel microRNA target transcript sequences are reported in Table 1.

Expression Analysis:

The relative expression levels of transcripts in infected versus uninfected CCL-141 cells were determined by deep-sequencing of RNA transcripts. Paired end 50 base reads from a total of four RNA samples (two infected, two uninfected) were sequenced using an Illumina IIG. The average number of reads per thousand base window of each transcript was determined after normalizing for the total number of reads obtained for the four samples.

Relative changes in expression levels for each transcript were determined by the ratio of average number of reads per thousand base window between infected and uninfected cells (See Table 7). Table 7 is list of the differentially expressed duck genes from cells infected with the B/Brisbane flu virus as measured by normalized counts of high-throughput sequencing reads. Transcripts with at least a two fold increase in expression levels in the infected versus uninfected cells are listed. Whereas, transcripts expressed in infected cells but not in uninfected cells are listed in Table 8. Fold Diff. stands for Fold differentially expressed.

Table 7: Differential Expression

Target SEQ ID	Target Transcript	Fold Diff.	Target SEQ ID	Target Transcript	Fold Diff.
1356	ENSAPLT00000001356	2129	14176	ENSAPLT000000014176	4
7416	ENSAPLT00000007416	1460	14494	ENSAPLT000000014494	4
16167	ENSAPLT000000016167	1117	12488	ENSAPLT000000012488	4
7867	ENSAPLT00000007867	1103	8196	ENSAPLT00000008196	4
16416	ENSAPLT000000016416	961	2796	ENSAPLT00000002796	4
4306	ENSAPLT00000004306	790	10791	ENSAPLT000000010791	4
11350	ENSAPLT000000011350	726	3869	ENSAPLT00000003869	4
14810	ENSAPLT000000014810	623	5715	ENSAPLT00000005715	4
14535	ENSAPLT000000014535	572	14553	ENSAPLT000000014553	4
6241	ENSAPLT00000006241	549	5530	ENSAPLT00000005530	4
16707	ENSAPLT000000016707	458	13884	ENSAPLT000000013884	4
9044	ENSAPLT00000009044	427	7905	ENSAPLT00000007905	4
16544	ENSAPLT000000016544	422	5661	ENSAPLT00000005661	4
9567	ENSAPLT00000009567	416	11853	ENSAPLT000000011853	4
7080	ENSAPLT00000007080	407	3754	ENSAPLT00000003754	4
11595	ENSAPLT000000011595	400	8556	ENSAPLT00000008556	4
15248	ENSAPLT000000015248	395	12213	ENSAPLT000000012213	4
13397	ENSAPLT000000013397	361	8610	ENSAPLT00000008610	4
12882	ENSAPLT000000012882	359	1958	ENSAPLT00000001958	4
2053	ENSAPLT00000002053	350	1169	ENSAPLT00000001169	4
1611	ENSAPLT00000001611	343	4296	ENSAPLT00000004296	4
16054	ENSAPLT000000016054	333	2309	ENSAPLT00000002309	4
9771	ENSAPLT00000009771	323	12883	ENSAPLT000000012883	4
1602	ENSAPLT00000001602	314	4500	ENSAPLT00000004500	4
8502	ENSAPLT00000008502	280	4882	ENSAPLT00000004882	4
3024	ENSAPLT00000003024	275	8812	ENSAPLT00000008812	4
2193	ENSAPLT00000002193	258	14867	ENSAPLT000000014867	4
6212	ENSAPLT00000006212	231	2922	ENSAPLT00000002922	4
10833	ENSAPLT000000010833	216	16144	ENSAPLT000000016144	4
13295	ENSAPLT000000013295	203	17103	ENSAPLT000000017103	4
13172	ENSAPLT000000013172	201	7525	ENSAPLT00000007525	4
12297	ENSAPLT000000012297	193	2477	ENSAPLT00000002477	4
10261	ENSAPLT000000010261	188	3611	ENSAPLT00000003611	4
3137	ENSAPLT00000003137	178	16805	ENSAPLT000000016805	4
16969	ENSAPLT000000016969	157	4631	ENSAPLT00000004631	4
13037	ENSAPLT000000013037	151	5790	ENSAPLT00000005790	4
2381	ENSAPLT00000002381	149	10558	ENSAPLT000000010558	4
15510	ENSAPLT000000015510	145	14934	ENSAPLT000000014934	4

15770	ENSAPLT00000015770	144	8008	ENSAPLT00000008008	4
2352	ENSAPLT00000002352	141	12603	ENSAPLT00000012603	4
10244	ENSAPLT00000010244	137	16154	ENSAPLT00000016154	4
882	ENSAPLT00000000882	135	7936	ENSAPLT00000007936	4
14453	ENSAPLT00000014453	134	8656	ENSAPLT00000008656	4
4188	ENSAPLT00000004188	134	17073	ENSAPLT00000017073	4
5288	ENSAPLT00000005288	132	11735	ENSAPLT00000011735	4
7802	ENSAPLT00000007802	130	11491	ENSAPLT00000011491	4
16189	ENSAPLT00000016189	121	17082	ENSAPLT00000017082	4
13762	ENSAPLT00000013762	121	13495	ENSAPLT00000013495	4
9077	ENSAPLT00000009077	118	3753	ENSAPLT00000003753	4
7157	ENSAPLT00000007157	117	12265	ENSAPLT00000012265	4
9570	ENSAPLT00000009570	112	11645	ENSAPLT00000011645	4
11330	ENSAPLT00000011330	111	15874	ENSAPLT00000015874	4
12824	ENSAPLT00000012824	108	11582	ENSAPLT00000011582	4
9829	ENSAPLT00000009829	108	11538	ENSAPLT00000011538	4
7705	ENSAPLT00000007705	107	2737	ENSAPLT00000002737	4
15846	ENSAPLT00000015846	105	10385	ENSAPLT00000010385	4
2403	ENSAPLT00000002403	104	16745	ENSAPLT00000016745	4
16124	ENSAPLT00000016124	104	11181	ENSAPLT00000011181	4
14920	ENSAPLT00000014920	104	4334	ENSAPLT00000004334	4
13923	ENSAPLT00000013923	103	861	ENSAPLT00000000861	4
4679	ENSAPLT00000004679	103	5591	ENSAPLT00000005591	4
5886	ENSAPLT00000005886	103	7406	ENSAPLT00000007406	4
11742	ENSAPLT00000011742	102	6732	ENSAPLT00000006732	4
6012	ENSAPLT00000006012	101	12984	ENSAPLT00000012984	4
5178	ENSAPLT00000005178	100	14081	ENSAPLT00000014081	4
10146	ENSAPLT00000010146	99	12936	ENSAPLT00000012936	4
10518	ENSAPLT00000010518	99	580	ENSAPLT00000000580	4
15150	ENSAPLT00000015150	99	9874	ENSAPLT00000009874	4
15791	ENSAPLT00000015791	98	2580	ENSAPLT00000002580	4
9406	ENSAPLT00000009406	97	16296	ENSAPLT00000016296	4
12926	ENSAPLT00000012926	96	8089	ENSAPLT00000008089	4
358	ENSAPLT00000000358	96	7691	ENSAPLT00000007691	4
16122	ENSAPLT00000016122	90	13310	ENSAPLT00000013310	4
6457	ENSAPLT00000006457	88	2486	ENSAPLT00000002486	4
15263	ENSAPLT00000015263	87	2874	ENSAPLT00000002874	4
4073	ENSAPLT00000004073	87	2409	ENSAPLT00000002409	4
10804	ENSAPLT00000010804	87	12996	ENSAPLT00000012996	4

10748	ENSAPLT00000010748	81	12448	ENSAPLT00000012448	4
4483	ENSAPLT00000004483	80	2043	ENSAPLT00000002043	4
5561	ENSAPLT00000005561	80	5002	ENSAPLT00000005002	4
7083	ENSAPLT00000007083	77	6508	ENSAPLT00000006508	4
13206	ENSAPLT00000013206	75	16169	ENSAPLT00000016169	4
12906	ENSAPLT00000012906	75	5729	ENSAPLT00000005729	4
5476	ENSAPLT00000005476	74	5958	ENSAPLT00000005958	4
11217	ENSAPLT00000011217	73	10811	ENSAPLT00000010811	4
697	ENSAPLT00000000697	72	2268	ENSAPLT00000002268	4
594	ENSAPLT00000000594	70	10109	ENSAPLT00000010109	4
16742	ENSAPLT00000016742	69	15450	ENSAPLT00000015450	4
1873	ENSAPLT00000001873	69	5068	ENSAPLT00000005068	3
13966	ENSAPLT00000013966	68	3624	ENSAPLT00000003624	3
4620	ENSAPLT00000004620	68	14084	ENSAPLT00000014084	3
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11601	ENSAPLT00000011601	4	16862	ENSAPLT00000016862	2
2624	ENSAPLT00000002624	4	10066	ENSAPLT00000010066	2
4024	ENSAPLT00000004024	4	6922	ENSAPLT00000006922	2
6827	ENSAPLT00000006827	4	8272	ENSAPLT00000008272	2
15153	ENSAPLT00000015153	4	2895	ENSAPLT00000002895	2
7002	ENSAPLT00000007002	4	14329	ENSAPLT00000014329	2
2736	ENSAPLT00000002736	4	8274	ENSAPLT00000008274	2
5595	ENSAPLT00000005595	4	3826	ENSAPLT00000003826	2
15945	ENSAPLT00000015945	4	1358	ENSAPLT00000001358	2
8041	ENSAPLT00000008041	4	11515	ENSAPLT00000011515	2
11409	ENSAPLT00000011409	4	13743	ENSAPLT00000013743	2
11295	ENSAPLT00000011295	4	1021	ENSAPLT00000001021	2
3427	ENSAPLT00000003427	4	12913	ENSAPLT00000012913	2
8792	ENSAPLT00000008792	4	9929	ENSAPLT00000009929	2
16542	ENSAPLT00000016542	4	5216	ENSAPLT00000005216	2
250	ENSAPLT00000000250	4	10569	ENSAPLT00000010569	2
679	ENSAPLT00000000679	4	4917	ENSAPLT00000004917	2
13925	ENSAPLT00000013925	4	7141	ENSAPLT00000007141	2
17162	ENSAPLT00000017162	4	2293	ENSAPLT00000002293	2
8585	ENSAPLT00000008585	4	15696	ENSAPLT00000015696	2
12137	ENSAPLT00000012137	4	10923	ENSAPLT00000010923	2
10575	ENSAPLT00000010575	4	9381	ENSAPLT00000009381	2
6655	ENSAPLT00000006655	4	16235	ENSAPLT00000016235	2
3879	ENSAPLT00000003879	4	9041	ENSAPLT00000009041	2
13597	ENSAPLT00000013597	4	15936	ENSAPLT00000015936	2
1364	ENSAPLT00000001364	4	16413	ENSAPLT00000016413	2
1477	ENSAPLT00000001477	4	5705	ENSAPLT00000005705	2
7112	ENSAPLT00000007112	4	10805	ENSAPLT00000010805	2
1419	ENSAPLT00000001419	4	4041	ENSAPLT00000004041	2

1111	ENSAPLT00000001111	4	10914	ENSAPLT000000010914	2
5749	ENSAPLT00000005749	4	11859	ENSAPLT000000011859	2
12493	ENSAPLT000000012493	4	14926	ENSAPLT000000014926	2
13016	ENSAPLT000000013016	4	626	ENSAPLT00000000626	2
9934	ENSAPLT00000009934	4	5572	ENSAPLT00000005572	2
1016	ENSAPLT00000001016	4	5360	ENSAPLT00000005360	2
3390	ENSAPLT00000003390	4	17084	ENSAPLT000000017084	2
6963	ENSAPLT00000006963	4	16844	ENSAPLT000000016844	2
17017	ENSAPLT000000017017	4	1647	ENSAPLT000000001647	2
2044	ENSAPLT00000002044	4	4454	ENSAPLT00000004454	2
4042	ENSAPLT00000004042	4	8772	ENSAPLT00000008772	2
9495	ENSAPLT00000009495	4	1853	ENSAPLT000000001853	2
12257	ENSAPLT000000012257	4	7397	ENSAPLT000000007397	2
2579	ENSAPLT00000002579	4	16732	ENSAPLT000000016732	2
910	ENSAPLT00000000910	4	3475	ENSAPLT000000003475	2
3563	ENSAPLT00000003563	4	5007	ENSAPLT000000005007	2
15743	ENSAPLT000000015743	4	9722	ENSAPLT000000009722	2
3494	ENSAPLT00000003494	4	4106	ENSAPLT000000004106	2
16325	ENSAPLT000000016325	4	3705	ENSAPLT000000003705	2
9589	ENSAPLT00000009589	4	6855	ENSAPLT000000006855	2
17083	ENSAPLT000000017083	4	5300	ENSAPLT000000005300	2
13222	ENSAPLT000000013222	4	8405	ENSAPLT000000008405	2
2235	ENSAPLT00000002235	4	7273	ENSAPLT000000007273	2
2278	ENSAPLT00000002278	4	10533	ENSAPLT000000010533	2
2752	ENSAPLT00000002752	4	4448	ENSAPLT000000004448	2
1313	ENSAPLT00000001313	4	6406	ENSAPLT000000006406	2
14914	ENSAPLT000000014914	4	12379	ENSAPLT000000012379	2
804	ENSAPLT00000000804	4	7543	ENSAPLT000000007543	2
11133	ENSAPLT000000011133	4	6263	ENSAPLT000000006263	2
5370	ENSAPLT00000005370	4	1605	ENSAPLT000000001605	2
16777	ENSAPLT000000016777	4	14677	ENSAPLT000000014677	2
6637	ENSAPLT00000006637	4	9158	ENSAPLT000000009158	2
5682	ENSAPLT00000005682	4	4287	ENSAPLT000000004287	2
6435	ENSAPLT00000006435	4	5560	ENSAPLT000000005560	2
11621	ENSAPLT000000011621	4	6334	ENSAPLT000000006334	2
10746	ENSAPLT000000010746	4	3183	ENSAPLT000000003183	2
13715	ENSAPLT000000013715	4	10156	ENSAPLT000000010156	2
3077	ENSAPLT00000003077	4	1060	ENSAPLT000000001060	2
7848	ENSAPLT00000007848	4	1458	ENSAPLT000000001458	2

12152	ENSAPLT00000012152	4	4927	ENSAPLT00000004927	2
13307	ENSAPLT00000013307	4	10034	ENSAPLT00000010034	2
716	ENSAPLT00000000716	4	5018	ENSAPLT00000005018	2
9985	ENSAPLT00000009985	4	8590	ENSAPLT00000008590	2
11738	ENSAPLT00000011738	4	11195	ENSAPLT00000011195	2
17143	ENSAPLT00000017143	4	12495	ENSAPLT00000012495	2
9591	ENSAPLT00000009591	4	15664	ENSAPLT00000015664	2
2802	ENSAPLT00000002802	4	6397	ENSAPLT00000006397	2
4257	ENSAPLT00000004257	4	16791	ENSAPLT00000016791	2
8254	ENSAPLT00000008254	4	9756	ENSAPLT00000009756	2
2584	ENSAPLT00000002584	4	7612	ENSAPLT00000007612	2
1713	ENSAPLT00000001713	4	135	ENSAPLT00000000135	2
5036	ENSAPLT00000005036	4	16098	ENSAPLT00000016098	2
4016	ENSAPLT00000004016	4	13331	ENSAPLT00000013331	2
2325	ENSAPLT00000002325	4	15140	ENSAPLT00000015140	2
12652	ENSAPLT00000012652	4	3422	ENSAPLT00000003422	2
14500	ENSAPLT00000014500	4	12194	ENSAPLT00000012194	2
14956	ENSAPLT00000014956	4	2324	ENSAPLT00000002324	2
6961	ENSAPLT00000006961	4	15103	ENSAPLT00000015103	2
5981	ENSAPLT00000005981	4			

From the table, it is evident that certain transcripts are expressed at much higher level in the CCL-1 cells upon viral infection. It will be advantageous to target these in order to optimize the production of biological products. Preferred targets are those that are upregulated by at least 20, 50, 100, 200, 500, 1000 or 2000 fold.

