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(54) Title: GEMININ INHIBITORS AS TUMOR TREATMENT

(57) Abstract: Disclosed herein are methods of selectively killing cancer cells. The methods include contacting a cancer cell with a compound that inhibits geminin. Disclosed herein are methods that selectively induce apoptosis in cancer cells in the absence of a cell cycle checkpoint inhibitor. Also disclosed are methods for identifying a compound that selectively kills cancer cells, including contacting a cancer cell with a test compound, determining the cell cycle status of the cancer cell, and determining geminin activity in the cancer cell, wherein a compound that induces DNA re-replication, cell cycle arrest, or apoptosis and inhibits geminin activity is a compound that selectively induces apoptosis of cancer cells.



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GEMININ INHIBITORS AS TUMOR TREATMENT

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No.
5 61/106,465, filed October 17, 2008, which is incorporated herein in its entirety.

FIELD

This disclosure relates to methods of selectively killing cancer cells,
particularly with inhibitors of geminin. Further, the disclosure relates to methods of
10 identifying compounds that selectively kill cancer cells by inducing DNA re-
replication, such as inhibitors of geminin.

BACKGROUND

Cell division throughout the eukaryotic kingdom is normally restricted to
15 duplicating the genome once and only once each time a cell divides (DePamphilis *et al.*, In *DNA Replication and Human Disease* (Cold Spring Harbor Laboratory Press, NY), 313-334, 2006). To this end, genome duplication is regulated through multiple convergent pathways. When these mechanisms are subverted, DNA re-replication occurs during genome duplication, resulting in abnormal replication structures and
20 DNA damage, events that are detected by the ATM, ATR-CHK1, CHK2-CDC25 DNA damage response pathways. Triggering these pathways rapidly arrests cell proliferation and, if the problem is not corrected, induces apoptosis.

Eukaryotic DNA replication requires assembly of pre-replication complexes (preRCs) at multiple sites throughout the genome (Aladjem *et al.*, In *DNA*
25 *Replication and Human Disease* (Cold Spring Harbor Laboratory Press, NY), 31-62, 2006; Costa and Blow, *EMBO Rep.* 8:332-334, 2007; Sivaprasad *et al.*, In *DNA Replication and Human Disease* (Cold Spring Harbor Laboratory Press, NY), 63-88, 2006). In mammalian cells, this process begins when the six subunit origin recognition complex (ORC) binds to newly replicated DNA during the M to G1-
30 phase transition in the cell division cycle (Noguchi *et al.*, *EMBO J.* 25:5372-5382, 2006). This event is accompanied by binding of CDC6 and then CDT1, both of

which are needed to recruit the minichromosome maintenance proteins (MCM2-7) for initiation of DNA replication. Once S-phase begins, further assembly of preRCs is prevented by phosphorylation, ubiquitination and degradation of preRC proteins, in particular ORC1, CDC6 and CDT1, and by geminin, a specific inhibitor of CDT1 activity that is unique to metazoa.

Geminin is a 25 kDa protein that binds specifically to CDT1 and inhibits its activity while simultaneously protecting it from ubiquitination and degradation. Once DNA replication begins, geminin binds to CDT1 and prevents reassembly of the preRC complex. Geminin levels remain high during G2 phase. Geminin is degraded by the anaphase promoting complex as cells exit metaphase, releasing CDT1 and allowing reassembly of the preRC complex and a new round of DNA synthesis. Thus, geminin prevents cells from re-replicating those portions of the genome that already have been duplicated, while at the same time preventing the complete loss of CDT1 from the cell.

SUMMARY

Disclosed herein are methods for selectively killing cancer cells, for example, without killing non-cancer cells. In particular examples, the methods include inducing apoptosis of cancer cells without inducing apoptosis of non-cancer cells. The methods include contacting a cancer cell with an inhibitor of geminin (such as a compound that induces DNA re-replication in cancer cells) in the absence of a cell cycle checkpoint inhibitor (such as without added or exogenous cell cycle checkpoint inhibitor, for example, less than 10 nM cell cycle checkpoint inhibitor (such as less than about 5 nM, less than about 1 nM, or less than about 0.1 nM cell cycle checkpoint inhibitor)).

In some embodiments, the method includes contacting a cancer cell with a compound that inhibits geminin in the absence of a cell cycle checkpoint inhibitor for a period of time sufficient to induce apoptosis of the cancer cell without inducing apoptosis of non-cancer cells, thereby selectively inducing apoptosis of the cancer cell. In some examples, the period of time sufficient to induce apoptosis of the cancer cell without inducing apoptosis of non-cancer cells is at least about two to

eight days (such as about four to eight days). In some examples, the inhibitor inhibits or decreases geminin expression or geminin activity. As disclosed herein, the geminin inhibitor selectively kills cancer cells in the absence of a cell cycle checkpoint inhibitor. In particular examples, the inhibitor of geminin includes an
5 RNAi molecule, a peptide, an antibody, a small organic molecule, an antisense molecule, or a combination of two or more thereof.

In particular examples, the cancer cell is a colon cancer cell, a breast cancer cell, a lung cancer cell, a kidney cancer cell, a bone cancer cell, or a brain cancer cell. In some examples, the method includes contacting the cancer cell with the
10 geminin inhibitor for at least four days. In some examples, the cancer cell is contacted with the geminin inhibitor for about two to about eight days.

In additional examples, the methods include contacting a cancer cell with an inhibitor of geminin by administering a therapeutically effective amount of the compound that inhibits geminin to an individual in need of treatment for cancer. For
15 example, the inhibitor of geminin can be administered to a subject (such as a subject with cancer) in a preselected dose for a preselected period of time to achieve the desired tissue concentration of the inhibitor at the target tissue (for example, a tumor). The methods also include administering the geminin inhibitor and a second anti-cancer therapeutic to a subject. In additional embodiments, the methods include
20 isolating a cancer cell from a subject, determining whether the cancer cell expresses or overexpresses geminin, and administering a therapeutically effective amount of the compound that inhibits geminin to the subject if the cancer cell expresses or overexpresses geminin.

Also disclosed are methods for identifying a compound that selectively kills
25 cancer cells, including contacting at least one test compound with a cancer cell expressing geminin in the absence of a cell cycle checkpoint inhibitor (such as without added or exogenous cell cycle checkpoint inhibitor, for example, less than 10 nM cell cycle checkpoint inhibitor (such as less than about 5 nM, less than about 1 nM, or less than about 0.1 nM cell cycle checkpoint inhibitor)), assessing the cell
30 cycle status of the cancer cell (such as DNA re-replication, cell cycle arrest, or apoptosis), and assessing geminin activity (such as geminin expression or geminin-

Cdt1 binding) wherein a compound that induces DNA re-replication, apoptosis, or cell cycle arrest in cancer cells (such as induces DNA re-replication, apoptosis, or cell cycle arrest more in cancer cells than in non-cancer cells) and inhibits geminin activity in cancer cells (such as inhibits geminin activity more in cancer cells than in non-cancer cells) indicates that the compound is a compound that selectively kills cancer cells. In some examples, the cancer cell is contacted with the geminin inhibitor for about two to about eight days. In particular examples, the test compound includes RNAi molecules, peptides, antibodies, small organic molecules, antisense molecules, or a combination of two or more thereof.

The foregoing and other features will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the effect of depletion of geminin on DNA re-replication in human colorectal cancer cell lines. FIG. 1A shows SW480 colon adenocarcinoma cells. FIG. 1B shows HCT116 colon carcinoma cells. FIG. 1C shows DLD-1 colon adenocarcinoma cells. FIG. 1D shows COLO 320 DM colon adenocarcinoma cells. Each panel shows quantification of DNA content by FACS analysis (top) with the percentage of cells with greater than 4N DNA content indicated in the FACS profile; analysis of nucleus size by DAPI staining (middle) with percentage of cells with giant nuclei (diameter greater than twice that of nuclei in siGL2 treated cells) indicated in the fluorescence microscope image; and Western blotting of geminin and actin proteins (bottom) in cells treated with control siRNA (siGL2) or anti-geminin siRNA (siGem).

FIG. 2 shows the effect of depletion of geminin on DNA re-replication in normal human fetal colon cell lines. FIG. 2A shows CCD 841 CoN cells. FIG. 2B shows FHC cells. Each panel shows quantification of DNA content by FACS analysis (top) with the percentage of cells with greater than 4N DNA content indicated in the FACS profile; analysis of nucleus size by DAPI staining (middle) with percentage of cells with giant nuclei (diameter greater than twice that of nuclei

in siGL2 treated cells) indicated in the fluorescence microscope image; and Western blotting of geminin and actin proteins (bottom) in cells treated with control siRNA (siGL2) or anti-geminin siRNA (siGem).

FIG. 3A shows a series of FACS analyses showing the percentage of cells with greater than 4N DNA content in the indicated cell lines 48 hours after transfection with siGL2 or geminin siRNA (siGem, siGem2, or siGem3). FIG. 3B shows a photographs of Western blots for geminin and actin in the indicated cell lines 48 hours after transfection with the indicated siRNA.

FIG. 4A shows the total number of HCT116 cells transfected with either siGem (closed squares) or siGL2 (open squares) at indicated times post-transfection. FIG. 4B shows geminin and control protein in HCT116 cells transfected with either siGem or siGL2 detected by Western immuno-blotting at the indicated times post-transfection. The control protein is a protein that cross-reacts with the anti-geminin antibody. FIG. 4C shows the fraction of total HCT116 cells with giant nuclei in cells transfected with either siGem (closed squares) or siGL2 (open squares) at indicated times post-transfection. FIG. 4D shows FACS analysis of HCT116 cells transfected with either siGem or siGL2. The percentage of cells with less than 2N DNA content and the percentage of cells with greater than 4N DNA content are shown at the indicated times post-transfection.

FIG. 5A shows photomicrographs of TUNEL assay of human colon carcinoma HCT116 and normal skin fibroblast D-1 cells 4 days post-transfection with either siGL2 or siGem (left panel) and cells treated with DNase I (right panel), which provided a TUNEL positive control. FIG. 5B shows phase contrast images of HCT116 cells 4 days after transfection with either siGem or siGL2. Giant cells that have rounded up and detached from the dish are indicated by light color arrows, and those undergoing apoptosis (blebbing) are indicated by darker arrows. Both images were taken at the same magnification. FIG. 5C shows a photograph of a Western blot for the indicated proteins in HCT116, AG11132, and MCF10A cells 4 days post-transfection with siGL2 or siGEM. ETP, cells treated with 3 μ M etoposide for 16 hours in parallel with cells treated with the same solvent (DMSO). p53-P is p53 phosphorylated on serine 15.

FIG. 6 shows the effect of geminin depletion in normal human skin fibroblast D-1 cells. FIG. 6A shows the total number of cells transfected with either siGem (closed squares) or siGL2 (open squares) at indicated times post-transfection. FIG. 6B shows geminin and control protein in cells transfected with either siGem or siGL2 detected by Western immuno-blotting at the indicated times post-transfection. FIG. 6C shows FACS analysis of cells transfected with either siGem or siGL2. The percentage of cells with greater than 4N DNA content are shown at the indicated times post-transfection. FIG. 6D shows the percentage of cells transfected with siGem relative to the number of cells transfected with siGL2 6 days post-transfection.

FIG. 7A shows Western blotting of asynchronously proliferating populations of the indicated cell lines for geminin and actin. FIG. 7B shows FACS analysis showing the fraction of proliferating cells (S+G2+M phases).

FIG. 8 shows the effect of geminin and cyclin A siRNA on DNA content in normal and cancer cell lines. FIG. 8A shows normal human skin fibroblast D-1 cells. FIG. 8B shows human colon carcinoma HCT116 cells. Cells were transfected with siGL2, siGem, siCcnA, or both siGem and siCcnA, two days post-transfection, analyzed by FACS analysis to quantitate DNA content with the percentage of cells with greater than 4N DNA content indicated in the FACS profile (left panel) and Western blotting to determine the relative levels of cyclin A, Cdt1, geminin, Orc1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (right panel).

FIG. 9 shows results from a plate-based assay for identifying compounds that induce DNA re-replication. FIG. 9A shows a plate view of SW480 cells treated with DMSO or 3-(2-chloro-3-indolylmethylene)-1,3-dihydroindol-2-one (CDK1 inhibitor) and Hoechst 33342 and scanned with an Acumen® X3 microplate cytometer. FIG 9B is the total fluorescence intensity histogram, showing the distribution of cells in the cell cycle (G1, S, G2/M, and re-replicated cells having >4N DNA content).

SEQUENCE LISTING

Any nucleic acid and amino acid sequences listed herein or in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R.

- 5 1.822. In at least some cases, only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand.

SEQ ID NOs: 1 and 2 are exemplary human geminin cDNA and amino acid sequences respectively.

- 10 SEQ ID NO: 3 is the sequence of human geminin siRNA siGem.
 SEQ ID NO: 4 is the sequence of human geminin siRNA siGem2.
 SEQ ID NO: 5 is the sequence of human geminin siRNA siGem3.
 SEQ ID NO: 6 is the sequence of an exemplary luciferase siRNA.
 SEQ ID NO: 7 is the sequence of an exemplary human cyclin A siRNA.
 15 SEQ ID NO: 8 is the sequence of human geminin shRNA shGem1.
 SEQ ID NO: 9 is the sequence of human geminin shRNA shGem2.
 SEQ ID NO: 10 is the sequence of an exemplary luciferase shRNA.

