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(54) Title: RETINOIC ACID-INDUCIBLE GENE I PROMOTER AND COMPOSITIONS AND METHODS RELATING TO SAME

(57) Abstract: The present disclosure provides a promoter having at least the core components of a duck retinoic acid-inducible gene I (RIG-I) promoter, as well as expression constructs having the duck RIG-I promoter operably linked to a gene product-encoding nucleic acid (e.g., an avian RIG-I protein), and recombinant host cells containing the duck RIG-I promoter, e.g., in such expression constructs. The present disclosure also provide animals genetically modified to have a gene encoding a duck RIG-I promoter operably linked to a gene product-encoding nucleic acid (e.g., an avian RIG-I protein, such as a duck RIG-I protein).



WO 2019/060993 A1

RETINOIC ACID-INDUCIBLE GENE I PROMOTER AND COMPOSITIONS AND
METHODS RELATING TO SAME

CROSS-REFERENCE

[0001] This application claims priority benefit of U.S. Provisional Patent Application No. 62/564,867, filed September 28, 2017, which application is incorporated herein by reference in its entirety.

INTRODUCTION

[0002] Influenza virus H5N1 causes 100% mortality in chickens within hours or days after infection (Barber et al. 2010, *Proc Natl Acad Sci* 107(13), 5913-5918; Kim J, et al., 2009 Influenza and Other Respiratory Diseases 3:121-128) and 60% mortality in humans. Innate immunity is the first line of defense against infectious diseases or tissue damage. A successful innate immune response to influenza infection involves a robust, yet transient induction of interferon-stimulated antiviral genes. Cytoplasmic RNA sensors recognize microbial products in the cytoplasm and trigger the production of INF- β and downstream interferon-stimulated antiviral genes.

[0003] Retinoic acid-inducible gene I (RIG-I) has been identified as a cytoplasmic RNA sensor that stimulates a downstream signaling pathway leading to inflammatory cytokine and Type-I IFN release, further inducing the production of antiviral factors. RIG-I is present in ducks but absent in chickens.

[0004] A gene edited chicken that exhibits resistance to disease following exposure to RNA viruses, such as highly pathogenic avian influenza H5N1 viruses, would be of great interest, particularly in the commercial setting.

SUMMARY

[0005] The present disclosure provides a promoter having at least the core components of a duck retinoic acid-inducible gene I (RIG-I) promoter, as well as expression constructs having the duck RIG-I promoter operably linked to a gene product-encoding nucleic acid (e.g., an avian RIG-I protein), and recombinant host cells containing the duck RIG-I promoter, e.g., in such expression constructs. The present disclosure also provides animals having modified genomes

(e.g., gene knockin chickens) expressing a gene construct encoding a duck RIG-I promoter operably linked to a gene product-encoding nucleic acid (e.g., an avian RIG-I protein, such as a duck RIG-I protein).

[0006] The present disclosure provides expression constructs comprising an operably linked promoter and a polypeptide-encoding nucleic acid for expression of the polypeptide in a host cell, where the promoter comprises, from 5' to 3', an Sp1/Sp2 transcription factor binding site and an IRF-1/STAT1/STAT2 transcription factor binding site, wherein the Sp1/Sp2 transcription factor binding site comprises the nucleotide sequence CGGCCAGAGGGCGGGC (SEQ ID NO:03) and wherein the IRF-1/STAT1/STAT2 transcription factor binding site is comprised of the nucleotide sequence GGCCGCTTTCGTTTTCCATTC (SEQ ID NO:04). The present disclosure also provides expression constructs wherein the promoter comprises, from 5' to 3', a NHLH1 transcription factor binding site comprising the nucleotide sequence ACGAAGCTGCGA (SEQ ID NO: 05), the Sp1/Sp2 transcription factor binding site comprising the nucleotide sequence CGGCCAGAGGGCGGGC (SEQ ID NO: 06), and the IRF-1/STAT1/STAT2 transcription factor binding site comprising the nucleotide sequence GGCCGCTTTCGTTTTCCATTC (SEQ ID NO:07).

[0007] The present disclosure also provides an expression construct containing a polypeptide encoding nucleic acid operably linked to a promoter to provide for expression in a host cell wherein the promoter nucleic acid sequence is of at least 85% sequence identity to the nucleic acid sequence:

CTGGACCCAGGCCCGTGTCTCGGTGTCCCGGTGAGGCTGTTGGTGGCGATCCGAGG
CTCACGAAGCTGCGAGCCGCTGCCTATGCCCGCTGGCGGCCAGAGGGCGGGCTGCG
GATGGGGCCGCGATCTCCACACCCCGCGGGGGCCGCTTTCGTTTTCCATCCCCGCC
GGGCCTCGCTGCCTTTCTGTGCCGAGCCGGTTGGGGGGCCGGGCGGGCCGGGCGG
GGCGGCGGCCGGCAGAGCCCAGCC (SEQ ID NO: 01)

[0008] In related embodiments, the polypeptide-encoding nucleic acid of the expression constructs disclosed herein encodes a retinoic acid-inducible gene I (RIG-I) protein. The RIG-I-encoding nucleic acid may encode a RIG-I protein having at least 75% sequence identity to a RIG-I protein having the amino acid sequence of:

MTADEKRSLQCYRRYIERSLNPVYVLGNMTDWLPDELRRERIRKEEERGVSAAALFLD
AVLQLEARGWFRGMLDAMLAAGYTGLAEAIENWDFSKLEKLELHRQLLKRIEATMLE

VDPVALIPYISTCLIDRECEEIQQISENRSKAAGITKLIIECLCRSDKEHWPKSLQLALDTTG
 YYRASELWDIREDNAKDVDSEMTDASEDCLEASMTYSEEAEPPDDNLSENLGSAAEIGIG
 KPPPVEYETKKARSYQIELAQPAINGKNALICAPTGSCKTFVSILICEHHFQNPAGRKAK
 VVFLATKVPVYEQQKNVFKHHFERQGYSVQGISGENFSNVSVVEKVIEDSDIIVVTPQILV
 NSFEDGTLTSLSIFTLMIFDECHNTTGNHPYNVLMTRYLEQKFNSASQLPQILGLTASVG
 VGNAKNIEETIEHICSLCSYLDIQAISTVRENIQELQRFMNKPEIDVRLVKRRIHNPFAAIIS
 NLMSETEALMRTIYSVDTLSQLNSKKDFGTQNYEHWIVVTQRKCRLQLLEDKEEESRICR
 ALFICTEHLRKYNDAIISEDARIIDALSYLTEFFTNVKNGPYTELEQHLTAKFQEKEPELI
 ALSKDETNNENPKLEELVCILDDAYRYNPQTRTLLFAKTRALVSALKKCMEEENPILNYIKP
 GVLMGRGRRDQTTGMTLPSQKGVLD AFKTSKDNRLLIATSVADEGIDIVQCNLVVLYE
 YSGNVTKMIQVRGRGRAAGSKCILVTSKTEVVENEKCNRYKEEMMNKAVEKIQKWDE
 ETFAKKIHNLMKERVLRDSRRKEIKPKVVEGQKNLLCGKCKAYACSTDDIRIHKDSHHI
 VLGEAFKERYTTKPHKKPMQFDGFEEKSKMYCRNNNCQHDWGITVKYLTFDNLPIVIKI
 KSFVMESTATGTQMDFFQKWSINSSLNFDVEEMSNLYPP (SEQ ID NO: 11).

[0009] In related embodiments, the promoter of the expression constructs disclosed herein comprises a nucleic acid sequence beginning at the 5' end of the Sp1/Sp2 binding site, having at least 80% sequence identity to the sequence

CGGCCAGAGGGCGGGCTGCGGATGGGGCCGCGATCTCCACACCCCGCGGGGGCCGC
 TTTCGTTTTCCATTCCCCGC||CGGGCCTCGCTGCCTTTCTGTGCCGAGCCGGTTGGGG
 GGCCGGGCGGGCCGGGCGGGCGGGCGGCCGGCAGAGCCCAGCC (SEQ ID NO: 08).

[0010] In related embodiments, the promoter of the expression constructs disclosed herein comprise a nucleic acid sequence having at least 80% sequence identity to the sequence of SEQ ID NO:02, or comprise a promoter wherein the promoter begins just 3' of the NHLH1 binding site and comprises a nucleic acid sequence having at least 80% sequence identity to the sequence:

GCCGCTGCCTATGCCCCGCTGGCGGCCAGAGGGCGGGCTGCGGATGGGGCCGCGATC
 TCCACACCCCGCGGGGGCCGCTTTTCGTTTTCCATTCCCCGCCGGGCCTCGCTGCCTTT
 CTGTGCCGAGCCGGTTGGGGGGCCGGGCGGGCCGGGCGGGCGGGCGGGCGGCCGGCAG
 AGCCCAGCC (SEQ ID NO: 09).

[0011] In related embodiments, the expression construct is a viral vector. Additionally, the viral vector may be a retroviral vector, an adenoviral vector or an adeno-associated viral vector.

[0012] The present disclosure also provides a recombinant cell comprising any of the expression constructs disclosed herein. The recombinant host cell may be an avian cell such as, for example, a chicken cell.

[0013] The present disclosure also provides a transgenic non-human animal, for example a chicken, comprising the genomic integration of any of the expression constructs disclosed herein. Also presently disclosed is a method for producing a chicken resistant to disease caused by avian influenza infection comprised of introducing into a chicken embryo any of the expression constructs disclosed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Figs. 1A-1D depict duck RIG-I promoter activity.

[0015] Fig. 1A is a schematic diagram for inserting duck RIG-I promoter segment into pGL3 basic vector to construct the full length promoter reporter vector (p2024) expressing firefly luciferase.

[0016] Fig. 1B shows the results of dual luciferase analysis of DF-1 cells transfected with a *Renilla* luciferase vector and either pGL3-Basic (negative control) or p2024. Firefly luciferase activity is normalized to *Renilla* luciferase activity in the dual luciferase assay. The mean of triplicate determinations ($\pm$ SD) is shown and significance was analyzed with Student's t-test (* $p < 0.05$).

[0017] Fig. 1C shows the results of dual luciferase analysis of DF-1 cells co-transfected pGL3-basic or p2024 with GST only or GST-2CARD as stimulator. RIG-I 2 CARD is the constitutively active N-terminal fragment of RIG-I. Significance was analyzed with multiple T-test (* $P < 0.05$) measured by Dual Luciferase analysis.

[0018] Fig. 1D shows the results of dual luciferase analysis of DF-1 cells transfected pGL3-basic or p2024 and treated with or without Poly I: C. Significance was analyzed with multiple T-test (* $P < 0.05$) measured by Dual Luciferase analysis.

[0019] Figs. 2A-2B provide evidence that the core promoter of duck RIG-I is 250bp upstream of TSS.

[0020] Fig. 2A provides a schematic of a series of deletion mutants of duck RIG-I promoter (upper panel) and the results of transfection of each of these deletion mutants into DF-1 cells with *Renilla* luciferase vector (lower panel). Relative luciferase activities were tested 24 hours post transfection and the mean ($\pm$ SD) of triplicate determinations for each transfection is shown.

[0021] Fig. 2B shows results using the serial deletion mutants of duck RIG-I promoter of Fig. 2A transfected into DF-1 cells with GST or GST-2CARD stimulator. RIG-I 2CARD is a constitutive activator of the MAVS signaling pathway. Relative luciferase activities were tested 24 hours post transfection and the mean ($\pm$ SD) of triplicate determinations for each transfection is shown.

[0022] Figs. 3A-3E depict the sequence and a schematic of the duck RIG-I core promoter and the results of experiments showing that deletion of the Sp1/Sp2 binding site largely decreased the basal RIG-I promoter activity, whereas, deletion of the IRF-1/STAT1/STAT2 binding site undermined RIG-I promoter interferon inducible activity.

[0023] Fig. 3A shows the sequence of the RIG-I core promoter showing the transcription factor binding sites in the core promoter of duck RIG-I (SEQ ID NO: 01) as predicted by JASPAR. The grey highlighted regions are the locations of transcription factor binding sites. The position of the nucleotides used in the constructs is shown. The box indicates the random sequence deleted in the control vector.

[0024] Fig. 3B. Shows a schematic of the constructs made with indicated deletions of the transcription factor binding sites.

[0025] Fig. 3C shows the results of experiments in which DF-1 cells were transfected with wild-type or control with random deletion or p250 with the indicated transcription factor binding sites deleted. Relative luciferase activities were tested 24 hours post transfection and the mean ($\pm$ SD) of triplicate determinations is shown for each assay. Significance was analyzed with t-test (* $P < 0.05$) Fig. 3D shows the results of experiments in which DF-1 cells were co-transfected with wild type p250 constructs with the control random deletion or transcription factor binding site deleted from p250 or p125 with GST or GST- 2CARD as stimulator. Relative luciferase activities were tested 24 hours post transfection and the mean ($\pm$ SD) for triplicate determinations for each transfection is shown

[0026] Fig. 3E DF-1 cells were transfected with the wild type p250 promoter, or p250 promoter with TF binding sites deleted or mutated to test the influence of the predicted TF binding sites on the basal promoter activity. Significance was analyzed with Student's t-test. (* $p < 0.05$)

[0027] Fig. 3F DF-1 cells were co-transfected with the indicated wild type p250 promoter or p250 promoter with TF binding sites deleted or mutated with or without GST-2CARD (stimulator) to assess which predicted TF binding sites affect the inducible promoter activity. For all experiments, firefly luciferase activities were normalized to *Renilla* activity at 24 h post transfection. The mean of triplicate determinations ($\pm$ SD) of relative luciferase activity is shown and each experiment was repeated at least twice.

[0028] Figs. 4A-4C depicts the results of experiments showing that chicken IRF-7 and duck IRF-1 can induce duck RIG-I promoter activity.

[0029] Fig. 4A shows the overexpression of mCherry tagged transcription factors in DF-1 cells. Cells that are visible reflect mCherry fluorescence.

[0030] Fig. 4B shows the results of dual luciferase assays to analyze the inducible activity of the duck RIG-I promoter in the indicated DF-1 cells overexpressing the mCherry-tagged transcription factors. Triplicate results are shown for each group. Significance was analyzed with one-way ANOVA. (* $P < 0.05$)

[0031] Fig. 4C shows the induction of p125 promoter in the presence or absence of the putative IRF-1 site.

[0032] Figs. 5A-5B provides the results of phylogenetic analysis of RIG-I proteins and promoters.

[0033] Fig. 5A is a schematic of a phylogenetic tree of RIG-I promoters was generated by MEGA7 with bootstrap analysis (Tree was generated using the Neighbor-Joining method, 1000 replicates, Maximum Composite Likelihood model). Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed.

[0034] Fig. 5B is a schematic of a phylogenetic tree of RIG-I proteins generated as above (Neighbor-Joining method, 1000 replicates, Poisson mode).

[0035] Fig. 5C shows a phylogenetic tree of all available avian RIG-I sequences and their relationship to duck RIG-I. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the

evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 19 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 719 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

[0036] Fig. 6 depicts the sequence of full-length Duck RIG-I promoter (SEQ ID NO:02).

[0037] Fig. 7A. depicts an amino acid sequence alignment of mallard duck (accession no. EU363349) (Seq. ID No. 11), zebra finch (accession no. XM_002194524) (SEQ. ID NO. 22), and human RIG-I (accession no. AF038963) (SEQ. ID NO. 23) performed using the ClustalW program and edited with Boxshade. Black shading indicates amino acid identity and gray shading indicates similarity (50% threshold). Plus signs indicate human residues involved in polyubiquitination and asterisks indicate residues involved in ligand binding. The ATP binding motif is boxed.

[0038] Fig. 7B depicts the results of assays that illustrate that the human RIG-I 2CARD domain is not able to stimulate IFN- β promoter activity in DF-1 cells, however, duck RIG-I 2CARD indeed stimulated IFN- β promoter activity in AD293T cells though its function is much weaker than human RIG-I 2CARD. In Panel A, DF-1 cells were transfected with GST, GST-duck 2CARD (GST-d2CARD) or GST-human 2CARD (GST-h2CARD) individually with chicken IFN- β firefly luciferase reporter vector (chIFN- β) and Renilla luciferase vector (the internal reference control). 24 hours post transfection, the cells were lysed and the chIFN- β activity was measured by dual luciferase assay. GST only is the control. In Panel B, AD293T cells were transfected with GST, GST-d2CARD or GST-h2CARD individually with human IFN- β firefly luciferase reporter vector (hIFN- β) and Renilla luciferase vector (the internal reference control). 24 hours post transfection, the cells were lysed and the hIFN- β reporter activity was measured by dual luciferase assay as above.

[0039] Fig. 7C. depicts an amino acid sequence alignment of a number of avian RIG-I proteins. *Anser anser* (Greylag Goose, GenBank: ADV58759.1) (SEQ ID NO.:18);

Anas platyrhynchos (mallard duck, NCBI Reference Sequence: NP_001297309.1)(SEQ ID No.: 11); *Cairina moschata* (GenBank: AGX27431.1) (SEQ ID NO.: 19); *Columba livia* (NCBI Reference Sequence: XP_013225185.1) (SEQ. ID NO. 20); and *Picoides pubescens* (GenBank: KJV61638.1) (SEQ. ID NO. 21).

DESCRIPTION OF EXAMPLES OF EMBODIMENTS

[0040] Before the present invention is described further, it is to be understood that this invention is not limited to particular embodiments described, and as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0041] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range is encompassed within the invention.

[0042] Unless defined otherwise, 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 any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

[0043] It must be noted that as used herein and in the appended claims, the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of cells and reference to "a candidate agent" includes reference to one or more candidate agents and equivalents thereof known to those skilled in the art, and so forth. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely", "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation.

[0044] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

[0045] All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference and are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

[0046] As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present invention. Any recited method can be carried out in the order of events recited or in any other order which is logically possible.

DEFINITIONS

[0047] The terms “retinoic acid-inducible gene I”, “RIG-I”, “RIG-I protein” and “RIG-I polypeptide” are used interchangeably herein refer to a protein which acts as a cytoplasmic RNA sensor and pattern recognition receptor (PRR) for detecting a variety of RNA viruses (including influenza A virus) and inducing Type I interferon (IFN), inflammatory cytokines and interferon stimulated genes (ISGs).

[0048] “RIG-I promoter” as used herein refers to the regulatory DNA region which, in nature, controls expression of a RIG-I gene.

[0049] A “duck RIG-I promoter”, in the context of the present invention and as used herein, is a nucleic acid compound having a sequence with at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO: 01.

[0050] The term “promoter” refers to a regulatory DNA region which controls transcription of a operably linked nucleic acid encoding a gene product, and which can be located adjacent to or overlapping a nucleotide or region of nucleotides at which RNA transcription is initiated. A promoter generally contains specific DNA sequences which bind protein factors, often referred to as transcription factors, which facilitate binding of RNA polymerase to the DNA leading to gene transcription.

[0051] A “core promoter”, usually refers to a promoter which contains the basic elements required to promote transcriptional expression of an operably linked polynucleotide. Eukaryotic basal promoters typically, though not necessarily, contain a TATA-box and/or a CAAT box.