Table 8: Infected Only Transcripts

Target SEQ ID	Target Transcript	Target SEQ ID	Target Transcript
1491	ENSAPLT00000001491	6577	ENSAPLT00000006577
13296	ENSAPLT00000013296	15322	ENSAPLT00000015322
12459	ENSAPLT00000012459	10516	ENSAPLT00000010516
11973	ENSAPLT00000011973	11609	ENSAPLT00000011609
15284	ENSAPLT00000015284	1608	ENSAPLT0000001608
15306	ENSAPLT00000015306	16072	ENSAPLT00000016072
10184	ENSAPLT00000010184	15451	ENSAPLT00000015451
13156	ENSAPLT00000013156	11694	ENSAPLT00000011694
1580	ENSAPLT00000001580	6395	ENSAPLT00000006395

16110	ENSAPLT00000016110	14311	ENSAPLT00000014311
537	ENSAPLT00000000537	9689	ENSAPLT00000009689
11168	ENSAPLT00000011168	5348	ENSAPLT00000005348
6118	ENSAPLT00000006118	1212	ENSAPLT00000001212
728	ENSAPLT00000000728	10709	ENSAPLT00000010709
11916	ENSAPLT00000011916	9485	ENSAPLT00000009485
5578	ENSAPLT00000005578	7234	ENSAPLT00000007234
13134	ENSAPLT00000013134	6605	ENSAPLT00000006605
895	ENSAPLT00000000895	16353	ENSAPLT00000016353
1990	ENSAPLT00000001990	12442	ENSAPLT00000012442
2321	ENSAPLT00000002321	11868	ENSAPLT00000011868
16266	ENSAPLT00000016266	9110	ENSAPLT00000009110
9566	ENSAPLT00000009566	11560	ENSAPLT00000011560
6604	ENSAPLT00000006604	9853	ENSAPLT00000009853
2464	ENSAPLT00000002464	14368	ENSAPLT00000014368
13970	ENSAPLT00000013970	13891	ENSAPLT00000013891
2038	ENSAPLT00000002038	4914	ENSAPLT00000004914
5608	ENSAPLT00000005608	1451	ENSAPLT00000001451
2609	ENSAPLT00000002609	16377	ENSAPLT00000016377
404	ENSAPLT00000000404	15644	ENSAPLT00000015644
220	ENSAPLT00000000220	1072	ENSAPLT00000001072
1863	ENSAPLT00000001863	5461	ENSAPLT00000005461
11996	ENSAPLT00000011996	13398	ENSAPLT00000013398
5709	ENSAPLT00000005709	14419	ENSAPLT00000014419
13960	ENSAPLT00000013960	3297	ENSAPLT00000003297
10540	ENSAPLT00000010540	15309	ENSAPLT00000015309
2300	ENSAPLT00000002300	2829	ENSAPLT00000002829
16878	ENSAPLT00000016878	2222	ENSAPLT00000002222
12991	ENSAPLT00000012991	8705	ENSAPLT00000008705
4154	ENSAPLT00000004154	1798	ENSAPLT00000001798
15445	ENSAPLT00000015445	1570	ENSAPLT00000001570
11958	ENSAPLT00000011958	8921	ENSAPLT00000008921
1249	ENSAPLT00000001249	10842	ENSAPLT00000010842
1965	ENSAPLT00000001965	7995	ENSAPLT00000007995
2683	ENSAPLT00000002683	15145	ENSAPLT00000015145
6050	ENSAPLT00000006050	15303	ENSAPLT00000015303
2333	ENSAPLT00000002333	8032	ENSAPLT00000008032
3021	ENSAPLT00000003021	12011	ENSAPLT00000012011
2610	ENSAPLT00000002610	4954	ENSAPLT00000004954
651	ENSAPLT00000000651	12631	ENSAPLT00000012631
4726	ENSAPLT00000004726	1160	ENSAPLT00000001160

3588	ENSAPLT00000003588	807	ENSAPLT00000000807
15890	ENSAPLT00000015890	14257	ENSAPLT00000014257
16108	ENSAPLT00000016108	2954	ENSAPLT00000002954
10289	ENSAPLT00000010289	5735	ENSAPLT00000005735
606	ENSAPLT00000000606	5340	ENSAPLT00000005340
4988	ENSAPLT00000004988	11577	ENSAPLT00000011577
2911	ENSAPLT00000002911	16140	ENSAPLT00000016140
14204	ENSAPLT00000014204	5230	ENSAPLT00000005230
2305	ENSAPLT00000002305	11419	ENSAPLT00000011419
223	ENSAPLT00000000223	6618	ENSAPLT00000006618
2639	ENSAPLT00000002639	16334	ENSAPLT00000016334
3840	ENSAPLT00000003840	14072	ENSAPLT00000014072
9555	ENSAPLT00000009555	8359	ENSAPLT00000008359
2118	ENSAPLT00000002118	14401	ENSAPLT00000014401
1283	ENSAPLT00000001283	5149	ENSAPLT00000005149
896	ENSAPLT00000000896	10946	ENSAPLT00000010946
11554	ENSAPLT00000011554	1436	ENSAPLT00000001436
2523	ENSAPLT00000002523	4939	ENSAPLT00000004939
10132	ENSAPLT00000010132	3350	ENSAPLT00000003350
476	ENSAPLT00000000476	9560	ENSAPLT00000009560
16602	ENSAPLT00000016602	15629	ENSAPLT00000015629
78	ENSAPLT00000000078	3301	ENSAPLT00000003301
15995	ENSAPLT00000015995	15952	ENSAPLT00000015952
464	ENSAPLT00000000464	5247	ENSAPLT00000005247
5982	ENSAPLT00000005982	1056	ENSAPLT00000001056
9437	ENSAPLT00000009437	16826	ENSAPLT00000016826
9	ENSAPLT00000000009	7387	ENSAPLT00000007387
15658	ENSAPLT00000015658	11134	ENSAPLT00000011134
14324	ENSAPLT00000014324	14839	ENSAPLT00000014839
16227	ENSAPLT00000016227	2722	ENSAPLT00000002722
125	ENSAPLT00000000125	12260	ENSAPLT00000012260
2918	ENSAPLT00000002918	12706	ENSAPLT00000012706
1601	ENSAPLT00000001601	16776	ENSAPLT00000016776
972	ENSAPLT00000000972	5785	ENSAPLT00000005785
2290	ENSAPLT00000002290	13356	ENSAPLT00000013356
3461	ENSAPLT00000003461	16939	ENSAPLT00000016939
3656	ENSAPLT00000003656	2696	ENSAPLT00000002696
7871	ENSAPLT00000007871	1950	ENSAPLT00000001950
816	ENSAPLT00000000816	8543	ENSAPLT00000008543
14465	ENSAPLT00000014465	3499	ENSAPLT00000003499
14552	ENSAPLT00000014552	12360	ENSAPLT00000012360

9763	ENSAPLT00000009763	5708	ENSAPLT00000005708
13091	ENSAPLT00000013091	4144	ENSAPLT00000004144
4468	ENSAPLT00000004468	14438	ENSAPLT00000014438
9051	ENSAPLT00000009051	11153	ENSAPLT00000011153
100	ENSAPLT00000000100	8202	ENSAPLT00000008202
2069	ENSAPLT00000002069	9245	ENSAPLT00000009245
2940	ENSAPLT00000002940	13272	ENSAPLT00000013272
451	ENSAPLT00000000451	12396	ENSAPLT00000012396
2210	ENSAPLT00000002210	4687	ENSAPLT00000004687
1386	ENSAPLT00000001386	5695	ENSAPLT00000005695
415	ENSAPLT00000000415	8566	ENSAPLT00000008566
16653	ENSAPLT00000016653	1933	ENSAPLT00000001933
10	ENSAPLT00000000010	7068	ENSAPLT00000007068
13051	ENSAPLT00000013051	11502	ENSAPLT00000011502
1424	ENSAPLT00000001424	14957	ENSAPLT00000014957
1359	ENSAPLT00000001359	16598	ENSAPLT00000016598
11670	ENSAPLT00000011670	4120	ENSAPLT00000004120
10873	ENSAPLT00000010873	8731	ENSAPLT00000008731
2545	ENSAPLT00000002545	3022	ENSAPLT00000003022
13614	ENSAPLT00000013614	3843	ENSAPLT00000003843
14357	ENSAPLT00000014357	10608	ENSAPLT00000010608
5229	ENSAPLT00000005229	10106	ENSAPLT00000010106
891	ENSAPLT00000000891	1145	ENSAPLT00000001145
676	ENSAPLT00000000676	13200	ENSAPLT00000013200
16724	ENSAPLT00000016724	675	ENSAPLT00000000675
397	ENSAPLT00000000397	15523	ENSAPLT00000015523
6269	ENSAPLT00000006269	2197	ENSAPLT00000002197
11089	ENSAPLT00000011089	4738	ENSAPLT00000004738
1548	ENSAPLT00000001548	5576	ENSAPLT00000005576
5748	ENSAPLT00000005748	11159	ENSAPLT00000011159
9164	ENSAPLT00000009164	16193	ENSAPLT00000016193
5066	ENSAPLT00000005066	16129	ENSAPLT00000016129
2567	ENSAPLT00000002567	8504	ENSAPLT00000008504
1195	ENSAPLT00000001195	4361	ENSAPLT00000004361
4297	ENSAPLT00000004297	1376	ENSAPLT00000001376
15178	ENSAPLT00000015178	5792	ENSAPLT00000005792
7934	ENSAPLT00000007934	10912	ENSAPLT00000010912
5076	ENSAPLT00000005076	13510	ENSAPLT00000013510
9016	ENSAPLT00000009016	11414	ENSAPLT00000011414
1751	ENSAPLT00000001751	919	ENSAPLT00000000919
12616	ENSAPLT00000012616	8434	ENSAPLT00000008434

12176	ENSAPLT00000012176	12221	ENSAPLT00000012221
1393	ENSAPLT00000001393	13722	ENSAPLT00000013722
4209	ENSAPLT00000004209	14189	ENSAPLT00000014189
14913	ENSAPLT00000014913	12932	ENSAPLT00000012932
15426	ENSAPLT00000015426	4821	ENSAPLT00000004821
2726	ENSAPLT00000002726	1529	ENSAPLT00000001529
2769	ENSAPLT00000002769	14430	ENSAPLT00000014430
4774	ENSAPLT00000004774	6766	ENSAPLT00000006766
3635	ENSAPLT00000003635	13063	ENSAPLT00000013063
13468	ENSAPLT00000013468	10550	ENSAPLT00000010550
8232	ENSAPLT00000008232	10674	ENSAPLT00000010674
11081	ENSAPLT00000011081	6673	ENSAPLT00000006673
13084	ENSAPLT00000013084	16097	ENSAPLT00000016097
17043	ENSAPLT00000017043	12796	ENSAPLT00000012796
2978	ENSAPLT00000002978	4125	ENSAPLT00000004125
420	ENSAPLT00000000420	10651	ENSAPLT00000010651
15614	ENSAPLT00000015614	15980	ENSAPLT00000015980
10076	ENSAPLT00000010076	9879	ENSAPLT00000009879
3941	ENSAPLT00000003941	9641	ENSAPLT00000009641
6327	ENSAPLT00000006327	2288	ENSAPLT00000002288
1282	ENSAPLT00000001282	10140	ENSAPLT00000010140
6445	ENSAPLT00000006445	12540	ENSAPLT00000012540
12328	ENSAPLT00000012328	3103	ENSAPLT00000003103
232	ENSAPLT00000000232	8782	ENSAPLT00000008782
14451	ENSAPLT00000014451	13009	ENSAPLT00000013009
7938	ENSAPLT00000007938	11791	ENSAPLT00000011791
15428	ENSAPLT00000015428	14929	ENSAPLT00000014929
2797	ENSAPLT00000002797	10888	ENSAPLT00000010888
543	ENSAPLT00000000543	13660	ENSAPLT00000013660
1024	ENSAPLT00000001024	8547	ENSAPLT00000008547
10051	ENSAPLT00000010051	16009	ENSAPLT00000016009
7451	ENSAPLT00000007451	11855	ENSAPLT00000011855
1155	ENSAPLT00000001155	15548	ENSAPLT00000015548
685	ENSAPLT00000000685	15326	ENSAPLT00000015326
4227	ENSAPLT00000004227	8123	ENSAPLT00000008123
7980	ENSAPLT00000007980	7668	ENSAPLT00000007668
4508	ENSAPLT00000004508	4063	ENSAPLT00000004063
5577	ENSAPLT00000005577	5840	ENSAPLT00000005840
2084	ENSAPLT00000002084	11768	ENSAPLT00000011768
4196	ENSAPLT00000004196	14393	ENSAPLT00000014393
6004	ENSAPLT00000006004	8053	ENSAPLT00000008053