DETAILED DESCRIPTION

20 I. Abbreviations

- BrdU:** 5-bromo-2-deoxyuridine
CcnA: cyclin A
DAPI: 4', 6'-diamidino-2-phenylindole
FACS: fluorescence-activated cell sorting
 25 **MCM:** minichromosome maintenance protein
ORC: origin recognition complex
Pre-RC: pre-replication complex
siCcnA: cyclin A siRNA
siGem: geminin siRNA
 30 **siGL2:** luciferase siRNA
siRNA: short interfering RNA
TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling
UCN-01: 7-hydroxystaurosporine

II. Terms

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

In order to facilitate review of the various embodiments of the disclosure, the following explanations of specific terms are provided:

Antibody: A protein (or protein complex) that includes one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the myriad of immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

The basic immunoglobulin (antibody) structural unit is generally a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" (about 50-70 kDa) chain. The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms "variable light chain" (V_L) and "variable heavy chain" (V_H) refer, respectively, to these light and heavy chains.

As used herein, the term "antibodies" includes intact immunoglobulins as well as a number of well-characterized functional fragments. For instance, Fabs, Fvs, and single-chain Fvs (SCFvs) that bind to target protein (or epitope within a protein or fusion protein) would also be specific binding agents for that protein (or epitope). These antibody fragments are defined as follows: (1) Fab, the fragment

which contains a monovalent antigen-binding fragment of an antibody molecule produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain; (2) Fab', the fragment of an antibody molecule obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule; (3) (Fab')₂, the fragment of the antibody obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; (4) F(ab')₂, a dimer of two Fab' fragments held together by two disulfide bonds; (5) Fv, a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and (6) single chain antibody, a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule. Methods of making these fragments are routine (see, for example, Harlow and Lane, *Using Antibodies: A Laboratory Manual*, CSHL, New York, 1999).

Antibodies for use in the methods of this disclosure can be monoclonal or polyclonal. Merely by way of example, monoclonal antibodies can be prepared from murine hybridomas according to the classical method of Kohler and Milstein (*Nature* 256:495-97, 1975) or derivative methods thereof. Detailed procedures for monoclonal antibody production are described in Harlow and Lane, *Using Antibodies: A Laboratory Manual*, CSHL, New York, 1999.

Antisense, Sense, and Antigene: Antisense molecules are molecules that are specifically hybridizable or specifically complementary to either RNA or the plus strand of DNA. Sense molecules are molecules that are specifically hybridizable or specifically complementary to the minus strand of DNA. Antigene molecules are either antisense or sense molecules directed to a dsDNA target.

Double-stranded DNA ("dsDNA") has two strands, a 5' → 3' strand, referred to as the plus strand, and a 3' → 5' strand (the reverse complement), referred to as the minus strand. Because RNA polymerase adds nucleic acids in a 5' → 3' direction, the minus strand of the DNA serves as the template for the RNA during

transcription. Thus, the RNA formed will have a sequence complementary to the minus strand and identical to the plus strand (except that U is substituted for T).

Apoptosis: A form of cell death induced by external stimuli that activates a proteolytic activation cascade of intracellular proteases called caspases. The
5 resulting signaling events lead (through the activation of caspases) to the digestion of numerous cellular protein substrates, such as permeabilization of the mitochondria with release of intramitochondrial components, including cytochrome C, and the fragmentation of genomic DNA into small molecules about hundreds of nucleotides long. These changes are incompatible with cellular viability, and create
10 distinct microscopic features, for example, chromatin condensation and fragmentation or rounded morphology (“blebbing”).

Cancer: The product of neoplasia is a neoplasm (a tumor or cancer), which is an abnormal growth of tissue that results from excessive cell division. A tumor that does not invade surrounding tissue and/or does not metastasize is referred to as
15 “benign.” A tumor that invades the surrounding tissue and/or can metastasize is referred to as “malignant.” Neoplasia is one example of a proliferative disorder. In particular examples, a “cancer cell” is a cell that is neoplastic, for example a cell or cell line isolated from a tumor. Neoplasia, malignancy, cancer and tumor are often used interchangeably and refer to abnormal growth of a tissue or cells that results
20 from excessive cell division.

Examples of hematological tumors include leukemias, including acute leukemias (such as acute lymphocytic leukemia, acute myelocytic leukemia, acute myelogenous leukemia and myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia), chronic leukemias (such as chronic myelocytic
25 (granulocytic) leukemia, chronic myelogenous leukemia, and chronic lymphocytic leukemia), polycythemia vera, lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma (indolent and high grade forms), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, myelodysplastic syndrome, hairy cell leukemia and myelodysplasia.

30 Examples of solid tumors, such as sarcomas and carcinomas, include fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma,

and other sarcomas, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, lymphoid malignancy, pancreatic cancer, breast cancer, lung cancers (such as small cell lung carcinoma and non-small cell lung carcinoma), ovarian cancer, prostate cancer, hepatocellular carcinoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, medullary thyroid carcinoma, papillary thyroid carcinoma, pheochromocytomas sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, Wilms' tumor, cervical cancer, testicular tumor, seminoma, bladder carcinoma, melanoma, and CNS tumors (such as a glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, neuroblastoma and retinoblastoma).

Cell cycle: The cycle of cellular division, which consists of M phase, G1 phase, S phase, and G2 phase. During S phase (synthesis), the cell replicates its DNA, doubling the cell DNA content. The period from the end of DNA replication until mitosis is G2 (gap 2) during which the cell nucleus contains two sets of chromosomes (also referred to as 4N state). M phase is mitosis, during which the duplicated chromosomes are separated and two daughter cells are formed. Once M phase is complete, the cell enters G1 phase (gap 1), during which RNAs and proteins are synthesized and cell size increases.

Progression through the cell cycle is tightly regulated by cell cycle checkpoints, control mechanisms that ensure that each phase of the cell cycle has been accurately completed prior to progression to the next phase. In particular, there are three major cell cycle checkpoints: G1/S checkpoint (also known as the "restriction point" in animal cells or the "start point" in yeast cells), G2/M checkpoint, and anaphase checkpoint. The restriction point is mainly controlled by action of the CKI-p16 (CDK inhibitor p16). This protein inhibits CDK4/6 and ensures that it can no longer interact with cyclin D1 to promote cell cycle progression. The G2/M checkpoint involves the ATM, ATR-CHK1, CHK2-CDC25 DNA damage response pathway, which prevents cells from entering mitosis until the

damage is repaired. The anaphase (mitotic) checkpoint primarily involves the anaphase promoting complex, which ensures that all chromosomes are lined up at the mitotic spindle before anaphase proceeds. For a general review of cell cycle, see DePamphilis, M.L. (ed.), "DNA Replication and Human Disease," chapters 17 and 18 (pp. 335 to 376), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; and Kastan and Bartek, *Nature* 432(7015):316-323, 2004.

Cells undergo "cell cycle arrest" when they are blocked from progression through the cell cycle. In a particular example, cells are arrested in the cell cycle prior to mitosis.

A "cell cycle checkpoint inhibitor" is a compound that blocks or inactivates the checkpoint mechanism(s), allowing a cell to proceed through the cell cycle that would otherwise have been arrested by the checkpoint mechanism. In particular examples, cell cycle checkpoint inhibitors such as caffeine or 7-hydroxystaurosporine (UCN-07) allow a cell to proceed through the G2/M checkpoint when they would otherwise be blocked (for example, when DNA is damaged), such as in the presence of DNA re-replication in non-cancer cells. In some examples, a cancer cell is contacted with an inhibitor of geminin in the absence of a cell cycle checkpoint inhibitor (such as in the substantial absence of a cell cycle checkpoint inhibitor, for example, without added or exogenous cell cycle checkpoint inhibitor, for example, less than 10 nM cell cycle checkpoint inhibitor (such as less than about 5 nM, less than about 1 nM, or less than about 0.1 nM cell cycle checkpoint inhibitor))

Contacting: Placement in direct physical association, including for example, a solid or liquid form. Contacting can occur *in vitro* with isolated cells or tissue or *in vivo* by administering to a subject (for example, administering a compound to a subject to achieve a desired concentration for a desired time at a target tissue in the body, for example, a tumor).

DNA (deoxyribonucleic acid): DNA is a long chain polymer which comprises the genetic material of most living organisms (some viruses have genes comprising ribonucleic acid (RNA)). The repeating units in DNA polymers are four different nucleotides, each of which comprises one of the four bases, adenine,

guanine, cytosine and thymine bound to a deoxyribose sugar to which a phosphate group is attached. Triplets of nucleotides (referred to as codons) code for each amino acid in a polypeptide, or for a stop signal. The term codon is also used for the corresponding (and complementary) sequences of three nucleotides in the mRNA
5 into which the DNA sequence is transcribed.

Unless otherwise specified, any reference to a DNA molecule is intended to include the reverse complement of that DNA molecule. Except where single-strandedness is required by the text herein, DNA molecules, though written to depict only a single strand, encompass both strands of a double-stranded DNA molecule.

10 For example, a reference to the DNA molecule that encodes geminin, or a fragment thereof, encompasses both the sense strand and its reverse complement. Thus, for instance, it is appropriate to generate probes or primers from the reverse complement sequence of the disclosed nucleic acid molecules.

DNA re-replication: Unscheduled initiation events that occur during a
15 single cell division cycle and cause part or all of the newly replicated DNA to replicate again. This results in multiple copies of a chromosome or portion of a chromosome that are generated during a single cell cycle, leading to cells in G2 phase with a DNA content greater than 4N. DNA re-replication activates cell cycle checkpoint pathways that can induce apoptosis.

20 **Geminin:** An approximately 25 kDa protein that contributes to the regulation of DNA replication during the cell cycle. Geminin binds to CDT1 and prevents CDT1 from recruiting the MCM proteins to the preRC. Geminin is degraded during M phase and remains low during G1 phase, permitting CDT1 to interact with the ORC and promote formation of the preRC for the next round of
25 DNA synthesis.

Geminin sequences are publicly available and known to one of skill in the art. For example, GenBank Accession numbers NC_000006.10 (region 24883143..24894257) and NC_000079.5 (region 24843714..24853806 (complement)) disclose human and mouse geminin gene sequences, respectively.
30 GenBank Accession numbers NM_015895.3 and NP_056979.1 disclose exemplary human geminin cDNA and protein sequences, respectively (provided herein as SEQ

ID NOs: 1 and 2) and GenBank Accession numbers NM_020567.2 and NP_065592.1 disclose exemplary mouse geminin cDNA and protein sequences, respectively. All GenBank sequences described herein are incorporated by reference in their entirety on October 17, 2008.

5 One skilled in the art will appreciate that geminin nucleic acid and protein molecules can vary from those publicly available, such as geminin sequences having one or more substitutions, deletions, insertions, or combinations thereof, while still retaining geminin biological activity. In addition, geminin molecules include alternatively spliced isoforms and fragments that retain the desired geminin
10 biological activity. In certain examples, geminin has at least 80% sequence identity, for example at least 85%, 90%, 95%, or 98% sequence identity to a publicly available geminin sequence.

 An “inhibitor of geminin” and a “compound that inhibits geminin” are used interchangeably herein and include a compound that inhibits or decreases
15 geminin expression (for example, mRNA or protein expression) or geminin activity (such as geminin protein activity, for example, Cdt1 binding). The inhibitor of geminin can be any type of compound that either reduces or eliminates mRNA or protein expression of geminin (for example by at least 10%, such as at least about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or more as compared to in
20 the absence of the inhibitor) or reduces or eliminates one or more activities of geminin (for example, geminin protein activity, such as Cdt1 binding), for example by at least 10%, such as at least about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or more as compared to in the absence of the inhibitor.

Hybridization: Oligonucleotides and their analogs hybridize by hydrogen
25 bonding, which includes Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary bases. Generally, nucleic acid consists of nitrogenous bases that are either pyrimidines (cytosine (C), uracil (U), and thymine (T)) or purines (adenine (A) and guanine (G)). These nitrogenous bases form hydrogen bonds between a pyrimidine and a purine, and the bonding of the
30 pyrimidine to the purine is referred to as “base pairing.” More specifically, A will hydrogen bond to T or U, and G will bond to C. “Complementary” refers to the base

pairing that occurs between two distinct nucleic acid sequences or two distinct regions of the same nucleic acid sequence.

“Specifically hybridizable” and “specifically complementary” are terms that indicate a sufficient degree of complementarity such that stable and specific binding occurs between the oligonucleotide (or its analog) and the DNA or RNA target. The oligonucleotide or oligonucleotide analog need not be 100% complementary to its target sequence to be specifically hybridizable. An oligonucleotide or analog is specifically hybridizable when binding of the oligonucleotide or analog to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide or analog to non-target sequences under conditions where specific binding is desired, for example under physiological conditions in the case of *in vivo* assays or systems. Such binding is referred to as specific hybridization.

Hybridization conditions resulting in particular degrees of stringency will vary depending upon the nature of the hybridization method of choice and the composition and length of the hybridizing nucleic acid sequences. Generally, the temperature of hybridization and the ionic strength (especially the Na⁺ concentration) of the hybridization buffer will determine the stringency of hybridization, though wash times also influence stringency. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed by Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, chapters 9 and 11, herein incorporated by reference.

For present purposes, “stringent conditions” encompass conditions under which hybridization will only occur if there is less than 25% mismatch between the hybridization molecule and the target sequence. “Stringent conditions” may be broken down into particular levels of stringency for more precise definition. Thus, as used herein, “moderate stringency” conditions are those under which molecules with more than 25% sequence mismatch will not hybridize; conditions of “medium stringency” are those under which molecules with more than 15% mismatch will not

hybridize, and conditions of “high stringency” are those under which sequences with more than 10% mismatch will not hybridize. Conditions of “very high stringency” are those under which sequences with more than 6% mismatch will not hybridize.