[0052] The terms “nucleic acid” and “polynucleotide” are used interchangeably herein.

[0053] The terms “protein” and “polypeptide” are used interchangeably herein.

[0054] A polynucleotide or polypeptide can be described as having a certain percent “sequence identity” to another polynucleotide or polypeptide, meaning that, when aligned, that percentage of bases or amino acids are the same, and in the same relative position, when comparing the two sequences. Sequence similarity can be determined in a number of different manners. To determine sequence identity, sequences can be aligned using the methods and computer programs, including BLAST, available over the World Wide Web at ncbi.nlm.nih.gov/BLAST. See, e.g., Altschul et al. (1990), J. Mol. Biol. 215:403-10. Other techniques for alignment are described in Methods in Enzymology, vol. 266: Computer Methods for Macromolecular Sequence Analysis (1996), ed. Doolittle, Academic Press, Inc., a division of Harcourt Brace & Co., San Diego, California, USA. Of particular interest are alignment programs that permit gaps in the sequence. The Smith-Waterman is one type of algorithm that permits gaps in sequence alignments. See Meth. Mol. Biol. 70: 173-187 (1997). CLUSTAL, MUSCLE, and T-COFFEE are additional examples of alignment programs.

[0055] The term “coding sequence” refers to a nucleic acid sequence that once transcribed and translated produces a protein, for example, *in vivo*, when placed under the control of appropriate regulatory elements. A coding sequence as used herein may have a continuous ORF or might have an ORF interrupted by the presence of introns or non-coding sequences. In this embodiment, the non-coding sequences are spliced out from the pre-mRNA to

produce a mature mRNA. A “non-human” animal refers to any animal of a species that is not human.

[0056] The term “operably-linked” refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably-linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., the coding sequence is under the transcriptional control of the promoter). Similarly, when an intron is operably-linked to a coding sequence, the intron is spliced out of the mRNA to provide for expression of the coding sequence. In the context of gene conversion, two nucleic acids sequences are operably linked if one sequence can “donate” sequence to the other by gene conversion. If two sequences are unlinked in that one can donate sequence to the other via gene conversion, the donating sequences may be upstream or downstream of the other, and the two sequences may be proximal to each other, i.e., in that there are no other intervening genes. “Unlinked” means that the associated genetic elements are not closely associated with one another and the function of one does not affect the other.

[0057] The terms “upstream” and “downstream” are used with reference to the direction of transcription.

[0058] The term “intron” refers to a sequence of DNA found in eukaryotic genes that may be transcribed, but are removed from the pre-mRNA transcript before the mRNA is translated into a protein. This process of intron removal occurs by splicing together of nucleic acid (exons) that flank the intron.

[0059] As used herein, the term "variant", as in “variant polypeptide” or “variant polynucleotide”, encompasses naturally-occurring variants (e.g., homologs and allelic variants) and non-naturally-occurring variants (e.g., muteins). Naturally-occurring variants include homologs, i.e., nucleic acids and polypeptides that differ in nucleotide or amino acid sequence, respectively, from one species to another. Naturally-occurring variants include allelic variants, i.e., nucleic acids and polypeptides that occur in nature and differ in nucleotide or amino acid sequence, respectively, from one individual to another within a species. Non-naturally-occurring variants include nucleic acids and polypeptides that comprise a change in nucleotide or amino acid sequence, respectively, where the change in sequence is artificially introduced and does not occur in nature, e.g., the change is generated in the laboratory or other facility by human intervention ("hand of man").

[0060] “Transgenic animal” refers to an animal comprising cells that are genetically modified by the hand of man so as to contain recombinant nucleic acid that is not native to the animal. The foreign nucleic acid may be present in all cells of the animal or in some, but not all, cells of the animal, and may be genomically integrated. The foreign nucleic acid may be referred to as a “transgene”. By inserting a transgene into a fertilized oocyte or cells from the early embryo, the resulting transgenic animal may be fully transgenic and able to transmit the foreign nucleic acid stably in its germline. Alternatively, a foreign nucleic acid may be introduced by transferring, e.g., implanting, a recombinant cell or tissue containing the same into an animal to produce a partially transgenic animal. “Transgenic animals”, e.g., “transgenic chickens”, include “gene edited animals” (e.g., “gene edited chickens”), which have a genome modified by use of a gene editing CRISPR systems, e.g., CRISPR/Cas9. “Gene edited” animals include both “knock in” and “knock out” gene- edited animals, i.e., animals having a genome modified to insert a coding sequence of interest (“knock in”) and animals having a genome modified to disrupt a native coding sequence (“knock out”). Knock in gene-edited chickens, e.g., produced using a CRISPR (e.g., CRISPR/Cas9) gene editing system are of particular interest.

[0061] The term “progeny” or “off-spring” refers to any and all future generations derived and descending from a particular animal. Thus, progeny of any successive generation are included herein such that the progeny, the F1, F2, F3, generations and so on are included in this definition.

[0062] The term “homozygous” indicates that a eukaryotic cell or animal has two copies of the same gene, with one on each chromosome. In contrast, “heterozygous” indicates that the cell or animal has dissimilar pairs of genes on each of the homologous chromosomes, e.g., one chromosome contains a transgene while the homologous chromosome does not. A transgenic animal may be homozygous or heterozygous for a transgene.

[0063] The term “endogenous”, with reference to a gene, indicates that the gene is native to a cell, i.e., the gene is present at a particular locus in the genome of a non-modified cell. An endogenous gene may be a wild type gene present at that locus in a wild type cell (as found in nature). An endogenous gene may be a modified endogenous gene if it is present at the same locus in the genome as a wild type gene. An example of such a modified endogenous gene is a gene into which a foreign nucleic acid is inserted. An endogenous gene may be present in the nuclear genome, mitochondrial genome etc.

[0064] The term “expression construct” or “expression vector” refers to a recombinant nucleic acid, generally recombinant DNA, that has been generated for the purpose of the expression of a selected nucleotide sequence(s), or is to be used in the construction of other recombinant nucleotide sequences. An expression construct can be present in a vector or in a genome.

[0065] A "vector" is a nucleic acid replicon, such as plasmid, phage nucleic acid, viral nucleic acid, or cosmid, to which another nucleic acid segment may be attached so as to bring about the replication of the attached segment in a cell.

[0066] The term “recombinant” refers to a polynucleotide, polypeptide, or host cell that has been manipulated by the hand of man, e.g., so as to provide a polynucleotide, polypeptide or host cell that does not occur in nature. A recombinant molecule may contain two or more naturally-occurring sequences that are linked together in a way that does not occur naturally. A recombinant cell contains a recombinant polynucleotide or polypeptide. If a cell is genetically modified to contain a recombinant nucleic acid, the nucleic acid is “exogenous” to the cell.

[0067] The term “selectable marker” refers to a protein capable of expression in a host that allows for ease of selection of those hosts containing an introduced nucleic acid or vector. Examples of selectable markers include, but are not limited to, proteins that confer resistance to antimicrobial agents (e.g., hygromycin, bleomycin, or chloramphenicol), proteins that confer a metabolic advantage, such as a nutritional advantage on the host cell, as well as proteins that confer a functional or phenotypic advantage (e.g., cell division) on a cell. Additionally, a florescent protein may be used as a selectable marker., such as GFP or mCherry.

[0068] The term “expression”, as used herein, refers to the process by which a polypeptide or other gene product is produced based on the nucleic acid sequence of a gene. The process includes both transcription and, if the gene product is a protein, translation.

[0069] The term “introduced” in the context of inserting a nucleic acid sequence into a cell, means “transfection”, or ‘transformation” or “transduction” and includes reference to the incorporation of a nucleic acid sequence into a eukaryotic or prokaryotic cell wherein the nucleic acid sequence may be incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid, or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

[0070] A "host cell" or "target cell" as used herein, denotes an *in vivo* or *in vitro* cell, e.g., an avian cell, that can be, or has been, genetically modified, e.g., to contain an expression construct as described herein, and include the progeny of such cells (e.g., when the cell has been genetically modified). It is understood that the progeny of a single cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation. A "recombinant host cell" (also referred to as a "genetically modified host cell") is a host cell into which has been manipulated by the hand of man so as to introduce a nucleic acid exogenous to the cell, e.g., an exogenous expression vector.

[0071] The term "stem cell" is used herein to refer to a cell (e.g., an avian stem cell) that has the ability both to self-renew and to generate a differentiated cell type (see Morrison et al. (1997) Cell 88:287-298). Stem cells of interest include pluripotent stem cells (PSCs). The term "pluripotent stem cell" or "PSC" is used herein to mean a stem cell capable of producing all cell types of the organism. Therefore, a PSC can give rise to cells of all germ layers of the organism (e.g., the endoderm, mesoderm, and ectoderm of a vertebrate). Pluripotent cells are capable of forming teratomas and of contributing to ectoderm, mesoderm, or endoderm tissues in a living organism.

[0072] The terms "treatment" "treating" and the like are used herein to refer to any treatment of any disease or condition in a mammal, e.g. particularly a human or a mouse, and includes: a) preventing a disease, condition, or symptom of a disease or condition from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it; b) inhibiting a disease, condition, or symptom of a disease or condition, e.g., arresting its development and/or delaying its onset or manifestation in the patient; and/or c) relieving a disease, condition, or symptom of a disease or condition, e.g., causing regression of the condition or disease and/or its symptoms.

[0073] The terms "subject," "host," "patient," and "individual" are used interchangeably herein to refer to any mammalian subject for whom diagnosis or therapy is desired, particularly humans. Other subjects may include cattle, dogs, cats, guinea pigs, rabbits, rats, mice, horses, and so on.

[0074] The term "plurality" refers to at least 2, at least 5, at least 10, at least 20, at least 50, at least 100, at least 200, at least 500, at least 1000, at least 2000, at least 5000, or at least

10,000 or at least 50,000 or more. In certain cases, a plurality includes at least 10 to 50. In other embodiments, a plurality may be at least 50 to 1,000.

DUCK RIG-I PROMOTER

[0075] The present disclosure provides a promoter having the minimal components of a duck RIG-I promoter, as well as constructs (e.g., expression constructions), recombinant host cells, and transgenic animals (e.g., transgenic avians, e.g., transgenic chickens) containing same.

[0076] As illustrated in the examples below, the In one embodiment, the RIG-I promoter comprises, from 5' to 3', an Sp1/Sp2 transcription factor binding site (CGGCCAGAGGGCGGGC (SEQ ID NO:03) and an IRF-1/STAT1/STAT2 transcription factor binding site (GGCCGCTTTCGTTTTCCATTC (SEQ ID NO:04)). In other embodiments, the RIG-I promoter comprises, from 5' to 3', a NHLH1 transcription factor binding site (ACGAAGCTGCGA (SEQ ID NO:05), an Sp1/Sp2 transcription factor binding site (CGGCCAGAGGGCGGGC (SEQ ID NO:06), and an IRF-1/STAT1/STAT2 transcription factor binding site (GGCCGCTTTCGTTTTCCATTC (SEQ ID NO:07)).

[0077] The promoter can be at least 160 nucleotides, 175 nucleotides, 180 nucleotides, 185 nucleotides, 190 nucleotides, 200 nucleotides, 225 nucleotides, or 250 nucleotides or more in length. The promoter of the present disclosure provides for Type I interferon (IFN)-inducible expression of an operably linked gene product, e.g., in response to a product of a IFN- stimulated antiviral gene.

[0078] The promoter comprises a nucleotide sequence of at least about 160 nucleotides and comprising a sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 99% or 100% identical to the sequence comprising the sequence:

[0079] CGGCCAGAGGGCGGGCTGCGGATGGGGCCGCGATCTCCACACCCCG
CGGGGGCCGCTTTCGTTTTCCATTCCCCGC¶CGGGCCTCGCTGCCTTTCTGTGCCG
 AGCCGTTGGGGGGCCGGGCCGGGCCGGGCCGGCCGGCCGGCAGAGCCCAGC
 C (SEQ ID NO: 08), where the underlined nucleotides are the Sp1/Sp2 and an IRF-1/STAT1/STAT2 transcription factor binding sites.

[0080] In some embodiments, the promoter comprises a sequence of at least about 180 nucleotides, and comprising a sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 99% or 100% identical to the sequence:

[0081] GCCGCTGCCTATGCCCCGCTGGCGGCCAGAGGGCGGGCTGCGGATGG
 GGCCGCGATCTCCACACCCCGCGGGGGCCGCTTTCGTTTTCCATTCCCCGCCGGGC
 CTCGCTGCCTTTCTGTGCCGAGCCGGTTGGGGGGCCGGGCCGGGCCGGGCCGGGGC
 GCGGCCGGCAGAGCCCAGCC (SEQ ID NO:09), where the underlined nucleotides are an
 Sp1/Sp2 transcription factor binding site and an IRF-1/STAT1/STAT2 transcription factor
 binding site.

[0082] In another embodiment, the promoter comprises a sequence of at least about 250
 nucleotides, and comprising a sequence at least 80%, at least 85%, at least 90%, at least 95%, at
 least 99% or 100% identical to the sequence:

[0083] CTGGACCCAGGCCCGTGTCTCGGTGTCCCGGTGAGGCTGTTGGTGGC
 GATCCGAGGCTCACGAAGCTGCGAGCCGCTGCCTATGCCCCGCTGGCGGCCAGAGG
GCGGGCTGCGGATGGGGCCGCGATCTCCACACCCCGCGGGGGGCCGCTTTCGTTTT
CCATTCCCCGCCGGGCTCGCTGCCTTTCTGTGCCGAGCCGGTTGGGGGGCCGGGCC
 GGGCCGGGCCGGGCGGCCGGCCGGCAGAGCCCAGCC (SEQ ID NO:01), where the
 underlined nucleotides are the Sp1/Sp2 transcription factor binding site, an
 IRF-1/STAT1/STAT2 transcription factor binding site, and an NHLH1 transcription factor
 binding site.

[0084] In another embodiment, the promoter comprises a sequence of at least about 2024
 nucleotides comprising at least 80%, at least 85%, at least 90%, at least 95%, at least 99% or
 100% identical to the sequence:

AGCTGATGACCTGCAAAAAGTTGGACATGCAAAATTTAAAGCTGAGGAACACA
 ACTACACACTATGCTCTGAAAAAGAAAAATAATAACAATCAAGAAAAGCATT
 TATCCCACCAGCTGGACCACACTCTCTAAGGCAGGACTTGGTGGGTCACACAGGG
 ACCCTTCCAGACAGCCACAAGTGATTTTGGGAAAAAGTAACATATTTCTACAC
 CCTTGCCTAGAAAGTTCTCCAGAAACTGCTCAGCACCATGGTTTGCAGTAGCACA
 AGCAGGTTAAGGTCTTCCCAGTGCTGCAGCTTCTTCTCACCCGATGTGCTTGGCC
 ACTGCAACCACACAGTTACGATGTAGCATAAAACAGCCACTGAGGATATGAGCAT
 GAGATAACCGCACAGGCCACAACCCATCCCCTGCACCACTGCCCTGCACCAACA
 AAGCCTGGGAGAGCCTCCTGTGCTCACCAGGTGCATCTCCAGCCTGGTGGGACT
 GATCCCTGCTGCCCTCAGTACCCCTCCTGCAGCCACCCTGCCAGCAGGTGCAT
 CCCACCTCCTGCATGCACGATCAGAGGTGCTATTCCCCCGTGCACTCCAGTGCC
 AGCTTTGTTGAAGAGCAGCAG

GTCCACTTCACTGGGCAGATTGACTTCTCCATCTAGGGCTGGAGAAGCAGCAAGCAA
GGAGTTTGATTTCTCCTCTTCTCAGGTGAAGGACAAGACTGGGTTTCTGCTTGGCA
GAGCAATTGCAACCCACCCTCAGCACTGATCTTTGAATCTTCGAACACTCTGTCTGT
GAGGAGCAGAACCTGAGTGCTAATATCACTCATTACTGAATGAAAGCTGTAATAGG
CATTTCTCAAGTGGAAATAGGCTCAACAGGCACTACCAAGTAAAAATGCCTCTCTGC
TATGTTTGTACCTGTCAGGCTAATCACCATGCTCTACAGGGTGTGTTGGGCAACTC
ACGTAGGGGGCTAACGCTTCTTCCTTGTTACTCACAAGCAAATTAAGCCCTAAGAGAT
TACCTTACTGAAAATGCAGGGTGGAGAGATGACTGACAGGGGGCTTGTTACCTGCCC
TTTTCTTGCTGCAGAAGATGTCCTCTCTCAGTTGTTTCAGCATTCCCCAGTCTAGCAGT
GGATAGCCTTTCTGTGTCTTGGTCAGCTGTGCATGATATTTCTCTGGTCCTTCTCATT
GCTCTGGTTAAGCTAACAGCCCAAATGCAAGTCTCTCCTAGCCTAGGGCCATTTGTA
GCCTAGGGCAACAGCAGCTGGGAGAGAGTGAGAAAATGTGAGAGAACCTTACATTT
GGGATCTTTGGGGAGCTGCTGCCCATGGGGGACCCGTGCTGGAGCAGTTTGCTCCTG
GGGGATGGATGGATGGACCCCGTGGGACGGAGCCGTGTGGGAGCAGTGCTTGAAGA
GCTGCTGCCTGTGGGCAGCCCCCGCGGGATCGGTTCCGGGAAGGACGGCATCCCTGG
GAGGGACCCCATGGGGAGCAGGGGCAGAGAGGGACCGTGAGGGAGAGGGAGCGGC
GGAGACAAAGTGCCAGGGACTGACCGCAGCCCCCATTCCCCTGCACTGCCTGGGGG
GGAAGAGGTAGAGGAGGGTGGATGGTGGGGAAGGTGTTTTTCATTTCTTTTATTTT
TTCTCACTGCTAGTCTGTTAGTGATAGGCAATTAATTCTATTCTCCCCATCCTTGTGC
CAACCCCTGAGCCCTTTCCATCGCATTTTCTCCCCGTTTCCCTTTGAGGAGGCGGAGT
GGGAGAGCGGCTGTGCTGGACCCAGGCCCCTGTCTCGGTGTCCCGGTGAGGCTGTT
GGTGGCGATCCGAGGCTCACGAAGCTGCGAGCCGCTGCCTATGCCCGCTGGCGGCC
AGAGGGCGGGCTGCGGATGGGGCCGCGATCTCCACACCCCGCGGGGGCCGCTTTCG
TTTTCCATTCCCCGCCGGGCTCGCTGCCTTTCTGTGCCGAGCCGTTGGGGGGCCG
GGCCGGGCGGGGCCGGGCGGCGGCCGGCAGAGCCCAGCC (SEQ ID NO:02)

RIG-I PROTEIN

[0085] In some embodiments, the RIG-I promoter of the present disclosure is operably linked to a nucleic acid encoding a gene product of interest, e.g., for expression in a host cell. In one embodiment of particular interest, the RIG-I promoter is operably link to a nucleic acid encoding a RIG-I protein. In some embodiments, the duck RIG-I promoter of the present

disclosure is operably linked to a nucleic acid encoding a retinoic acid-inducible gene I (“RIG-I”) protein. As noted above, the terms “retinoic acid-inducible gene I”, “RIG-I”, “RIG-I protein” and “RIG-I polypeptide” are used interchangeably herein refer to a protein which acts as a cytoplasmic RNA sensor and pattern recognition receptor (PRRvh) for detecting a variety of RNA viruses (including influenza viruses (e.g., influenza A viruses, such as H5N1 virus), Sendai virus, and flavivirus) and inducing Type I interferon (IFN), inflammatory cytokines and interferon stimulated genes (ISGs). RIG-I typically recognizes short (< 4000nt) 5'triphosphate uncapped double stranded or single stranded RNA. RIG-I is also able to detect non-self 5'-triphosphorylated dsRNA transcribed from AT-rich dsDNA by DNA-dependent RNA polymerase III (Pol III).