2081	ENSAPLT00000002081	16987	ENSAPLT00000016987
11985	ENSAPLT00000011985	11891	ENSAPLT00000011891
12199	ENSAPLT00000012199	12710	ENSAPLT00000012710
448	ENSAPLT00000000448	12419	ENSAPLT00000012419
8988	ENSAPLT00000008988	9363	ENSAPLT00000009363
11541	ENSAPLT00000011541	1577	ENSAPLT00000001577
6972	ENSAPLT00000006972	854	ENSAPLT00000000854
1314	ENSAPLT00000001314	5543	ENSAPLT00000005543
14542	ENSAPLT00000014542	743	ENSAPLT00000000743
3161	ENSAPLT00000003161	1698	ENSAPLT00000001698
15625	ENSAPLT00000015625	8410	ENSAPLT00000008410
3842	ENSAPLT00000003842	9783	ENSAPLT00000009783
3781	ENSAPLT00000003781	5265	ENSAPLT00000005265
270	ENSAPLT00000000270	13600	ENSAPLT00000013600
1671	ENSAPLT00000001671	6228	ENSAPLT00000006228
12032	ENSAPLT00000012032	6616	ENSAPLT00000006616
354	ENSAPLT00000000354	5833	ENSAPLT00000005833
690	ENSAPLT00000000690	15151	ENSAPLT00000015151
13261	ENSAPLT00000013261	16539	ENSAPLT00000016539
11424	ENSAPLT00000011424	16900	ENSAPLT00000016900
12543	ENSAPLT00000012543	3523	ENSAPLT00000003523
495	ENSAPLT00000000495	8811	ENSAPLT00000008811
16908	ENSAPLT00000016908	10654	ENSAPLT00000010654
1973	ENSAPLT00000001973	6792	ENSAPLT00000006792
8833	ENSAPLT00000008833	16221	ENSAPLT00000016221
10526	ENSAPLT00000010526	15157	ENSAPLT00000015157
9981	ENSAPLT00000009981	5081	ENSAPLT00000005081
16093	ENSAPLT00000016093	16002	ENSAPLT00000016002
11192	ENSAPLT00000011192	9741	ENSAPLT00000009741
887	ENSAPLT00000000887	982	ENSAPLT00000000982
1807	ENSAPLT00000001807	14217	ENSAPLT00000014217
1703	ENSAPLT00000001703	2202	ENSAPLT00000002202
371	ENSAPLT00000000371	3065	ENSAPLT00000003065
15159	ENSAPLT00000015159	14796	ENSAPLT00000014796
8840	ENSAPLT00000008840	4947	ENSAPLT00000004947
923	ENSAPLT00000000923	3978	ENSAPLT00000003978
213	ENSAPLT00000000213	8260	ENSAPLT00000008260
2870	ENSAPLT00000002870	7972	ENSAPLT00000007972
14077	ENSAPLT00000014077	1946	ENSAPLT00000001946
1353	ENSAPLT00000001353	12343	ENSAPLT00000012343
1999	ENSAPLT00000001999	7277	ENSAPLT00000007277

14115	ENSAPLT00000014115	4163	ENSAPLT00000004163
14060	ENSAPLT00000014060	2150	ENSAPLT00000002150
4142	ENSAPLT00000004142	8036	ENSAPLT00000008036
12432	ENSAPLT00000012432	8541	ENSAPLT00000008541
1445	ENSAPLT00000001445	11641	ENSAPLT00000011641
3661	ENSAPLT00000003661	6298	ENSAPLT00000006298
15683	ENSAPLT00000015683	1884	ENSAPLT00000001884
3768	ENSAPLT00000003768	5468	ENSAPLT00000005468
9739	ENSAPLT00000009739	16564	ENSAPLT00000016564
1084	ENSAPLT00000001084	5814	ENSAPLT00000005814
7968	ENSAPLT00000007968	8470	ENSAPLT00000008470
1825	ENSAPLT00000001825	11379	ENSAPLT00000011379
4924	ENSAPLT00000004924	1164	ENSAPLT00000001164
12841	ENSAPLT00000012841	5526	ENSAPLT00000005526
11730	ENSAPLT00000011730	575	ENSAPLT00000000575
14479	ENSAPLT00000014479	8667	ENSAPLT00000008667
2094	ENSAPLT00000002094	8951	ENSAPLT00000008951
6209	ENSAPLT00000006209	3370	ENSAPLT00000003370
4176	ENSAPLT00000004176	1563	ENSAPLT00000001563
4520	ENSAPLT00000004520	15232	ENSAPLT00000015232
621	ENSAPLT00000000621	2261	ENSAPLT00000002261
39	ENSAPLT00000000039	11750	ENSAPLT00000011750
11760	ENSAPLT00000011760	10016	ENSAPLT00000010016
3093	ENSAPLT00000003093	5310	ENSAPLT00000005310
15399	ENSAPLT00000015399	656	ENSAPLT00000000656
16196	ENSAPLT00000016196	5963	ENSAPLT00000005963
15703	ENSAPLT00000015703	7030	ENSAPLT00000007030
7586	ENSAPLT00000007586	773	ENSAPLT00000000773
14463	ENSAPLT00000014463	16929	ENSAPLT00000016929
10825	ENSAPLT00000010825	1406	ENSAPLT00000001406
15489	ENSAPLT00000015489	6797	ENSAPLT00000006797
16593	ENSAPLT00000016593	15175	ENSAPLT00000015175
5087	ENSAPLT00000005087	16992	ENSAPLT00000016992
6148	ENSAPLT00000006148	6742	ENSAPLT00000006742
13165	ENSAPLT00000013165	1847	ENSAPLT00000001847
2208	ENSAPLT00000002208	11656	ENSAPLT00000011656
6800	ENSAPLT00000006800	13329	ENSAPLT00000013329
9173	ENSAPLT00000009173	4915	ENSAPLT00000004915
11091	ENSAPLT00000011091	10104	ENSAPLT00000010104
8374	ENSAPLT00000008374	8396	ENSAPLT00000008396
1213	ENSAPLT00000001213	17054	ENSAPLT00000017054

2211	ENSAPLT00000002211	6279	ENSAPLT00000006279
6240	ENSAPLT00000006240	12871	ENSAPLT00000012871
1294	ENSAPLT00000001294	15304	ENSAPLT00000015304
15907	ENSAPLT00000015907	15139	ENSAPLT00000015139
3545	ENSAPLT00000003545	11366	ENSAPLT00000011366
15149	ENSAPLT00000015149	5006	ENSAPLT00000005006
8176	ENSAPLT00000008176	3528	ENSAPLT00000003528
12545	ENSAPLT00000012545	1327	ENSAPLT00000001327
10073	ENSAPLT00000010073	16578	ENSAPLT00000016578
11167	ENSAPLT00000011167	4398	ENSAPLT00000004398
9134	ENSAPLT00000009134	7081	ENSAPLT00000007081
115	ENSAPLT00000000115	10340	ENSAPLT00000010340
2929	ENSAPLT00000002929	14575	ENSAPLT00000014575
10390	ENSAPLT00000010390	1488	ENSAPLT00000001488
16372	ENSAPLT00000016372	4618	ENSAPLT00000004618
16362	ENSAPLT00000016362	1480	ENSAPLT00000001480
6252	ENSAPLT00000006252	3990	ENSAPLT00000003990
430	ENSAPLT00000000430	8618	ENSAPLT00000008618
7103	ENSAPLT00000007103	11754	ENSAPLT00000011754
9546	ENSAPLT00000009546	15574	ENSAPLT00000015574
1299	ENSAPLT00000001299	16789	ENSAPLT00000016789
17069	ENSAPLT00000017069	1486	ENSAPLT00000001486
16070	ENSAPLT00000016070	9159	ENSAPLT00000009159
10359	ENSAPLT00000010359	8516	ENSAPLT00000008516
306	ENSAPLT00000000306	8256	ENSAPLT00000008256
11829	ENSAPLT00000011829	3265	ENSAPLT00000003265
1663	ENSAPLT00000001663	8210	ENSAPLT00000008210
273	ENSAPLT00000000273	7606	ENSAPLT00000007606
6251	ENSAPLT00000006251	14414	ENSAPLT00000014414
13399	ENSAPLT00000013399	5448	ENSAPLT00000005448
1746	ENSAPLT00000001746	3899	ENSAPLT00000003899
349	ENSAPLT00000000349	15580	ENSAPLT00000015580
1633	ENSAPLT00000001633	4119	ENSAPLT00000004119
10906	ENSAPLT00000010906	10933	ENSAPLT00000010933
4321	ENSAPLT00000004321	2855	ENSAPLT00000002855
1389	ENSAPLT00000001389	11519	ENSAPLT00000011519
16496	ENSAPLT00000016496	6482	ENSAPLT00000006482
11373	ENSAPLT00000011373	12553	ENSAPLT00000012553
12945	ENSAPLT00000012945	6721	ENSAPLT00000006721
8476	ENSAPLT00000008476	6799	ENSAPLT00000006799
3145	ENSAPLT00000003145	8461	ENSAPLT00000008461

5574	ENSAPLT00000005574	3767	ENSAPLT00000003767
15144	ENSAPLT00000015144	2976	ENSAPLT00000002976
1972	ENSAPLT00000001972	4813	ENSAPLT00000004813
3204	ENSAPLT00000003204	6938	ENSAPLT00000006938
1040	ENSAPLT00000001040	3349	ENSAPLT00000003349
721	ENSAPLT00000000721	8689	ENSAPLT00000008689
13028	ENSAPLT00000013028	6200	ENSAPLT00000006200
1597	ENSAPLT00000001597	16670	ENSAPLT00000016670
2289	ENSAPLT00000002289	15295	ENSAPLT00000015295
329	ENSAPLT00000000329	1826	ENSAPLT00000001826
720	ENSAPLT00000000720	9111	ENSAPLT00000009111
9526	ENSAPLT00000009526	8992	ENSAPLT00000008992
12136	ENSAPLT00000012136	3983	ENSAPLT00000003983
16386	ENSAPLT00000016386	6211	ENSAPLT00000006211
11246	ENSAPLT00000011246	16078	ENSAPLT00000016078
1690	ENSAPLT00000001690	14228	ENSAPLT00000014228
200	ENSAPLT00000000200	10163	ENSAPLT00000010163
729	ENSAPLT00000000729	13534	ENSAPLT00000013534
1066	ENSAPLT00000001066	15335	ENSAPLT00000015335
10884	ENSAPLT00000010884	10610	ENSAPLT00000010610
2991	ENSAPLT00000002991	4765	ENSAPLT00000004765
7645	ENSAPLT00000007645	627	ENSAPLT00000000627
1843	ENSAPLT00000001843	10239	ENSAPLT00000010239
372	ENSAPLT00000000372	13414	ENSAPLT00000013414
522	ENSAPLT00000000522	9184	ENSAPLT00000009184
4692	ENSAPLT00000004692	16198	ENSAPLT00000016198
1998	ENSAPLT00000001998	11454	ENSAPLT00000011454
13404	ENSAPLT00000013404	10177	ENSAPLT00000010177
13926	ENSAPLT00000013926	986	ENSAPLT00000000986
1764	ENSAPLT00000001764	11536	ENSAPLT00000011536
9638	ENSAPLT00000009638	3932	ENSAPLT00000003932
12053	ENSAPLT00000012053	11204	ENSAPLT00000011204
16632	ENSAPLT00000016632	15573	ENSAPLT00000015573
7472	ENSAPLT00000007472	13946	ENSAPLT00000013946
4604	ENSAPLT00000004604	3934	ENSAPLT00000003934
776	ENSAPLT00000000776	7306	ENSAPLT00000007306
12594	ENSAPLT00000012594	15013	ENSAPLT00000015013
1727	ENSAPLT00000001727	12370	ENSAPLT00000012370
14256	ENSAPLT00000014256	14252	ENSAPLT00000014252
1494	ENSAPLT00000001494	14763	ENSAPLT00000014763
8248	ENSAPLT00000008248	11972	ENSAPLT00000011972

8528	ENSAPLT00000008528	4795	ENSAPLT00000004795
94	ENSAPLT0000000094	4565	ENSAPLT00000004565
16165	ENSAPLT00000016165	12310	ENSAPLT00000012310
4562	ENSAPLT00000004562	5188	ENSAPLT00000005188
8807	ENSAPLT00000008807	1993	ENSAPLT00000001993
3153	ENSAPLT00000003153	6377	ENSAPLT00000006377
830	ENSAPLT00000000830	12188	ENSAPLT00000012188
1308	ENSAPLT00000001308	12718	ENSAPLT00000012718
9847	ENSAPLT00000009847	11364	ENSAPLT00000011364
15154	ENSAPLT00000015154	13290	ENSAPLT00000013290
8560	ENSAPLT00000008560	13888	ENSAPLT00000013888
14903	ENSAPLT00000014903	9906	ENSAPLT00000009906
12569	ENSAPLT00000012569	7074	ENSAPLT00000007074
1334	ENSAPLT00000001334	2706	ENSAPLT00000002706
14107	ENSAPLT00000014107	13631	ENSAPLT00000013631
1377	ENSAPLT00000001377	2461	ENSAPLT00000002461
1132	ENSAPLT00000001132	11235	ENSAPLT00000011235
1	ENSAPLT00000000001	732	ENSAPLT00000000732
10877	ENSAPLT00000010877	5884	ENSAPLT00000005884
8574	ENSAPLT00000008574	15016	ENSAPLT00000015016
3054	ENSAPLT00000003054	16370	ENSAPLT00000016370
3704	ENSAPLT00000003704	10007	ENSAPLT00000010007
15947	ENSAPLT00000015947	16626	ENSAPLT00000016626
184	ENSAPLT00000000184	758	ENSAPLT00000000758
9904	ENSAPLT00000009904	7826	ENSAPLT00000007826
3192	ENSAPLT00000003192	2205	ENSAPLT00000002205
10727	ENSAPLT00000010727	4205	ENSAPLT00000004205
4335	ENSAPLT00000004335	5878	ENSAPLT00000005878
4226	ENSAPLT00000004226	15740	ENSAPLT00000015740
7571	ENSAPLT00000007571	7564	ENSAPLT00000007564
9244	ENSAPLT00000009244	13903	ENSAPLT00000013903
12456	ENSAPLT00000012456	4506	ENSAPLT00000004506
15310	ENSAPLT00000015310	2851	ENSAPLT00000002851
6724	ENSAPLT00000006724	5515	ENSAPLT00000005515
8983	ENSAPLT00000008983	702	ENSAPLT00000000702
274	ENSAPLT00000000274	6461	ENSAPLT00000006461
13415	ENSAPLT00000013415	10490	ENSAPLT00000010490
4728	ENSAPLT00000004728	7444	ENSAPLT00000007444
2854	ENSAPLT00000002854	7824	ENSAPLT00000007824
2434	ENSAPLT00000002434	1925	ENSAPLT00000001925
2245	ENSAPLT00000002245	12009	ENSAPLT00000012009