Interfering with or inhibiting expression of a target gene: The ability of
5 an agent, such as an interfering nucleotide sequence such as a dsRNA, siRNA, antisense, or other molecules, to measurably reduce the expression of a target gene (for example, geminin). It contemplates reduction of the end-product of the gene, such as the expression or function of the encoded protein, and thus includes reduction in the amount or longevity of the mRNA transcript.

10 Inhibition does not require absolute suppression of the gene. Thus, in certain examples, following application of an interfering agent or inhibitor, the gene is expressed at least 5% less than prior to application of the agent, for example at least 10% less, at least 15% less, at least 20% less, at least 25% less, at least 50% less, at least 75% less, at least 95% less, or even at least 99% less.

15 **Isolated:** An “isolated” biological component (such as a cell, nucleic acid molecule, protein, or organelle) has been substantially separated or purified away from other biological components in the cell or the organism in which the component naturally occurs, such as other cells, chromosomal and extra-chromosomal DNA and RNA, proteins, and organelles. Nucleic acids and proteins
20 that have been “isolated” include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids. In some examples, an isolated cancer cell includes a cancer cell that has been substantially purified from non-cancer cells of the same tissue or organism where
25 the cancer (or tumor) is located.

Oligonucleotide: An oligonucleotide is a plurality of joined nucleotides joined by native phosphodiester bonds, between about 6 and about 300 nucleotides in length. An oligonucleotide analog refers to moieties that function similarly to oligonucleotides but have non-naturally occurring portions. For example,
30 oligonucleotide analogs can contain non-naturally occurring portions, such as altered sugar moieties or inter-sugar linkages, such as a phosphorothioate

oligodeoxynucleotide. Functional analogs of naturally occurring polynucleotides can bind to RNA or DNA, and include peptide nucleic acid (PNA) molecules.

Particular oligonucleotides and oligonucleotide analogs can include linear sequences up to about 200 nucleotides in length, for example a sequence (such as
5 DNA or RNA) that is at least 6 bases, for example at least 8, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100 or even 200 bases long, or from about 6 to about 70 bases, for example about 10-25 bases, such as 12, 15 or 20 bases.

Polypeptide: A polymer in which the monomers are amino acid residues which are joined together through amide bonds. When the amino acids are alpha-
10 amino acids, either the L-optical isomer or the D-optical isomer can be used. The terms “polypeptide,” “peptide,” or “protein” as used herein are intended to encompass any amino acid sequence and include modified sequences such as glycoproteins. The term “polypeptide” is specifically intended to cover naturally occurring proteins, as well as those which are recombinantly or synthetically
15 produced.

Probes and primers: A probe comprises an isolated nucleic acid capable of hybridizing to a target nucleic acid (such as geminin nucleic acid molecule). A detectable label or reporter molecule can be attached to a probe or primer. Typical labels include radioactive isotopes, enzyme substrates, co-factors, ligands,
20 chemiluminescent or fluorescent agents, haptens, and enzymes. Methods for labeling and guidance in the choice of labels appropriate for various purposes are discussed, for example in Sambrook *et al.* (In *Molecular Cloning: A Laboratory Manual*, CSHL, New York, 1989) and Ausubel *et al.* (In *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1998).

25 In a particular example, a probe includes at least one fluorophore, such as an acceptor fluorophore or donor fluorophore. For example, a fluorophore can be attached at the 5'- or 3'-end of the probe. In specific examples, the fluorophore is attached to the base at the 5'-end of the probe, the base at its 3'-end, the phosphate group at its 5'-end or a modified base, such as a T internal to the probe.

30 Probes are generally at least 15 nucleotides in length, such as at least 15, at least 16, at least 17, at least 18, at least 19, least 20, at least 21, at least 22, at least

23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, at least 30,
at least 31, at least 32, at least 33, at least 34, at least 35, at least 36, at least 37, at
least 38, at least 39, at least 40, at least 41, at least 42, at least 43, at least 44, at least
45, at least 46, at least 47, at least 48, at least 49, at least 50 at least 51, at least 52, at
5 least 53, at least 54, at least 55, at least 56, at least 57, at least 58, at least 59, at least
60, at least 61, at least 62, at least 63, at least 64, at least 65, at least 66, at least 67,
at least 68, at least 69, at least 70, or more contiguous nucleotides complementary to
the target nucleic acid molecule, such as 20-70 nucleotides, 20-60 nucleotides, 20-50
nucleotides, 20-40 nucleotides, or 20-30 nucleotides.

10 Primers are short nucleic acid molecules, for instance DNA oligonucleotides
10 nucleotides or more in length, which can be annealed to a complementary target
nucleic acid molecule by nucleic acid hybridization to form a hybrid between the
primer and the target nucleic acid strand. A primer can be extended along the target
nucleic acid molecule by a polymerase enzyme. Therefore, primers can be used to
15 amplify a target nucleic acid molecule (such as a portion of a geminin nucleic acid
molecule).

The specificity of a primer increases with its length. Thus, for example, a
primer that includes 30 consecutive nucleotides will anneal to a target sequence with a
higher specificity than a corresponding primer of only 15 nucleotides. Thus, to obtain
20 greater specificity, probes and primers can be selected that include at least 15, 20, 25,
30, 35, 40, 45, 50, 55, 60, 65, 70 or more consecutive nucleotides. In particular
examples, a primer is at least 15 nucleotides in length, such as at least 15 contiguous
nucleotides complementary to a target nucleic acid molecule. Particular lengths of
primers that can be used to practice the methods of the present disclosure (for
25 example, to amplify a region of a geminin nucleic acid molecule) include primers
having at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21,
at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least
29, at least 30, at least 31, at least 32, at least 33, at least 34, at least 35, at least 36, at
least 37, at least 38, at least 39, at least 40, at least 45, at least 50, at least 55, at least
30 60, at least 65, at least 70, or more contiguous nucleotides complementary to the target

nucleic acid molecule to be amplified, such as a primer of 15-70 nucleotides, 15-60 nucleotides, 15-50 nucleotides, or 15-30 nucleotides.

Primer pairs can be used for amplification of a nucleic acid sequence, for example, by PCR, real-time PCR, or other nucleic-acid amplification methods known
5 in the art. An “upstream” or “forward” primer is a primer 5' to a reference point on a nucleic acid sequence. A “downstream” or “reverse” primer is a primer 3' to a reference point on a nucleic acid sequence. In general, at least one forward and one reverse primer are included in an amplification reaction.

Nucleic acid probes and primers can be readily prepared based on the
10 nucleic acid molecules provided herein. It is also appropriate to generate probes and primers based on fragments or portions of these disclosed nucleic acid molecules, such as a geminin nucleic acid molecule.

PCR primer pairs can be derived from a known sequence (such as a gene encoding a geminin protein), by using computer programs intended for that purpose
15 such as Primer (Version 0.5, © 1991, Whitehead Institute for Biomedical Research, Cambridge, MA) or PRIMER EXPRESS® Software (Applied Biosystems, AB, Foster City, CA).

RNA (ribonucleic acid): RNA is a long chain polymer which consists of nucleic acids joined by 3'-5' phosphodiester bonds. The repeating units in RNA
20 polymers are four different nucleotides, each of which comprises one of the four bases, adenine, guanine, cytosine, and uracil bound to a ribose sugar to which a phosphate group is attached. In general, DNA is transcribed to RNA by an RNA polymerase. RNA transcribed from a particular gene contains both introns and exons of the corresponding gene; this RNA is also referred to as pre-mRNA. RNA
25 splicing subsequently removes the intron sequences and generates a messenger RNA (mRNA) molecule, which can be translated into a polypeptide. Triplets of nucleotides (referred to as codons) in an mRNA molecule code for each amino acid in a polypeptide, or for a stop signal.

Except where single-strandedness is required by the text herein, RNA
30 molecules, though written to depict only a single strand, encompass both strands of a double-stranded RNA molecule.

RNA interference (RNAi): Refers to a cellular process that inhibits expression of genes, including cellular and viral genes. RNAi is a form of antisense-mediated gene silencing involving the introduction of double stranded RNA-like oligonucleotides leading to the sequence-specific reduction of RNA transcripts.

- 5 Double-stranded RNA molecules that inhibit gene expression through the RNAi pathway include siRNAs, miRNAs, and shRNAs.

Selectively killing cancer cells: Causing cancer cells to die or be in the process of dying without causing non-cancer cells to die or be in the process of dying. For example, contacting cancer cells with an inhibitor of geminin (such as in
10 the absence of a cell cycle checkpoint inhibitor) results in cell death of the cancer cells, however, contacting non-cancer cells with the inhibitor of geminin (such as in the absence of a cell cycle checkpoint inhibitor) does not result in a significant amount of cell death of the non-cancer cells (for example, compared to untreated non-cancer cells or non-cancer cells treated with a control compound). In particular
15 examples, the cancer cell death occurs by the process of apoptosis.

Sequence identity: The similarity between two nucleic acid sequences, or two amino acid sequences, is expressed in terms of the similarity between the sequences, otherwise referred to as sequence identity. Sequence identity is frequently measured in terms of percentage identity (or similarity or homology); the
20 higher the percentage, the more similar the two sequences are. Homologs or orthologs of geminin protein, and the corresponding cDNA or gene sequence(s), will possess a relatively high degree of sequence identity when aligned using standard methods. This homology will be more significant when the orthologous proteins or genes or cDNAs are derived from species that are more closely related (*e.g.*, human
25 and chimpanzee sequences), compared to species more distantly related (*e.g.*, human and *C. elegans* sequences).

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith & Waterman, *Adv. Appl. Math.* 2: 482, 1981; Needleman & Wunsch, *J. Mol. Biol.* 48: 443, 1970;
30 Pearson & Lipman, *Proc. Natl. Acad. Sci. USA* 85: 2444, 1988; Higgins & Sharp, *Gene*, 73: 237-244, 1988; Higgins & Sharp, *CABIOS* 5: 151-153, 1989; Corpet *et*

al., *Nuc. Acids Res.* 16, 10881-90, 1988; Huang *et al.*, *Computer Appls. in the Biosciences* 8, 155-65, 1992; and Pearson *et al.*, *Meth. Mol. Bio.* 24:307-31, 1994. Altschul *et al.* (*J. Mol. Biol.* 215:403-410, 1990) presents a detailed consideration of sequence alignment methods and homology calculations.

5 The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, *J. Mol. Biol.* 215:403-410, 1990) is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, MD) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. By way of example, for comparisons of amino acid
10 sequences of greater than about 30 amino acids, the Blast 2 sequences function is employed using the default BLOSUM62 matrix set to default parameters (gap existence cost of 11, and a per residue gap cost of 1). When aligning short peptides (fewer than around 30 amino acids), the alignment is performed using the Blast 2 sequences function, employing the PAM30 matrix set to default parameters (open
15 gap 9, extension gap 1 penalties).

 An alternative indication that two nucleic acid molecules are closely related is that the two molecules hybridize to each other under stringent conditions. Stringent conditions are sequence-dependent and are different under different environmental parameters. Generally, stringent conditions are selected to be about
20 5° C to 20° C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence remains hybridized to a perfectly matched probe or complementary strand. Conditions for nucleic acid hybridization and calculation of stringencies can be found in Sambrook *et al.* (In
25 *Molecular Cloning: A Laboratory Manual*, CSHL, New York, 1989) and Tijssen (*Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes* Part I, Chapter 2, Elsevier, New York, 1993). Nucleic acid molecules that hybridize under stringent conditions to a human geminin protein-encoding sequence will typically hybridize to a probe based on either an entire
30 human geminin protein-encoding sequence or selected portions of the encoding sequence under wash conditions of 2x SSC at 50° C.

Nucleic acid sequences that do not show a high degree of sequence identity may nevertheless encode similar amino acid sequences, due to the degeneracy of the genetic code. It is understood that changes in nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid molecules that all encode
5 substantially the same protein.

Short (or Small) Interfering Nucleotide Sequence (siRNA): A nucleotide sequence capable of interfering with gene expression, for instance by inducing gene-specific inhibition of expression. Typically, the sequence of a siRNA is substantially identical to a portion of a transcript of a target gene (mRNA) for which
10 interference or inhibition of expression is desired. For example, small, double stranded RNAs of about 15 to about 40 nucleotides in length (the length of each of the individual strands of the dsRNA), such as about 15 to about 25 nucleotides in length (for example, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides), that interfere with, or inhibit, expression of a target sequence.

15 The RNA backbone and/or component nucleotides can be unmodified or modified. For instance, the dsRNA can contain one or more deoxynucleic acids. Synthetic small dsRNAs may be used to induce gene-specific inhibition of expression. The dsRNAs can be formed from complementary single stranded RNAs ("ssRNAs") or from a ssRNA that forms a hairpin or from expression from a DNA
20 vector. In certain examples, these small interfering nucleotide sequences have 3' and/or 5' overhangs on each strand of the duplex. These overhangs can be 0 nucleotides (that is, blunt ends) to 5 nucleotides in length.

Such siRNA molecules can be used as reverse genetic and therapeutic tools in mammalian cells, including human cells, both *in vitro* and *in vivo*. These small
25 interfering nucleotide sequences are suitable for interference or inhibition of expression of a target gene wherein the sequence of the small interfering nucleotide sequence is substantially identical to a portion of an mRNA or transcript of the target gene for which interference or inhibition of expression is desired.