[0086] The overall structure and function of RIG-I is conserved across phylogeny, despite significant protein sequence divergence. RIG-I is composed of N-terminal two caspase activation and recruitment domains (“2CARDs”), a central DExD/H box helicase domain (characterized by the conserved motif Asp-Glu-Ala-Asp (SEQ ID NO:10)), and a C-terminal regulatory domain (CTD). In the absence of RNA trigger, 2CARDs fold back to helicase domain and CTD to form an auto-repressed state. Upon binding of its ligand (short dsRNA or 5'-triphosphate ssRNA) (Hornung et al., (2006) *Science*, 314(5801), 994-997; Lu et al., 2010, *Structure*, 18(8), 1032-1043; Luo et al., 2011, *Cell*, 147(2), 409-422; Yoneyama et al., 2005, *J Immunol*, 175(5), 2851-2858) to the helicase domain and CTD, RIG-I undergoes extensive rearrangement to expose 2CARDs (Luo et al., 2011, supra). Upon ubiquitination by TRIM25, an E3 ubiquitin ligase, RIG-I is activated and its 2CARDs interact with the CARD domain of the adaptor protein, mitochondria antiviral-signaling protein (MAVS), to stimulate a downstream signaling pathway leading to inflammatory cytokine and Type I IFN release, further inducing the production of interferon stimulated genes (ISG), which are antiviral factors (Gack et al., 2007, *Nature*, 446(7138), 916-920; Jiang et al., 2011, *Nature*, 479(7373), 423-427; Kawai et al., 2005, *Nat Immunol*, 6(10), 981-988; Seth, et al., 2005, *Cell*, 122(5), 669-682).

[0087] Fig. 7A provides an alignment of mallard duck (accession no. EU363349), zebra finch (accession no. XM_002194524), and human RIG-I (accession no. AF038963). Mallard duck RIG-I and zebra finch RIG-I share about 78% amino acid sequence identity. In contrast, human RIG-I, which does not initiate signaling in chicken cells, has only about 54% amino acid sequence identity to mallard duck RIG-I (**Fig. 7B**). Fig. 7C provides an alignment of avian RIG-I

proteins of *Anser anser* (graylag goose; GenBank: ADV58759.1, SEQ ID No. 18); *Anas platyrhynchos* (mallard duck; NCBI Reference Sequence: NP_001297309.1, SEQ ID No. 11); *Cairina moschata* (Muscovy duck; GenBank: AGX27431.1, SEQ ID No. 20); *Columba livia* (Rock dove; NCBI Reference Sequence: XP_013225185.1, SEQ ID No. 21); and *Picoides pubescens* (GenBank: KfV61638.1, SEQ ID No. 22). Guidance for amino acid changes can be provided by, for example, reference to the alignments of Figs. 7A and 7B. For example, amino acid substitution from the mallard duck RIG-I can be based on substitutions of amino acids in corresponding positions of another avian RIG-I, while avoiding the amino acid substitution to human RIG-I.

[0088] In certain embodiments, the RIG-I protein of the expression constructs of the present disclosure is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% identical in amino acid sequence to a RIG-I protein having the amino acid sequence of mallard duck RIG-I (*Anas platyrhynchos*, Genbank accession no. EU363349.1), which amino acid sequence is provided below:

MTADEKRSLQCYRRYIERSLNPVYVLGNMTDWLPDELRRERIRKEEERGVSGAAA
 LFLDAVLQLEARGWFRGMLDAML AAGYTGLAEAIENWDFSKLEKLELHRQLLK
 RIEATMLEVDPVALIPYISTCLIDRECEEIQQISENRSKAAGITKLIECLCRSDKEHW
 PKSLQLALD TTGYRASELWDIREDNAKDVDSEMTDASEDCLEASMTYSEEAEPP
 DDNLSENLSAAEGIGKPPPVEYETKKARSYQIELAQPAINGKNALICAPTGSGKTF
 VSILICEHHFQNM PAGRKAKVVFLATKVPVYEQQKNVFKHHFERQGYSVQGIGS
 ENFSNVSVKEVIEDSDIIVVTPQILVNSFEDGTLTSLSIFTLMIFDECHNTTGNHPY
 NVLMTRYLEQKFNSASQLPQILGLTASVGVGNAKNIETIEHICSLCSYLDIQAIST
 VRENIQELQRFMNKPEIDVRLVKRRIHNPFAAIISNLMSETEALMRTIYSVDTLSQ
 NSKKDFGTQNYEHWIVVTQRKCRLQLLEDKEEESRICRALFICTEHLRKYNDALII
 SEDARIIDALSYLEFFTNVKNGPYTELEQHLTAKFQEKEPELIALSKDETNNPK
 LEELVCILDDAYRYNPQTRTLLFAKTRALVSALKKCMEEENPILNYIKPGVLMGRG
 RRDQTTGMTLPSQKGVLD AFKTSKDNRLLIATSV ADEGIDIVQC NLVVL YEYS
 NVTKMIQVRGRGRAAGSKCILVTSKTEVVENEKCNRYKEEMMNKAVEKIQKW
 DEETFAKKIHNLMKERVLRDSRRKEIKPKVVEGQKNLLCGKCKAYACSTDDIRI
 IKDSHHIVLGEAFKERYTTKPHKKPMQFDGFEKKSKMYCRNNNCQHDWGITVK

YLTFDNLPVLIKISFVMESTATGTQMDFAQKWSINSSLKNFDVEEMSNLYPP
(SEQ ID NO: 11)

[0089] The RIG-I protein can be an avian RIG-I protein. Avian RIG-I proteins suitable for use in this invention may include, but are not necessarily limited to, duck, (e.g., mallard duck (*Anas platyrhynchos*; NCBI Reference Sequence: NP_001297309.1), Muscovy duck (*Cairina moschata*; GenBank Accession No. AGX27431.1); goose (e.g., swan goose (*Anser cygnoides deomsticus*; GenBank Accession No. KJ124555), graylag goose (*Anser anser*; GenBank Accession No. ADV58759.1); eagle (e.g., golden eagle (*Aquila chrysaetos canadensis*; GenBank Accession No. KF905228.1), bald eagle (*Haliaeetus leucocephalus*); pigeon, and zebra finch (e.g., NCBI Reference Sequence: XM_002194524), little egret (*Egretta garzetta*; GenBank Accession No. KFP10984.1), Asian Houbara (*Chlamydotis macqueenii*; GenBank Accession No. KFP36728.1); crowned crane (*Balearica regulorum gibbericeps*; GenBank Accession No. KFO06723.1), American crow (*Corvus brachyrhynchos*; GenBank Accession No. KFO59778.1), Golden-collared Manakin (*Manacus vitellinus*; GenBank Accession No. KFW80420.1); rifleman (*Acanthisitta chloris*; GenBank Accession No. KFP82392.1); Northern Carmine Bee-eater (*Merops nubicus*; GenBank Accession No. KFQ30270.1) Anna's Hummingbird (*Calypte anna*; GenBank Accession No. KFP02435.1); Downy woodpecker (*Picoides pubescens*; GenBank Accession No. KJV61638.1); Hoatzin (*Opisthocomus hoazin*; GenBank Accession No. KFR01539.1); Great Crested Grebe (*Podiceps cristatus*; GenBank Accession No. KFZ58430.1); Red-crested Turaco (*Tauraco erythrolophus*; GenBank Accession No. KJV18917.1); South African ostrich (*Struthio camelus australis*; GenBank Accession No. KJV82733.1); and Rock dove (*Columba livia*; NCBI Reference Sequence: XP_013225185.1).

VECTORS, EXPRESSION CONSTRUCTS AND HOST CELLS

[0090] The RIG-I promoter of the present disclosure can be provided in a vector, which may provide for replication of nucleic acid of interest (e.g., for recombination production of nucleic acid encoding a RIG-I promoter of the present disclosure, optionally operably linked to a gene product-encoding nucleic acid) and, optionally, for RIG-I-promoted expression of an operably linked gene product-encoding nucleic acid in a host cell). In one embodiment of particular interest, the RIG-I promoter is operably linked to a nucleic acid encoding a RIG-I protein. RIG-I proteins of interest are discussed in more detail below.

[0091] A variety of host- expression and/or replication vector systems may be used in connection with the RIG-I promoter of the present disclosure. These include but are not limited to CRISPR/Cas9 systems, replication defective viral vectors, lentiviral vectors, and the Piggyback transposon.

[0092] In one embodiment, the recombinant nucleic acid is a recombinant expression vector, such as a plasmid or viral vector. Plasmid and viral vectors useful in creating transgenic chickens are readily known in the art.

[0093] In one embodiment, the vector is a bacterial plasmid vector expressing Cas9 and appropriate guide RNA sequences targeting the insertion site under the control of the chicken U6 promoter. This vector can express Cas9 in chicken cells and generate single strand breaks in the target intergenic region.

[0094] In one embodiment, the vector is a viral vector, optionally a retroviral, adenoviral or adeno-associated viral vector. Such vectors are able to deliver the recombinant nucleic acid into the cells and the nucleic acids are then stably integrated into the chromosomal DNA of the host.

[0095] In an embodiment, the retroviral vector is a replication defective retrovirus vector. The term "replication defective retrovirus vector" as used herein refers to a retrovirus that is capable of insertion of a foreign gene into cells in an initial round of infection but where the vectors would not permit re-infection of the cells after the initial round of infection. For example, a replication defective retrovirus may have the retroviral coding sequences, such as gag, pol and env, replaced by other nucleic acids or deleted, which render the virus unable to replicate. Replication defective virus vectors have been described in Bosselman et al. Mol. Cell. Biol. 7: 1797-1806 (1987); U.S. Pat. No. 5,162,215; Cone and Mulligan, PNAS USA 81:6349-6353 (1984); Mann et al. Cell 33:153-159 (1983); Miller and Buttimore, Mol. Cell. Biol. 6:2895-2902 (1986); Watanabe and Temin, Mol. Cell. Biol. 3:2441-2449 (1983); Stoker and Bissell, J. Virol. 62: 1008-1015 (1988), U.S. Pat. No. 4,650,764 and Emerman and Temin, Cell, 39:459-467 (1984), all of which are herein incorporated by reference. For example, replication defective retroviral vectors include, without limitation, replication defective retrovirus vectors derived from Moloney murine leukemia virus, Moloney murine sarcoma virus, reticuloendotheliosis virus type A and Avian leukemia virus.

[0096] In yet another embodiment, an adenovirus-derived vector is used. The adenovirus vector can be modified such that its ability to replicate in a normal lytic viral life cycle is inactivated. Introduced adenoviral vectors are not integrated into the genome of the host cell but instead remain episomal.

[0097] In yet another embodiment, an adeno-associated virus is used. Such viruses can be packaged and can integrate.

[0098] Non-viral expression vectors can also be used. For example, a RIG-I promoter and operably linked RIG-I encoding nucleic acid can be entrapped in liposomes for delivery into to cells.

[0099] The recombinant expression vectors of the present disclosure may also contain a selectable marker gene which facilitates the selection of host cells transformed or transfected with a recombinant molecule disclosed herein. Examples of selectable marker genes are genes encoding a protein such as G418 and hygromycin which confer resistance to certain drugs, beta-galactosidase, chloramphenicol acetyltransferase, firefly luciferase, and the like. Transcription of the selectable marker gene is monitored by changes in the concentration of the selectable marker protein such as beta-galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. If the selectable marker gene encodes a protein conferring antibiotic resistance such as neomycin resistance transformant cells can be selected with G418. Cells that have incorporated the selectable marker gene will survive, while the other cells die. This makes it possible to visualize and assay for expression of the recombinant expression vectors disclosed herein and in particular to determine the effect of a mutation on expression and phenotype. It will be appreciated that selectable markers can be introduced on a separate vector from the nucleic acid of interest.

[00100] Host cells for use with the vectors and expression construct of the present disclosure can be selected from any suitable host cell. For example, where the host cell is to be used for replication of a vector of the present disclosure, the host cell can be bacteria, yeast, insect, or any other suitable host cell. Where inducible expression from a RIG-I promoter of the present disclosure is of interest, then the host cell can be a mammalian host cell, e.g., an avian cell, e.g., a chicken cell.

TRANSGENIC ANIMALS

[00101] The present disclosure provides non-human transgenic animals that are transgenic for a RIG-I promoter of the present disclosure, which promoter is optionally operably linked to a gene product-encoding nucleic acid of interest, e.g., an avian RIG-I protein. Transgenic non-human animals (e.g., transgenic chickens) in which the transgene contains a RIG-I promoter of the present disclosure operably linked to an avian RIG-I-encoding nucleic acid so as to facilitate an immune response to an RNA virus infection through induction of IFN-stimulated antiviral genes are of particular interest.

[00102] In one embodiment, the transgenic animal is a transgenic bird. The term “bird” as used herein refers to any avian species, subspecies or race of organism of the taxonomic Class Aves, such as, but not limited to, such organisms as chicken, turkey, duck (including mallard duck (*Anas platyrhynchos*), goose, quail, pheasants, parrots, finches, hawks, crows and ratites including ostrich, emu and cassowary. The term includes the various known strains of *Gallus gallus* (chickens), for example, White Leghorn, Brown Leghorn, Barred-Rock, Sussex, New Hampshire, Rhode Island, Australorp, Cornish, Minorca, Amrox, California Gray, Italian Partridge-colored, as well as strains of turkeys, pheasants, quails, duck, ostriches and other poultry commonly bred in commercial quantities.

[00103] As used herein, a “genetically modified bird” or “transgenic bird” refers to any bird in which one or more of the cells of the bird contains heterologous nucleic acid introduced by way of human intervention. Accordingly, the term “transgenic chicken” as used herein refers to a chicken that contains a heterologous gene in at least a portion of its cells. Such transgenic chickens can be produced by introduction of a nucleic acid into a cell, directly or indirectly by introduction into a precursor of the cell, by genetic manipulation, including without limitation, sperm-mediated or restriction enzyme mediated integration, microinjection or viral infection. In one embodiment, the recombinant nucleic acid is stably integrated into the genome of the avian, e.g., chicken. In another embodiment, the recombinant nucleic acid is expressed in the germ cells and/or somatic cells of the avian, e.g., chicken. In a related embodiment, the present disclosure provides a recombinant cell or cell line obtained from a transgenic avian (e.g., transgenic chicken) of the present disclosure.

[00104] In particular, the present disclosure provides transgenic avians, e.g., transgenic chickens, which are transgenic for a RIG-I promoter of the present disclosure, which RIG-I

promoter is operably linked to a gene product-encoding nucleic acid, e.g., a RIG-I protein as described herein, particularly a duck RIG-I protein as described herein. Transgenic avians of the order *Galliformes*, e.g., a chicken, turkey, grouse, New World quail and Old World quail, ptarmigan, partridge, or pheasant which are transgenic for a RIG-I promoter of the present disclosure, which RIG-I promoter is operably linked to a gene product-encoding nucleic acid, e.g., a RIG-I protein as described herein, particularly a duck RIG-I protein as described herein, which transgenic chickens being of particular interest.

[00105] In some embodiments, the transgenic bird is a chicken transgenic for a RIG-I promoter of the present disclosure, operably linked to a nucleic acid encoding a RIG-I protein (e.g., a RIG-I protein having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% identical in amino acid sequence to a RIG-I protein having the amino acid sequence of mallard duck RIG-I (Genbank accession no. EU363349.1)). In such transgenic chickens, the transgene facilitates resistance to disease caused by viral infection, e.g., caused by infection by an RNA virus, e.g., an influenza virus (e.g., an influenza A virus, such as an H5N1 virus) or Newcastle Disease virus. Such transgenic chickens can initiate a successful immune response to RNA virus infection by recognizing RNA virus infection and inducing IFN-stimulated antiviral genes.

[00106] Accordingly, the present disclosure also provides a method for conferring resistance to a disease in a bird (e.g., chicken) caused by an RNA virus, where the method comprises genetically modifying the genome of a chicken so as to be transgenic for a RIG-I promoter-avian RIG-I protein-encoding expression construct of the present disclosure. The present disclosure also provide a method for reducing RNA virus replication in a chicken, where the method comprises genetically modifying the genome of a chicken so as to be transgenic for a RIG-I promoter-avian RIG-I protein-encoding expression construct of the present disclosure.

[00107] Chickens having genetically modified genomes have been generated in a variety of ways. The production of transgenic chickens through various strategies including lentiviral and viral vectors, transposons, transcription activator effector nuclease (TALEN), and zinc-finger nucleases (ZFN) has recently been thoroughly reviewed (Doran et al., 2016, *Transgenic Res* 25, 307-319). The TALEN approach has recently been successful to generate gene knockouts in chickens (Park et al., 2014, *Proc Natl Acad Sci USA* 111, 12716-12721). Gene editing of chicken genomes can be achieved using a CRISPR system, such as CRISPR/Cas9,

which provides for versatility and efficiency (see, e.g., Hsu et al., 2014, *Cell* 157, 1262-1278). CRISPR/Cas9 can be used efficiently to modify chicken embryonic DF-1 cells and make alterations in somatic (Abu-Bonsrah et al., 2016, *Sci Rep* 6, 3452) and embryonic germ cells in the chicken embryo (Dimitrov et al., 2016, *PloS One* 11, e0154303). Repair through homologous recombination has been used to insert genes into primordial germ cells of chickens (knockins) and clonal lines of PGCs can be used to generate gene knockin birds (Dimitrov et al., 2016, *infra*). Examples of methods of generating genetically modified birds, including genetically modified chickens, have been described, for example, in U.S. Pat. No. 5,162,215 (Bosselman et al.), US 2002/0108132 (Rapp), US 2009/0158449 (Nakaishi et al.), Tyack et. al, Transgenic Res. 2013 June 17; 22:1257-1264; and Doran et. al, Transgenic Res. 2013 June 25(3):307-19, and in US Patent No. 7,375,258, US Patent No. 7,323,619, US Patent No. 7,312,374, US Patent No. 7,375,258, US Patent No. 7,507,873, US Patent No. 7,550,650, 8,507,749, and US Patent No. 9,510,571, which are hereby incorporated by reference in their entirety.

[00108] For example, transgenic chickens may be produced by using a retrovirus vector, embryonic stem cells, primordial germ cells or spermatozoa. Methods of producing the transgenic chickens having a RIG-I promoter/RIG-I protein-encoding expression construct include, without limitation, introducing the transgene to the chicken using, a viral or a non-viral vector; sperm-mediated gene transfer; restriction enzyme-mediated integration; nuclear transfer; ovum transfer and the like. Transgenic chickens produced by the present methods will be able to lay eggs and/or produce offspring containing the RIG-I heterologous protein.