5336	ENSAPLT00000005336	1265	ENSAPLT00000001265
10125	ENSAPLT000000010125	15812	ENSAPLT000000015812
1565	ENSAPLT000000001565	3655	ENSAPLT000000003655
16046	ENSAPLT000000016046	6123	ENSAPLT000000006123
8083	ENSAPLT000000008083	3392	ENSAPLT000000003392
16407	ENSAPLT000000016407	9092	ENSAPLT000000009092
16976	ENSAPLT000000016976	17138	ENSAPLT000000017138
3573	ENSAPLT000000003573	1423	ENSAPLT000000001423
4834	ENSAPLT000000004834	14932	ENSAPLT000000014932
2110	ENSAPLT000000002110	2620	ENSAPLT000000002620
14936	ENSAPLT000000014936	12904	ENSAPLT000000012904
254	ENSAPLT000000000254	2792	ENSAPLT000000002792
14187	ENSAPLT000000014187	7844	ENSAPLT000000007844
10196	ENSAPLT000000010196	8001	ENSAPLT000000008001
10069	ENSAPLT000000010069	831	ENSAPLT000000000831
797	ENSAPLT000000000797	16142	ENSAPLT000000016142
870	ENSAPLT000000000870	16675	ENSAPLT000000016675
9278	ENSAPLT000000009278	9478	ENSAPLT000000009478
15045	ENSAPLT000000015045	3874	ENSAPLT000000003874
12933	ENSAPLT000000012933	12085	ENSAPLT000000012085
14406	ENSAPLT000000014406	10398	ENSAPLT000000010398
7978	ENSAPLT000000007978	940	ENSAPLT000000000940
16669	ENSAPLT000000016669	3417	ENSAPLT000000003417
268	ENSAPLT000000000268	13548	ENSAPLT000000013548
15350	ENSAPLT000000015350	15578	ENSAPLT000000015578
11974	ENSAPLT000000011974	12515	ENSAPLT000000012515
10848	ENSAPLT000000010848	12291	ENSAPLT000000012291
8501	ENSAPLT000000008501	6967	ENSAPLT000000006967
8825	ENSAPLT000000008825	3084	ENSAPLT000000003084
6328	ENSAPLT000000006328	14838	ENSAPLT000000014838
6141	ENSAPLT000000006141	4233	ENSAPLT000000004233
12094	ENSAPLT000000012094	2301	ENSAPLT000000002301
4519	ENSAPLT000000004519	3057	ENSAPLT000000003057
5368	ENSAPLT000000005368	5915	ENSAPLT000000005915
5424	ENSAPLT000000005424	3323	ENSAPLT000000003323
10488	ENSAPLT000000010488	7654	ENSAPLT000000007654
11884	ENSAPLT000000011884	13712	ENSAPLT000000013712
8400	ENSAPLT000000008400	10127	ENSAPLT000000010127
8044	ENSAPLT000000008044	5250	ENSAPLT000000005250
7304	ENSAPLT000000007304	16888	ENSAPLT000000016888
6787	ENSAPLT000000006787	8442	ENSAPLT000000008442

3844	ENSAPLT00000003844	585	ENSAPLT00000000585
14787	ENSAPLT000000014787	3509	ENSAPLT000000003509
9445	ENSAPLT000000009445	3993	ENSAPLT000000003993
2804	ENSAPLT000000002804	15355	ENSAPLT000000015355
2249	ENSAPLT000000002249	10977	ENSAPLT000000010977
4566	ENSAPLT000000004566	5653	ENSAPLT000000005653
15025	ENSAPLT000000015025	4705	ENSAPLT000000004705
1776	ENSAPLT000000001776	10314	ENSAPLT000000010314
14545	ENSAPLT000000014545	10854	ENSAPLT000000010854
2774	ENSAPLT000000002774	8415	ENSAPLT000000008415
5024	ENSAPLT000000005024	10941	ENSAPLT000000010941
1220	ENSAPLT000000001220	13889	ENSAPLT000000013889
13601	ENSAPLT000000013601	16612	ENSAPLT000000016612
12767	ENSAPLT000000012767	13617	ENSAPLT000000013617
596	ENSAPLT00000000596	7449	ENSAPLT000000007449
9115	ENSAPLT000000009115	11151	ENSAPLT000000011151
5359	ENSAPLT000000005359	8148	ENSAPLT000000008148
13056	ENSAPLT000000013056	9473	ENSAPLT000000009473
6063	ENSAPLT000000006063	12038	ENSAPLT000000012038
16026	ENSAPLT000000016026	1576	ENSAPLT000000001576
2049	ENSAPLT000000002049	1828	ENSAPLT000000001828
2961	ENSAPLT000000002961	13938	ENSAPLT000000013938
2435	ENSAPLT000000002435	6176	ENSAPLT000000006176
14090	ENSAPLT000000014090	5596	ENSAPLT000000005596
9905	ENSAPLT000000009905	15166	ENSAPLT000000015166
579	ENSAPLT00000000579	6225	ENSAPLT000000006225
14314	ENSAPLT000000014314	11389	ENSAPLT000000011389
1672	ENSAPLT000000001672	12371	ENSAPLT000000012371
16790	ENSAPLT000000016790	12916	ENSAPLT000000012916
1054	ENSAPLT000000001054	4124	ENSAPLT000000004124
7579	ENSAPLT000000007579	3028	ENSAPLT000000003028
7458	ENSAPLT000000007458	12709	ENSAPLT000000012709
9860	ENSAPLT000000009860	3562	ENSAPLT000000003562
4998	ENSAPLT000000004998	9006	ENSAPLT000000009006
11250	ENSAPLT000000011250	5346	ENSAPLT000000005346
12277	ENSAPLT000000012277	6408	ENSAPLT000000006408
14390	ENSAPLT000000014390	5108	ENSAPLT000000005108
1033	ENSAPLT000000001033	11936	ENSAPLT000000011936
1382	ENSAPLT000000001382	1700	ENSAPLT000000001700
787	ENSAPLT00000000787	9072	ENSAPLT000000009072
8450	ENSAPLT000000008450	571	ENSAPLT00000000571

5598	ENSAPLT00000005598	753	ENSAPLT00000000753
12592	ENSAPLT00000012592	15143	ENSAPLT00000015143
1269	ENSAPLT00000001269	1831	ENSAPLT00000001831
2621	ENSAPLT00000002621	3521	ENSAPLT00000003521
1381	ENSAPLT00000001381	10006	ENSAPLT00000010006
3256	ENSAPLT00000003256	14206	ENSAPLT00000014206
14619	ENSAPLT00000014619	12707	ENSAPLT00000012707
3321	ENSAPLT00000003321	16121	ENSAPLT00000016121
9189	ENSAPLT00000009189	1385	ENSAPLT00000001385
16465	ENSAPLT00000016465	1036	ENSAPLT00000001036
15533	ENSAPLT00000015533	1403	ENSAPLT00000001403
9165	ENSAPLT00000009165	3965	ENSAPLT00000003965
3803	ENSAPLT00000003803	14445	ENSAPLT00000014445
11446	ENSAPLT00000011446	3069	ENSAPLT00000003069
15011	ENSAPLT00000015011	11604	ENSAPLT00000011604
1869	ENSAPLT00000001869	942	ENSAPLT00000000942
8857	ENSAPLT00000008857	9664	ENSAPLT00000009664
235	ENSAPLT00000000235	2694	ENSAPLT00000002694
4875	ENSAPLT00000004875	12098	ENSAPLT00000012098
6132	ENSAPLT00000006132	3262	ENSAPLT00000003262
4474	ENSAPLT00000004474	11870	ENSAPLT00000011870
4538	ENSAPLT00000004538	8694	ENSAPLT00000008694
2821	ENSAPLT00000002821	8144	ENSAPLT00000008144
15264	ENSAPLT00000015264	8348	ENSAPLT00000008348
10191	ENSAPLT00000010191	10260	ENSAPLT00000010260
1350	ENSAPLT00000001350	4216	ENSAPLT00000004216
1603	ENSAPLT00000001603	8251	ENSAPLT00000008251
945	ENSAPLT00000000945	15588	ENSAPLT00000015588
3159	ENSAPLT00000003159	12267	ENSAPLT00000012267
15183	ENSAPLT00000015183	5406	ENSAPLT00000005406
8149	ENSAPLT00000008149	16846	ENSAPLT00000016846
988	ENSAPLT00000000988	1095	ENSAPLT00000001095
6615	ENSAPLT00000006615	14966	ENSAPLT00000014966
13609	ENSAPLT00000013609	8767	ENSAPLT00000008767
16036	ENSAPLT00000016036	8245	ENSAPLT00000008245
3140	ENSAPLT00000003140	1329	ENSAPLT00000001329
1296	ENSAPLT00000001296	8942	ENSAPLT00000008942
12799	ENSAPLT00000012799	10910	ENSAPLT00000010910
12289	ENSAPLT00000012289	14447	ENSAPLT00000014447
10971	ENSAPLT00000010971	8675	ENSAPLT00000008675
15554	ENSAPLT00000015554	6013	ENSAPLT00000006013

12428	ENSAPLT00000012428	5151	ENSAPLT00000005151
13029	ENSAPLT00000013029	6745	ENSAPLT00000006745

It has surprisingly been discovered that certain transcripts are expressed in infected avian cells but not in uninfected cells. These avian transcripts represent an opportunity to modulate the overall biological product production in cells upon infection by modulating the transcripts which will be expressed upon infection. As such any one or more of these transcripts represents a unique target for the alteration of the avian cellular phenotype to improve the methods disclosed herein.

Example 7: Screening Targeting Constructs

A subset of avian transcript targets was selected for screening. Table 9 lists the siRNA duplex IDs, target (including transcript name), and the synthesized sense and antisense sequence IDs for the targeting constructs designed against the subset of nine avian target genes. These genes are bcl2 (B-cell CLL/lymphoma 2), Dicer1 (ribonuclease type III), eIF2AK2 (eukaryotic translation initiation factor 2-alpha kinase 2), IFNAR(interferon (alpha, beta and omega) receptor 1), IFNB (interferon beta), IRF7 (interferon regulatory factor 7), MAVS (mitochondrial antiviral signaling protein), MX1 (myxovirus resistance 1, interferon-inducible protein p78) and TLR3 (toll-like receptor 3). Endolight chemistry was applied as described herein.

Table 9: Targeting Constructs

Duplex Name	Target Gene	Target Transcript	Sense SEQ ID	Antisense SEQ ID
AD-49232.1	BCL2	ENSAPLT00000007572	755528	755529
AD-49238.1	BCL2	ENSAPLT00000007572	755530	755531
AD-49244.1	BCL2	ENSAPLT00000007572	755532	755533
AD-49250.1	BCL2	ENSAPLT00000007572	755534	755535
AD-49256.1	BCL2	ENSAPLT00000007572	755536	755537
AD-49262.1	BCL2	ENSAPLT00000007572	755538	755539
AD-49267.1	BCL2	ENSAPLT00000007572	755540	755541
AD-49272.1	BCL2	ENSAPLT00000007572	755542	755543
AD-49233.1	BCL2	ENSAPLT00000007572	755544	755545
AD-49239.1	BCL2	ENSAPLT00000007572	755546	755547
AD-49245.1	BCL2	ENSAPLT00000007572	755548	755549
AD-49251.1	BCL2	ENSAPLT00000007572	755550	755551

AD-49257.1	BCL2	ENSAPLT00000007572	755552	755553
AD-49263.1	BCL2	ENSAPLT00000007572	755554	755555
AD-49268.1	BCL2	ENSAPLT00000007572	755556	755557
AD-49273.1	BCL2	ENSAPLT00000007572	755558	755559
AD-49234.1	BCL2	ENSAPLT00000007572	755560	755561
AD-49240.1	BCL2	ENSAPLT00000007572	755562	755563
AD-49246.1	BCL2	ENSAPLT00000007572	755564	755565
AD-49252.1	BCL2	ENSAPLT00000007572	755566	755567
AD-49258.1	BCL2	ENSAPLT00000007572	755568	755569
AD-49264.1	BCL2	ENSAPLT00000007572	755570	755571
AD-49417.1	DICER1	ENSAPLT00000012965	1288360	1288361
AD-49423.1	DICER1	ENSAPLT00000012965	1288362	1288363
AD-37986.2	DICER1	ENSAPLT00000012965	1288366	1288367
AD-37986.1	DICER1	ENSAPLT00000012965	1288366	1288367
AD-49432.1	DICER1	ENSAPLT00000012965	1288368	1288369
AD-49438.1	DICER1	ENSAPLT00000012965	1288370	1288371
AD-49444.1	DICER1	ENSAPLT00000012965	1288372	1288373
AD-49450.1	DICER1	ENSAPLT00000012965	1288374	1288375
AD-49456.1	DICER1	ENSAPLT00000012965	1288376	1288377
AD-49418.1	DICER1	ENSAPLT00000012965	1288378	1288379
AD-49424.1	DICER1	ENSAPLT00000012965	1288380	1288381
AD-49428.1	DICER1	ENSAPLT00000012965	1288382	1288383
AD-49433.1	DICER1	ENSAPLT00000012965	1288384	1288385
AD-49439.1	DICER1	ENSAPLT00000012965	1288386	1288387
AD-49445.1	DICER1	ENSAPLT00000012965	1288388	1288389
AD-49451.1	DICER1	ENSAPLT00000012965	1288390	1288391
AD-49457.1	DICER1	ENSAPLT00000012965	1288392	1288393
AD-49419.1	DICER1	ENSAPLT00000012965	1288394	1288395
AD-49425.1	DICER1	ENSAPLT00000012965	1288396	1288397
AD-49429.1	DICER1	ENSAPLT00000012965	1288398	1288399
AD-49434.1	DICER1	ENSAPLT00000012965	1288400	1288401
AD-49440.1	DICER1	ENSAPLT00000012965	1288402	1288403
AD-49446.1	DICER1	ENSAPLT00000012965	1288404	1288405
AD-49452.1	DICER1	ENSAPLT00000012965	1288406	1288407
AD-49458.1	DICER1	ENSAPLT00000012965	1288408	1288409
AD-49420.1	DICER1	ENSAPLT00000012965	1288410	1288411
AD-49426.1	DICER1	ENSAPLT00000012965	1288412	1288413
AD-49430.1	DICER1	ENSAPLT00000012965	1288414	1288415
AD-49435.1	DICER1	ENSAPLT00000012965	1288416	1288417