In addition to native nucleotide molecules, nucleotides suitable for inhibiting
30 or interfering with the expression of a target sequence include nucleotide derivatives and analogs. For example, a non-natural linkage between nucleotide residues can be

used, such as a phosphorothioate linkage. The nucleotide strand can be derivatized with a reactive functional group or a reporter group, such as a fluorophore. For example, the 2'-hydroxyl at the 3' terminus can be readily and selectively derivatized with a variety of groups. Other useful nucleotide derivatives incorporate nucleotides
5 having modified carbohydrate moieties, such as 2'-O-alkylated residues or 2'-deoxy-2'-halogenated derivatives. Particular examples of such carbohydrate moieties include 2'-O-methyl ribosyl derivatives and 2'-O-fluoro ribosyl derivatives.

The nucleotide bases can be modified. Any modified base useful for inhibiting or interfering with the expression of a target sequence can be used. For
10 example, halogenated bases, such as 5-bromouracil and 5-iodouracil can be incorporated. The bases may also be alkylated, for example, 7-methylguanosine may be incorporated in place of a guanosine residue. Non-natural bases that yield successful inhibition can also be incorporated.

Subject: Living multi-cellular vertebrate organisms, a category that includes
15 both human and non-human mammals. Subjects include veterinary subjects, including livestock such as cows and sheep, rodents (such as mice and rats), and non-human primates.

Therapeutically effective amount: A quantity of an agent or compound sufficient to achieve a desired effect in a subject or a cell being treated. For
20 instance, this can be the amount necessary to selectively kill cancer cells without killing non-cancer cells, for example to increase apoptosis or DNA re-replication.

A desired response can be an increase in apoptosis. One example of a therapeutic effect is an increase in the number of cancer cells undergoing apoptosis. One example of a therapeutic effect is a decrease in the number of cells, such as
25 cancer cells, which can lead to a regression of a cancer, such as colon cancer, breast cancer, lung cancer, kidney cancer, bone cancer, or brain cancer.

Transfected: A transfected cell is a cell into which has been introduced a nucleic acid molecule by molecular biology techniques. As used herein, the term transfection encompasses all techniques by which a nucleic acid molecule (such as a
30 DNA or siRNA) might be introduced into such a cell, including transfection with

viral vectors, transformation with plasmid vectors, and introduction of naked nucleic acid by electroporation, lipofection, and particle gun acceleration.

III. Methods for Selectively Killing Cancer Cells

5 Disclosed herein are methods for selectively killing cancer cells without killing non-cancer cells. In particular examples, the methods include inducing apoptosis of cancer cells without inducing apoptosis of non-cancer cells. These methods include contacting cancer cells with a compound that inhibits geminin in the absence of a cell cycle checkpoint inhibitor, for a period of time sufficient to
10 induce apoptosis of cancer cells without inducing apoptosis of non-cancer cells, thereby selectively killing cancer cells.

 In particular embodiments, the compound that inhibits geminin is a compound that inhibits or decreases geminin expression (for example, mRNA or protein expression) or geminin activity (such as Cdt1 binding). The compound that
15 inhibits expression or activity of geminin can be any type of compound that either reduces or eliminates mRNA or protein expression of geminin, or reduces or eliminates one or more activities of geminin (such as Cdt1 binding activity). In some embodiments, the compound is an antisense compound, a small molecule, a peptide, or an antibody. In some embodiments, the antisense compound is a shRNA,
20 antisense oligonucleotide, siRNA, miRNA or ribozyme. In one embodiment, the antisense compound is a siRNA. In particular, non-limiting examples, the compound is a siRNA that includes the nucleic acid sequence of SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5. In another embodiment, the antisense compound is a shRNA. In particular non-limiting examples, the compound is an shRNA that
25 includes the nucleic acid sequence of SEQ ID NO: 8 or SEQ ID NO: 9.

 In the disclosed methods, the cancer cells are contacted (for example, *in vitro* or *in vivo*) with a compound that inhibits geminin in the absence of a cell cycle checkpoint inhibitor (such as in the substantial absence of a cell cycle checkpoint inhibitor, for example, without added or exogenous cell cycle checkpoint inhibitor,
30 for example, less than 10 nM cell cycle checkpoint inhibitor (such as less than about 5 nM, less than about 1 nM, or less than about 0.1 nM cell cycle checkpoint

inhibitor)). Cell cycle checkpoint inhibitors are compounds that inactivate or override the cell cycle checkpoints at the G1/S transition, the G2/M transition, and the anaphase checkpoint. Compounds that inhibit the G2/M cell cycle checkpoint include caffeine, 7-hydroxystaurosporine (UCN-01), SB202190, and CHIR124.

5 The methods described herein include contacting cancer cells with a compound that inhibits geminin for a period of time sufficient to induce apoptosis of the cancer cell. The period of time may vary, depending on the type of compound (such as antisense compound, antibody, peptide, small molecule). One of skill in the art can determine the appropriate time period of treatment for a particular
10 compound. In general, the cancer cell is contacted with the compound for about one to ten days, such as at about two to eight days, for example, at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 days. In a particular example, the cancer cell is contacted with the compound for about four days.

 In a particular example, the compound is an antisense compound (such as
15 siRNA, such as geminin siRNA) and the cancer cells are contacted with the compound for more than two days, such as at least about four days, at least about six days, at least about eight days, or at least about ten days. In some examples, the period of time sufficient to induce apoptosis of the cancer cell is equal to or greater than the period of time sufficient to induce DNA re-replication in the cancer cells
20 contacted with the compound.

 Cancer is the result of abnormal and uncontrolled growth of cells. Neoplasia, malignancy, cancer and tumor are often used interchangeably and refer to abnormal growth of tissue or cells resulting from excessive cell division. The amount of a tumor in an individual is the “tumor burden” which can be measured as
25 the number, volume, or weight of the tumor. A tumor that does not invade surrounding tissue and/or does not metastasize is referred to as “benign.” A tumor that invades the surrounding tissue and/or can metastasize is referred to as “malignant.” The cancer cells described refer to malignant or benign cancer cells such as a solid tumor or hematological malignancy, metastasis from a tumor, cells
30 isolated or purified from a tumor, or cell lines established from a tumor. In particular non-limiting examples, the cancer cells include cells from a colon cancer

(such as colon carcinoma or colon adenocarcinoma), breast cancer (such as breast adenocarcinoma or breast carcinoma), lung cancer (such as non-small cell lung carcinoma or small cell lung carcinoma), kidney cancer (such as renal cell adenocarcinoma), bone cancer (such as osteosarcoma), and brain cancer (such as glioma). In additional examples, the cancer cells include cells from hematological malignancies (such as leukemias), lymphoma, prostate cancer, bladder cancer, and pancreatic cancer.

In some examples, the method includes contacting a cancer cell with a compound that inhibits geminin by administering a compound that inhibits geminin in the absence of a cell cycle checkpoint inhibitor (for example, without administering a cell cycle checkpoint inhibitor) to a subject in need of treatment for cancer. In particular examples, the subject has not received, and will not receive, a cell cycle checkpoint inhibitor while being administered the geminin inhibitor.

A. *Inhibition of geminin expression*

In particular embodiments, the geminin inhibitor is a compound that inhibits geminin expression, for example an antisense compound, a small molecule, a peptide, or an antibody. In some embodiments, the antisense compound is a shRNA, antisense oligonucleotide, siRNA, miRNA or ribozyme. In one embodiment, the antisense compound is a siRNA.

Generally, the principle behind antisense technology is that an antisense compound hybridizes to a target nucleic acid and effects the modulation of gene expression activity, or function, such as transcription, translation or splicing. The modulation of gene expression can be achieved by, for example, target RNA degradation or occupancy-based inhibition. An example of modulation of target RNA function by degradation is RNase H-based degradation of the target RNA upon hybridization with a DNA-like antisense compound, such as an antisense oligonucleotide. Antisense oligonucleotides can also be used to modulate gene expression, such as splicing, by occupancy-based inhibition, such as by blocking access to splice sites.

Another example of modulation of gene expression by target degradation is RNA interference (RNAi) using small interfering RNAs (siRNAs). RNAi is a form of antisense-mediated gene silencing involving the introduction of double stranded (ds)RNA-like oligonucleotides leading to the sequence-specific reduction of targeted endogenous mRNA levels. In particular examples, the siRNA is a geminin siRNA. Geminin siRNAs include siRNAs that target a geminin nucleic acid, such as a human geminin nucleic acid, for example, a nucleic acid having the nucleic acid sequence of SEQ ID NO: 1. Exemplary geminin siRNAs include, but are not limited to, siRNAs including the nucleic acid sequence of SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5. Additional geminin siRNAs can be designed and synthesized utilizing methods well known to one of skill in the art.

Another type of antisense compound that utilizes the RNAi pathway is a microRNA (miRNA). miRNAs are naturally occurring RNAs involved in the regulation of gene expression. However, these compounds can be synthesized to regulate gene expression via the RNAi pathway. Similarly, shRNAs are RNA molecules that form a tight hairpin turn and can be used to silence gene expression via the RNAi pathway. The shRNA hairpin structure is cleaved by the cellular machinery into siRNA. Geminin shRNAs include shRNAs that target a geminin nucleic acid, such as a human geminin nucleic acid, for example, a nucleic acid having the nucleic acid sequence of SEQ ID NO: 1. Exemplary geminin shRNAs include, but are not limited to, shRNAs including the nucleic acid sequence of SEQ ID NO: 8 or SEQ ID NO: 9.

Other compounds that are often classified as antisense compounds are ribozymes. Ribozymes are catalytic RNA molecules that can bind to specific sites on other RNA molecules and catalyze the hydrolysis of phosphodiester bonds in the RNA molecules. Ribozymes modulate gene expression by direct cleavage of a target nucleic acid, such as a messenger RNA.

Each of the above-described antisense compounds provides sequence-specific target gene regulation. This sequence-specificity makes antisense compounds effective tools for the selective modulation of a target nucleic acid of interest. In one embodiment, the target nucleic acid is geminin. Methods of

designing and synthesizing antisense compounds are well known in the art. In some cases, the antisense compounds comprise one or more modifications to improve nuclease resistance or increase binding specificity of the compound. For example, an antisense compound can comprise a modified base, sugar and/or internucleoside linkage, examples of each of which are well known to one of skill in the art.

Complete inhibition of geminin expression is not required in the methods disclosed herein. In particular examples, the expression of geminin is decreased by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, or even at least about 99% compared to expression of geminin in an untreated cell. In particular examples, geminin expression is decreased at least about 70% relative to untreated cells.

1. Geminin nucleic acid

In some examples, geminin expression is assessed by measuring the amount of geminin nucleic acid (such as mRNA or cDNA) present in a sample, such as a cancer cell. Methods of detecting a target nucleic acid molecule (such as RNA or DNA, for example mRNA or cDNA) in a sample are well known in the art. For example, nucleic acid amplification methods (with the appropriate probes and primers), as well as nucleic acid arrays (containing the appropriate probes), can be used. For example, the level of geminin gene expression can be determined or even quantified utilizing methods well known in the art, such as Northern blots, RNase protection assays, nucleic acid arrays, reverse transcription-PCR, quantitative PCR (such as quantitative real-time PCR or TaqMan® assays), dot blot assays, in-situ hybridization, or combinations thereof.

In one example, the method includes contacting nucleic acid molecules (which can be isolated) from a biological sample (such as a sample containing cancer cells or nucleic acid molecules obtained from such a sample) with a geminin-specific nucleic acid probe under conditions sufficient for the probe to specifically bind to geminin nucleic acid molecules (such as mRNA molecule) in the sample, thereby forming geminin-nucleic acid molecules complexes. The resulting

complexes are then detected using any standard detection system, for example by detecting a label on the probe.

A variety of known hybridization solvents can be employed, the choice being dependent on considerations known to one of skill in the art (see U.S. Patent 5,981,185). Detecting a hybridized complex in an array of oligonucleotide probes has been previously described (see U.S. Patent No. 5,985,567). In one example, detection includes detecting one or more labels present on the oligonucleotides, the sequences obtained from the subject, or both. Detection can further include treating the hybridized complex with a conjugating solution to effect conjugation or coupling of the hybridized complex with the detection label, and treating the conjugated, hybridized complex with a detection reagent. In particular examples, the method further includes quantification, for instance by determining the amount of hybridization.

Methods for labeling nucleic acid molecules so that they can be detected are well known. Examples of such labels include non-radiolabels and radiolabels. Non-radiolabels include, but are not limited to enzymes, chemiluminescent compounds, fluorophores, metal complexes, haptens, colorimetric agents, dyes, or combinations thereof. Radiolabels include, but are not limited to, ^{32}P . Radioactive and fluorescent labeling methods, as well as other methods known in the art, are suitable for use with the present disclosure. In one example, the primers used to amplify the nucleic acids are labeled (such as with biotin, a radiolabel, or a fluorophore). In another example, the amplified nucleic acid samples are end-labeled to form labeled amplified material. For example, amplified nucleic acid molecules can be labeled by including labeled nucleotides in the amplification reactions. In another example, nucleic acid molecules obtained from a subject are labeled, and applied to an array containing oligonucleotides.

2. Geminin protein

Expression of geminin can also be assessed by determining the amount of geminin protein present in a sample, such as a cancer cell. Methods of detecting a protein in a sample are well known in the art. For example, immunoassays and immunocytology methods can be used. However, the disclosure is not limited to

particular methods of detection. The availability of antibodies specific for geminin facilitates the detection and quantitation of geminin protein. Exemplary immunoassay methods are presented in Harlow and Lane (*Antibodies, A Laboratory Manual*, CSHL, New York, 1988), Bancroft and Stevens (*Theory and Practice of Histological Techniques*, Churchill Livingstone, 1982) and Ausubel *et al.* (*Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1998). Geminin antibodies are commercially available, including, but not limited to, anti-geminin antibodies from Santa Cruz Biotechnology, Santa Cruz, CA (*e.g.*, Cat. Nos. sc-13015 and sc-74456); Abcam, Cambridge, MA (*e.g.*, Cat. Nos. ab12147, ab84423, and ab37138); and Bethyl Laboratories, Montgomery, TX (*e.g.*, Cat. Nos. A300-934A and A300-935A).