[00109] For example, Bosselman et al. (U.S. Pat. No. 5,162,215) describe introduction of a replication defective retroviral vector into pluripotent stem cells of a chick embryo. Briefly, an opening, for example an opening about 5 mm in diameter, is made in a laid chicken egg which is not more than two days old. Typically the opening in the laid chicken egg is made using a drilling tool with an abrasive rotating tip to drill a hole in the eggshell but maintaining the underlying shell membrane intact. The membrane is cut out by a scalpel to expose the embryo which can be visualized with an optical dissecting microscope having 6X – 50X magnification. The solution comprising the DNA or vector can be any suitable medium, such as tissue culture medium, which is then microinjected into an area beneath and around the blastoderm. Typically 5-20 µl of solution is microinjected. Microinjection is carried out using a micromanipulator and a very small diameter needle, optionally a glass needle with 40 to 60 µm outer diameter. The egg

is then sealed with shell membrane and a sealing material, such as glue, cement or paraffin. The sealed microinjected egg is incubated at 37degreeC. to allow development of the embryo until the egg hatches. The hatched chickens are then genotyped, for example, by analysis of blood DNA and positive chickens are bred to produce progeny transgenic chickens.

[00110] Accordingly, in one embodiment, the present disclosure provides a method of producing a transgenic chicken with increased resistance to RNA virus infection, comprising: inserting into a chicken embryo a recombinant nucleic acid molecule comprising a RIG-I promoter/RIG-I protein-encoding expression construct of the present disclosure; and incubating the egg so as to allow the embryo to hatch.

[00111] Alternatively, the DNA or vector comprising the DNA may be injected into chicken embryonic stem cells or primordial germ cells, which are then injected into recipient embryos. See for example, US 2003/0115622 to Ponce de Leon et al., US 2006/0075513 to Ivarie et al. and US 2006/0206952 to Van de Lavoie and Leighton, all of which are incorporated herein by reference. Briefly, Ponce de Leon et al. describe excising blastodermal cells containing presumptive primordial germ cells (PGCs) from donor eggs, typically at stage XII to XIV. The PGCs are cultured in suitable culture medium containing factors to promote proliferation, such as LIF, bFGF, SCF and IGF to produce embryonic germ cells, which are typically produced after 28 days of culture. The cultured PGCs are introduced with a transgene by any suitable means, such as lipofection, transfection, microinjection, transformation, etc. The transgenic PGCs are then introduced into the dorsal aorta of recipient embryos, typically around stage 13-14. Similar to the above protocol from Bosselman et al., a small opening in shell is made, typically with forceps, and the PGCs are injected using a micropipette. Typically 200 donor PGCs are injected. After injection, the opening is sealed as described herein. The transfected donor cells become incorporated into the recipient embryos, some of which will produce chickens with the transgene in the germline.

[00112] Alternatively, WO87/05325 to Rottman and Hofer describe transferring material into sperm or egg cells by using liposomes containing the recombinant DNA or vector. The transgenic donor egg can then be transferred to the oviduct of a recipient hen (see Tanaka et al. 1994, J. Reprod. And Fertility, 100:447-449, incorporated herein by reference) or male germ cells can be transferred to a recipient testis. Restriction enzyme mediated integration is an

alternative method for creating transgenic sperm (see Shemesh et al. in WO 99/42569, incorporated herein by reference).

[00113] Accordingly, in another embodiment, the present disclosure provides a method of producing a transgenic chicken with increased resistance to RNA virus infection, comprising: inserting into a chicken germline or embryonic stem cell a vector comprising a a RIG-I promoter/RIG-I protein-encoding expression construct of the present disclosure to produce a genetically modified chicken; incubating the genetically modified chicken germline or embryonic stem cell; culturing the genetically modified chicken germline or embryonic stem cell so as to produce a zygote, and incubating the zygote to form an embryo, and incubating the embryo to allow the embryo to hatch.

[00114] It should be noted that the term “germline cell” as used herein refers to a germ cell, such as a primordial germ cell, or sex cell, such as sperm or egg, that have DNA that is passed on to offspring. The term “zygote” as used herein refers to the cell resulting from the union of sperm and egg. The phrase “producing a zygote from a germline or embryonic stem cell” as used herein includes, without limitation, fertilization of a non-transgenic egg with a transgenic sperm cell or fertilization of a transgenic egg with a non-transgenic sperm cell or microinjection of a transgenic germline cell or embryonic stem cell or culture of cells into a non-transgenic zygote or ovum or sperm transfer. The term “embryo” as used herein refers to the product of the zygote inside the avian (e.g., chicken) egg.

EXAMPLES

[00115] The following examples are provided in order to demonstrate and further illustrate certain embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

METHODS AND MATERIALS

Identification and cloning of duck RIG-I promoter TOPO vector.

[00116] The sequence of duck RIG-I has been previously described (Barber et al., Proc. Natl. Acad. Sci. USA 2010 107(3): 5913-18) and a sequence of a duck genome described (see,

2013 Nat. Genet. 2013 45(7): 776-83). However, the transcription start site (TSS) and promoter region of duck RIG-I could not be identified in the genome sequence.

[00117] Using a forward primer based on the genome sequence (5'-CCAAGTAAAAATGCCTCTCTGCT-3' SEQ ID NO:12) and a reverse primer in exon 2 (5'-GCATCGCGTCCAGCATCCCTCGGA-3' SEQ ID NO:13), a 1kb PCR fragment was amplified from blood genomic DNA. Sequencing of this product identified exon 1 and sequences approximately 2 kb upstream. A forward primer was designed further upstream based on genome sequence (5'-AGCTGATGACCTGCAAAAAGTT-3' SEQ ID NO:14) and a reverse primer within exon 1 (5'-GTTGAGGCTCCGCTCGATGTA-3' SEQ ID NO:15) resulting in the amplification of approximately 2 kb upstream from the first exon of duck RIG-I (from -2024 to +63) using duck genomic DNA as template and the KAPA Hi Fi™ PCR Kit (Kapa Biosystems). The product was cloned into PCR2.1 TOPO vector (Invitrogen) to sequence as per the manufacture's protocol.

[00118] Finally, taking the 2 kb fragment in TOPO vector as template, the full length of duck RIG-I promoter (-2024 to -1) was amplified using the Q5 High-Fidelity PCR Kit (NEB) and primers 5'-AGCTGATGACCTGCAAAAAGTT-3' (SEQ ID NO:16) and 5'-GGCTGGGCTCTGCCGGCCG-3' (SEQ ID NO:17), then ligated it into PCR 2.1 TOPO vector with T4 DNA ligase (NEB) as the manufacture's instruction.

Construction of wild type and mutant duck RIG-I promoter reporter plasmids

[00119] The full-length duck RIG-I promoter sequence was digested out of PCR2.1 TOPO vector by KpnI and XhoI (NEB) at 37°C for 30 minutes, followed by electrophoresis and gel extraction. The extracted product was ligated to pGL3-basic luciferase reporter vector (Promega; Madison, WI), to generate p2024 vector as illustrated in Figure 1A. This construct was then used as the backbone plasmid in all duck RIG-I promoter constructs discussed below

[00120] A series of deletion mutants and specific transcription factor binding site deletion mutants of duck RIG-I promoter were generated using a site-directed mutagenesis protocol (NEB; Ipswich, MA). In brief, p2024 was used to synthesize the additional truncation constructs, p1000, p500, p250, p125, p73 using primers based on the sequence. The specific truncated promoter construct (p250 and p125) was used as the template to synthesize the transcription binding site deletion mutants using Phusion High-Fidelity PCR kit (NEB; Ipswich, MA). The extracted products were treated with Dpn I (NEB; Ipswich, MA) to eliminate the original

template plasmid. The treated products were further purified with the QIAquick™ PCR purification kit (QIAGEN; Mississauga, ON) and the two ends of amplified mutant strands were ligated together with T4 DNA ligase following addition of 5'-phosphates with T4 Polynucleotide Kinase (NEB) to form the mutant constructs.

Cell culture and transfection

[00121] DF-1 cells are spontaneously immortalized chicken embryo fibroblast cells, derived from East Lansing Line (ELL-0) chicken embryos previously described in Schaefer-Klein (Virology 1998 248(2): 305-11) and were purchased from ATCC (ATCC® CRL-12203™). They were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) in a 5% CO₂ incubator at 39 °C.

[00122] DF-1 cells were seeded into 24-well plates at 2×10⁵/well. 24 h later, the cells were transfected with the indicated DNA constructs using LIPOFECTAMINE 2000™ reagent (Invitrogen) at a ratio of 1:2.5 (DNA: Lipofectamine). 24 h post transfection, the cells were lysed for the dual luciferase assay. The indicated RIG-I promoter vectors, the Renilla control vector and the RIG-I 2CARD construct were transfected at 150 ng/well, 10 ng/well and 20 ng/well, respectively.

Dual Luciferase Assay

[00123] DF-1 cells in a 24-well plate were transfected with the indicated duck RIG-I promoter plasmid and the Renilla luciferase control vector with or without the stimulators (20 ng/well). The constitutively active duck RIG-I N-terminal domain (2CARD) or 2 µg/ml high molecular weight (HMW) polyinosinic-polycytidylic acid, poly (I: C)) were used to activate the MAVS signalling pathway. The promoter activity of duck RIG-I was measured using the Dual Luciferase Reporter Assay System (Promega) 24 h post transfection according to manufacturer's instructions. Briefly, the cells were lysed with 100 µl 1X passive lysis buffer at room temperature for 15 min. 20 µl cell lysate was transferred to 1.5 ml Eppendorf tube, followed by measuring the luciferase activity using the GloMax 20/20 Luminometer (Promega) after adding 100 µl luciferase assay reagent II and 20 µl 1×stop and glo reagent into cell lysate, in sequence. The firefly luminescence from the test reporters was normalized to the Renilla luminescence from the control vector and the normalized relative luciferase units (RLU) were further divided by the mean RLU of the control group. For each group, the experiment was done in triplicate. Assays were performed at least twice.

Statistical analysis

[00124] Values indicated the means $\pm$ standard deviation and the statistical analysis among multiple experimental groups was performed by Tukey test under one-way analysis of variance (ANOVA) using GraphPad Prism 6 software. $P < 0.05$ was considered significant.

EXAMPLE 1: CLONING AND SEQUENCING THE DUCK RIG-I PROMOTER REGION

[00125] To recover the RIG-I promoter sequence, which was lacking exon 1 in the duck genome sequence, two fragments upstream of RIG-I were amplified. The first 1 kb fragment spanned from upstream to exon 2 of RIG-I and contained part of the promoter sequence, the complete 5'UTR, exon 1 and intron 1 and a part of exon 2. Using a forward primer based on a region further upstream of RIG-I in the annotated duck genome sequence and a reverse primer within exon 1 of the amplified sequence, approximately 2kb of the RIG-I promoter sequence was amplified and sequenced. The sequence of this approximately 2kb of the RIG-I promoter is provided in Fig. 6 (SEQ ID NO: 02). The promoter sequence was confirmed to be identical in the overlap between both independent amplified regions.

EXAMPLE 3: THE DUCK PROMOTER IS INTERFERON INDUCIBLE

[00126] To determine whether the amplified 2024 bp sequence (SEQ ID NO:02) upstream of the duck RIG-I gene has promoter activity, this segment was inserted into the multiple cloning site (MCS) in the pGL3-basic vector to generate a promoter reporter vector, p2024 (Fig. 1A). P2024 was transfected into DF-1 cells to test its promoter activity using the dual luciferase assay. The empty pGL3-basic vector was transfected as negative control. The inserted 2024bp promoter sequence significantly increased the relative luciferase activity by 17 times compared to the empty pGL3-basic vector (Fig. 1B), indicating that the inserted segment has promoter activity.

[00127] It has been previously shown that duck RIG-I is significantly upregulated during an innate immune response by Barber et al. (Proc. Natl. Acad. Sci. USA 2010 107(3): 5913-18). The activation of RIG-I or MDA-5 induces type-I IFN production and both RIG-I and MDA5 are ISGs and also regulated by type-I IFN in a positive feedback loop (Kang et al., Oncogene 2004 23(9): 1789-1800; Kang et al., Proc. Natl. Acad. Sci. 2002 99(2): 637-42; Su et al., J. Cell Physiol. 2007 213(2): 502-10). It was also previously shown that overexpression of the constitutively active N-terminal region of duck RIG-I, the two CARD domains (2CARD), can

stimulate the MAVS signaling pathway and induce type-I IFN and ISG upregulation in chicken DF-1 cells (Miranzo-Navarro, D. and K.E. Magor, 2014. Activation of Duck RIG-I by TRIM25 is Independent of Anchored Ubiquitin. PLoS One 9: e86968.

[00128] In view of this, in order to determine whether the promoter activity of duck RIG-I is inducible by type-I IFN, RIG-I promoter vector (p2024) was co-transfected with or without duck RIG-I 2CARD. The relative activity of the RIG-I promoter upon stimulation for 24 hours following transfection with RIG-I 2CARD was approximately two fold higher than the p2014 promoter alone (Fig. 1C). Though chickens lack the RIG-I gene, they have MDA-5, which can be effectively activated by longer RNA molecules (>2kb) such as HMW poly (I: C) (Kato et al., J. Exp Med. 2008 205(7): 1601-10) Chicken MDA5 can compensate, at least partially, for the lack of RIG-I (Liniger et al., 2012, J Virol. 2012 Jan;86(2):705-17 and Karpala et al., 2011, J Immunol. 2011 May 1;186(9):5397-405). The relative luciferase activity of duck RIG-I promoter co-transfected with poly (I: C) was also approximately two-fold higher than the p2024 RIG-I promoter alone, thus significantly induced (Fig. 1D). The firefly luciferase activities remain unchanged for the pGL-3 basic control, either stimulated or unstimulated by co-transfection with 2CARD or poly (I: C), (Fig. 1D). Chicken cells sense influenza A virus infection through MDA5 and CARDIF signaling involving LGP2.

EXAMPLE 4: THE CORE PROMOTER OF DUCK RIG-I IS INTERFERON-INDUCIBLE

[00129] In order to identify the core promoter and the essential elements for duck RIG-I inducible activity, a series of deletion mutants of the duck RIG-I promoter were constructed (Fig. 2A) and transfected into DF-1 cells. At 24 hours post transfection, the relative promoter activity was tested by dual luciferase assay. Compared with the full-length promoter p2024 and deletion mutants, p250 had the highest basal promoter activity, whereas p73, the most proximal region to the transcription start site (TSS), had the lowest activity (Fig. 2B). This indicated that p250 contained the core promoter of duck RIG-I. One or more suppressive elements may be located between -250 bp and -2024 bp, because several longer fragments had lower activity than the p250 fragment. All promoter constructs were inducible by RIG-I 2CARD except p73 (Fig. 2C). Construct p125 had the highest inducible activity following transfection of RIG-I 2CARD in DF-1 cells (Fig. 2C). This result demonstrates that the core promoter region contains

all the necessary elements for the interferon inducible expression of duck RIG-I expression. The sequence of the core promoter region (SEQ ID NO:01) is provided in Fig. 3A.

EXAMPLE 5: IDENTIFICATION OF PUTATIVE TRANSCRIPTION FACTOR BINDING SITES

[00130] To identify transcription factor (TF) binding sites in the core promoter sequence, the proximal promoter sequence was examined with the TF search program, “JASPAR”. Two transcription factor binding sites (Sp1 and Sp2) were predicted to bind between -125bp and -250bp, and (Sp1 and Sp2) bind the same position in this region. Additionally, three TFs (IRF1, STAT1 and STAT2) were predicted to bind the same position between -125bp and -73bp. There were no predicted TF binding sites in the -73bp region (Fig. 3A).

To confirm the importance of these binding sites to the basal and inducible expression of duck RIG-I, promoter deletion mutants with the Sp1/Sp2 or IRF1/ STAT1/STAT2 binding sites deleted, and a control construct deleting a random sequence were constructed from the p250 promoter construct. Additionally, promoter mutation constructs were generated by mutating the SP1/SP2 transcription factor binding site comprising the nucleotide sequence: GAGGGCGGGC (SEQ ID NO: 24) to GAGTTCTTGC (SEQ ID NO: 25). Additionally we generated a mutated IRF-1 site, GCTTTCGTTTCCA (SEQ ID NO: 26) to GCTAACGTGAGCCA (SEQ ID NO: 27).

[00131] The mutant or wild-type promoter constructs were transfected into DF-1 cells, alone or co-transfected with duck RIG-I 2CARD. At 24 h post transfection the relative promoter activities were determined by dual luciferase assay. Compared to the wild type promoter vectors (p250 or p125), the deletion of the Sp1/Sp2 binding site significantly decreased the basal promoter activity (Fig. 3B). The deletion of the IRF1/STAT1/STAT2 binding site resulted in the loss of the inducible activity. Because all deletion mutants of p250 still bear IRF1/STAT1/STAT2 binding site, their promoter activities could be induced by RIG-I 2CARD (Figure 3C). The comparison of wild-type and deletion and mutation constructs of predicted Sp1/Sp2 binding site and the IRF1/STAT1/STAT2 support that these binding sites contribute to duck RIG-I basal and inducible expression, respectively.

EXAMPLE 6: CHICKEN IRF7 AND DUCK IRF1 INDUCE PROMOTER ACTIVITY OF DUCK RIG-I IN DF-1 CELLS

[00132] To investigate whether *IRF1*, *IRF7*, *STAT1* or *STAT2* and *IFNB* are upregulated in RIG-I 2CARD stimulated cells, mRNA levels were measured in DF-1 cells transfected with p2024 or pGL-3 basic vector with or without co-transfected RIG-I 2CARD using qPCR. The housekeeping gene (*GAPDH*) was used as the internal reference. Transcript levels for IRF1, STAT1 and STAT2, IFNB were low and were not significantly different in RIG-I 2CARD treated and untreated groups at 24 h post-transfection (Fig. 4A). Upon phosphorylation, TFs are activated and translocated from cytoplasm to nucleus to perform their function of regulating gene transcription. It is not necessary to increase their expression level to regulate gene transcription. IRF1 was not induced, even though IRF1 has been reported to control inducible expression of human RIG-I. The upregulation of *IRF7* and the *MX* genes demonstrated that an interferon response was stimulated.

[00133] To determine whether chicken IRF factors can induce the duck RIG-I gene in chicken cells, chicken IRF1, chicken IRF7 and duck IRF1 were cloned. Because of the lack of effective antibodies to chicken IRFs, mCherry constructs were generated for chicken IRF1 (pmC-chIRF1) and chicken IRF7 (pmC-chIRF7), duck IRF1 (pmC-dIRF1) and mCherry (vector only) expression constructs and their expression was confirmed by fluorescence. All constructs were expressed, and staining was evident in both cytoplasm and nucleus (Figure 4B).