AD-49441.1	DICER1	ENSAPLT00000012965	1288418	1288419
AD-49447.1	DICER1	ENSAPLT00000012965	1288420	1288421
AD-49453.1	DICER1	ENSAPLT00000012965	1288422	1288423
AD-49459.1	DICER1	ENSAPLT00000012965	1288424	1288425
AD-49421.1	DICER1	ENSAPLT00000012965	1288426	1288427
AD-49431.1	DICER1	ENSAPLT00000012965	1288428	1288429
AD-49436.1	DICER1	ENSAPLT00000012965	1288430	1288431
AD-49448.1	DICER1	ENSAPLT00000012965	1288432	1288433
AD-49454.1	DICER1	ENSAPLT00000012965	1288434	1288435
AD-49460.1	DICER1	ENSAPLT00000012965	1288436	1288437
AD-49422.1	DICER1	ENSAPLT00000012965	1288438	1288439
AD-38003.2	DICER1	ENSAPLT00000012965	1288442	1288443
AD-38003.1	DICER1	ENSAPLT00000012965	1288442	1288443
AD-38009.1	DICER1	ENSAPLT00000012965	1288444	1288445
AD-49437.1	DICER1	ENSAPLT00000012965	1288446	1288447
AD-49443.1	DICER1	ENSAPLT00000012965	1288448	1288449
AD-49449.1	DICER1	ENSAPLT00000012965	1288450	1288451
AD-49455.1	DICER1	ENSAPLT00000012965	1288452	1288453
AD-49269.1	EIF2AK2	ENSAPLT00000002924	296860	296861
AD-49274.1	EIF2AK2	ENSAPLT00000002924	296862	296863
AD-49235.1	EIF2AK2	ENSAPLT00000002924	296864	296865
AD-49241.1	EIF2AK2	ENSAPLT00000002924	296866	296867
AD-49247.1	EIF2AK2	ENSAPLT00000002924	296868	296869
AD-49253.1	EIF2AK2	ENSAPLT00000002924	296870	296871
AD-49259.1	EIF2AK2	ENSAPLT00000002924	296872	296873
AD-49265.1	EIF2AK2	ENSAPLT00000002924	296874	296875
AD-49270.1	EIF2AK2	ENSAPLT00000002924	296876	296877
AD-49275.1	EIF2AK2	ENSAPLT00000002924	296878	296879
AD-49236.1	EIF2AK2	ENSAPLT00000002924	296880	296881
AD-49242.1	EIF2AK2	ENSAPLT00000002924	296882	296883
AD-49248.1	EIF2AK2	ENSAPLT00000002924	296884	296885
AD-49254.1	EIF2AK2	ENSAPLT00000002924	296886	296887
AD-49260.1	EIF2AK2	ENSAPLT00000002924	296888	296889
AD-49266.1	EIF2AK2	ENSAPLT00000002924	296890	296891
AD-49271.1	EIF2AK2	ENSAPLT00000002924	296892	296893
AD-49276.1	EIF2AK2	ENSAPLT00000002924	296894	296895
AD-49237.1	EIF2AK2	ENSAPLT00000002924	296896	296897
AD-49249.1	EIF2AK2	ENSAPLT00000002924	296898	296899
AD-49255.1	EIF2AK2	ENSAPLT00000002924	296900	296901

AD-49461.1	IFNAR1	ENSAPLT00000011786	1171716	1171717
AD-49467.1	IFNAR1	ENSAPLT00000011786	1171718	1171719
AD-49473.1	IFNAR1	ENSAPLT00000011786	1171720	1171721
AD-49479.1	IFNAR1	ENSAPLT00000011786	1171722	1171723
AD-49485.1	IFNAR1	ENSAPLT00000011786	1171724	1171725
AD-49491.1	IFNAR1	ENSAPLT00000011786	1171726	1171727
AD-49497.1	IFNAR1	ENSAPLT00000011786	1171728	1171729
AD-49503.1	IFNAR1	ENSAPLT00000011786	1171730	1171731
AD-49462.1	IFNAR1	ENSAPLT00000011786	1171732	1171733
AD-49468.1	IFNAR1	ENSAPLT00000011786	1171734	1171735
AD-49474.1	IFNAR1	ENSAPLT00000011786	1171736	1171737
AD-49480.1	IFNAR1	ENSAPLT00000011786	1171738	1171739
AD-49486.1	IFNAR1	ENSAPLT00000011786	1171740	1171741
AD-49492.1	IFNAR1	ENSAPLT00000011786	1171742	1171743
AD-49498.1	IFNAR1	ENSAPLT00000011786	1171744	1171745
AD-49504.1	IFNAR1	ENSAPLT00000011786	1171746	1171747
AD-49463.1	IFNAR1	ENSAPLT00000011786	1171748	1171749
AD-49469.1	IFNAR1	ENSAPLT00000011786	1171750	1171751
AD-49475.1	IFNAR1	ENSAPLT00000011786	1171752	1171753
AD-49481.1	IFNAR1	ENSAPLT00000011786	1171754	1171755
AD-49487.1	IFNAR1	ENSAPLT00000011786	1171756	1171757
AD-49493.1	IFNAR1	ENSAPLT00000011786	1171758	1171759
AD-49499.1	IFNAR1	ENSAPLT00000011786	1171760	1171761
AD-49505.1	IFNAR1	ENSAPLT00000011786	1171762	1171763
AD-49464.1	IFNAR1	ENSAPLT00000011786	1171764	1171765
AD-49470.1	IFNAR1	ENSAPLT00000011786	1171766	1171767
AD-49476.1	IFNAR1	ENSAPLT00000011786	1171768	1171769
AD-49482.1	IFNAR1	ENSAPLT00000011786	1171770	1171771
AD-49488.1	IFNAR1	ENSAPLT00000011786	1171772	1171773
AD-49494.1	IFNAR1	ENSAPLT00000011786	1171774	1171775
AD-49500.1	IFNAR1	ENSAPLT00000011786	1171776	1171777
AD-49506.1	IFNAR1	ENSAPLT00000011786	1171778	1171779
AD-49465.1	IFNAR1	ENSAPLT00000011786	1171780	1171781
AD-49471.1	IFNAR1	ENSAPLT00000011786	1171782	1171783
AD-49477.1	IFNAR1	ENSAPLT00000011786	1171784	1171785
AD-49483.1	IFNAR1	ENSAPLT00000011786	1171786	1171787
AD-49495.1	IFNAR1	ENSAPLT00000011786	1171788	1171789
AD-49501.1	IFNAR1	ENSAPLT00000011786	1171790	1171791
AD-49466.1	IFNAR1	ENSAPLT00000011786	1171792	1171793

AD-49472.1	IFNAR1	ENSAPLT00000011786	1171794	1171795
AD-49484.1	IFNAR1	ENSAPLT00000011786	1171796	1171797
AD-49490.1	IFNAR1	ENSAPLT00000011786	1171798	1171799
AD-49496.1	IFNAR1	ENSAPLT00000011786	1171800	1171801
AD-49502.1	IFNAR1	ENSAPLT00000011786	1171802	1171803
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AD-49740.1	IFNB	ENSAPLT00000001356	143550	143551
AD-49746.1	IFNB	ENSAPLT00000001356	143552	143553
AD-49752.1	IFNB	ENSAPLT00000001356	143554	143555
AD-49757.1	IFNB	ENSAPLT00000001356	143556	143557
AD-49762.1	IFNB	ENSAPLT00000001356	143558	143559
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AD-49763.1	IFNB	ENSAPLT00000001356	143574	143575
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AD-49831.1	IRF7	ENSAPLT00000013271	1318852	1318853
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AD-49843.1	IRF7	ENSAPLT00000013271	1318856	1318857
AD-49848.1	IRF7	ENSAPLT00000013271	1318858	1318859
AD-49853.1	IRF7	ENSAPLT00000013271	1318860	1318861
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AD-49793.1	MAVS	ENSAPLT00000003383	341910	341911
AD-49799.1	MAVS	ENSAPLT00000003383	341912	341913
AD-49805.1	MAVS	ENSAPLT00000003383	341914	341915
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AD-49782.1	MAVS	ENSAPLT00000003383	341920	341921
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AD-49794.1	MAVS	ENSAPLT00000003383	341924	341925
AD-49800.1	MAVS	ENSAPLT00000003383	341926	341927
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AD-49879.1	MX1	ENSAPLT00000016707	1658382	1658383
AD-49885.1	MX1	ENSAPLT00000016707	1658384	1658385
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AD-49938.1	TLR3	ENSAPLT00000009346	930230	930231
AD-49943.1	TLR3	ENSAPLT00000009346	930232	930233
AD-49948.1	TLR3	ENSAPLT00000009346	930234	930235
AD-49910.1	TLR3	ENSAPLT00000009346	930236	930237
AD-49916.1	TLR3	ENSAPLT00000009346	930238	930239
AD-49922.1	TLR3	ENSAPLT00000009346	930240	930241
AD-49928.1	TLR3	ENSAPLT00000009346	930242	930243
AD-49934.1	TLR3	ENSAPLT00000009346	930244	930245
AD-49939.1	TLR3	ENSAPLT00000009346	930246	930247
AD-49944.1	TLR3	ENSAPLT00000009346	930248	930249
AD-49949.1	TLR3	ENSAPLT00000009346	930250	930251
AD-49917.1	TLR3	ENSAPLT00000009346	930252	930253
AD-49923.1	TLR3	ENSAPLT00000009346	930254	930255
AD-49929.1	TLR3	ENSAPLT00000009346	930256	930257
AD-49935.1	TLR3	ENSAPLT00000009346	930258	930259
AD-49940.1	TLR3	ENSAPLT00000009346	930260	930261
AD-49945.1	TLR3	ENSAPLT00000009346	930262	930263
AD-49950.1	TLR3	ENSAPLT00000009346	930264	930265
AD-49912.1	TLR3	ENSAPLT00000009346	930266	930267
AD-49918.1	TLR3	ENSAPLT00000009346	930268	930269
AD-49924.1	TLR3	ENSAPLT00000009346	930270	930271
AD-49930.1	TLR3	ENSAPLT00000009346	930272	930273
AD-49936.1	TLR3	ENSAPLT00000009346	930274	930275
AD-49941.1	TLR3	ENSAPLT00000009346	930276	930277
AD-49946.1	TLR3	ENSAPLT00000009346	930278	930279
AD-49951.1	TLR3	ENSAPLT00000009346	930280	930281
AD-49913.1	TLR3	ENSAPLT00000009346	930282	930283
AD-49919.1	TLR3	ENSAPLT00000009346	930284	930285
AD-49925.1	TLR3	ENSAPLT00000009346	930286	930287
AD-49931.1	TLR3	ENSAPLT00000009346	930288	930289
AD-49937.1	TLR3	ENSAPLT00000009346	930290	930291
AD-49942.1	TLR3	ENSAPLT00000009346	930292	930293
AD-49947.1	TLR3	ENSAPLT00000009346	930294	930295

AD-49952.1	TLR3	ENSAPLT00000009346	930296	930297
AD-49914.1	TLR3	ENSAPLT00000009346	930298	930299
AD-49920.1	TLR3	ENSAPLT00000009346	930300	930301
AD-49926.1	TLR3	ENSAPLT00000009346	930302	930303
AD-49932.1	TLR3	ENSAPLT00000009346	930304	930305

EXAMPLE 8: Synthesis of cationic lipids

Any of the compounds, e.g., cationic lipids and the like, used in the nucleic acid-lipid particles of the invention may be prepared by known organic synthesis techniques.

5 Synthesis of Formula A

In one embodiment, nucleic acid-lipid particles of the invention are formulated using a cationic lipid of formula A:

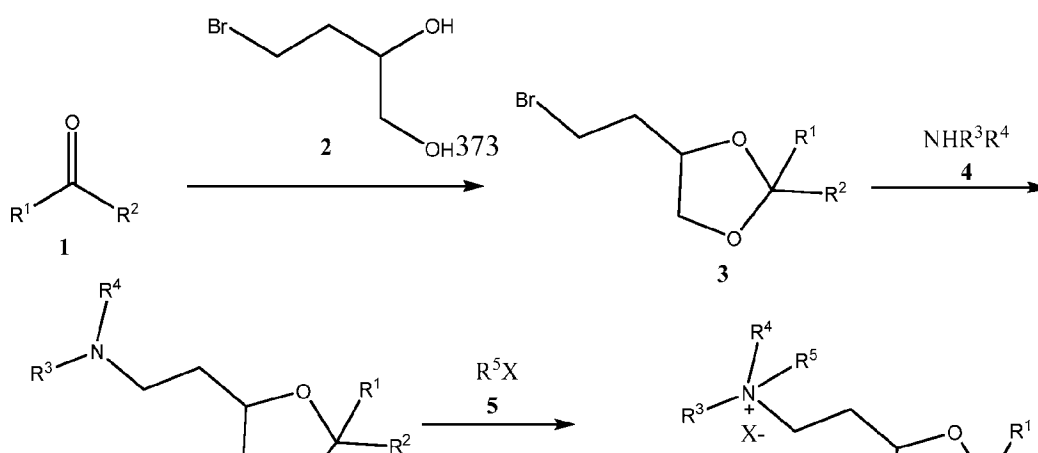


where R1 and R2 are independently alkyl, alkenyl or alkynyl, each can be optionally substituted, and R3 and R4 are independently lower alkyl or R3 and R4 can be taken together to form an optionally substituted heterocyclic ring. In some embodiments, the cationic lipid is XTC (2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane). In general, the lipid of formula A above may be made by the following Reaction Schemes 1 or 2, wherein all substituents are as defined above unless indicated otherwise.

15

Scheme 1

20



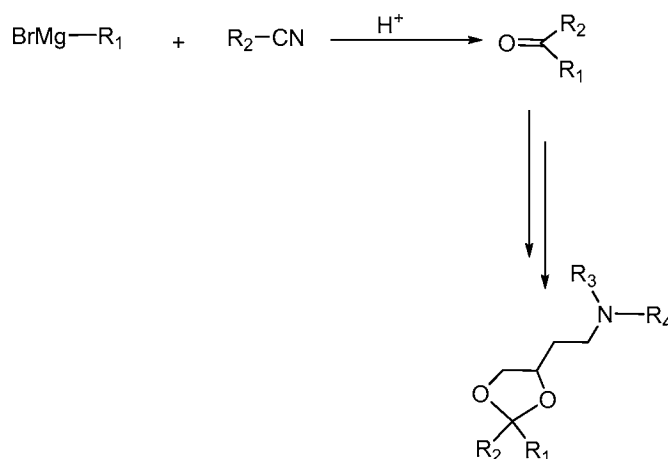
5

10

Lipid A, where R_1 and R_2 are independently alkyl, alkenyl or alkynyl, each can be optionally substituted, and R_3 and R_4 are independently lower alkyl or R_3 and R_4 can be taken together to form an optionally substituted heterocyclic ring, can be prepared according to Scheme 1. Ketone 1 and bromide 2 can be purchased or prepared according to methods known to those of ordinary skill in the art. Reaction of 1 and 2 yields ketal 3. Treatment of ketal 3 with amine 4 yields lipids of formula A. The lipids of formula A can be converted to the corresponding ammonium salt with an organic salt of formula 5, where X is anion counter ion selected from halogen, hydroxide, phosphate, sulfate, or the like.