Generally, the method includes contacting a sample (such as a sample containing cancer cells or proteins isolated from such a sample) with a geminin-specific antibody under conditions sufficient for the antibody to specifically bind to geminin proteins in the sample, thereby forming geminin-antibody complexes. The resulting geminin-antibody complexes are then detected using any standard detection system. For example, the anti-geminin antibody can include a label, thereby permitting detection of the complexes. In some examples, the geminin-antibody complexes are contacted with an appropriate labeled secondary antibody under conditions sufficient to permit specific binding of the secondary antibody to geminin-antibody complexes, thereby forming labeled geminin-antibody complexes. The label associated with the secondary label can then be detected.

Methods for labeling antibodies so that they can be detected are well known. Exemplary labels include fluorophores (such as Cy3, FITC, BODIPY, and Cy5), enzymes (such as horseradish peroxidase or alkaline phosphatase), or radiolabels. Methods of detecting labels are known, and include detection using microscopy and flow cytometry.

In some examples, the biological sample includes proteins isolated from a cancer cell. In such examples, any standard immunoassay format (such as ELISA, Western blot, or RIA assay) can be used to measure geminin protein levels. In addition, geminin proteins can be detected and quantified using antibody probe

arrays, quantitative spectroscopic methods (for example mass spectrometry, such as surface-enhanced laser desorption/ionization (SELDI)-based mass spectrometry), or combinations thereof.

5 *B. Inhibition of geminin activity*

 In some embodiments, the compound that inhibits geminin is a compound that inhibits or decreases geminin protein activity. In particular examples, the inhibitor is an anti-geminin antibody, a peptide, or a small organic molecule. In some examples, geminin protein activity is inhibited as a result of inhibition or a
10 decrease in geminin expression as described above.

 Methods of assessing geminin activity are well known in the art. Geminin prevents DNA re-replication at least in part by binding to the protein Cdt1 and preventing the incorporation of Cdt1 into the preRC complex. In particular embodiments, geminin activity is assessed by determining its interaction with Cdt1.
15 A decrease of geminin-Cdt1 interaction in the presence of a compound (such as a decrease of at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, or even at least about 99%) as compared to in the absence of the compound indicates that the compound is an inhibitor of geminin
20 activity. Methods to assess geminin-Cdt1 interaction or binding include immunoassay techniques, such as ELISA (for example sandwich ELISA) and co-immunoprecipitation. In some examples, geminin-Cdt1 interaction is assessed by co-immunoprecipitation of Cdt1 with geminin, for example from cells or cell lysates containing both proteins or using purified geminin and Cdt1. For example, geminin
25 and Cdt1 are incubated in the presence and absence of a compound (such as a geminin inhibitor) and geminin is immunoprecipitated with an anti-geminin antibody. The immunoprecipitate can be assessed for the presence of Cdt1, for example by Western blotting. A decreased amount of Cdt1 (for example as compared to in the absence of the compound or in the presence of a control
30 compound) indicates that the compound inhibits geminin binding to Cdt1.

In additional examples, geminin activity is assessed by measuring DNA re-replication, for example in a cancer cell that is susceptible to DNA re-replication in the presence of a geminin inhibitor. An increase of DNA re-replication in the presence of a compound (such as an increase of at least about 10%, at least about 25%, at least about 50%, at least about 75%, at least about 2-fold, at least about 5-fold, at least about 10-fold, or at least about 20-fold or more) as compared to in the absence of the compound indicates that the compound is an inhibitor of geminin activity. Methods of measuring DNA re-replication are well known in the art. In some examples, DNA re-replication is measured by determining the presence of cells with >4N DNA content. In particular examples, DNA content of cells (such as cells treated with a geminin inhibitor) is assessed by FACS analysis (for example by staining with a fluorescent DNA dye such as propidium iodide) and the percentage of cells having >4N content reflects the percentage of cells that have undergone DNA re-replication. In additional examples, DNA content is assessed by fluorescence microscopy, wherein cells (such as cells treated with a geminin inhibitor) are stained with a fluorescent DNA dye (such as Hoechst 33342 or DAPI) and the percentage of cells with giant nuclei is counted. Cells with giant nuclei are defined as those having a nucleus with a diameter more than twice that of control cells that are not treated with the compound or are treated with a control compound.

20

IV. Administration of Compounds that Selectively Kill Cancer Cells

The disclosed methods include selectively killing cancer cells by administering a compound that inhibits geminin in the absence of a cell cycle checkpoint inhibitor (for example, without administering a cell cycle checkpoint inhibitor) to a subject in need of treatment for cancer. In particular examples, the subject has not received, and will not receive, a cell cycle checkpoint inhibitor while being administered the geminin inhibitor. The inhibitor of geminin can be administered to the subject in a preselected dose for a preselected period of time to achieve the desired tissue concentration of the inhibitor at a target tissue (for example, a tumor).

30

In particular examples, the subject in need of treatment has a cancer that is selectively killed by treatment with an inhibitor of geminin, such as colon cancer, breast cancer, lung cancer, kidney cancer, bone cancer, or brain cancer. In additional examples, the subject in need of treatment may have a leukemia, lymphoma, prostate cancer, bladder cancer, or pancreatic cancer.

In additional embodiments, the disclosed methods include isolating at least one cancer cell from a subject, determining if the cancer cell overexpresses geminin as compared to a non-cancer cell (such as adjacent unaffected tissue from the subject, or normal tissue from the same organ from another subject), and administering a therapeutically effective amount of a geminin inhibitor in the absence of a cell cycle checkpoint inhibitor, thereby selectively killing cancer cells, but not killing non-cancer cells. Methods of determining expression of geminin, such as those described in Section III (above), are well known to one of skill in the art. In particular examples, a cancer cell overexpresses geminin if the amount of geminin is increased (such as an increase of about 2-fold to about 50-fold, for example, about 2-fold, about 5-fold, about 10-fold, about 15-fold, about 20-fold, about 30-fold, about 40-fold, about 50-fold) compared to a non-cancer cell.

Geminin inhibitors are administered in any suitable manner, preferably with pharmaceutically acceptable carriers. Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions of the present disclosure. The pharmaceutically acceptable carriers useful in this disclosure are conventional. *Remington: The Science and Practice of Pharmacy*, The University of the Sciences in Philadelphia, Editor, Lippincott, Williams, & Wilkins, Philadelphia, PA, 21st Edition (2005), describes compositions and formulations suitable for pharmaceutical delivery of the compounds herein disclosed.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water,

alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based
5 on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

Formulations for topical administration may include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional
10 pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, or tablets. Thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders may
15 be desirable.

In some embodiments, the geminin inhibitor is administered to a subject in a single dose. In other embodiments, the geminin inhibitor is administered to a subject in multiple doses. When administered in multiple doses, the time period between each administration can vary and will depend in part on the subject being
20 treated and the type of cancer being treated. In some examples, the geminin inhibitor is administered daily, bi-weekly, weekly, bi-monthly or monthly. When administered in multiple doses, the time period between each administration can vary and will depend in part on the subject being treated and the type of cancer being treated. One of skill in the art can determine an appropriate dosing schedule
25 for each subject.

In one example, such administration decreases the volume or number of cells of a tumor or a metastatic tumor, or both. Decreasing the volume of a tumor or a metastatic tumor does not require a 100% reduction in the volume, and in some examples includes decreasing the volume or number of cells by at least 10%, for
30 example by at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or more as compared to a volume in the absence of the therapeutic agent. In another example,

such administration enhances apoptosis of the cancer cells, or metastatic tumor cells, or both. Increasing the apoptosis of cancer cells, or metastatic tumor cells, does not require that 100% of the cells undergo apoptosis, and in some examples includes increasing the number of cells undergoing apoptosis by at least 10%, for example by
5 at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or more as compared to an amount of apoptosis in the absence of the therapeutic agent. In addition, the disclosed methods can result in a decrease in the symptoms associated with a tumor or a metastatic tumor.

10 A. *Administration of siRNA or shRNA*

In particular examples, the geminin inhibitor is a siRNA molecule or an shRNA molecule that is converted to a siRNA in a cell. In certain examples, expression vectors are employed to express the at least one siRNA or shRNA molecule. For example, an expression vector can include a nucleic acid sequence
15 encoding at least one siRNA or shRNA molecule corresponding to geminin. In a particular example, the vector contains a sequence(s) encoding both strands of a siRNA molecule comprising a duplex. In another example, the vector also contains sequence(s) encoding a single nucleic acid molecule that is self-complementary and thus forms a siRNA molecule. Non-limiting examples of such expression vectors
20 are described in Paul *et al.*, *Nature Biotechnology* 19:505, 2002; Miyagishi and Taira, *Nature Biotechnology* 19:497, 2002; Lee *et al.*, *Nature Biotechnology* 19:500, 2002; and Novina *et al.*, *Nature Medicine*, online publication Jun. 3, 2003.

In other examples, siRNA or shRNA molecules include a delivery vehicle, including for example, liposomes, for administration to a subject, carriers and
25 diluents and their salts, and can be present in pharmaceutical compositions. Nucleic acid molecules can be administered to cells by a variety of methods known to those of skill in the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other delivery vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres, or by
30 proteinaceous vectors (see, for example, O'Hare and Normand, International PCT Publication No. WO 00/53722).

Alternatively, the nucleic acid/vehicle combination can be locally delivered by direct injection or by use of an infusion pump. Direct injection of the siRNA, whether subcutaneous, intramuscular, or intradermal, can take place using standard needle and syringe methodologies, or by needle-free technologies such as those described by Barry *et al.*, International PCT Publication No. WO 99/31262. Other delivery routes include, but are not limited to, oral delivery (such as in tablet or pill form), intrathecal or intraperitoneal delivery. For example, intraperitoneal delivery can take place by injecting the treatment into the peritoneal cavity of the subject in order to directly deliver the molecules to tumor sites in the peritoneum, such as disseminated intraperitoneal metastases. Intrathecal delivery can be used for tumors in contact with cerebrospinal fluid. More detailed descriptions of nucleic acid delivery and administration are provided in Sullivan *et al.*, PCT WO 94/02595, Draper *et al.*, PCT WO93/23569, Beigelman *et al.*, PCT WO99/05094, and Klimuk *et al.*, PCT WO99/04819, all of which are incorporated by reference herein.

Alternatively, certain siRNA or shRNA molecules can be expressed within cells from eukaryotic promoters. Those skilled in the art will recognize that any nucleic acid can be expressed in eukaryotic cells using the appropriate DNA/RNA vector. The activity of such nucleic acids can be augmented by their release from the primary transcript by an enzymatic nucleic acid (Draper *et al.*, PCT WO 93/23569, and Sullivan *et al.*, PCT WO 94/02595).

In other examples, siRNA or shRNA molecules can be expressed from transcription units (see for example, Couture *et al.*, 1996, TIG 12:510) inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or viral vectors. siRNA expressing viral vectors can be constructed based on, for example, but not limited to, adeno-associated virus, retrovirus, adenovirus, lentivirus or alphavirus. In another example, pol III based constructs are used to express nucleic acid molecules of the invention (see for example, Thompson, U.S. Pat. Nos. 5,902,880 and 6,146,886).

The recombinant vectors capable of expressing the siRNA or shRNA molecules can be delivered as described above, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of

nucleic acid molecules. Such vectors can be repeatedly administered as necessary. Once expressed, the siRNA molecule interacts with the target mRNA and generates an RNAi response. Delivery of siRNA or shRNA molecule expressing vectors can be systemic, such as by intravenous or intramuscular administration, by
5 administration to target cells ex-planted from a subject followed by reintroduction into the subject, or by any other means that would allow for introduction into the desired target cell.

B. Additional Treatments

10 In some embodiments, the methods include administering a second anti-cancer therapeutic to the subject in addition to the inhibitor of geminin, wherein the second anti-cancer therapeutic is not a geminin inhibitor or an agent that specifically inhibits geminin. Anti-cancer therapeutics include, but are not limited to, chemotherapeutic drug treatment, radiation, gene therapy, hormonal manipulation,
15 immunotherapy and antisense oligonucleotide therapy. Chemotherapeutic agents that do not specifically inhibit geminin (for example, agents that do not specifically inhibit geminin expression or geminin activity) include, but are not limited to alkylating agents, such as nitrogen mustards (for example, chlorambucil, chlormethine, cyclophosphamide, ifosfamide, and melphalan), nitrosoureas (for
20 example, carmustine, fotemustine, lomustine, and streptozocin), platinum compounds (for example, carboplatin, cisplatin, oxaliplatin, and bbr3464), busulfan, dacarbazine, mechlorethamine, procarbazine, temozolomide, thiotepa, and uramustine; antimetabolites, such as folic acid (for example, methotrexate, pemetrexed, and raltitrexed), purine (for example, cladribine, clofarabine,
25 fludarabine, mercaptopurine, and tioguanine), pyrimidine (for example, capecitabine), cytarabine, fluorouracil, and gemcitabine; plant alkaloids, such as podophyllum (for example, etoposide, and teniposide), taxane (for example, docetaxel and paclitaxel), vinca (for example, vinblastine, vincristine, vindesine, and vinorelbine); cytotoxic/antitumor antibiotics, such as anthracycline family members
30 (for example, daunorubicin, doxorubicin, epirubicin, idarubicin, mitoxantrone, and valrubicin), bleomycin, hydroxyurea, and mitomycin; topoisomerase inhibitors, such

as topotecan and irinotecan; monoclonal antibodies, such as alemtuzumab, bevacizumab, cetuximab, gemtuzumab, rituximab, and trastuzumab; photosensitizers, such as aminolevulinic acid, methyl aminolevulinate, porfimer sodium, and verteporfin; and other agents, such as alitretinoin, altretamine, 5 amsacrine, anagrelide, arsenic trioxide, asparaginase, bexarotene, bortezomib, celecoxib, denileukin diftitox, erlotinib, estramustine, gefitinib, hydroxycarbamide, imatinib, pentostatin, masoprocol, mitotane, pegaspargase, and tretinoin.