[00134] To determine which IRFs could drive expression of duck RIG-I, each mCherry tagged protein was overexpressed in DF-1 cells with the RIG-I promoter constructs to investigate whether the promoter activity of duck RIG-I would be upregulated. The overexpression of chIRF-1 did not induce RIG-I promoter activity. However, overexpression of either chIRF-7 or dIRF-1 significantly induced duck RIG-I promoter activity (Figure 4C). P73, which was not inducible in stimulated chicken cells, was induced by duck IRF1, and not chicken IRF1 or IRF7 (Figure 4C). These results demonstrate that chicken IRF-7 induces duck RIG-I promoter activity in DF-1 cells. In contrast, it was previously shown by Su et al (J Cell Physiol 2001 213(2):502-10) that human IRF-1 is induced by type-I IFN and that it directly controlled human RIG-I expression.

EXAMPLE 6: RIG-I PROMOTERS DO NOT SHOW EXPECTED PHYLOGENETIC RELATIONSHIP

[00135] To determine whether the evolutionary relationship of RIG-I promoters was consistent to that of the RIG-I proteins, phylogenetic trees between duck and other species were created using MEGA7. RIG-I protein and promoter sequences were downloaded from the NCBI protein database and UCSC database, respectively. The mallard duck RIG-I is closer in evolutionary distance to other bird RIG-I sequences than to mammalian proteins (Fig. 5B). In contrast, RIG-I promoters do not follow the expected phylogenetic relationship between different species. Mouse RIG-I promoter appears to be closest to the duck RIG-I (Fig. 5A; see also FIG.). This result differs from the phylogenetic analysis of MDA-5 promoters, which demonstrated taxonomic relationship among different vertebrates as described by Zhang et al. (Mol. Immunol. 2016 76:1-6), even though RIG-I and MDA-5 are in the same PRR family and their structures and functions are very similar. A phylogenetic tree of all avian RIG-I proteins is shown in Fig. 5C which indicates that Muscovy duck and goose are closest in evolutionary distance, as expected.

EXAMPLE 7: PRODUCTION OF GENE-EDITED KNOCKIN CHICKEN CELLS

[00136] Transgenic chickens have been generated in a variety of ways. The production of transgenic chickens through various strategies including lentiviral and viral vectors, transposons, transcription activator effector nuclease (TALEN), and zinc-finger nucleases (ZFN) has been reviewed (Doran et al., 2016, *Transgenic Res* 25, 307-319). The TALEN approach has recently been successful to generate gene knockouts in chickens (Park et al., 2014, Proc Natl Acad Sci U S A 111, 12716-12721).

[00137] **Gene editing of chicken genomes can be achieved through CRISPR/Cas9, which provides both a versatile and efficient** approach (Hsu et al., 2014, *Cell* 157, 1262-1278). CRISPR/Cas9 can be used efficiently to modify chicken embryonic DF-1 cells and make alterations in somatic (Abu-Bonsrah et al., 2016, *Sci Rep* 6, 34524) and embryonic germ cells in the chicken embryo (Dimitrov et al., 2016, *PloS One* 11, e0154303). Repair through homologous recombination has been used to insert genes into primordial germ cells of chickens (knockins) and clonal lines of PGCs can be used to generate gene knockin birds (Dimitrov et al., 2016, *infra*).

[00138] Constructs of duck RIG-I under the control of full and partial promoters for expression in chicken cells are designed. One strategy involves the core promoter of duck RIG-I

as described herein operably linked to drive expression of a spliced RIG-I gene coding sequence (corePR-RIG-Ics).

[00139] To identify a unique coding sequence that will allow targeting of the gene to the region of the chicken genome where the *DDX58* gene is missing, a fragment corresponding to this region is amplified using the available chicken genome sequence. Based on comparative genomics the chicken is missing the *DDX58* gene because of a large-scale rearrangement that reversed the arms of the Z chromosome. This rearrangement happened in an ancestor to the entire galliform lineage (chickens, turkeys, quails). The gene can be inserted in the Z chromosome. The Z chromosome contains many innate immune genes including the interferons, thus the chromosome may be upregulated during an interferon response through chromatin accessibility through epigenetic effects.

[00140] Using CRISPR technology optimized for gene editing in chickens (Zuo et al., 2016, Site-Directed Genome Knockout in Chicken Cell Line and Embryos Can Use CRISPR/Cas Gene Editing Technology. *G3* (Bethesda) 6, 1787-1792) the duck *DDX58* gene is inserted into the chicken Z chromosome. Targeting to this region is done using the vector with the chicken U6 promoter for driving expression of the CRISPR/Cas9, which provides both the Cas9 protein and the guide RNAs. Several cell lines are available to do this, including the chicken embryonic fibroblast DF-1 cells (Schaefer-Klein et al., 1998, *Virology* 248, 305-311), or a chicken lung cell line (Esnault et al., 2011, *Virus Res* 159, 32-42). Alternately, chicken DT40 B cells can be used, which have a known high success rate of homologous recombination (Harris et al., 2002, *Curr Biol* 12, 435-438). One approach involves using double-strand breaks made with CRISPR/Cas9 RNA using guide RNAs targeted to the Z chromosome sequence in the gene interval adjacent to the acontinase I gene (Fig X). The targeting region is first amplified to confirm the sequence of the targeting region. The amplified sequence can then be used to attach 1 kb regions of homology to flank the RIG-I gene in the repair vector for directing repair of the double-strand breaks. This strategy has been successfully used to insert EGFP into the chicken IgH locus (Dimitrov et al., 2016, *PLoS One* 11, e0154303). RIG-I can be epitope-tagged or RIG-I expression confirmed in chicken cells with reverse-transcription PCR of DNase-treated RNA. The generation of a *DDX58* chicken cell line can be tested to confirm regulation of RIG-I in response to interferon, and influenza virus infection. The effect of RIG-I on the viral titre by plaque assay, TCID₅₀ or an examination of percent infected cells using high content microscopy,

can be performed according to techniques previously described (Blyth et al., 2016, *Virology* 90, 103-116).

EXAMPLE 8: PRODUCTION OF GENE-EDITED KNOCKIN CHICKEN

[00141] Production of transgenic chickens using cultured primordial germ cells as intermediates, and injection of clones of altered primordial germ cells (**PGCs**) has been described (Dimitrov et al., 2016, *PloS One* 11, e0154303). Lipofectamine 3000 reagent can be used to facilitate more efficient transfection of chicken primary cells. Alternatively, DNA and lipofectamine can be applied *in vivo* in the developing chick.

[00142] The CRISPR/Cas9 system is used for targeting genomic location and cleavage and the strategy of homologous recombination or non-homologous end joining (He et al., 2016, *Nucleic Acids Res* 44, e85) along with co-transfection of the repair construct to introduce either the promoter and DDX58 (RIG-I coding sequence) in chicken primordial germ cells or, alternatively, with the intact 20kb *DDX58* gene.

[00143] Cas9 nuclease generates site-specific breaks in double-strand DNA using a 20 nt guide RNA (gRNA). Efficient knock-in of large genes into human germ cells has been accomplished using either homology directed repair (which is inefficient) and non-homologous end joining pathways which is much more efficient and allows a larger insert (He et al., 2016, *Nucleic Acids Res* 44, e85). This strategy is used to knock in the duck *DDX58* gene in chicken PGCs isolated and cultured as previously described (Song et al., 2014, *Biol Reprod* 90, 15). To produce gene edited birds, PGCs are selected that have been modified (Dimitrov et al., 2016), by incorporating the puromycin or neomycin resistance gene for selection and cloning of PGC lines. This approach achieved 100% targeting in one clone from transfected chicken PGCs (Dimitrov et al., 2016, *PloS One* 11, e0154303). In subsequent experiments, the selectable marker may be omitted and PGCs cloned to single cells by limiting dilution. Alternatively, the construct can be introduced to the PGCs *in ovo* in the embryo along with CRISPR/Cas9 targeting to generate chimeric germ line. To determine whether chicken cells expressing duck RIG-I have been generated, PGCs and offspring are screened for RIG-I using PCR and/or using specific polyclonal anti-duck RIG-I antibodies.

CLAIMS

What is claimed is:

1. An expression construct comprising:
 - a promoter comprising, from 5' to 3',
 - an Sp1/Sp2 transcription factor binding site comprising the nucleotide sequence CGGCCAGAGGGCGGGC (SEQ ID NO:03), and
 - an IRF-1/STAT1/STAT2 transcription factor binding site comprising the nucleotide sequence GGCCGCTTTCGTTTTCCATTC (SEQ ID NO:04);
 - and
 - a polypeptide-encoding nucleic acid;
 - wherein the promoter and the polypeptide -encoding nucleic acid are operably linked to provide for expression of the polypeptide in a host cell.
2. The expression construct of claim 1, wherein the promoter comprises, from 5' to 3'
 - a NHLH1 transcription factor binding site comprising the nucleotide sequence ACGAAGCTGCGA (SEQ ID NO:05),
 - the Sp1/Sp2 transcription factor binding site comprising the nucleotide sequence CGGCCAGAGGGCGGGC (SEQ ID NO:06), and
 - the IRF-1/STAT1/STAT2 transcription factor binding site comprising the nucleotide sequence GGCCGCTTTCGTTTTCCATTC (SEQ ID NO:07).
3. An expression construct comprising:
 - a promoter comprising a nucleic acid sequence of at least 85% sequence identity to the nucleic acid sequence:

CTGGACCCCAGGCCCGTGTCTCGGTGTCCCGGTGAGGCTGTTGGTGGCGATCCGAGG
 CTCACGAAGCTGCGAGCCGCTGCCTATGCCCGCTGGCGGCCAGAGGGCGGGCTGCG
 GATGGGGCCGCGATCTCCACACCCCGCGGGGGCCGCTTTCGTTTTCCATTCCCCGCC
 GGGCCTCGCTGCCTTTCTGTGCCGAGCCGGTTGGGGGGCCGGGCCGGGCCGGGCCG
 GGCGGCGGCCGGCAGAGCCCAGCC (SEQ ID NO: 01); and
 - a polypeptide-encoding nucleic acid;

wherein the promoter and the polypeptide -encoding nucleic acid are operably linked to provide for expression of the polypeptide in a host cell.

4. The expression construct of any one of claims 1-3, wherein the polypeptide-encoding nucleic acid encodes a retinoic acid-inducible gene I (RIG-I) protein.
5. The expression construct of any one of claims 1-4, wherein the RIG-I-encoding nucleic acid encodes a RIG-I protein having at least 75% sequence identity to a RIG-I protein having the amino acid sequence of

MTADEKRSLQCYRRYIERSLNPVYVLGNMTDWLPDELRRERIRKEEERGVSAAA
 LFLDAVLQLEARGWFRGMLDAMLAAGYTGLAEAIENWDFSKLEKLELHRQLK
 RIEATMLEVDPVALIPYISTCLIDRECEEIQQISENRSKAAGITKLIECLCRSDKEHW
 PKSLQLALD TTGYRASELWDIREDNAKDVDSEMTDASEDCLEASMTYSEEAE
 DDNLSENLSAAEGIGKPPVYETKKARSYQIELAQPAINGKNALICAPTGSGKTF
 VSILICEHHFQNPAGRKAKVVFLATKVPVYEQQKNVFKHHFERQGYSVQGISG
 ENFSNVSVVEKVIEDSDIIVVTPQILVNSFEDGTLTSLSIFTLMIFDECHNTTGNHPY
 NVLMTRYLEQKFNSASQLPQILGLTASVGVGNNAKNEETIEHICSLCSYLDIQAIST
 VRENIQELQRFMNKPEIDVRLVKRRIHNPFAAISNLMSETEALMRTIYSVDTL
 NSKKDFGTQNYEHWIVVTQRKCRLQLLEDKEEESRICRALFICTEHLRKYNDALII
 SEDARIIDALSYLTEFFT NVKNGPYTELEQHLTAKFQEKEPELIALSKDETNP
 LEELVCILDDAYRYNPQTRTLLFAKTRALVSALKKCMEENPILNYIKPGVLMGRG
 RRDQTTGMTLPSQKGVLD AFKTSKDNRLLIATSV ADEGIDIVQCNLVVLYEYSG
 NVTKMIQVRGRGRAAGSKCILVTSKTEVVENEKCNRYKEEMMNKAVEKIQKW
 DEETFAKKIHNLMKERVLRDSRRKEIKPKVVEGQKNLLCGKCKAYACSTDDIRI
 IKDSHHIVLGEAFKERYTTKPHKKPMQFDGFEKKSKMYCRNNNCQHDWGIVK
 YLTFDNLPVIKISFVMESTATGTQMD FQKWSINSSLKNFDVEEMSNLYPP
 (SEQ ID NO: 11).

6. The expression construct of any one of claims 1 to 5, wherein the promoter comprises a nucleic acid sequence having at least 80% sequence identity to the sequence

CGGCCAGAGGGCGGGCTGCGGATGGGGCCGCGATCTCCACACCCCGCGGGG
 GCCGCTTTCGTTTTCCATTCCCCGC||CGGGCCTCGCTGCCTTTCTGTGCCGAGCCGGT
 TGGGGGGCCGGGCCGGGCCGGGCCGGCGGCGGCCGAGAGCCAGCC (SEQ ID
 NO: 08).

7. The expression construct of any one of claims 1 to 5, wherein the promoter comprises a nucleic acid sequence having at least 80% sequence identity to the sequence

GCCGCTGCCTATGCCCCGCTGGCGGCCAGAGGGCGGGCTGCGGATGGGGCCGCGATC
TCCACACCCCGCGGGGGCCGCTTTCGTTTTCCATTCCCCGCCGGGCCTCGCTGCCTTT
CTGTGCCGAGCCGGTTGGGGGGCCGGGCCGGGCCGGGCCGGGCCGGCCGGCCGGCAG
AGCCCAGCC (SEQ ID NO:09).

8. The expression construct of any one of claims 1 to 5, wherein the promoter comprises a nucleic acid sequence having at least 80% sequence identity to the sequence of SEQ ID NO:02.

9. The expression construct of any of claims 1 to 8, wherein the expression construct is a viral vector.

10. The expression construct of claim 9, wherein the viral vector is a retroviral vector, an adenoviral vector or an adeno-associated viral vector.

11. A recombinant host cell comprising the expression construct of any one of claims 1-10.

12. The recombinant host cell of claim 11, wherein the host cell is an avian cell.

13. The recombinant host cell of claim 12, wherein the avian cell is a chicken cell.

14. A transgenic non-human animal comprising a genomically integrated expression construct of any one of claims 1-10.

15. The transgenic animal of claim 14, wherein the transgenic non-human animal is a chicken.

16. A method for producing a chicken resistant to disease caused by avian influenza infection, the method comprising:

introducing into a chicken embryo an expression construct of any one of claims 1-10.