20

Scheme 2



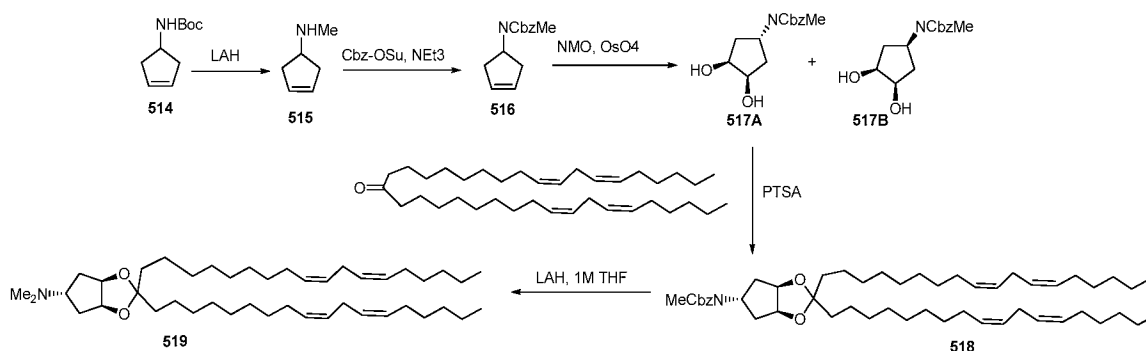
Alternatively, the ketone 1 starting material can be prepared according to Scheme 2. Grignard reagent 6 and cyanide 7 can be purchased or prepared according to methods known to those of ordinary skill in the art. Reaction of 6 and 7 yields ketone 1. Conversion of ketone 1 to the corresponding lipids of formula A is as described in Scheme 1.

Synthesis of MC3

Preparation of DLin-M-C3-DMA (i.e., (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate) is as follows. A solution of (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-ol (0.53 g), 4-N,N-dimethylaminobutyric acid hydrochloride (0.51 g), 4-N,N-dimethylaminopyridine (0.61 g) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.53 g) in dichloromethane (5 mL) is stirred at room temperature overnight. The solution is washed with dilute hydrochloric acid followed by dilute aqueous sodium bicarbonate. The organic fractions are dried over anhydrous magnesium sulphate, filtered and the solvent removed on a rotovap. The residue is passed down a silica gel column (20 g) using a 1-5% methanol/dichloromethane elution gradient. Fractions containing the purified product are combined and the solvent removed, yielding a colorless oil (0.54 g).

Synthesis of ALNY-100

Synthesis of ketal 519 [ALNY-100] is performed using the following scheme 3:



Synthesis of 515:

- 5 To a stirred suspension of LiAlH_4 (3.74 g, 0.09852 mol) in 200 ml anhydrous THF in a two neck RBF (1L), is added a solution of 514 (10g, 0.04926mol) in 70 mL of THF slowly at 0 °C under nitrogen atmosphere. After complete addition, reaction mixture is warmed to room temperature and then heated to reflux for 4 h. Progress of the reaction is monitored by TLC. After completion of reaction (by TLC) the mixture is
- 10 cooled to 0 °C and quenched with careful addition of saturated Na_2SO_4 solution. Reaction mixture is stirred for 4 h at room temperature and filtered off. Residue is washed well with THF. The filtrate and washings are mixed and diluted with 400 mL dioxane and 26 mL conc. HCl and stirred for 20 minutes at room temperature. The volatilities are stripped off under vacuum to furnish the hydrochloride salt of 515 as a
- 15 white solid. Yield: 7.12 g $^1\text{H-NMR}$ (DMSO , 400MHz): δ = 9.34 (broad, 2H), 5.68 (s, 2H), 3.74 (m, 1H), 2.66-2.60 (m, 2H), 2.50-2.45 (m, 5H).

Synthesis of 516:

- To a stirred solution of compound 515 in 100 mL dry DCM in a 250 mL two neck RBF, is added NEt_3 (37.2 mL, 0.2669 mol) and cooled to 0 °C under nitrogen
- 20 atmosphere. After a slow addition of N-(benzyloxy-carbonyloxy)-succinimide (20 g, 0.08007 mol) in 50 mL dry DCM, reaction mixture is allowed to warm to room temperature. After completion of the reaction (2-3 h by TLC) mixture is washed successively with 1N HCl solution (1 x 100 mL) and saturated NaHCO_3 solution (1 x 50 mL). The organic layer is then dried over anhyd. Na_2SO_4 and the solvent is evaporated

to give crude material which was purified by silica gel column chromatography to get 516 as sticky mass. Yield: 11 g (89%). ¹H-NMR (CDCl₃, 400MHz): δ = 7.36-7.27(m, 5H), 5.69 (s, 2H), 5.12 (s, 2H), 4.96 (br., 1H) 2.74 (s, 3H), 2.60(m, 2H), 2.30-2.25(m, 2H). LC-MS [M+H] -232.3 (96.94%).

5 Synthesis of 517A and 517B:

The cyclopentene 516 (5 g, 0.02164 mol) is dissolved in a solution of 220 mL acetone and water (10:1) in a single neck 500 mL RBF and to it is added N-methyl morpholine-N-oxide (7.6 g, 0.06492 mol) followed by 4.2 mL of 7.6% solution of OsO₄ (0.275 g, 0.00108 mol) in tert-butanol at room temperature. After completion of the
10 reaction (~ 3 h), the mixture is quenched with addition of solid Na₂SO₃ and resulting mixture is stirred for 1.5 h at room temperature. Reaction mixture is diluted with DCM (300 mL) and washed with water (2 x 100 mL) followed by saturated NaHCO₃ (1 x 50 mL) solution, water (1 x 30 mL) and finally with brine (1x 50 mL). Organic phase is dried over an.Na₂SO₄ and solvent was removed in vacuum. Silica gel column
15 chromatographic purification of the crude material was afforded a mixture of diastereomers, which are separated by prep HPLC. Yield: - 6 g crude

517A - Peak-1 (white solid), 5.13 g (96%). ¹H-NMR (DMSO, 400MHz): δ= 7.39-7.31(m, 5H), 5.04(s, 2H), 4.78-4.73 (m, 1H), 4.48-4.47(d, 2H), 3.94-3.93(m, 2H), 2.71(s, 3H), 1.72- 1.67(m, 4H). LC-MS - [M+H]-266.3, [M+NH₄ +]-283.5 present,
20 HPLC-97.86%. Stereochemistry confirmed by X-ray.

Synthesis of 518:

Using a procedure analogous to that described for the synthesis of compound 505, compound 518 (1.2 g, 41%) is obtained as a colorless oil. ¹H-NMR (CDCl₃, 400MHz): δ= 7.35-7.33(m, 4H), 7.30-7.27(m, 1H), 5.37-5.27(m, 8H), 5.12(s, 2H), 4.75(m,1H),
25 4.58-4.57(m,2H), 2.78-2.74(m,7H), 2.06-2.00(m,8H), 1.96-1.91(m, 2H), 1.62(m, 4H), 1.48(m, 2H), 1.37-1.25(br m, 36H), 0.87(m, 6H). HPLC-98.65%.

General Procedure for the Synthesis of Compound 519:

A solution of compound 518 (1 eq) in hexane (15 mL) is added in a drop-wise fashion to an ice-cold solution of LAH in THF (1 M, 2 eq). After complete addition, the
30 mixture is heated at 40°C over 0.5 h then cooled again on an ice bath. The mixture is

carefully hydrolyzed with saturated aqueous Na₂SO₄ then filtered through celite and reduced to an oil. Column chromatography provided the pure 519 (1.3 g, 68%) which was obtained as a colorless oil. Formulations prepared by either the standard or extrusion-free method can be characterized in similar manners. For example, formulations are

5 typically characterized by visual inspection. They should be whitish translucent solutions free from aggregates or sediment. Particle size and particle size distribution of lipid-nanoparticles can be measured by light scattering using, for example, a Malvern Zetasizer Nano ZS (Malvern, USA). Particles should be about 20-300 nm, such as 40-100 nm in size. The particle size distribution should be unimodal. The total dsRNA, e.g., siRNA

10 concentration in the formulation, as well as the entrapped fraction, is estimated using a dye exclusion assay. A sample of the formulated dsRNA can be incubated with an RNA-binding dye, such as Ribogreen (Molecular Probes) in the presence or absence of a formulation disrupting surfactant, e.g., 0.5% Triton-X100. The total dsRNA in the formulation can be determined by the signal from the sample containing the surfactant,

15 relative to a standard curve. The entrapped fraction is determined by subtracting the “free” dsRNA content (as measured by the signal in the absence of surfactant) from the total dsRNA content. Percent entrapped dsRNA is typically >85%. For SNALP formulation, the particle size is at least 30 nm, at least 40 nm, at least 50 nm, at least 60 nm, at least 70 nm, at least 80 nm, at least 90 nm, at least 100 nm, at least 110 nm, and at

20 least 120 nm. The suitable range is typically about at least 50 nm to about at least 110 nm, about at least 60 nm to about at least 100 nm, or about at least 80 nm to about at least 90 nm.

Example 9. MAVS targeting siRNA

A series of siRNA was designed to target the 3' UTR of the target gene, MAVS

25 (mitochondrial antiviral signaling protein; SEQ ID NO: 3383; ENSAPLT00000003383). These siRNA are shown in Table 10 below. Identified in the name of the siRNA duplex is the start and stop site of the sense strand.

Table 10. siRNA duplexes targeting MAVS 3'UTR

Duplex	Sense Sequence (5'-3')	Sense	Antisense Sequence (5'-3')	Antisens

		SEQ ID		e SEQ ID
MAVS 3UTR_714 -732	CUUUGUAUUUCCACUUGA A	176313 0	UUCAAGUGGAAAUACAAA G	1763173
MAVS 3UTR_214 -232	CCCCAAACCCAAUUAUU U	176313 1	AAUUUAAUUGGGUUUGG GG	1763174
MAVS 3UTR_529 -547	UUAAGGAAUUCUGUCUGU G	176313 2	CACAGACAGAAUUCUUA A	1763175
MAVS 3UTR_125 -143	AAAUUUGGGUCCCGAAU U	176313 3	AAAUUCGGGACCCAAAU U	1763176
MAVS 3UTR_507 -525	CAGUGAAACAUUUUGCUC A	176313 4	UGAGCAAAAUGUUUCACU G	1763177
MAVS 3UTR_582 -600	GCUUUUAACACUCGGAU U	176313 5	AAAUCCGAGUGUAAAAG C	1763178
MAVS 3UTR_298 -316	GUCUGAUGGGGCUGAUUU U	176313 6	AAAAUCAGCCCCAUCAGA C	1763179
MAVS 3UTR_505 -523	CCCAGUGAAACAUUUUGC U	176313 7	AGCAAAAUGUUUCACUGG G	1763180
MAVS 3UTR_520 -538	UGCUCAGCCUUAAGGAAU U	176313 8	AAUCCUUAAGGCUGAGC A	1763181
MAVS 3UTR_568 -586	CCACGGAAGCCGCAGCUU U	176313 9	AAAGCUGCGGCUUCCGUG G	1763182
MAVS 3UTR_213 -231	ACCCCAAACCCAAUUAUU U	176314 0	AUUUAAUUGGGUUUGGG GU	1763183
MAVS 3UTR_212 -230	AACCCCAAACCCAAUUAU A	176314 1	UUUAAUUGGGUUUGGGG UU	1763184
MAVS 3UTR_195	GCUUCCAAACAAGCUCAA A	176314	UUUGAGCUUGUUUGGAA GC	1763185

-213		2		
MAVS 3UTR_277 -295	GAAACAAACAGCGGGAUU U	176314 3	AAAUCCCGCUGUUUGUUU C	1763186
MAVS 3UTR_196 -214	CUUCCAAACAAGCUCAAA C	176314 4	GUUUGAGCUUGUUUGGA AG	1763187
MAVS 3UTR_621 -639	GCGGAAAAUCUUCCCAA U	176314 5	AUUUGGGAAGAUUUUCCG C	1763188
MAVS 3UTR_429 -447	CUCAAAAAGCCGCGGUGA A	176314 6	UUCACCGCGGCUUUUGA G	1763189
MAVS 3UTR_502 -520	GUGCCCAGUGAAACAUUU U	176314 7	AAAUGUUUCACUGGGCA C	1763190
MAVS 3UTR_248 -266	CAAAGUGCCUUCGAAAA A	176314 8	UUUUUCGGAAGGCACUUU G	1763191
MAVS 3UTR_244 -262	UGCACAAAGUGCCUUCG A	176314 9	UCGGAAGGCACUUUGUGC A	1763192
MAVS 3UTR_104 -122	CCUGAGGAGCAGCCUUAU U	176315 0	AAUAAGGCUGCUCCUCAG G	1763193
MAVS 3UTR_622 -640	CGGAAAAUCUUCCCAAU G	176315 1	CAUUUGGGAAGAUUUUCC G	1763194
MAVS 3UTR_656 -674	GGCUCUUAGUGGGAUUCU U	176315 2	AAGAAUCCACUAAGAGC C	1763195
MAVS 3UTR_705 -723	UCUCAUUCACUUUGUAUU U	176315 3	AAAUACAAAGUGAAUGA GA	1763196
MAVS 3UTR_711 -729	UCACUUUGUAUUUCCACU U	176315 4	AAGUGGAAAUACAAAGU GA	1763197

MAVS 3UTR_593 -611	UCGGAUUUCCUCUGAAUU U	176315 5	AAAUUCAGAGGAAAUCCG A	1763198
MAVS 3UTR_463 -481	CCCCAAGCAGGGAGAAGA G	176315 6	CUCUUCUCCUGCUUGGG G	1763199
MAVS 3UTR_518 -536	UUUGCUCAGCCUUAAGGA A	176315 7	UUCCUUAAGGCUGAGCAA A	1763200
MAVS 3UTR_569 -587	CACGGAAGCCGCAGCUUU U	176315 8	AAAAGCUGCGGCUUCCGU G	1763201
MAVS 3UTR_93- 111	AUGUCUGAAACCCUGAGG A	176315 9	UCCUCAGGGUUUCAGACA U	1763202
MAVS 3UTR_124 -142	CAAAUUUGGGUCCCGAAU U	176316 0	AAUUCGGGACCCAAAUUU G	1763203
MAVS 3UTR_496 -514	CCAGCAGUGCCCAGUGAA A	176316 1	UUUCACUGGGCACUGCUG G	1763204
MAVS 3UTR_704 -722	GUCUCAUUCACUUUGUAU U	176316 2	AAUACAAAGUGAAUGAG AC	1763205
MAVS 3UTR_657 -675	GCUCUUAGUGGGAUUCUU C	176316 3	GAAGAAUCCACUAAGAG C	1763206
MAVS 3UTR_245 -263	GCACAAAGUGCCUCCGA A	176316 4	UUCGGAAGGCACUUUGUG C	1763207
MAVS 3UTR_310 -328	UGAUUUUGGGGCUGGACG U	176316 5	ACGUCCAGCCCCAAAUC A	1763208
MAVS 3UTR_362 -380	GGCAGAGGGCUUUCAGAA C	176316 6	GUUCUGAAAGCCCUCUGC C	1763209
MAVS 3UTR_109 -127	GGAGCAGCCUUAUCCAA A	176316 7	UUUGGAAUAAGGCUGCUC C	1763210

MAVS 3UTR_301 -319	UGAUGGGGCUGAUUUUGG G	176316 8	CCCAAAAUCAGCCCCAUC A	1763211
MAVS 3UTR_591 -609	ACUCGGAUUUCCUCUGAA U	176316 9	AUUCAGAGGAAAUCCGAG U	1763212
MAVS 3UTR_276 -294	GGAAACAAACAGCGGGAU U	176317 0	AAUCCCGCUGUUUGUUUC C	1763213
MAVS 3UTR_224 -242	AAUUAUUUCCGCAGCCA A	176317 1	UUGGCUGCGGAAUUUAAU U	1763214
MAVS 3UTR_710 -728	UUCACUUUGUAUUUCCAC U	176317 2	AGUGGAAAUAACAAAGUG AA	1763215

LENGTHY TABLE

The patent application contains a lengthy table section. A copy of the table is
5 available in electronic form from the USPTO web site. An electronic copy of the table
will also be available from the USPTO upon request and payment of the fee set forth in
37 CFR 1.19(b)(3).