The geminin inhibitor and the second anti-cancer therapeutic can be delivered at the same time (such as part of the same composition or as separate 10 compositions), or can be administered at different times. When administered at different times, the second anti-cancer therapeutic can either be administered before the geminin inhibitor or after the geminin inhibitor. The time between administration of the geminin inhibitor and the second anti-cancer therapeutic can vary and will depend on the type of second anti-cancer therapy selected, the cancer being treated 15 and the subject being treated. Similarly, the second anti-cancer therapeutic can be administered in a single dose or in multiple doses. One of skill in the art can determine an appropriate dosing schedule for each subject.

V. Methods for Identifying Compounds That Selectively Kill Cancer Cells

20 Disclosed herein are methods for identifying a compound that selectively kills cancer cells. The methods include contacting a test compound with a cancer cell expressing geminin in the absence of a cell cycle checkpoint inhibitor (such as in the substantial absence of a cell cycle checkpoint inhibitor, for example, without added or exogenous cell cycle checkpoint inhibitor, for example, less than 10 nM 25 cell cycle checkpoint inhibitor (such as less than about 5 nM, less than about 1 nM, or less than about 0.1 nM cell cycle checkpoint inhibitor)), assessing the cell cycle status of the cancer cell, and assessing geminin activity in the cancer cell. In some examples, the cell cycle status is assessed by determining DNA re-replication, apoptosis, or cell cycle arrest, and the geminin activity is assessed by determining 30 geminin expression (such as mRNA or protein expression) or geminin-Cdt1 binding. In particular examples, a compound that induces DNA re-replication, apoptosis, or

cell cycle arrest in cancer cells (such as induces DNA re-replication, apoptosis, or cell cycle arrest more in cancer cells than in non-cancer cells) and inhibits geminin activity in cancer cells (such as inhibits geminin activity more in cancer cells than in non-cancer cells) indicates that the compound is a compound that selectively kills
5 cancer cells.

The cancer cells useful for the screening methods described herein include any malignant or benign cancer cells such as a solid tumor or hematological malignancy, metastasis from a tumor, cells isolated or purified from a tumor, or cell lines established from a tumor. Such cells include, but are not limited to, colorectal
10 cancer cells, breast cancer cells, lung cancer cells, kidney cancer cells, bone cancer cells, brain cancer cells, cells from hematological malignancies, lymphoma cells, prostate cancer cells, bladder cancer cells, and pancreatic cancer cells. In particular, non-limiting examples, the cancer cells include HCT116 human colorectal carcinoma cells, SW480 human colorectal adenocarcinoma cells, COLO 320DM
15 human colorectal adenocarcinoma cells, DLD-1 colorectal adenocarcinoma cells, MCF7 human mammary adenocarcinoma cells, H1299 non-small cell lung carcinoma cells, 786-O renal cell adenocarcinoma cells, U-1 OS osteosarcoma cells, U-87 MG glioblastoma cells, and T98G glioblastoma cells.

In some examples, the activity of the test compound (such as DNA re-replication, apoptosis, inhibition of geminin) in the cancer cells is compared to the
20 activity of the test compound in non-cancer cells. In particular examples, the cancer cells are compared to non-cancer cells that are derived from the same tissue as the cancer cells. For example, colorectal carcinoma or adenocarcinoma cells are compared to cells from normal (non-cancerous) colon tissue. Similarly, breast
25 cancer cells are compared to normal (non-cancerous or non-tumorigenic) mammary gland or breast tissue. In particular, non-limiting, examples, the non-cancer cells include CCD-841 CoN or FHC human normal fetal colon cells, AG11132 or AG11134 normal human breast cells, MCF10A non-tumorigenic mammary gland cells, WI-38 normal human fetal lung fibroblasts, hFOB 1.19 normal human
30 osteoblast cells (immortalized), 293T normal human fetal kidney epithelial cells

(immortalized), D-1 normal human dermal fibroblast cells, and K-1 normal human epidermal keratinocytes.

The screening methods described herein include contacting cancer cells with one or more test compounds (in the presence or absence of a cell cycle checkpoint inhibitor) for a period of time sufficient to induce DNA re-replication, apoptosis, or cell cycle arrest of the cancer cell. The period of time may vary, depending on the type of compound (such as antisense compound, antibody, peptide, small molecule). One of skill in the art can determine the appropriate time period of treatment for a particular compound. In general, the cancer cell is contacted with the compound for about one to ten days, such as at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 days. In a particular example, the compound is an antisense compound (such as siRNA, such as geminin siRNA) and the cancer cells are contacted with the compound for more than two days, such as at least about four days, about six days, or about eight days.

15 A. *Screening methods*

The screening methods disclosed herein include assessing the cell cycle status of a cell, for example, assessing DNA re-replication, apoptosis, or cell cycle arrest. Methods of measuring DNA re-replication are known in the art, such as by analysis of DNA content of a cell. Cells that have >4N DNA content are considered to be undergoing DNA re-replication. The percentage of cells with >4N DNA content in the population of cells can be calculated. A compound is considered to induce DNA re-replication if it increases the percentage of cells with >4N DNA content (for example, an increase of about 3-fold to about 30-fold, such as about 3-fold to about 25-fold, about 4-fold to about 20-fold, or about 5-fold to about 15-fold) as compared to cells that are not treated with the test compound or cells treated with a control compound. In particular examples, DNA content in the presence and absence of a test compound is determined by FACS analysis. DNA is stained with a fluorescent dye (such as propidium iodide, ethidium bromide, Hoechst dyes (for example, Hoechst 33342 and Hoechst 33258), mithramycin, DAPI, 7-aminoactinomycin D, To-Pro-3, or chromomycin) and DNA content is measured. In

a particular example, DNA content is assessed using FACS analysis and Becton Dickinson CellQuest™ software.

In additional examples, DNA re-replication is measured by determining the percentage of cells with giant nuclei in a population of cells that have been treated in the presence or absence of a test compound. A compound is considered to induce DNA re-replication if it increases the percentage of cells with giant nuclei (for example, an increase of about 3-fold to about 35-fold, such as about 3-fold to about 30-fold, about 4-fold to about 25-fold, or about 5-fold to about 30-fold) as compared to cells that are not treated with the test compound or cells treated with a control compound. In particular examples cells are stained with a fluorescent DNA dye (such as propidium iodide, ethidium bromide, Hoechst dyes (for example, Hoechst 33342 and Hoechst 33258), mithramycin, DAPI, 7-aminoactinomycin D, To-Pro-3, or chromomycin) and the diameter of nuclei are measured using fluorescence microscopy. Cells having a giant nucleus are those cells having a nuclear diameter at least about two times greater than the nuclear diameter of cells that are treated in the absence of the test compound or are treated with a control compound. Nucleus diameter can be measured by, for example, using MetaMorph™ Imaging System software. In addition, Acumen Explorer microplate cytometer and the accompanying software can automatically measure the nucleus size of each cell, and calculate the percentage of cells with giant nuclei.

In additional examples, cell cycle status is assessed in the presence and absence of a test compound by measuring apoptosis. Methods of measuring apoptosis are well known in the art. For example, apoptotic cell death can be characterized by cell shrinkage, membrane blebbing and chromatin condensation culminating in cell fragmentation. Cells undergoing apoptosis also display a characteristic pattern of internucleosomal DNA cleavage. A compound is considered to induce apoptosis if it increases the number or percentage of cells in a population undergoing apoptosis (for example, an increase of about 2-fold to about 50-fold, such as about 3-fold to about 30-fold, about 4-fold to about 25-fold, or about 5-fold to about 30-fold) as compared to cells that are not treated with the test compound or cells treated with a control compound.

Apoptosis can be measured in the presence or the absence of Fas-mediated signals. In another example, cytochrome C release from mitochondria during cell apoptosis can be detected (see, for example, Bossy-Wetzel *et al.*, *Methods in Enzymol.* 322:235-42, 2000). Other assays include cytofluorometric quantitation of
5 nuclear apoptosis induced in a cell-free system (see, for example, Lorenzo *et al.*, *Methods in Enzymol.* 322:198-201, 2000), apoptotic nuclease assays (see, for example, Hughes, FM, *Methods in Enzymol.* 322:47-62, 2000), microscopic analysis of apoptotic cells by flow and laser scanning cytometry (see, for example, Darzynkiewicz *et al.*, *Methods in Enzymol.* 322:18-39, 2000), annexin-V/propidium
10 iodide labeling, transient transfection assays for cell death genes (see, for example, Miura *et al.*, *Methods in Enzymol.* 322:480-92, 2000), and assays that detect DNA cleavage (see, for example, Kauffman *et al.*, *Methods in Enzymol.* 322:3-15, 2000).

Apoptosis can also be measured by TdT incorporation of labeled nucleotides into DNA strand breaks (TUNEL assay). This system is a fluorescent TUNEL assay
15 that measures apoptotic DNA fragmentation by directly incorporating fluorescein-12-dUTP at the 3'-OH DNA ends using Terminal Deoxynucleotidyl Transferase (TdT), which forms a polymeric tail. The fluorescein-dUTP-labeled DNAs from cells (such as cells in the presence or absence of one or more test compounds) are then visualized directly by fluorescence microscope or quantified by flow cytometry.

20 Apoptosis can also be measured by determining the DNA content of cells, for example by FACS analysis of cells stained with a fluorescent DNA dye. Cells having less than 2N DNA content are cells which are undergoing apoptosis. A compound is considered to induce apoptosis if it increases the number or percentage of cells in a population with less than 2N DNA (for example, an increase of about 2-
25 fold to about 50-fold, such as about 3-fold to about 30-fold, about 4-fold to about 25-fold, or about 5-fold to about 30-fold) as compared to cells that are not treated with the test compound or cells treated with a control compound.

In additional examples, cell cycle status is assessed in the presence and absence of a test compound by measuring cell cycle arrest before mitosis. Methods
30 of measuring cell cycle arrest before mitosis are well known in the art. In one example, a compound is considered to induce cell cycle arrest before mitosis if it

decreases the amount of phosphorylation of histone H3 (Ser 10) (for example, a decrease of at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, or even at least about 99%) as compared to

5 cells that are not treated with the test compound or cells treated with a control compound. Phosphorylation of histone H3 can be determined by immunostaining for phosphorylated histone H3 (such as by ELISA or Western blotting). Antibodies that specifically detect phosphorylated histone H3, but not non-phosphorylated histone H3 are commercially available (for example, Sigma-Aldrich, St. Louis, MO;

10 Cell Signaling Technology, Danvers, MA; Abcam, Cambridge, MA; Santa Cruz Biotechnology, Santa Cruz, CA).

In further examples, cell cycle arrest is measured by determining cell proliferation, such as by incorporation of a DNA label (for example 5-bromo-2-deoxyuridine (BrdU) or [³H]thymidine). In the presence of label, cells which are in

15 S-phase incorporate the label. After an incubation period, cells which were in S-phase during the labeling period can be detected, such as by autoradiography (for cells labeled with [³H]thymidine or with fluorescently-labeled antibodies specific to BrdU (for cells labeled with BrdU). A decrease in the number of cells in S-phase (such as a decrease of at least about 10%, about 20%, about 30%, about 40%, about

20 50%, about 60%, about 70%, about 80%, about 90%, about 95%, or even about 99%) in the presence of a test compound as compared to in the absence of the test compound or in the presence of a control compound indicates that the compound induced cell cycle arrest.

In a particular example, the methods for identifying a compound that induces

25 DNA re-replication and/or apoptosis in cancer cells can be performed in a high throughput format. For example, cancer cells and non-cancer cells can be plated in a multi-well plate (such as 24-well, 48-well, 96-well, 384-well, or 1536 well microtiter plates). Test compounds can be applied to the plate for a period of time (such as about 1 day, about 2 days, about 3 days, about 4 days, about 6 days, or

30 about 8 days). DNA can be stained with a fluorescent DNA dye (such as propidium iodide, ethidium bromide, Hoechst dyes (for example, Hoechst 33342 and Hoechst

33258), mithramycin, DAPI, 7-aminoactinomycin D, To-Pro-3, or chromomycin) and the fluorescence can be read with a microplate cytometer (such as Acumen® X3, TTP LabTech, Cambridge, MA). Cells with re-replicated DNA can be identified based on DNA content $>4N$ or the presence of giant nuclei; cells
5 undergoing apoptosis can be identified based on DNA content $<2N$.

The screening methods disclosed herein include assessing geminin activity of a cell. In particular examples, geminin activity is assessed by determining geminin expression (such as mRNA or protein expression) or geminin-Cdt1 binding. Methods of determining geminin expression or geminin-Cdt1 binding are well
10 known to one of skill in the art; exemplary methods are described in Section III, above. In some examples, the expression of geminin is decreased by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, or even at least about 99% compared to expression of geminin in
15 an untreated cell or a cell treated with a control compound. In other examples, geminin-Cdt1 interaction in the presence of a compound is decreased (such as a decrease of at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, or even at least about 99%) as compared to in
20 an untreated cell or a cell treated with a control compound.