Figure 1A

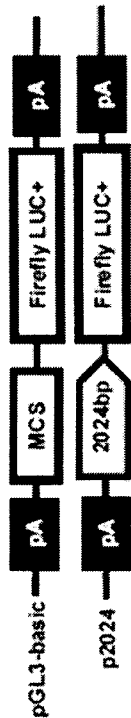


Figure 1B

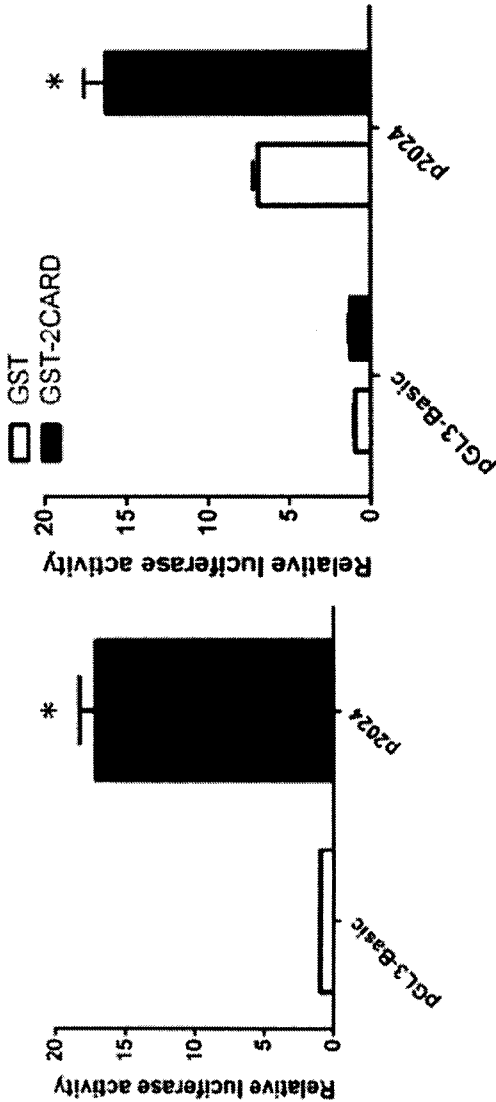


Figure 1C

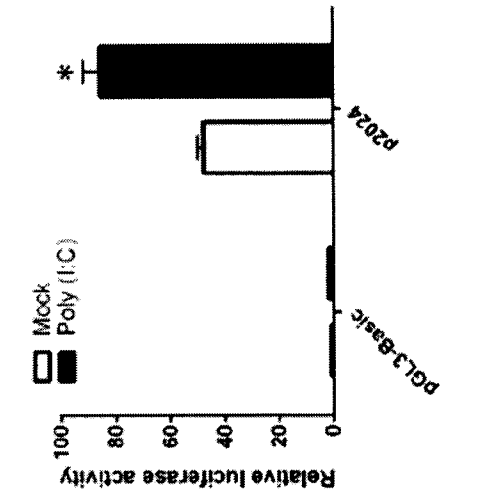


Figure 1D

Figure 2B

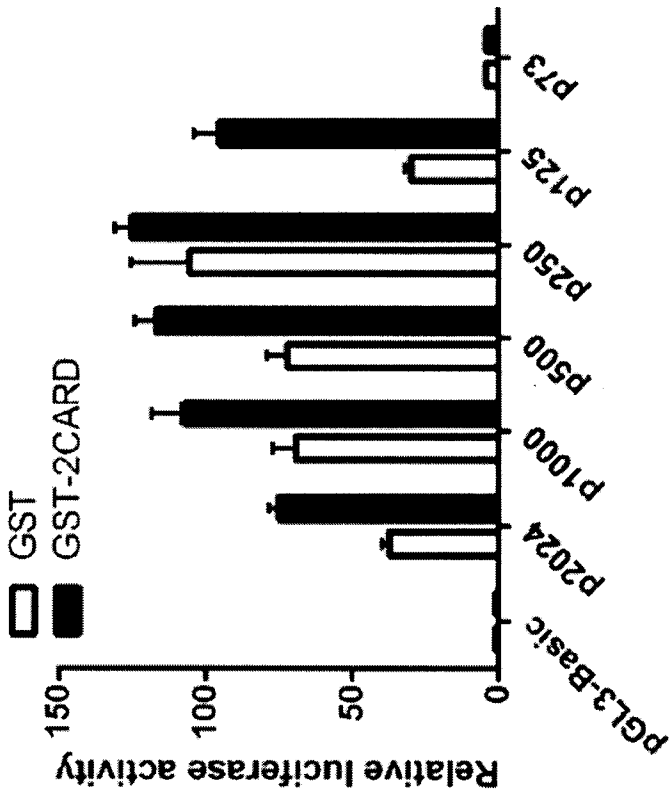


Figure 2A

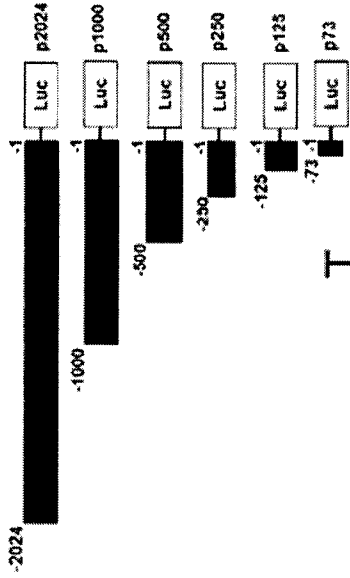
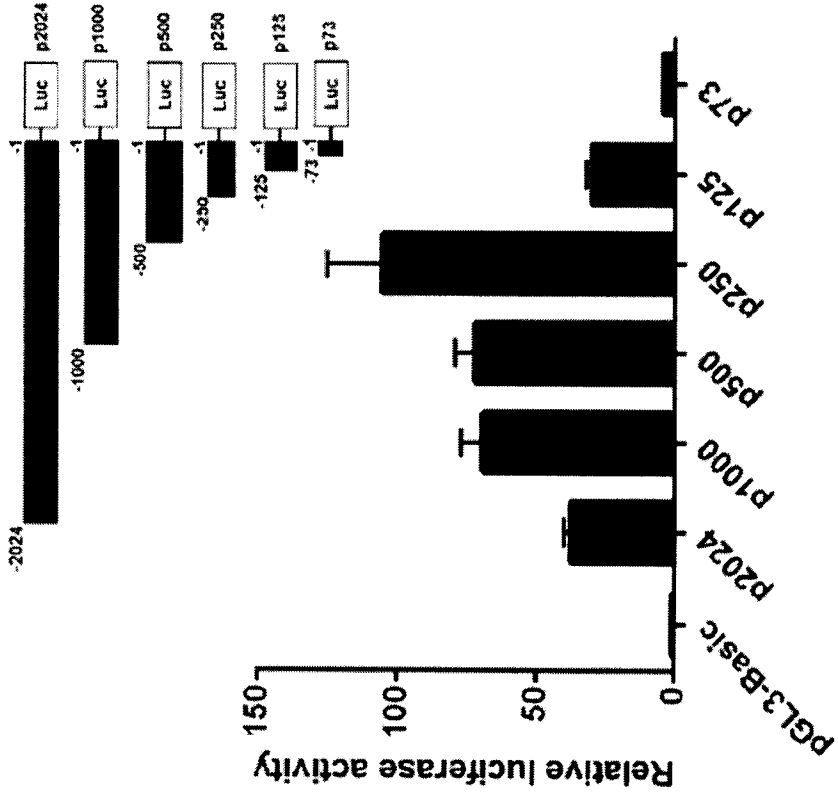


Figure 3A

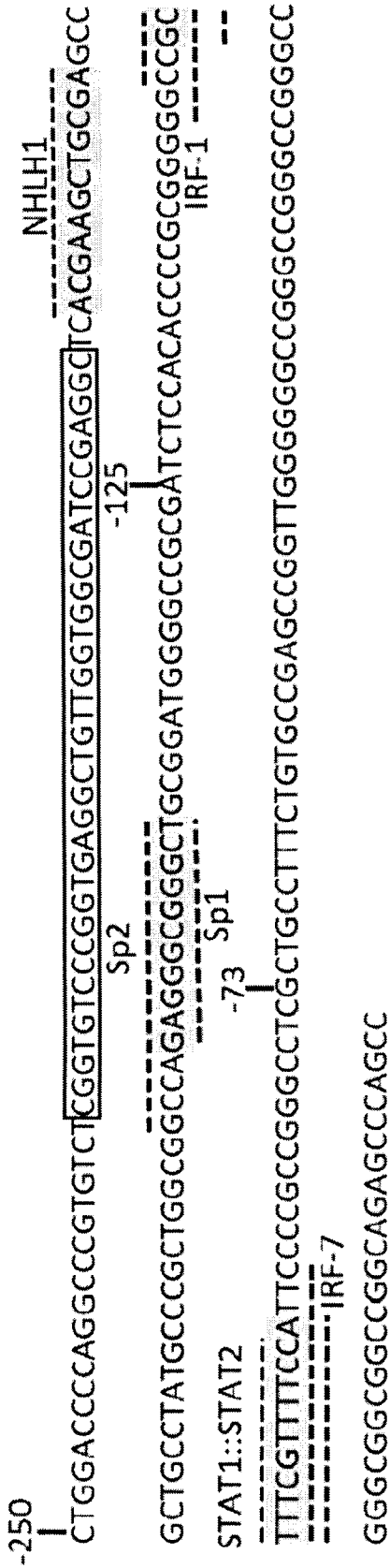


Figure 3B

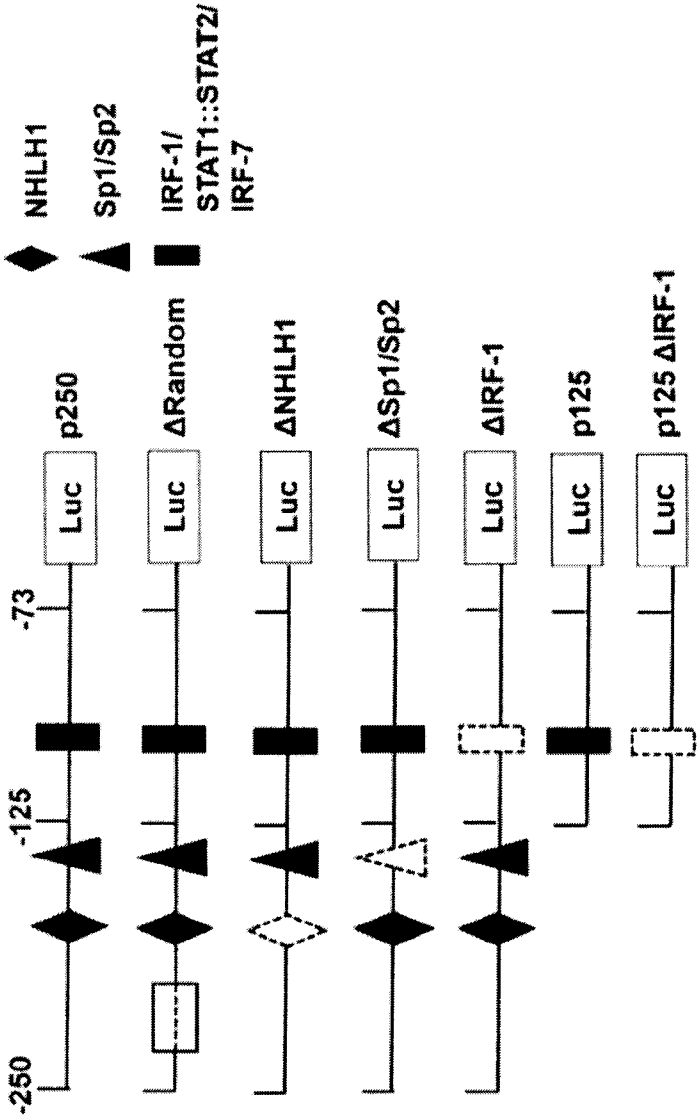


Figure 3D

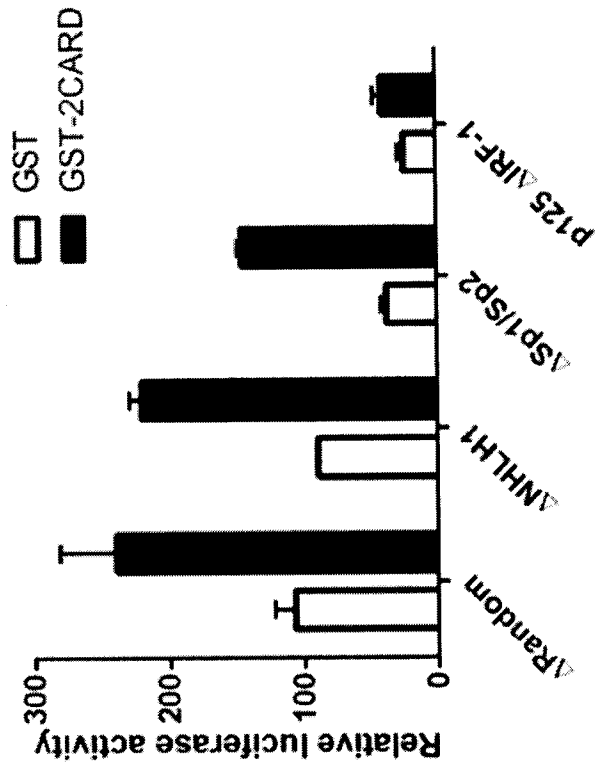


Figure 3C

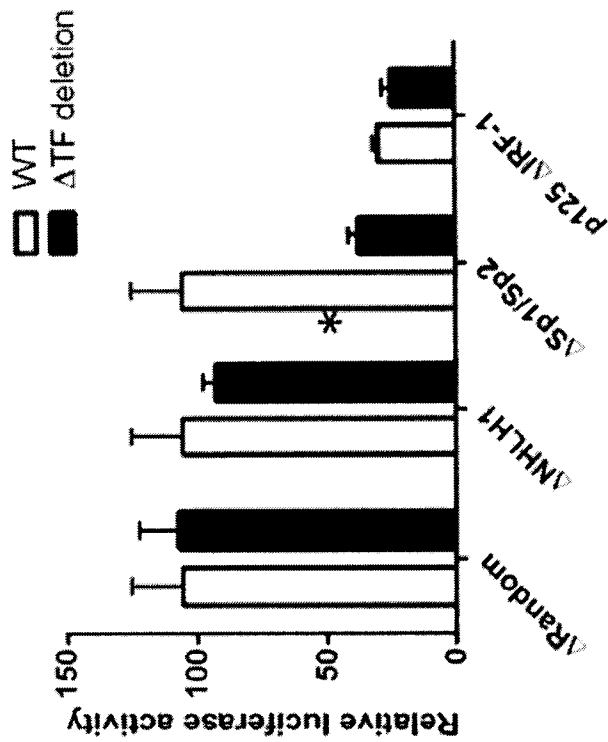


Figure 3F

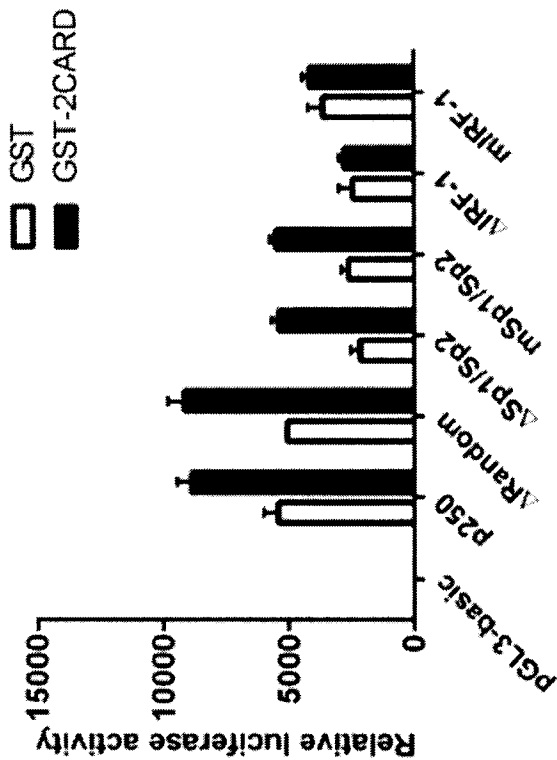


Figure 3E

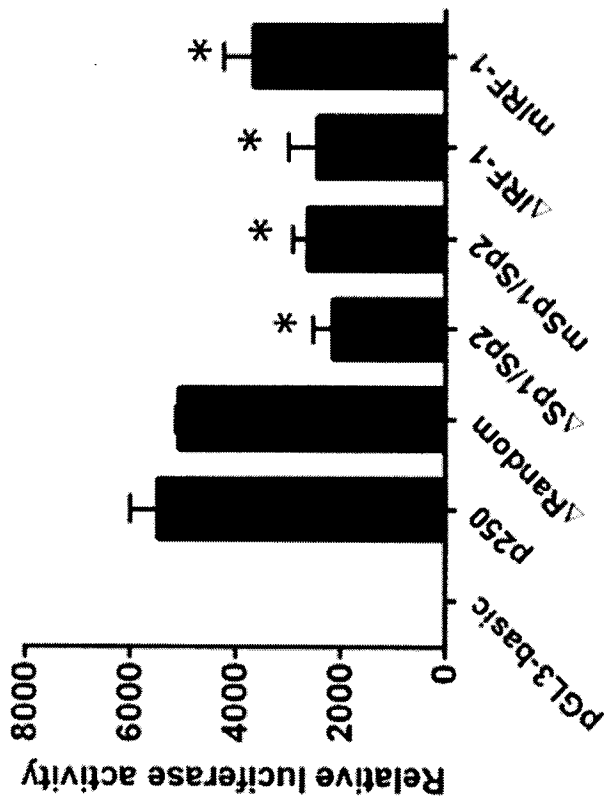


Figure 4A

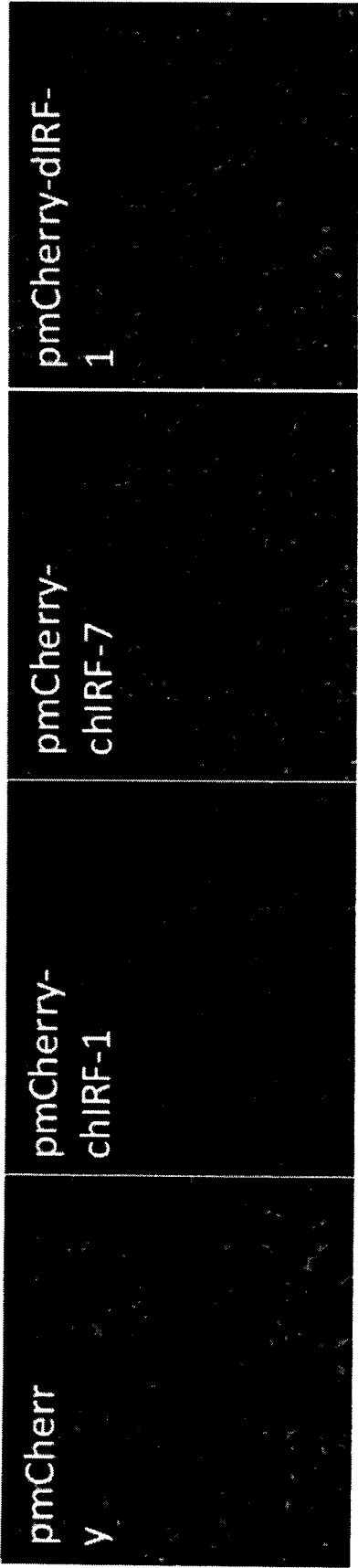


Figure 4B

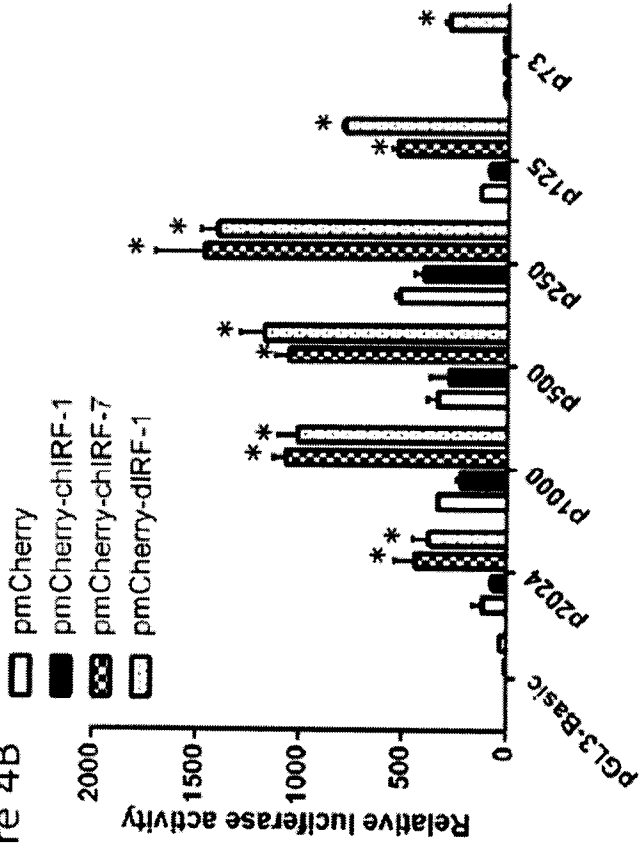


Figure 4C

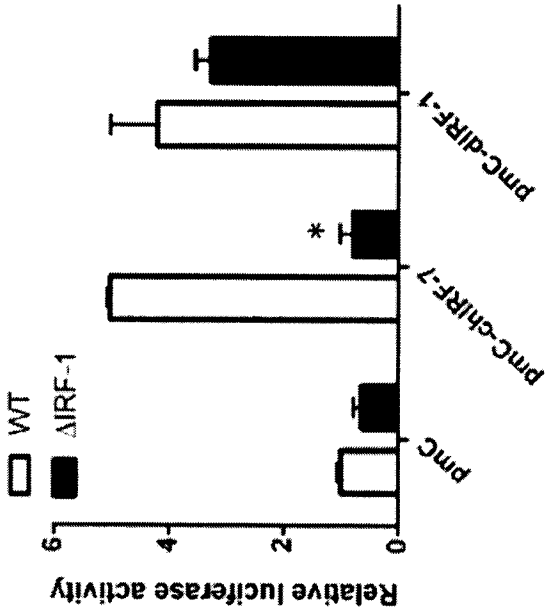


Figure 5A RIG-I promoter phylogenetic tree

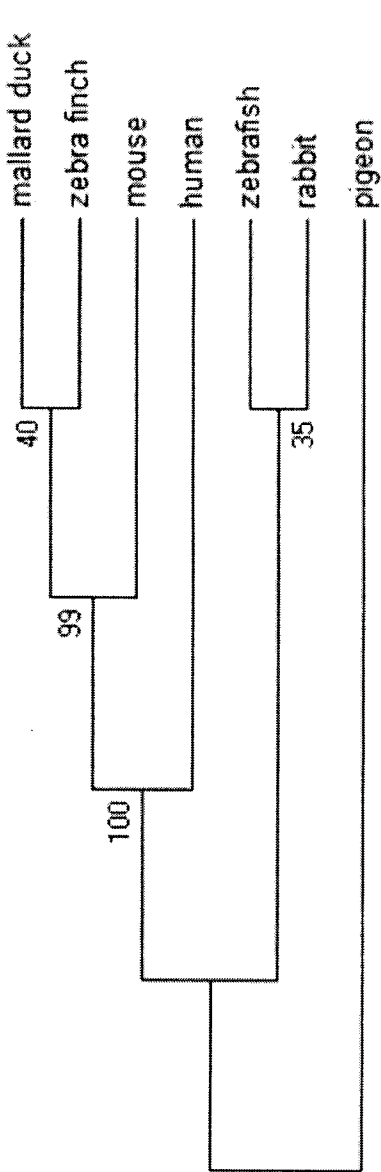
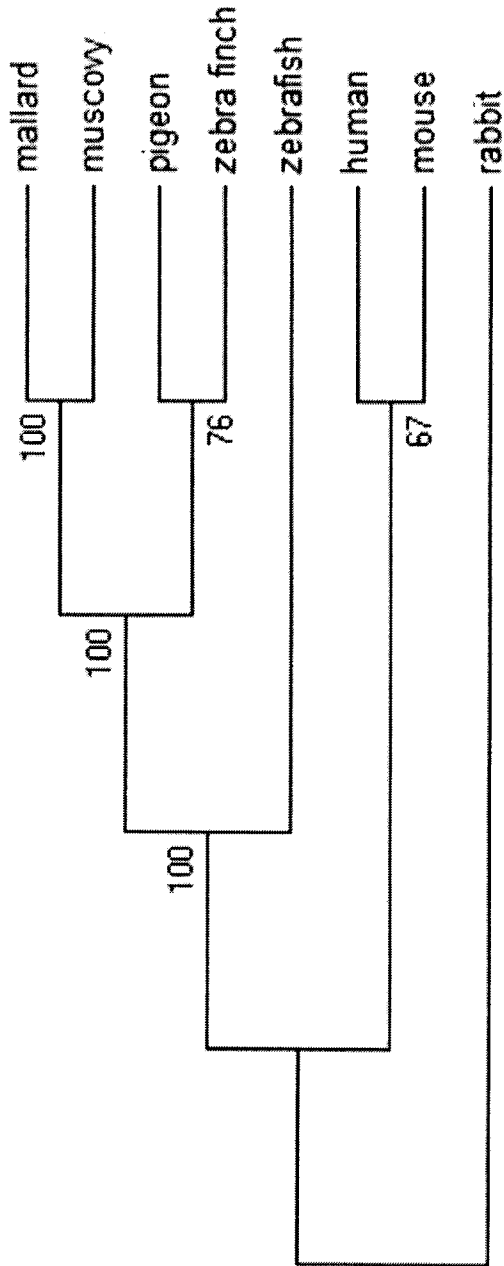