All publications, patent applications, patents, and other references mentioned
herein are incorporated by reference in their entirety. In case of conflict, the present
10 specification, including definitions, will control. In addition, the materials, methods, and
examples are illustrative only and not intended to be limiting.

Claims

1. A double-stranded ribonucleic acid (dsRNA) for inhibiting expression of an avian transcript, wherein said dsRNA comprises a sense strand and an antisense strand, the antisense strand comprising a region of complementarity to an avian transcript listed in Table 1, which antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from one of the antisense sequences selected from SEQ ID NO 18040 through SEQ ID NO 1762259.
2. The dsRNA of claim 1, wherein said dsRNA comprises at least one modified nucleotide.
3. The dsRNA of claim 2, wherein at least one of said modified nucleotides is chosen from the group consisting of: a 2'-O-methyl modified nucleotide, a nucleotide comprising a 5'-phosphate group, a locked nucleotide, a 2'-deoxy-2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, an abasic nucleotide, 2'-amino-modified nucleotide, 2'-alkyl-modified nucleotide, morpholino nucleotide, a phosphoramidate, and a non-natural base comprising nucleotide.
4. The dsRNA of claim 1, wherein the region of complementarity is at least 17 nucleotides in length.
5. The dsRNA of claim 1, wherein the region of complementarity is between 19 and 21 nucleotides in length.
6. The dsRNA of claim 5, wherein the region of complementarity is 19 nucleotides in length.
7. The dsRNA of claim 1, wherein each strand is no more than 30 nucleotides in length.
8. The dsRNA of claim 1, wherein at least one strand comprises a 3' overhang of at least 1 nucleotide.
9. The dsRNA of claim 1, wherein at least one strand comprises a 3' overhang of at least 2 nucleotides.
10. The dsRNA of claim 1, further comprising a ligand.
11. The dsRNA of claim 10, wherein the ligand is conjugated to the 3' end of the sense strand of the dsRNA.
12. A cell containing the dsRNA of claim 1.
13. A composition comprising the dsRNA of claim 1.
14. The composition of claim 13, further comprising a lipid formulation.

15. The composition of claim 14, wherein the lipid formulation is a SNALP, or XTC-containing formulation.
16. A method of inhibiting avian transcript expression in a cell, the method comprising:
 - (a) introducing into the cell the dsRNA of claim 1; and
 - (b) maintaining the cell produced in step (a) for a time sufficient to obtain degradation of the RNA of said avian transcript, thereby inhibiting expression of the avian transcript in the cell.
17. The method of claim 16, wherein the expression of the avian transcript is inhibited by at least 30%.
18. A vector encoding at least one strand of a dsRNA, wherein said dsRNA comprises a region of complementarity to at least a part of an avian transcript of Table 1, wherein said dsRNA is 30 base pairs or less in length, and wherein said dsRNA targets the avian transcript for cleavage.
19. The vector of claim 18, wherein the region of complementarity is at least 15 nucleotides in length.
20. The vector of claim 18, wherein the region of complementarity is 19 to 21 nucleotides in length.
21. A cell comprising the vector of claim 18.
22. A method of altering the path of lineage of an avian cell comprising,
 - (a) contacting said avian cell with a targeting construct and allowing said first cell to undergo cellular division to produce daughter cells,
 - (c) determining the RNA population signature of the daughter cells produced in (a),
 - (d) comparing the RNA population signature of the avian cell with the daughter cells wherein a difference in the RNA population signatures between the avian cell and the daughter cells indicates an alteration in the path of lineage of the avian cell.
23. The method of claim 22, further comprising confirming the lineal alteration of the avian cells by determining the cell type of the daughter cells.
24. The method of claim 23, wherein the cell type of the daughter cells is determined by measuring cell type specific markers.
25. The method of claim 22, wherein the avian cell is an avian gamete.
26. The method of claim 22, wherein the avian cell is an avian somatic cell.

27. The method of claim 22, wherein the avian cell is an avian stem cell.
28. The method of claim 27, wherein the stem cell is selected from the group consisting of adult, embryonic, pluripotent, and induced pluripotent.
29. The method of claim 26, wherein the somatic cell is a cell selected from the group consisting of endodermal-derived, ectodermal-derived, and mesodermal-derived.
30. The method of claim 22, wherein the targeting construct is encoded in a vector.
31. A method of altering the level of an avian transcript in an avian cell or cell culture comprising contacting said cell or culture with a targeting construct.
32. The method of claim 31, wherein the targeting construct is selected from the group consisting of iRNA agents, antisense molecules, ribozymes, aptamers, small molecules, antibodies, peptides, proteins, enzymes or fragments thereof, and vitamins.
33. A method of altering the expression of a gene comprising contacting an avian cell with a synthetic isolated targeting construct which interacts with chromatin modifying proteins or protein complexes.
34. A method of altering the epigenetic signature of an avian cell comprising contacting the cell with an artificial nucleosome protein.
35. A method of altering the methylation status or pattern of a chromosome locus comprising contacting an avian cell containing said chromosome locus with a targeting construct.
36. A method of altering the methylation status or pattern of a nucleosome protein in an avian cell comprising contacting said cell containing said nucleosome protein with an targeting construct comprising an avian transcript structural feature.
37. The method of claim 36, wherein nucleosome protein is a histone.
38. A method of altering avian cellular protein trafficking comprising contacting the avian cell with one or more targeting constructs.
39. A synthetic isolated RNA molecule comprising a structural feature of an avian transcript, wherein the avian transcript is selected from the group consisting of avian transcripts of Table 1.
40. The synthetic isolated RNA molecule of claim 40, wherein said structural feature is at least 50 nucleotides in length.
41. The synthetic isolated RNA molecule of claim 41, wherein said structural feature is from about 200 to about 500 nucleotides in length.

42. The synthetic isolated RNA molecule of claim 42, wherein said structural feature is from about 200 to about 300 nucleotides in length.
43. The synthetic isolated RNA molecule of claim 41, wherein said structural feature is from about 50 to about 100 nucleotides in length.
44. A system for selecting a sequence of at least one targeting construct suitable for modulating protein expression in an avian cell, the system comprising:
- (a) a computer system including at least one processor and associated memory, the memory storing at least one computer program for controlling the operation of the computer system;
 - (b) a database, connected to the computer system, comprising avian transcriptome information, the information comprising, a plurality of transcript sequences of the transcriptome, and optionally, a name of the transcript, and a pathway the transcript plays a role; and targeting construct information, the information comprising at least the sequence of the targeting construct and optionally target specificity of the targeting construct, wherein each targeting construct is designed to match at least one or more sequences in the avian transcriptome;
 - (c) a user interface program module executed by the computer system and configured to receive user parameters comprising at least one of, a cell type selection, a target organism selection, a cellular pathway selection, a cross-reactivity selection, an amount of transcript selection, a target gene name and/or sequence selection, and optionally a method of delivery selection comprising either in vivo or in vitro delivery options; and further optionally user address information;
 - (d) a first module executed by the computer system and configured to check the parameters against the sequences in the database for a matching combination of the parameters and transcriptome transcript sequences; and
 - (e) a second module executed by the computer system and configured to display a selected sequence of at least one targeting construct suitable for modulating protein expression in the cell.
45. The system of claim 44 further comprising a storage module for storing the at least one targeting construct in a container, wherein if there are two or more targeting constructs, each targeting construct is stored in a separate container, and a robotic handling module,

- which upon selection of the matching combination, selects a matching container, and optionally adds to the container additives based on a user selection for in vivo or in vitro delivery, and optionally further packages the container comprising the matching targeting construct to be sent to the user address.
46. The system of claim 44, wherein the avian transcriptome sequence information consists essentially of a plurality of avian transcripts listed in Table 1.
 47. The system of claim 44, wherein the targeting construct is selected from the group consisting of siRNA, miRNA, dsRNA, saRNA, shRNA, piRNA, tkRNAi, eiRNA, pdRNA, a gapmer, an antagomir, a ribozyme and any combination thereof.
 48. The system of claim 44, wherein the targeting construct is selected from the group consisting of an siRNA, a formulated siRNA, an siRNA mixture, and any combination thereof.
 49. The system of claim 44, wherein the targeting construct comprises an antisense strand comprising at least 16 contiguous nucleotides of the nucleotide sequence selected from SEQ ID NO 18040 through SEQ ID NO 1763215.
 50. A method for improving an avian cell line, the method comprising modulating at least one protein translated from an avian transcript selected from Table 1.
 51. A method for improving an avian cell line, the method comprising modulating at least two avian transcripts using a targeting construct, wherein a first transcript affects a first avian cell culture phenotype and a second transcript affects a second, different avian cell culture phenotype, wherein said first and second avian cell culture phenotypes are independently selected from the group consisting of a cell growth rate, a cellular productivity, a peak cell density, a sustained cell viability, a rate of ammonia production or consumption, and rate of lactate production or consumption.
 52. The method of claim 51, further comprising modulating a third avian transcript affecting a third avian cell culture phenotype different from the first and second cell culture phenotypes.
 53. An engineered avian cell line with an improved cellular productivity, improved cell growth rate, or improved cell viability, comprising a population of engineered avian cells, each of which comprises at least one targeting construct modulating one or more avian transcripts selected from Table 1.

54. The engineered avian cell line of claim 54, wherein the targeting construct is selected from the group consisting of siRNA, miRNA, dsRNA, saRNA, shRNA, piRNA, tkRNAi, eiRNA, pdRNA, a gapmer, an antagomir, a ribozyme and any combination thereof.
55. A method for producing a biological product in a large scale avian host cell culture, comprising:
- (a) contacting an avian host cell in a large scale avian host cell culture with at least a first targeting construct, a portion of which is complementary to at least one avian transcript of the avian host cell,
 - (b) maintaining the avian host cell culture for a time sufficient to modulate expression of the at least one first target gene, wherein the modulation of expression improves production of a biological product in the avian host cell;
 - (c) isolating the biological product from the host cell;
- wherein the large scale avian host cell culture is at least 1 liter in size, and wherein the avian host cell is contacted with at least a first targeting construct by addition of the targeting construct to a culture medium of the large scale avian host cell culture such that the target gene expression is transiently inhibited.
56. The method of claim 55, wherein the avian host cell in the large scale avian host cell culture is contacted with a plurality of targeting constructs, wherein the plurality of targeting constructs modulate expression of at least one avian transcript, at least two avian transcripts or a plurality of avian transcripts.
57. The method of claim 55, wherein the targeting construct, comprises a double-stranded ribonucleic acid (dsRNA), wherein said dsRNA comprises at least two sequences that are complementary to each other and wherein a sense strand comprises a first sequence and an antisense strand comprises a second sequence comprising a region of complementarity which is substantially complementary to at least part of an avian transcript of Table 1, and wherein said region of complementarity is 10-30 nucleotides in length.
58. The method of claim 55, wherein the contacting step is performed by continuous infusion of the targeting construct into the culture medium used for maintaining the avian host cell culture to produce the biological product.

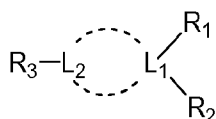
59. The method of claim 55, wherein the modulation of expression is inhibition of expression, and wherein the inhibition is a partial inhibition.
60. The method of claim 59, wherein the partial inhibition is no greater than a percent inhibition selected from the group consisting of: 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, and 85%.
61. The method of claim 55, wherein the contacting step is repeated at least once.
62. The method of claim 55, wherein the contacting step is repeated multiple times at a frequency selected from the group consisting of: 6 hr, 12 hr, 24 hr, 36 hr, 48 hr, 72 hr, 84 hr, 96 hr, and 108 hr.
63. The method of claim 55, wherein the modulation of expression is inhibition of expression and wherein the contacting step is repeated multiple times, or continuously infused, to maintain an average percent inhibition of at least 50% for the target gene(s) throughout the production of the biological product.
64. The method of claim 63, wherein the average percent inhibition is selected from the group consisting of at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, or 100%.
65. The method of claim 55, wherein the targeting construct is contacted at a concentration of less than 100 nM.
66. The method of claim 66, wherein the targeting construct is contacted at a concentration of less than 20 nM.
67. The method of any of claim 55, wherein said contacting an avian host cell in a large scale avian host cell culture with a targeting construct is done at least 6 hr, at least 12 hr, at least 18 hr, at least 36 hr, at least 48 hr, at least 60 hr, at least 72 hr, at least 96 hr, or at least 120 hr, or at least 1 week, before isolation of the biological product or prior to harvesting the supernatant.
68. The method of claim 55, wherein the targeting construct is composition formulated in a lipid formulation.
69. The method of claim 55, wherein the targeting construct is an siRNA.
70. The method of claim 69, wherein the targeting construct is chemically modified.

71. The method of claim 55, further comprising monitoring at least one measurable parameter selected from the group consisting of cell density, medium pH, oxygen levels, glucose levels, lactic acid levels, temperature, and protein production.
72. The method of claim 56, wherein each of the plurality of different targeting constructs is added simultaneously or at different times.
73. The method of claim 56, wherein each of the plurality of different targeting constructs is added at the same or different concentrations.
74. The method of claim 56, wherein the plurality of different targeting constructs is added at the same or different frequencies.
75. The method of claim 55, further comprising contacting the cell with a second agent.
76. The method of claim 75, wherein the second agent is selected from the group consisting of: an antibody, a growth factor, an apoptosis inhibitor, a kinase inhibitor, a phosphatase inhibitor, a protease inhibitor, and a histone demethylating agent.
77. The method of claim 55, wherein the biological product is a polypeptide.
78. The method of claim 55, wherein the biological product is a metabolite.
79. The method of claim 55, wherein the biological product is a nutraceutical.
80. The method of claim 55, wherein the avian cell is contacted with the targeting construct at a phase of cell growth selected from the group consisting of: stationary phase, early log phase, mid-log phase, late-log phase, lag phase, and death phase.
81. The method of claim 56, wherein the at least first targeting construct, or at least one of the plurality of targeting constructs, is 17-28 nucleotides in length.
82. The method of claim 56, wherein the at least first targeting construct, or at least one of the plurality of targeting constructs, comprises at least one modified nucleotide.
83. The method of claim 56, wherein the avian cell is a duck cell from the species *Anas platyrhynchos domesticus*; Pekin duck.
84. The method of claim 55, wherein the cell further comprises a genetic construct encoding the biological product.
85. The method of claim 55, wherein the cell further comprises a genetic construct encoding a viral receptor.
86. The method of claim 55, wherein the target gene encodes a protein that affects protein glycosylation.