B. Test compounds

The methods disclosed herein are of use for identifying compounds that can be used to selectively kill cancer cells. A “compound” or “test compound” is any
25 substance or any combination of substances that is useful for achieving an end or result. The compounds identified using the methods disclosed herein can be of use for selectively killing cancer cells, but not killing non-cancer cells, for example by inducing DNA re-replication and/or inhibiting geminin. Any compound that has potential (whether or not ultimately realized) to affect DNA re-replication or
30 geminin expression or activity can be tested using the methods of this disclosure.

Exemplary compounds include, but are not limited to, peptides, such as soluble peptides, including but not limited to members of random peptide libraries (see, *e.g.*, Lam *et al.*, *Nature*, 354:82-84, 1991; Houghten *et al.*, *Nature*, 354:84-86, 1991), and combinatorial chemistry-derived molecular libraries made of D-and/or L-
5 configuration amino acids, phosphopeptides (including, but not limited to, members of random or partially degenerate, directed phosphopeptide libraries; see, *e.g.*, Songyang *et al.*, *Cell*, 72:767-778, 1993), antibodies (including, but not limited to, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies, and Fab, F(ab')₂ and Fab expression library fragments, and epitope-
10 binding fragments thereof), small organic or inorganic molecules (such as, so-called natural products or members of chemical combinatorial libraries), molecular complexes (such as protein complexes), or nucleic acids (such as antisense compounds).

Appropriate compounds can be contained in libraries, for example, synthetic
15 or natural compounds in a combinatorial library. Numerous libraries are commercially available or can be readily produced; means for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides, such as antisense oligonucleotides and oligopeptides, also are known. Alternatively, libraries of natural compounds in the
20 form of bacterial, fungal, plant and animal extracts are available or can be readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Such libraries are useful for the screening of a large number of different compounds.

25 Libraries (such as combinatorial chemical libraries) useful in the disclosed methods include, but are not limited to, peptide libraries (see, *e.g.*, U.S. Pat. No. 5,010,175; Furka, *Int. J. Pept. Prot. Res.*, 37:487-493, 1991; Houghton *et al.*, *Nature*, 354:84-88, 1991; PCT Publication No. WO 91/19735), encoded peptides (*e.g.*, PCT Publication WO 93/20242), random bio-oligomers (*e.g.*, PCT Publication
30 No. WO 92/00091), benzodiazepines (*e.g.*, U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs *et al.*, *Proc. Natl. Acad.*

5 *Sci. USA*, 90:6909-6913, 1993), vinylogous polypeptides (Hagihara *et al.*, *J. Am. Chem. Soc.*, 114:6568, 1992), nonpeptidal peptidomimetics with glucose scaffolding (Hirschmann *et al.*, *J. Am. Chem. Soc.*, 114:9217-9218, 1992), analogous organic syntheses of small compound libraries (Chen *et al.*, *J. Am. Chem. Soc.*, 116:2661, 1994), oligocarbamates (Cho *et al.*, *Science*, 261:1303, 1003), and/or peptidyl phosphonates (Campbell *et al.*, *J. Org. Chem.*, 59:658, 1994), nucleic acid libraries (see Sambrook *et al. Molecular Cloning, A Laboratory Manual*, Cold Springs Harbor Press, N.Y., 1989; Ausubel *et al., Current Protocols in Molecular Biology*, Green Publishing Associates and Wiley Interscience, N.Y., 1989), peptide nucleic acid libraries (see, *e.g.*, U.S. Pat. No. 5,539,083), antibody libraries (see, *e.g.*, Vaughn *et al., Nat. Biotechnol.*, 14:309-314, 1996; PCT App. No. PCT/US96/10287), carbohydrate libraries (see, *e.g.*, Liang *et al., Science*, 274:1520-1522, 1996; U.S. Pat. No. 5,593,853), small organic molecule libraries (see, *e.g.*, benzodiazepines, Baum, C&EN, Jan 18, page 33, 1993; isoprenoids, U.S. Pat. No. 5,569,588; thiazolidionones and methathiazones, U.S. Pat. No. 5,549,974; pyrrolidines, U.S. Pat. Nos. 5,525,735 and 5,519,134; morpholino compounds, U.S. Pat. No. 5,506,337; benzodiazepines, 5,288,514) and the like.

20 Libraries useful for the disclosed screening methods can be produced in a variety of manners including, but not limited to, spatially arrayed multipin peptide synthesis (Geysen, *et al., Proc. Natl. Acad. Sci.*, 81(13):3998-4002, 1984), "tea bag" peptide synthesis (Houghten, *Proc. Natl. Acad. Sci.*, 82(15):5131-5135, 1985), phage display (Scott and Smith, *Science*, 249:386-390, 1990), spot or disc synthesis (Dittrich *et al., Bioorg. Med. Chem. Lett.*, 8(17):2351-2356, 1998), or split and mix solid phase synthesis on beads (Furka *et al., Int. J. Pept. Protein Res.*, 37(6):487-493, 1991; Lam *et al., Chem. Rev.*, 97(2):411-448, 1997). Libraries may include a varying number of compositions (members), such as up to about 100 members, such as up to about 1000 members, such as up to about 5000 members, such as up to about 10,000 members, such as up to about 100,000 members, such as up to about 500,000 members, or even more than 500,000 members.

30 In one convenient embodiment, high throughput screening methods involve providing a combinatorial chemical or peptide library containing a large number of

potential therapeutic compounds. Such combinatorial libraries are then screened in one or more assays as described herein to identify those library members (particularly chemical species or subclasses) that display a desired characteristic activity (such as inducing DNA re-replication or apoptosis of cancer cells). In one
5 example an compound is identified that increases the amount of DNA re-replication in cancer cells relative to non-cancer cells.

The compounds identified using the methods disclosed herein can serve as conventional “lead compounds” or can themselves be used as potential or actual therapeutics. In some instances, pools of candidate agents may be identified and
10 further screened to determine which individual or subpools of agents in the collective have a desired activity.

The present disclosure is illustrated by the following non-limiting Examples.

15 **EXAMPLES**

Example 1

Geminin Depletion Selectively Induces DNA Re-Replication in Cancer Cells

This example describes induction of DNA re-replication in cancer cells treated with geminin siRNA.

20 *Methods*

Cell culture and cells: All the human cells used in this study are listed in Table 1. Cells were grown in medium recommended by the providers shown in Table 1. D-1 cells were maintained in DMEM medium supplemented with 10% FBS. K-1 cells were maintained in Keratinocyte Medium (ScienCell, Carlsbad, CA; Cat. No. 2101). HI299 cells were maintained in DMEM medium supplemented with
25 10% FBS, and MCF10A cells were grown in a serum-free MEGM (Mammary Epithelial Growth Medium) supplemented with a MEGM bullet kit (Lonza, Basel, Switzerland; Cat. No. cc3150)

siRNA: Short interfering (siRNA) oligonucleotides were made to the
30 following target sequences (sense) for geminin: siGem,
UGCCAACUCUGGAAUCAAA (SEQ ID NO: 3); siGem2,

AACUUCCAGCCCUGGGGUAAU (SEQ ID NO: 4); and siGem3,
AAUCACUGGAUAAUCAGGAAU (SEQ ID NO: 5). siRNAs were also made to
luciferase (siGL2): AACGUACGCGGAAUACUUCGA (SEQ ID NO: 6) and cyclin
A (siCcnA): AGCCAGUGAGUGUAAUGA (SEQ ID NO: 7). Transfections were
5 performed with 20 nM siRNA oligonucleotide duplexes with Oligofectamine™
(Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

FACS analysis: Cells were collected by trypsinization and fixed with 70%
ethanol overnight at 4°C. After fixation, cells were centrifuged and stained in 1 ml
of propidium iodide solution (0.05% NP-40, 50 ng of propidium iodide per ml, and
10 10 µg of RNase A per ml). The labeled cells were analyzed on a Becton Dickinson
flow cytometer with CellQuest™ software.

Western blotting: Rabbit anti-geminin (Cat. No. sc-13015, Santa Cruz
Biotechnology, Inc., Santa Cruz, CA), rabbit anti-actin (Cat. No. A2066, Sigma-
Aldrich, St. Louis, MO), rabbit anti-GAPDH (Cat. No. G9545, Sigma-Aldrich),
15 mouse anti-p53 (Sigma-Aldrich), rabbit anti-p53 phosphorylated at serine15 (Cell
Signaling Technology, Danvers, MA), rabbit anti-p21 (Cat. No. sc-397, Santa Cruz
Biotechnology), and rabbit anti-cyclin A (Cat. No. sc-751, Santa Cruz
Biotechnology) were used for immunoblotting. For Western blotting, cells were
washed twice in PBS buffers and pellets were incubated with RIPA buffer, followed
20 by sonication. Proteins were resolved by sodium dodecylsulfate polyacrylamide gel
electrophoresis (SDS-PAGE), transferred to nitrocellulose, and probed.

Immunofluorescence: Cells were fixed with 2% paraformaldehyde in
phosphate-buffered saline (PBS) for 10 min and permeabilized with 0.2% Triton X-
100 for 10 min at room temperature. Cells were then washed and mounted with
25 solution containing 4', 6'-diamidino-2-phenylindole (DAPI) (Vector Laboratories,
Burlingame, CA) before examination under the microscope. Cells were analyzed
using MetaMorph™ Imaging System software and defined as having a “giant”
nucleus if the nucleus diameter was greater than twice that of nuclei in cells
transfected with siGL2 control siRNA.

30

Table 1. Human cell lines utilized

Cell Line	Source	Derivation	Morphology
CCD-841 CoN	CRL-1790	Normal fetal colon (21 weeks gestation)	Adherent epithelial
FHC	CRL-1831	Normal fetal colon (13 weeks gestation)	Adherent epithelial
HCT116	CCL-247	Colorectal carcinoma	Adherent epithelial
SW480	CCL-228	Colorectal adenocarcinoma	Adherent epithelial
COLO 320DM	CCL-220	Colorectal adenocarcinoma	Adherent and suspension
DLD-1	CCL-221	Colorectal adenocarcinoma	Adherent epithelial
AG11132	NIACR*	Normal breast tissue (from 16 year old female)	Adherent epithelial
AG11134	NIACR*	Normal breast tissue (from 28 year old female)	Adherent epithelial
MCF10A	CRL-10317	Non-tumorigenic mammary gland	Adherent epithelial
MCF7	HTB-22	Mammary gland adenocarcinoma	Adherent epithelial
WI-38	CCL-75	Normal fetal lung fibroblast (3 months gestation)	Adherent fibroblast
H1299	CRL-5803	Non-small cell lung carcinoma (p53-)	Adherent epithelial
293T	CRL-11268	Normal embryonic kidney, immortalized with SV40 T-ag	Adherent epithelial
786-O	CRL-1932	Kidney renal cell adenocarcinoma	Adherent epithelial
hFOB 1.19	CRL-11372	Normal osteoblast, immortalized with SV40 T-ag	Adherent
U-2 OS	HTB-96	Osteosarcoma	Adherent epithelial
U-87 MG	HTB-14	Glioblastoma	Adherent epithelial
T98G	CRL-1690	Glioblastoma	Adherent fibroblast
D-1		Normal dermal fibroblast	Adherent
K-1		Normal epidermal keratinocytes	Adherent
WM-266-4	CRL-1676	Metastatic site of malignant melanoma	Adherent epithelial
A375	CRL-1619	Metastatic site of malignant melanoma	Adherent epithelial
HeLa	CCL-2	Adenocarcinoma of cervix	Adherent epithelial

*National Institute of Aging Cell Repository, Coriell Institute for Medical Research. All other cells (except D-1 and K-1) were obtained from the American Type Culture Collection.

Results

The response of colon cancer cell lines to transfection with siRNA targeted against geminin (siGem) was evaluated. The fraction of cells that contained greater than 4N DNA content increased by about 10-fold within 2 days of siGem treatment, as evidenced by a change in their fluorescence activated flow cytometry (FACS) profiles (Fig. 1). Moreover, the number of giant nuclei detected by microscopy also increased by about 10-fold (Fig. 1). Analysis of protein levels by Western blotting confirmed that the average level of geminin in each cell population was reduced at least 10-fold as a result of treatment with siGem (Fig. 1). In contrast, treatment with a control siRNA targeted against firefly luciferase (siGL2), a gene that is not encoded by mammals, did not affect these parameters.

To determine whether or not siGem had the same effect on cells from non-carcinogenic tissues, this experiment was repeated with cells derived from normal human fetal colon tissue (CCD841 CoN and FHC cells). No change was detected either in the fraction of cells with greater than 4N DNA content, or in the fraction of giant nuclei, despite the fact that geminin protein was again reduced by at least 10-fold (Fig. 2). Thus, geminin depletion induced DNA re-replication in colorectal cancer cells, but not in their normal epithelial counterparts.