Figure 5B RIG-I protein phylogenetic tree



8/17

Figure 5C. Phylogenetic tree of avian RIG-I proteins

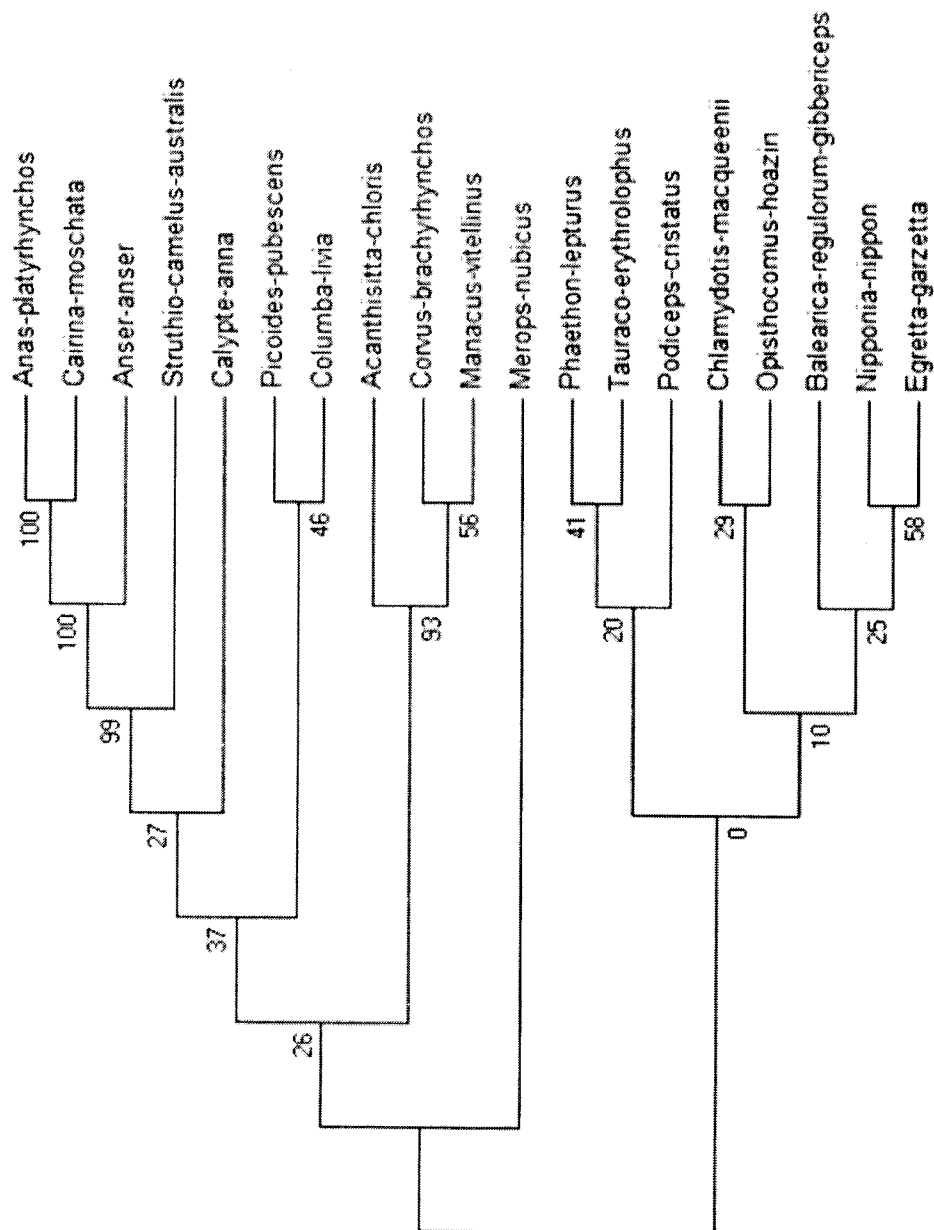


Figure 6

>duck RIG-I promoter (-204 to -1)
AGCTGATGACCTGCAAAAAGTTGGACATGCAAAATTAAGCTGAGGAACACAACACTACACTATGCTCTGAAAAAGAA
AAATAATAATAACAATCAAGAAAGCATTTATCCACCAGCTGGACCACACTCTTAAGGCAGGACTTGGTGGTGCACAC
AGGACCCCTTCAGACAGCCACAAGTGATTTTGGGAAAAGTAACATAATTTCTACACCCCTTGCCTAGAAAAGTTCTCCAGA
AAACTGCTCAGCACCATGGTTTGAGTAGCACAGCAGGTTAAGGTCTTCCAGTCTTCCAGCTTCTTCTACCCGATGTG
CTTGCCACTGCAACCCACCAAGTTACGATGTAGCATAAACAGCCACTGAGGATATGAGCATGAGATACCGACAGGCCAC
AACCCATCCCACTGCACCACTGCCCTGCACCAACAAGCCTGGGAGAGCCTCTGTGCTCACCAAGGTGCATCTCCAGCCTG
GTGGGACTGATCCCTGCTGCCCTCAGTACCCCTCCTGCAGCCACCTGCCAGAGGTGCATCCACCTCTCTGCATGCACGA
TCAGAGGTGCTATTCCTCCCTGCACTCCAGTCCAGCTTTGTTGAAGAGCAGCAGGTCCACTTCACTGGGCAGATTGACTT
CTCCATCTAGGGCTGGAGAGCAGCAAGCAAGGAGTTTGATTTCTCTCTCTCAGGTGAAGGACAAGACTGGGTTTCT
GCTTGGCAGAGCAATTGCAACCCACCTCAGCACTGATCTTTGAATCTTCGAACACTCTGTCTGTGAGGAGCAGAACCTGA
GTGCTAATATCACTTACTGAAATGAAGCTGTAATAGGCAATTTCTCAAGTGGAATAGGCTCAACAGGCACCTACCAAGTA
AAATGCCCTCTCTGTAITTTTGTACCTGTGAGGCTAATCACCATGCTCTACAGGGTGTGTGGGCAACTCACGTAGGGGC
TAACGCTTCTTCTTGTTACTCACAAAGCAATTAAGCCCTAAGAGATTACCTTACTGAAAATGCAGGGTGGAGAGATGACTG
ACAGGGGCTTGTTCACCTGCCCTTTCTTGTGCAGAAAGATGTCCTCTCTCAGTTGTTCAAGCATTCCTCCAGTCTAGCAGTGG
ATAGCCTTCTGTGCTTGTGTCAGCTGTGCATGATATTTCTCTGGTCTCTCTCAATTGCTCTGTTAAGCTAACAGCCCAATGC
AAGTCTCTCCTAGCCTAGGGCCATTTGTAGCCTAGGGCAACAGCAGCTGGGAGAGAGTGAGAAATGTGAAGAACTTA
CATTTGGGATCTTTGGGAGCTGCTGCCCATGGGGACCCGTGCTGGAGCAGTTTGTCTCTGGGATGGATGGATGGA
CCCCGTGGACGGAGCCGTGTGGGAGCAGTGCTTGAAGAGCTGTGCTGTGGGAGCCCTCCGCGGGATCGTTCCGGG
AAGGACGGCATCCCTGGAGGGACCCCATGGGGAGCAGGGGAGGACCGTGAGGGAGAGGGGCGGGA
GACAAAGTCCAGGGACTGACCGCAGCCCCCATTCCTGCACTGCTGGGGGGAAGAGGTAGAGGAGGGTGGATGG
TGGGGAAGGTGTTTTCAATTCCTTTTATTTTCTCACTGCTAGTCTGTTAGTAGGCAATTAATTCTCTCCCATCTCT
TGTGCCAACCCCTGAGCCCTTTCATCGCATTTTCTCCCGTTTCCCTTGGAGGCGGGAGTGGGAGAGCGGCTGTGCTG
GACCCAGGCGGTGTCTCGGTCTCCGGTGAGGCTGTGGTGCGATCCGAGGCTCACGAAGCTGCGAGCCGCTGCCTA
TGCCCGCTGGCGGCCAGAGGGCGGTGCGGATGGGCGCGATCTCCACCCCGGGGGCGGCTTTCGTTTTCCAT
TCCCGCGGGGCTCGCTGCTTCTGTGCGCAGCCGTTGGGGGCGGGCCGGCGGGCGGGCGGGCGGGCGGGCGGCA
GAGCCCCAGCC

Figure 6
(continued)

[illegible]

Ric-1 protein alignment

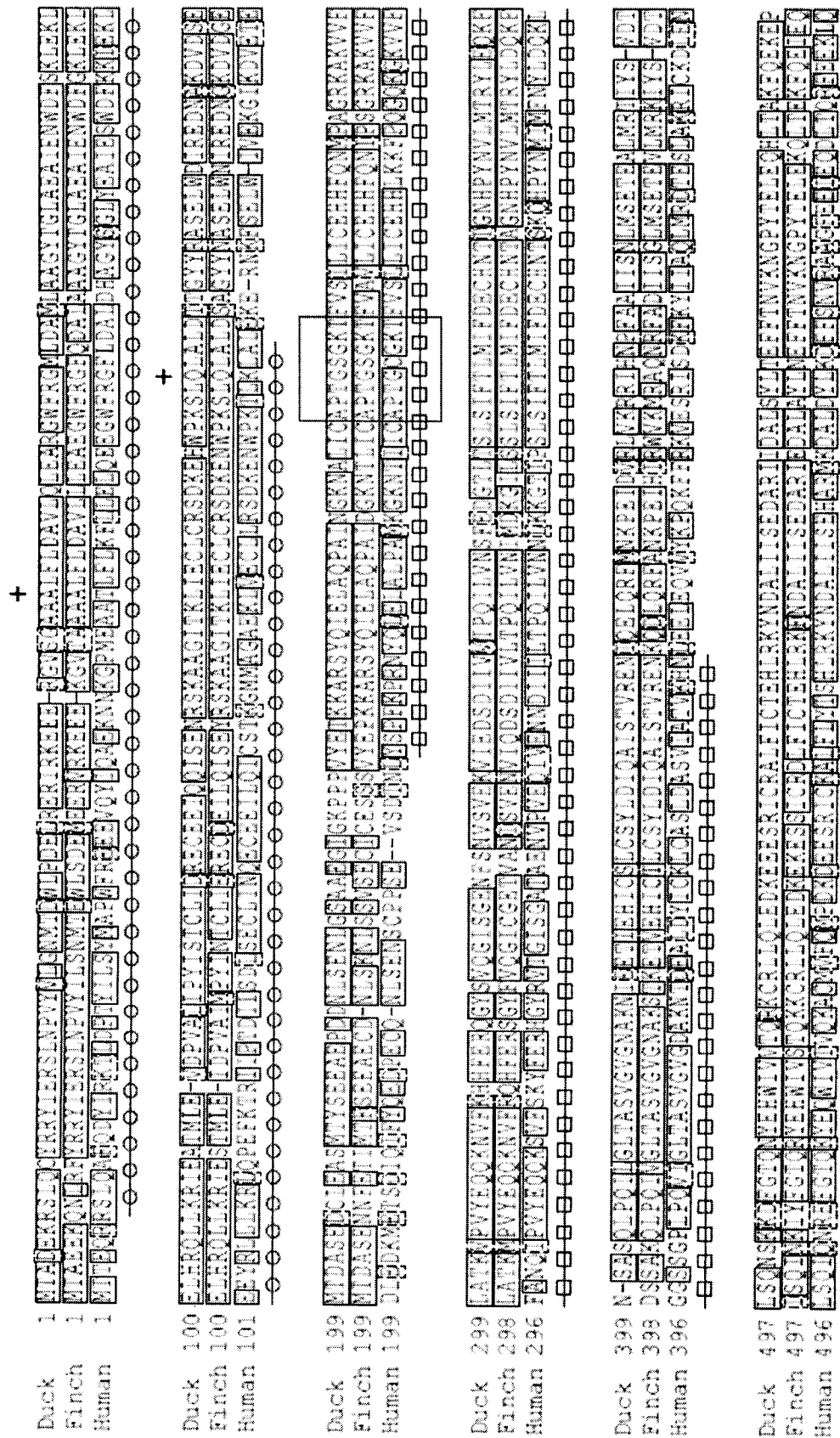


Figure 7A (continued)

	597	598	599	600	601	602	603	604	605	606	607	608	609	610	611	612	613	614	615	616	617	618	619	620	621	622	623	624	625	626	627	628	629	630	631	632	633	634	635	636	637	638	639	640	641	642	643	644	645	646	647	648	649	650	651	652	653	654	655	656	657	658	659	660	661	662	663	664	665	666	667	668	669	670	671	672	673	674	675	676	677	678	679	680	681	682	683	684	685	686	687	688	689	690	691	692	693	694	695	696	697	698	699	700
Duck	EL	AL	SK	DE	EN	EN	PK	TE	ED	IL	DA	YV	NP	OT	RT	IL	FA	K	TR	AL	VS	AL	X	CO	EE	NE	PI	IN	K	P	GM	MG	RR	DO	TC	HT	LE	PS	OK	GV	DA	RT	SK	IN	RU	IL	AL	SK	DE	EN	EN	PK	TE	ED	IL	DA	YV	NP	OT	RT	IL	FA	K	TR	AL	VS	AL	X	CO	EE	NE	PI	IN	K	P	GM	MG	RR	DO	TC	HT	LE	PS	OK	GV	DA	RT	SK	IN	RU														
Finch	EL	AL	SK	DE	EN	EN	PK	TE	ED	IL	DA	YV	NP	OT	RT	IL	FA	K	TR	AL	VS	AL	X	CO	EE	NE	PI	IN	K	P	GM	MG	RR	DO	TC	HT	LE	PS	OK	GV	DA	RT	SK	IN	RU	IL	AL	SK	DE	EN	EN	PK	TE	ED	IL	DA	YV	NP	OT	RT	IL	FA	K	TR	AL	VS	AL	X	CO	EE	NE	PI	IN	K	P	GM	MG	RR	DO	TC	HT	LE	PS	OK	GV	DA	RT	SK	IN	RU														
Human	EL	ES	IS	DE	EN	EN	PK	TE	ED	IL	DA	YV	NP	OT	RT	IL	FA	K	TR	AL	VS	AL	X	CO	EE	NE	PI	IN	K	P	GM	MG	RR	DO	TC	HT	LE	PS	OK	GV	DA	RT	SK	IN	RU	IL	AL	SK	DE	EN	EN	PK	TE	ED	IL	DA	YV	NP	OT	RT	IL	FA	K	TR	AL	VS	AL	X	CO	EE	NE	PI	IN	K	P	GM	MG	RR	DO	TC	HT	LE	PS	OK	GV	DA	RT	SK	IN	RU														

[illegible]

	797	797	796
Duck	LEP	LEP	LEP
Finch	NEP	NEP	NEP
Human	HEP	HEP	HEP

Duck	697	DEETATIG	QVDFCKWKX	SIN	KNED	VEEMS	NTLYRPI
Finch	697	DEETESG	QVDFCKA	SIN	KNED	ETCS	-----
Human	694	DEETATIG	QVDFCKA	DEETESG	QVDFCKA	DEETESG	-----