87. The method of claim 55, wherein the target gene encodes the biological product.
88. The method of claim 56 or 57, wherein the at least first targeting construct, or at least one of the plurality of targeting constructs, is added at a concentration selected from the group consisting of 0.1 nM, 0.5 nM, 0.75 nM, 1nM, 2 nM, 5 nM, 10 nM, 20 nM, 30 nM, 40 nM, 50 nM, 75 nM, and 100 nM.
89. The method of claim 56, wherein the at least first targeting construct, or at least one of the plurality of targeting constructs, is added at an amount of 50 molecules per cell, 100 molecules/cell, 200 molecules/cell, 300 molecules/cell, 400 molecules/cell, 500 molecules/ cell, 600 molecules/cell, 700 molecules/ cell, 800 molecules/cell, 900 molecules/cell, 1000 molecules/cell, 2000 molecules/cell, or 5000 molecules/cell.
90. The method of claim 56, wherein the at least first targeting construct, or at least one of the plurality of targeting constructs, is added at a concentration selected from the group consisting of: 0.01 fmol/ 10^6 cells, 0.1 fmol/ 10^6 cells, 0.5 fmol/ 10^6 cells, 0.75 fmol/ 10^6 cells, 1 fmol/ 10^6 cells, 2 fmol/ 10^6 cells, 5 fmol/ 10^6 cells, 10 fmol/ 10^6 cells, 20 fmol/ 10^6 cells, 30 fmol/ 10^6 cells, 40 fmol/ 10^6 cells, 50 fmol/ 10^6 cells, 60 fmol/ 10^6 cells, 100 fmol/ 10^6 cells, 200 fmol/ 10^6 cells, 300 fmol/ 10^6 cells, 400 fmol/ 10^6 cells, 500 fmol/ 10^6 cells, 700 fmol/ 10^6 cells, 800 fmol/ 10^6 cells, 900 fmol/ 10^6 cells, and 1 pmol/ 10^6 cells.
91. The method of claim 56, wherein the at least first targeting construct, or at least one of the plurality of targeting constructs, is selected from the group consisting of siRNA, miRNA, dsRNA, saRNA, shRNA, piRNA, tkRNAi, eiRNA, pdRNA, a gapmer, an antagomir, a ribozyme, and any combination thereof.
92. The method of claim 55, wherein the method further comprises contacting the avian cell with at least one additional targeting construct, or agent, that modulates a cellular process selected from the group consisting of: carbon metabolism and transport, apoptosis, RNAi uptake and/or efficiency, reactive oxygen species production, control of cell cycle, protein folding, protein pyroglutamation, protein deamidation, protein glycosylation, disulfide bond formation, protein secretion, gene amplification, viral replication, viral infection, viral particle release, control of cellular pH, and protein production.
93. A kit for enhancing production of a biological product by a cultured avian host cell, comprising:

- (a) a substrate comprising one or more assay surfaces suitable for culturing the avian host cell under conditions in which the biological product is produced;
 - (b) one or more targeting constructs, wherein at least a portion of each targeting construct is complementary to a target gene; and
 - (c) a reagent for detecting the biological product or production thereof by the avian cell, wherein the one or more targeting constructs is an siRNA comprising an antisense strand that comprises at least 16 contiguous nucleotides of the nucleotide sequence selected from the group consisting of SEQ ID NO 18040 through SEQ ID NO 1763215.
94. The kit of claim 93, wherein the one or more assay surfaces further comprises a matrix for supporting the growth and maintenance of avian host cells.
95. The kit of claim 93, wherein the one or more targeting constructs are deposited on the substrate.
96. The kit of claim 93, further comprising a carrier for promoting uptake of the targeting constructs by the avian host cell.
97. The kit of claim 93, further comprising cell culture media suitable for culturing the avian host cell.
98. The kit of claim 93, further comprising instructions for culturing an avian host cell in the presence of one or more targeting constructs and assaying the cell for production of the biological product.
99. A kit for optimizing production of a biological product by cultured avian cells, comprising:
- (a) a microarray substrate comprising a plurality of assay surfaces, the assay surfaces being suitable for culturing the cells under conditions in which the biological product is produced;
 - (b) one or more targeting constructs, wherein at least a portion of each targeting construct is complementary to a target gene; and
 - (c) a reagent for detecting the effect of the one or more targeting constructs on production of the biological product wherein the one or more targeting constructs is an siRNA comprising an antisense strand that comprises at least 16 contiguous nucleotides of the nucleotide sequence selected from the group consisting of SEQ ID NO 18040 through SEQ ID NO 1763215.

100. The kit of claim 99, wherein the substrate is a multi-well plate or biochip.
101. The kit of claim 99, wherein the substrate is a two-dimensional microarray plate or biochip.
102. The kit of claim 99, wherein the one or more targeting constructs are deposited on the assay surfaces of the substrate.
103. The kit of claim 99, wherein a plurality of different targeting constructs are deposited on assay surfaces across a first dimension of the microarray.
104. The kit of claim 103, wherein the plurality of targeting constructs are each complementary to a different target gene.
105. The method of claim 68, wherein the lipid formulation comprises a lipid having the following formula:



wherein:

R_1 and R_2 are each independently for each occurrence optionally substituted $\text{C}_{10}\text{--C}_{30}$ alkyl, optionally substituted $\text{C}_{10}\text{--C}_{30}$ alkoxy, optionally substituted $\text{C}_{10}\text{--C}_{30}$ alkenyl, optionally substituted $\text{C}_{10}\text{--C}_{30}$ alkenyloxy, optionally substituted $\text{C}_{10}\text{--C}_{30}$ alkynyl, optionally substituted $\text{C}_{10}\text{--C}_{30}$ alkynyloxy, or optionally substituted $\text{C}_{10}\text{--C}_{30}$ acyl;



represents a connection between L_2 and L_1 which is:

(1) a single bond between one atom of L_2 and one atom of L_1 , wherein

L_1 is $\text{C}(\text{R}_x)$, O, S or $\text{N}(\text{Q})$;

L_2 is $-\text{CR}_5\text{R}_6-$, $-\text{O}-$, $-\text{S}-$, $-\text{N}(\text{Q})-$, $=\text{C}(\text{R}_5)-$, $-\text{C}(\text{O})\text{N}(\text{Q})-$, $-\text{C}(\text{O})\text{O}-$, $-\text{N}(\text{Q})\text{C}(\text{O})-$, $-\text{OC}(\text{O})-$, or $-\text{C}(\text{O})-$;

(2) a double bond between one atom of L_2 and one atom of L_1 ; wherein

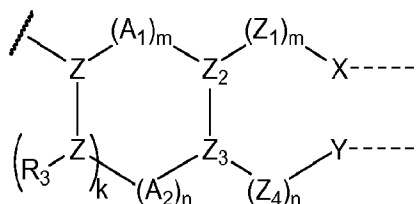
L_1 is C;

L_2 is $-\text{CR}_5=$, $-\text{N}(\text{Q})=$, $-\text{N}-$, $-\text{O}-\text{N}=$, $-\text{N}(\text{Q})-\text{N}=$, or $-\text{C}(\text{O})\text{N}(\text{Q})-\text{N}=$;

(3) a single bond between a first atom of L_2 and a first atom of L_1 , and a single bond between a second atom of L_2 and the first atom of L_1 , wherein

L_1 is C;

L_2 has the formula



wherein

X is the first atom of L_2 , Y is the second atom of L_2 , - - - - represents a single bond to the first atom of L_1 , and X and Y are each, independently, selected from the group consisting of -O-, -S-, alkylene, -N(Q)-, -C(O)-, -O(CO)-, -OC(O)N(Q)-, -N(Q)C(O)O-, -C(O)O-, -OC(O)O-, -OS(O)(Q₂)O-, and -OP(O)(Q₂)O-;

Z_1 and Z_4 are each, independently, -O-, -S-, -CH₂-, -CHR⁵-, or -CR⁵R⁵-;

Z_2 is CH or N;

Z_3 is CH or N;

or Z_2 and Z_3 , taken together, are a single C atom;

A_1 and A_2 are each, independently, -O-, -S-, -CH₂-, -CHR⁵-, or -CR⁵R⁵-;

each Z is N, C(R₅), or C(R₃);

k is 0, 1, or 2;

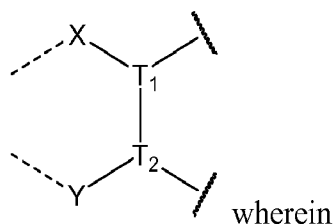
each m, independently, is 0 to 5;

each n, independently, is 0 to 5;

where m and n taken together result in a 3, 4, 5, 6, 7 or 8 member ring;

(4) a single bond between a first atom of L_2 and a first atom of L_1 , and a single bond between the first atom of L_2 and a second atom of L_1 , wherein

(A) L_1 has the formula:



wherein

X is the first atom of L_1 , Y is the second atom of L_1 , - - - - represents a single bond to the first atom of L_2 , and X and Y are each, independently, selected from the group consisting of -O-, -S-, alkylene, -N(Q)-, -C(O)-, -O(CO)-, -OC(O)N(Q)-, -N(Q)C(O)O-, -C(O)O-, -OC(O)O-, -OS(O)(Q₂)O-, and -OP(O)(Q₂)O-;

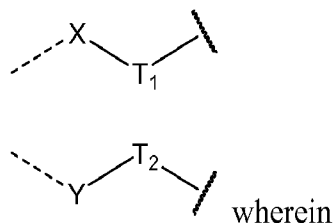
T_1 is CH or N;

T_2 is CH or N;

or T_1 and T_2 taken together are C=C;

L_2 is CR_5 ; or

(B) L_1 has the formula:



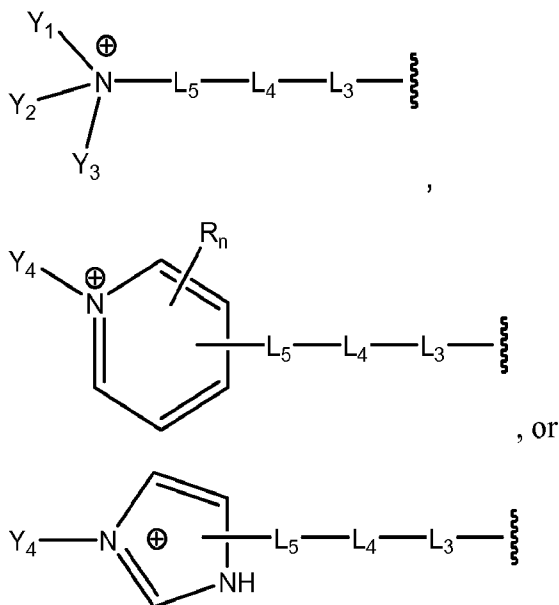
X is the first atom of L_1 , Y is the second atom of L_1 , - - - - represents a single bond to the first atom of L_2 , and X and Y are each, independently, selected from the group consisting of -O-, -S-, alkylene, -N(Q)-, -C(O)-, -O(CO)-, -OC(O)N(Q)-, -N(Q)C(O)O-, -C(O)O-, -OC(O)O-, -OS(O)(Q₂)O-, and -OP(O)(Q₂)O-;

T_1 is -CR₅R₅-, -N(Q)-, -O-, or -S-;

T_2 is -CR₅R₅-, -N(Q)-, -O-, or -S-;

L_2 is CR_5 or N;

R_3 has the formula:



wherein

each of Y_1 , Y_2 , Y_3 , and Y_4 , independently, is alkyl, cycloalkyl, aryl, aralkyl, or alkynyl;

or any two of Y_1 , Y_2 , and Y_3 are taken together with the N atom to which they are attached to form a 3- to 8- member heterocycle; or

Y_1 , Y_2 , and Y_3 are all be taken together with the N atom to which they are attached to form a bicyclic 5- to 12- member heterocycle;
 each R_n , independently, is H, halo, cyano, hydroxy, amino, alkyl, alkoxy, cycloalkyl, aryl, heteroaryl, or heterocyclyl;
 L_3 is a bond, -N(Q)-, -O-, -S-, -(CR₅R₆)_a-, -C(O)-, or a combination of any two of these;
 L_4 is a bond, -N(Q)-, -O-, -S-, -(CR₅R₆)_a-, -C(O)-, or a combination of any two of these;
 L_5 is a bond, -N(Q)-, -O-, -S-, -(CR₅R₆)_a-, -C(O)-, or a combination of any two of these;
 each occurrence of R_5 and R_6 is, independently, H, halo, cyano, hydroxy, amino, alkyl, alkoxy, cycloalkyl, aryl, heteroaryl, or heterocyclyl; or two R_5 groups on adjacent carbon atoms are taken together to form a double bond between their respective carbon atoms; or two R_5 groups on adjacent carbon atoms and two R_6 groups on the same adjacent carbon atoms are taken together to form a triple bond between their respective carbon atoms;
 each a , independently, is 0, 1, 2, or 3;
 wherein
 an R_5 or R_6 substituent from any of L_3 , L_4 , or L_5 is optionally taken with an R_5 or R_6 substituent from any of L_3 , L_4 , or L_5 to form a 3- to 8- member cycloalkyl, heterocyclyl, aryl, or heteroaryl group; and
 any one of Y_1 , Y_2 , or Y_3 , is optionally taken together with an R_5 or R_6 group from any of L_3 , L_4 , and L_5 , and atoms to which they are attached, to form a 3- to 8- member heterocyclyl group;
 each Q, independently, is H, alkyl, acyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl or heterocyclyl; and
 each Q_2 , independently, is O, S, N(Q)(Q), alkyl or alkoxy.

106. A method of doing a business for the production of a biological product, comprising
 - (i) providing a service for the rapid production of a biological product from an avian host cell using one or more iRNA targeting constructs; and
 - (ii) assessing the efficacy of the biological product.
107. The method of claim 106, wherein the biological product is useful in the treatment of an infection.
108. The method of claim 107, wherein the assessment of efficacy is against one or more infective agents suspected of causing said infection.

109. The method of claim 108 further comprising providing the effective biological product to a consumer or customer.
110. The method of claim 106, wherein the biological product is useful in the manufacture of textiles.
111. The method of claim 106, wherein the biological product is useful in the chemicals industry.
112. The method of claim 106, wherein the biological product is useful in the manufacture, cleaning or production of electronic devices or components.