○ ○ ○ ○ CARD
□ □ □ □ DEXD box
◇ ◇ ◇ ◇ Helicase
× × × × C-terminal Regulatory

Figure 7B

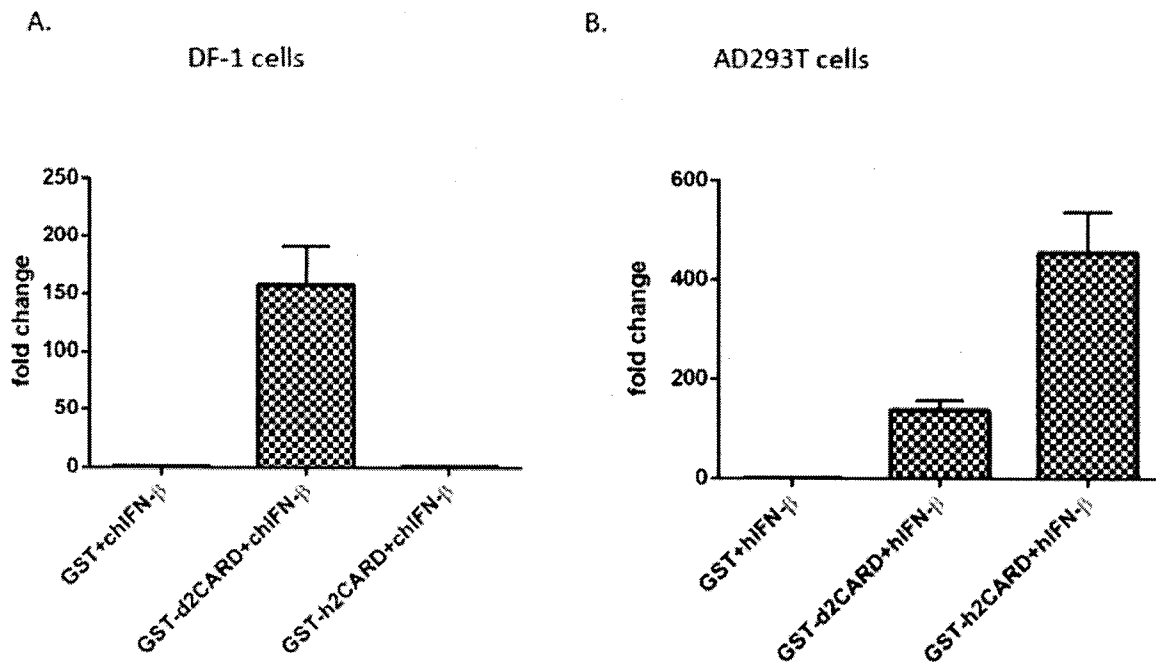


Figure 7C

Anser-anser----	1	MTADEKRSLOCYRRYIERSLNPVYVLGNMADWLPDELRRIRKEEERGVSGAAALFLDAV
Anas-platyrhync	1	MTADEKRSLOCYRRYIERSLNPVYVLGNMTDWPDELRRIRKEEERGVSGAAALFLDAV
Cairina-moschat	1	MTADEKRSLOCYRRYIERSLNPVYVLGNMTDWPDELRRIRKEEERGVSGAAALFLDAV
Columba-livia--	1	-----MKRERVRKEEEKGVTAATAALFLDIT
Picoides-pubesc	1	MTAEKRSLOCYRRYIEKSLNPVYIISNMTDWTSDERVRKEEEKGVTAATAAMFLDVT
Anser-anser----	61	LQLEARGWFRGMLDAMLAAGHTGLAEAEIENWDFSKLEKLELHRQLLKRIEATMLEVDPVV
Anas-platyrhync	61	LQLEARGWFRGMLDAMLAAGYTGLAEAEIENWDFSKLEKLELHRQLLKRIEATMLEVDPVA
Cairina-moschat	61	LQLEARGWFRGMLDAMLAAGYTGLAEAEIENWDFSKLEKLELHRQLLKRIEATMLEVDPVA
Columba-livia--	25	LQLEARGWLRGFLDAIAAAGYTGLAEAEIENWDFSKLEKLELYRQLLKRIEATMLEVDPVA
Picoides-pubesc	61	LELEERMLRGFLDAIAAAGYTGLAEAEIENWDFSKLEKLELHRFLLKRIEATMLEVDPVM
Anser-anser----	121	LIPYINTCLIDRECEEEIQIQLSESRSKAAGITKLTCLCRSDKEHWPKSLQLALDNTGYR
Anas-platyrhync	121	LIPYISTCLIDRECEEEIQIQLSENRSKAAGITKLTCLCRSDKEHWPKSLQLALDTTGYR
Cairina-moschat	121	LIPYINTCLIDRECEEEIQIQLSENRSKAAGITKLTCLCRSDKEHWPKSLQLALDTTGYR
Columba-livia--	85	LPYMTSCLIIFRECEEEIQIQLSEMYRSKAAGITKLTCLCRSDKENWPKSLQLALDNTGYN
Picoides-pubesc	121	MPYINTCLIFRECEEEIQIQLSEMRSKAAGITKLTCLCRSDKENWPKSLQLALDNAGYNN
Anser-anser----	181	ASELWDIREDNAKDVDSEMTDASEDCLETSMTYSEEAEPDDNLSENLSGAAEGIDKPPPV
Anas-platyrhync	181	ASELWDIREDNAKDVDSEMTDASEDCLEASMTYSEEAEPDDNLSENLSGAAEGICKPPPV
Cairina-moschat	181	ASELWDIREDNAKDVDSEMTDASEDCLEASMTYSEEAEPDDNLSENLSGAAEGICKPPPV
Columba-livia--	145	ASELWDIREDNAKDVDCEMA DASESSFEIRMTIFSEEAECNNLSENLSGAAEGICKPPPV
Picoides-pubesc	181	ASELWDIREDNAKDVDCEMCDASENSFEAMTIFSEEMECDFNFSENLSSASGCTNEPPAV

Figure 7C (continued)

Anser-anser----	241	YEAKKARSYQIELAQPAINGKNALICAPTSGGKTFEALILICEHHFQNMMPAGRKQKVVFLA
Anas-platyrhync	241	YETKKARSYQIELAQPAINGKNALICAPTSGGKTFVSILICEHHFQNMMPAGRKAKVVFLA
Cairina-moschat	241	YETKKARSYQIELAQPAINGKNALICAPTSGGKTFVSILICEHHFQNMMPAGRKAKVVFLA
Columba-livia--	204	YEPKARSYQIELAQPAINGKNALICAPTSGGKTFVAILICEHHFQNMFTQKAKVVFLA
Picoides-pubesc	240	YEAKEARSYQIELAQPAINGKNALICAPTSGGKTFVALICEHHFQNMVEPERKAKIVFLA
Anser-anser----	301	TKVPVYEQQKNVFKQHFERQGYSGISGENFSNVSVENVEDNDIIVLTPOILVNSFED
Anas-platyrhync	301	TKVPVYEQQKNVFKHFERQGYSVQGISGENFSNVSEKVIEDSDIIVMTPPOILVNSFED
Cairina-moschat	301	TKVPVYEQQKNVFKHFERQGYSVQGISGENFSNVSEKVIEDSDIIVMTPPOILVNSFED
Columba-livia--	264	TKVPVYEQQKNVFKQHFERQGYSVQGISGENFSNVSEKVIQDSDIIVLTPOILVNIKK
Picoides-pubesc	300	TKVPVYEQQKNVFKQHFERQGYSVRGICGETVANPEVENVEDSDIIVLTPOILVNCQKQ
Anser-anser----	361	GTLTSLSMFTLMIFDECHNTTGNHPYNVLMTRYLEQKFNSPASQLPQILGLTASVGVGNA
Anas-platyrhync	361	GTLTSLSIFTLMIFDECHNTTGNHPYNVLMTRYLEQKFNSPASQLPQILGLTASVGVGNA
Cairina-moschat	361	GTLTSLSIFTLMIFDECHNTTGNHPYNVLMTRYLEQKFNSPASQLPQILGLTASVGVGNA
Columba-livia--	324	GTLSSLSIFTLMIFDECHNTTGNHPYNVLMTRYLEQKFSSANQLPQIMGLTASVGVGNA
Picoides-pubesc	360	GTLTSLSMFTLMIFDECHNTTGNHPYNVLMTRYLEQKFSSASQLPQIMGLTASVGVGNA
Anser-anser----	421	KNIEETIEHICSLCSYLDIQAISTVRENIQDLQRFMKNKPEIDVRLVKRRMENEFAMIIISD
Anas-platyrhync	420	KNIEETIEHICSLCSYLDIQAISTVRENIQELQRFMKNKPEIDVRLVKRRMENEFAMIIISN
Cairina-moschat	421	KNIEETIEHICSLCSYLDIQAISTVRENIQELQRFMKNKPEIDVRLVKRRMENEFAMIIISD
Columba-livia--	384	KDITTTIEHICSLCSYLDIQAISTVRENKEELQRFMKNKPEIDVRLVKRRMENEFAMIIISG
Picoides-pubesc	420	RSITKEAIEHICSLCSYLDIQAISTVRENKEELQRFMKNKPEIDVRLVKRRMENEFAMIIISG

Figure 7C (continued)

Anser-anser----	481	LMSETEALMRKIYSVCT	MSONSP	KDFGTQ	YEHWIVVTQ	KCRLLQ	LEDKEE	ESRICRA	
Anas-platyrhync	480	LMSETEALMRTIYSVD	TLSONSKK	DFGTQ	YEHWIVVTQ	KCRLLQ	LEDKEE	ESRICRA	
Cairina-moschat	481	LMSETEALMRTIYSVD	TIQNSKK	DFGTQ	YEHWIVVTQ	KCRLLQ	LEDKEE	ESRICRA	
Columba-livia--	444	LMSETEALMRRIYS	TDISQMN	KNDFGTQ	YEHWIVVTQ	KYRLLQ	LADKEE	ESSICRDL	
Picoides-pubesc	480	LMSETEALMRRIYSVD	TIQNSKN	DFGTQS	YEQWIVVTQ	KCRLLQ	LADKEE	ESSMCRDL	
Anser-anser----	541	FICTEHLRKYNDA	LIISEDARI	IDS	SYLT	TEFTNV	KNGPYTE	LEQHLTAKEQEKEPELT	
Anas-platyrhync	540	FICTEHLRKYNDA	LIISEDARI	IDS	SYLT	TEFTNV	KNGPYTE	LEQHLTAKEQEKEPELT	
Cairina-moschat	541	FICTEHLRKYNDA	LIISEDARI	IDS	SYLT	TEFTNV	KNGPYTE	LEQHLTAKEQEKEPELT	
Columba-livia--	504	FICTEHLRKFNDA	LIISEDARI	IDS	SYLT	TEFTNV	KNGPFF	TELEKQLTARFOEKEPELT	
Picoides-pubesc	540	FICTEHLRKFNDA	LIISEDARI	IDS	SYLT	TEFTNV	KNGPYT	QLEKRLTARFOEKEPELT	
Anser-anser----	601	ALSKDET	NENPKLEELAC	ILDDAY	CYNPQ	TRTLL	FAKTRAL	VAALKKCM	EENPILSYIKP
Anas-platyrhync	600	ALSKDET	NENPKLEELAC	ILDDAY	RYNPQ	TRTLL	FAKTRAL	VAALKKCM	EENPIIN
Cairina-moschat	601	ALSKDET	NENPKLEELAC	ILDDAY	RYNPQ	TRTLL	FAKTRAL	VAALKKCM	EENPILSYIKP
Columba-livia--	564	ALSKDQ	SNENPKLEELAC	ILDDAY	RYNPQ	TRTLL	FAKTRAL	VAALKKCM	EENKANPILSHIKP
Picoides-pubesc	600	ALSKDET	SENPKLEELAC	ILDDAY	RYNSD	TRTLL	FAKTRAL	VAALKKCM	EENPILSHMKP
Anser-anser----	661	DVLMGR	RRDQKTG	MTLPSQ	KGVLDA	FAKTSKD	SRLLI	ATSVADE	GIDIAQC
Anas-platyrhync	660	DVLMGR	RRDQKTG	MTLPSQ	KGVLDA	FAKTSKD	SRLLI	ATSVADE	GIDIMQC
Cairina-moschat	661	DVLMGR	RRDQKTG	MTLPSQ	KGVLDA	FAKTSKD	SRLLI	ATSVADE	GIDIMQC
Columba-livia--	624	DVLMG	HGGRDQKTG	MTLPSQ	KGVLDA	FAKTSKD	SRLLI	ATSVADE	GIDISECN
Picoides-pubesc	660	DVLMG	HGGRDQKTG	MTLPSQ	KGVLDA	FAKTSKD	SRLLI	ATSVADE	GIDISECN

Figure 7C (continued)

Anser-anser----	721	GNVT	KMIQVR	GRGR	AGSK	CLLV	TSKTE	VVEN	EKCN	RYKE	EMMN	KAL	EKLQ	E	WDE	ETFAK
Anas-platyrhync	720	GNVT	KMIQVR	GRGR	AGSK	CLLV	TSKTE	VVEN	EKCN	RYKE	EMMN	KAVE	KIQ	K	WDE	ETFAK
Cairina-moschat	721	GNVT	KMIQVR	GRGR	AGSK	CLLV	TSKTE	VVEN	EKCN	RYKE	EMMN	TAVE	KIQ	K	WDE	ETFAK
Columba-livia--	684	GN	TKMIQVR	GRGR	ARD	SKCL	LVTS	RAT	VVENE	KHN	CYKE	EMMN	EAT	ENLQ	NWDE	TI FAR
Picoides-pubesc	720	GNVT	KMIQVR	GRGR	ARN	SKCL	LVTS	KTE	VVEN	EKHN	RYKE	EMMN	EAVE	ECLQ	NWDE	AA FAR
Anser-anser----	781	KIHNLQ	MKE	VLRD	SRKKE	IKPK	VVEG	QKNLL	CGKCK	KAYAC	STDD	IRI	IK	ESH	IVL	GEA
Anas-platyrhync	780	KIHNLQ	MKE	VLRD	SRKKE	IKPK	VVEG	QKNLL	CGKCK	KAYAC	STDD	IRI	IK	ESH	IVL	GEA
Cairina-moschat	781	KIHNLQ	MKE	VLRD	SRKKE	IKPK	VVEG	QKNLL	CGKCK	KAYAC	STDD	IRI	IK	ESH	IVL	GEA
Columba-livia--	744	KIQE	LQMKEN	VLRD	SRKKE	IKPK	GAEK	KNLL	CGKCK	KAYAC	STDD	IRI	IK	ESH	IVL	GEA
Picoides-pubesc	780	KIHNLQ	MKE	VLRD	SRKKE	IKPK	VVEG	QKNLL	CGKCK	KAYAC	STDD	IRI	IK	ESH	IVL	GEA
Anser-anser----	841	EKERY	ITKPH	KKPMQ	FDGFE	KKSKM	HC	RNNN	CQHD	WGIT	VKYL	TFD	NLP	VIKIK	SFV	VS
Anas-platyrhync	840	EKERY	ITKPH	KKPMQ	FDGFE	KKSKM	HC	RNNN	CQHD	WGIT	VKYL	TFD	NLP	VIKIK	SFV	VS
Cairina-moschat	841	EKERY	ITKPH	KKPMQ	FDGFE	KKSKM	HC	RNNN	CQHD	WGIT	VKYL	TFD	NLP	VIKIK	SFV	VS
Columba-livia--	804	EKERY	ITKPH	KKPMQ	FDGFE	KKSKM	HC	RNNN	CQHD	WGIT	VKYL	TFD	NLP	VIKIK	SFV	VS
Picoides-pubesc	840	EKERY	ITKPH	KKPMQ	FDGFE	KKSKM	HC	RNNN	CQHD	WGIT	VKYL	TFD	NLP	VIKIK	SFV	VS
Anser-anser----	901	AATG	QMD	FQK	WKS	INSS	LKNF	DVE	EMS	NLY	PPE					
Anas-platyrhync	900	TATG	QMD	FQK	WKS	INSS	LKNF	DVE	EMS	NLY	PPE					
Cairina-moschat	901	TATG	QMD	FQK	WKS	INSS	LKNF	DVE	EMS	NLY	PPE					
Columba-livia--	864	VE	TRQMD	FQK	WKS	INSS	LKNF	DVE	EMS	NLY	PPE					
Picoides-pubesc	900	VE	TRQMD	FQK	WKS	INSS	LKNF	DVE	EMS	NLY	PPE					

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2018/051216

A. CLASSIFICATION OF SUBJECT MATTER

IPC: **C12N 15/85** (2006.01), **A01K 67/02** (2006.01), **A01K 67/027** (2006.01), **C07K 14/465** (2006.01),
C12N 15/113 (2010.01), **C12N 15/12** (2006.01) (more IPCs on the last page)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 Keywords searched across all IPCs.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Databases: Questel Orbit, Scopus, Genome Quest

Keywords: retinoic acid inducible gene I, RIG-I, RIG I, duck+, chicken+, transgenic, Magor, Katharine, Xiao, Yanna, SEQ ID NOs: 1-9

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO2005084430 A1 (CHRISTMANN, L. et al.) 15 September 2005 (15-09-2005) *entire document*	14
A	US20110247091 A1 (MAGOR, K. et al.) 6 October 2011 (06-10-2011) *entire document*	1-16

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* "A" "E" "L" "O" "P"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance earlier application or patent but published on or after the international filing date document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	"T" "X" "Y" "&"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art document member of the same patent family
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Date of the actual completion of the international search
 16 November 2018 (16-22-2018)

Date of mailing of the international search report
 04 January 2019 (04-01-2019)

Name and mailing address of the ISA/CA
 Canadian Intellectual Property Office
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 Gatineau, Quebec K1A 0C9
 Facsimile No.: 819-953-2476

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/CA2018/051216

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO2005084430A1	15 September 2005 (15-09-2005)	AU2002351192A1 AU2002351192B2 CA2467648A1 CA2467648C EP1461430A2 EP1461430A4 EP1608219A2 EP1608219A4 EP1633781A2 EP1708564A2 EP1708564A4 EP1725091A1 JP2005536984A US2003126628A1 US6875588B2 US2005003414A1 US7294507B2 US2005176047A1 US7335761B2 US2005050581A1 US7375258B2 US2004226057A1 US7381712B2 US2007113299A1 US7507873B2 US2009193534A1 US7795496B2 US2009182130A1 US7812215B2 US2004210954A1 US2004255345A1 US2005034186A1 US2005066383A1 US2005198700A1 US2005273873A1 US2006123504A1 US2006174364A1 US2008222744A1 US2009188002A1 US2010333219A1 WO03048364A2 WO03048364A3 WO2004080162A2 WO2004080162A3 WO2004092351A2 WO2004092351A3 WO2005040215A2 WO2005040215A3 WO2006093847A1	17 June 2003 (17-06-2003) 14 December 2006 (14-12-2006) 12 June 2003 (12-06-2003) 12 June 2012 (12-06-2012) 29 September 2004 (29-09-2004) 13 April 2005 (13-04-2005) 28 December 2005 (28-12-2005) 14 March 2007 (14-03-2007) 15 March 2006 (15-03-2006) 11 October 2006 (11-10-2006) 07 March 2007 (07-03-2007) 29 November 2006 (29-11-2006) 08 December 2005 (08-12-2005) 03 July 2003 (03-07-2003) 05 April 2005 (05-04-2005) 06 January 2005 (06-01-2005) 13 November 2007 (13-11-2007) 11 August 2005 (11-08-2005) 26 February 2008 (26-02-2008) 03 March 2005 (03-03-2005) 20 May 2008 (20-05-2008) 11 November 2004 (11-11-2004) 03 June 2008 (03-06-2008) 17 May 2007 (17-05-2007) 24 March 2009 (24-03-2009) 30 July 2009 (30-07-2009) 14 September 2010 (14-09-2010) 16 July 2009 (16-07-2009) 12 October 2010 (12-10-2010) 21 October 2004 (21-10-2004) 16 December 2004 (16-12-2004) 10 February 2005 (10-02-2005) 24 March 2005 (24-03-2005) 08 September 2005 (08-09-2005) 08 December 2005 (08-12-2005) 08 June 2006 (08-06-2006) 03 August 2006 (03-08-2006) 11 September 2008 (11-09-2008) 23 July 2009 (23-07-2009) 30 December 2010 (30-12-2010) 12 June 2003 (12-06-2003) 12 September 2003 (12-09-2003) 23 September 2004 (23-09-2004) 31 March 2005 (31-03-2005) 28 October 2004 (28-10-2004) 31 March 2005 (31-03-2005) 06 May 2005 (06-05-2005) 30 June 2005 (30-06-2005) 08 September 2006 (08-09-2006)
US2011247091A1	06 October 2011 (06-10-2011)	None	

CONTINUATION OF CLASSIFICATION OF SUBJECT MATTER:

C12N 5/10 (2006.01)