To determine whether or not siGem could distinguish between normal cells and cancer cells derived from tissues other than colon, the same experiment was carried out with cells representing human breast, lung, kidney, bone, brain, skin and cervix (Table 1). These included cells from both epithelial and fibroblast tissues. In each case, siGem reduced geminin levels by about 10-fold, but in normal cells no change was detected either in the fraction of cells with greater than 4N DNA, or in the fraction of giant nuclei (Table 2). Two cell lines that were originally derived from normal tissues, but later immortalized by constitutive expression of the large T-antigen oncogene from SV40 (293T kidney epithelial cells and hFOB 1.19 osteoblasts) also were not induced to re-replicate their DNA in response to geminin depletion. 293T cells are tumorigenic but non-metastatic (Yan and Shao, *J. Biol. Chem.* 281:19700-19708, 2006). Furthermore, two skin melanoma cell lines (WM-266-4 and A375), as well as the frequently studied cervix adenocarcinoma HeLa cell

line were also resistant to induction of DNA re-replication in response to geminin depletion. In contrast, cells derived from a breast adenocarcinoma (MCF7), lung carcinoma (H1299), kidney adenocarcinoma (786-O), osteosarcoma (U2OS), and glioblastoma (U-87 MG and T98G) were as sensitive to geminin depletion as were
 5 colon carcinoma (HCT116) and colon adenocarcinoma (SW480, COLO 320DM, and DLD-1) cell lines.

Table 2. Effect of siGem on DNA Re-replication in Human Cells

Tissue	Cell Type	Cell Line	DNA Re-replication (%)		
			siGL2	siGem	siGem + siCcnA
Colon	Normal epithelial	CCD-841 CoN	4	2	29
	Normal epithelial	FHC	2	3	
	Carcinoma	HCT116	4	44	50
	Adenocarcinoma	SW480	6	43	
	Adenocarcinoma	COLO 320DM	4	30	
	Adenocarcinoma	DLD-1	3	28	
Breast	Normal epithelial	AG11132	1	2	30
	Normal epithelial	AG11134	6	5	
	Normal epithelial	MCF10A	4	4	35
	Adenocarcinoma	MCF7	6	36	
Lung	Normal fibroblast	WI-38	4	3	
	Carcinoma	H1299	2	33	
Kidney	Immortalized epithelial	293T	3	4	
	Adenocarcinoma	786-O	1	28	
Bone	Immortalized osteoblast	hFOB 1.19	5	3	
	Osteosarcoma	U-2 OS	2	42	
Brain	Glioblastoma	U-87 MG	4	30	
	Glioblastoma	T98G	2	39	
Skin	Normal fibroblast	D-1	1	1	44
	Normal fibroblast	K-1	3	4	
	Melanoma	WM-266-4	2	2	5
	Melanoma	A375	1	2	4
Cervix	Adenocarcinoma	HeLa	2	2	

10 Two additional geminin siRNAs (siGem2 and siGem3) were developed that were directed against different sites within the geminin gene than the siGem siRNA. All three siRNAs induced DNA re-replication to a similar extent in HCT116 cells, but had no effect in HeLa and D-1 cells (FIGS. 3A and 3B).

Example 2

DNA Re-Replication Induces Apoptosis in Cancer Cells

This example describes the induction of apoptosis in cancer cells, but not
5 non-cancer cells, treated with geminin siRNA.

Methods

TUNEL assay was performed using *In Situ* Cell Death Detection Kit according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IN). Briefly, harvested cells were washed three times in PBS and then fixed in
10 freshly prepared fixation solution (4% paraformaldehyde in PBS, pH 7.4) for 1 hour at room temperature. Cells were then washed with PBS and incubated in permeabilization solution (0.1 % triton X-100 in 0.1% sodium citrate). Permeabilized cells were then incubated with TUNEL reaction mixture for 60 minutes at 37°C in a humidified atmosphere in the dark. After washing twice with
15 PBS, cells were mounted with solution containing DAPI (Vector Laboratories) before examination under the microscope. As a positive control, permeabilized cells were incubated with DNase I (1 U/μl) for 10 minutes at room temperature to induce DNA strand breaks prior to labeling procedures.

Transfection with siRNA, FACS analysis, and Western blotting were as
20 described in Example 1.

Results

To determine the effect of DNA re-replication on cell proliferation, colon carcinoma HCT116 cells were transfected either with siGL2 or with siGem and then harvested at various times post-transfection. The number of control cells increased
25 ~350-fold by 8 days with a doubling time of 18-20 hours, and geminin levels in these cells remained constant (FIGS. 4A and 4B). In contrast, level of geminin in siGem treated cells was suppressed within two days of treatment (FIG. 4B). Similarly, depletion of geminin suppressed cell proliferation in colon adenocarcinoma DLD-1 cells, non-small lung carcinoma H1299 cells, and mammary
30 gland adenocarcinoma MCF7 cells. By six days post-transfection, cells had recovered from geminin depletion, as evidenced by the restoration of geminin

protein to normal levels (FIG. 4B), a reduction in the fraction of cells with giant nuclei (FIG. 4C) and renewed cell proliferation (FIG. 4A).

The lack of cell proliferation in geminin depleted cancer cells resulted from induction of apoptosis. FACS analysis revealed that geminin depletion induced
5 DNA re-replication within two days, but within four days, cells with greater than 4N DNA content disappeared from the population and cells containing less than 2N DNA content appeared in their place (FIG. 4D). Moreover, the fraction of HCT116 cells with giant nuclei increased up to 2 days post-transfection, and then declined as they lost cell adhesion and detached from the culture dish (FIG. 4C). In contrast,
10 control cells continued to exhibit FACS profiles typical of proliferating cell populations (FIG. 4D) and did not accumulate cells with giant nuclei (FIG. 4C).

Previous studies reported that addition of either caffeine (an inhibitor of ATM and ATR protein kinases) or UCN-01 (an inhibitor of CHK1 protein kinase) two days after siGem treatment of HCT116 cells rapidly induced apoptosis in cells
15 that had undergone DNA re-replication (Zhu *et al.*, *Mol. Cell. Biol.* 24:7140-7150, 2004). However, the results presented in FIG. 4 revealed that direct inhibition of the DNA damage response pathway was not necessary to induce apoptosis in these cells, because they underwent apoptosis spontaneously within eight days of treatment with siGem.

20 To confirm that siGem induced apoptosis in cancer cells, geminin depleted colon carcinoma HCT116 cells were assayed 4 days post-transfection for nuclear DNA fragmentation and for rounded cell morphology with blebbing, two additional characteristics of apoptosis (Otsuki *et al.*, *Prog. Histochem. Cytochem.* 38:275-339, 2003). HCT116 cells with giant nuclei were TUNEL positive, but cells with normal
25 nuclei were not (FIG. 5A). The TUNEL assay detects free 3'-OH terminated DNA fragments that can be labeled with fluorescein-conjugated dUTP by terminal deoxynucleotidyl transferase. Moreover, cells with giant nuclei exhibited rounded cell morphology with multiple blebs, but cells with normal nuclei did not (FIG. 5B). These results confirmed that siGem-induced DNA re-replication in cells derived
30 from malignant cancers resulted eventually in apoptosis. Geminin depletion induced DNA re-replication in cancer cells, regardless of the presence or absence of the

tumor suppressor protein p53, but p53^{+/+} cancer cells induced expression and phosphorylation of p53, as well as expression of the CDK-specific inhibitor p21 whose transcription is p53 dependent (FIG. 5C). These events are part of the DNA damage response in mammalian cells, as shown by treatment of cells with etoposide,

5 a specific inhibitor of topoisomerase II, that induces DNA breaks (FIG. 5C). Thus, induction of DNA re-replication by siGem in cells from cancers resulted in apoptosis within 4 to 8 days post-transfection.

Normal somatic cells generally proliferate slowly compared with cancer
10 cells. For example, normal breast epithelial AG11132 cells had a doubling time of 38-40 hours, whereas breast adenocarcinoma MCF7 cells had a doubling time of 30-32 hours. Similarly, normal colon epithelial FHC cells doubled their number in 39 hours, whereas colon carcinoma HCT116 cells required only 18-20 hours. Since differences in the time required for cell division in mammalian cells is reflected in
15 differences in the length of G1-phase, the absence of DNA re-replication in normal cells treated with siGem may result simply from the presence of fewer cells in the population undergoing division (S+G2+M phase cells). In that case, normal cells may require more time to respond to geminin depletion. To test this possibility, the experiments described above for HCT116 cells were repeated using normal skin D-1
20 fibroblasts. The rate of D-1 cell proliferation was not affected significantly by siGem relative to siGL2 (FIG. 6A), despite the fact that geminin levels were markedly reduced in cells transfected with siGem, but not with cells transfected with siGL2 (FIG. 6B). Furthermore, recovery of D-1 cells from siGem induced geminin depletion (FIG. 6B) followed the same time course as observed with HCT116 cells
25 (FIG. 4B). FACS profiles for both siGL2 and siGem treated D-1 cells were characteristic of proliferating cell populations, with little change in the fraction of cells with >4N DNA (FIG. 6C). Moreover, no TUNEL positive D-1 cells were detected in populations treated with either siGL2 or siGem (FIG. 5A). Similar results were obtained for other noncarcinogenic cells such as colon epithelial
30 CCD841 CoN cells, lung fibroblast WI-38 cells, breast epithelial AG11132 and MCF10A cells (FIG. 6D).

Example 3

Relationship of Geminin Expression Level and Sensitivity to Geminin Depletion

This example describes the analysis of the relationship between the level of
5 geminin protein expression and sensitivity to geminin depletion in cancer and non-
cancer cells.

Western blotting and FACS analysis were performed as described in
Example 1.

Geminin levels are generally high in tumor cells relative to normal cells,
10 suggesting that only cells with high levels of geminin may be sensitive to geminin
depletion. To test this possibility, geminin protein levels in normal cells and cancer
cells were compared relative to actin levels and to the fraction of cells in S-phase.
As expected, normal colon epithelial cells (CCD841 CoN and FHC cells) contained,
on average, about 20% as much geminin protein as colon carcinoma and
15 adenocarcinoma cells (HCT116, DLD-1, SW480, COLO 320DM) (FIG. 7A), and
the FACS profiles for these populations revealed smaller populations of dividing
cells (FIG. 7B). However, normal breast epithelial cells (AG11132, AG11134,
MCF10A) exhibited geminin levels comparable to breast adenocarcinoma MCF7
cells (FIG. 7A), consistent with the relative fraction of cells in these populations
20 undergoing cell division (FIG. 7B). Normal skin D-1 fibroblast populations,
however, contained a low level of geminin protein relative to both normal breast
cells and breast adenocarcinoma cells, consistent with the small fraction of
proliferating cells. Thus, cell populations with low geminin levels were
predominantly G1-phase cells, whereas cell populations with high geminin levels
25 contained about half the cells in S, G2 or M phases. Therefore, although there
existed a simple inverse correlation between geminin levels in cell populations and
the fraction of cells in G1-phase, no simple correlation existed between the geminin
levels and sensitivity to geminin depletion.

Example 4

Induction of DNA Re-Replication in Normal Cells Required Suppression of Multiple Regulatory Pathways

This example describes induction of DNA re-replication in non-cancer cells
5 by depletion of both geminin and cyclin A.

Cells were transfected with siRNA and FACS analysis and Western blotting were as described in Example 1.

To test whether one or more additional regulatory pathways exist that prevents reinitiation of DNA replication during proliferation of normal cells, both
10 geminin and cyclin A were simultaneously depleted. Cyclin A-dependent protein kinase activities are involved in regulating of several DNA replication events. The results revealed that depletion of either geminin or cyclin A alone had little effect on DNA re-replication in normal skin D-1 fibroblasts, whereas co-depletion of geminin and cyclin A induced DNA re-replication to the same extent observed in cancer cells
15 treated with siGem alone (Fig. 8A). Similar results were obtained with normal breast AG11132 and MCF10A cells and with normal colon CCD841 CoN cells (Table 2). Therefore, both CDK and geminin-dependent regulatory pathways prevent DNA re-replication in normal cells, whereas cancer cells rely primarily, if not exclusively, on geminin to prevent DNA re-replication. Accordingly, the extent
20 of DNA re-replication in HCT116 cells was the same, regardless of whether they were treated with siGem alone or together with siCcnA (FIG. 8B).

Example 5

High Throughput Screening Assay for Identifying Molecules that Induce DNA Re-Replication

This example describes a high throughput screening assay that can be used to identify small molecules that induce DNA re-replication.

Methods

SW480 human colon adenocarcinoma cells were dispensed into 1536 well
30 plates (250 cells/well) by a multi-drop dispenser robot. Once cells attached to the plate (about 3-4 hours), cells were incubated with DMSO or the CDK1/CDK5

inhibitor 3-(2-chloro-3-indolylmethylene)-1,3-dihydroindol-2-one for 47 hours using a pin tool robot. Cells were then incubated with Hoechst 33342 dye for 1 hour to stain cell nuclei. Cells were washed once with PBS to remove excess Hoechst dye and scanned with the Acumen® X3 microplate cytometer. The Acumen® X3 is
5 able to scan all the cells in each well and display DNA content or calculate the size of each nucleus based on its fluorescent signal.

Results

As shown in Figure 9, cells treated with the CDK1/CDK5 inhibitor 3-(2-chloro-3-indolylmethylene)-1,3-dihydroindol-2-one (which induces DNA re-
10 replication in both cancer and non-cancer cells) exhibit dramatically increased DNA content (more than 4N DNA content). The percentage of cells with giant nuclei or with more than 4N DNA content increased to 50-60% (on average) after drug treatment compared to 4% (on average) for control treated cells. The quality and performance of this assay was evaluated by examining screen window coefficient
15 called the “Z-factor,” a characteristic of the capability of hit identification for each assay under a specific set of conditions. The Z-value for this assay was 0.51, and coefficient of variation (CV) was less than 10%, which is considered as an excellent HTS assay.

20 **Example 6**

High Throughput Screening Assay for Identifying Geminin Inhibitors

This example describes a high throughput screening assay that can be used to identify small molecules that inhibit geminin.

The screening method is carried out in two rounds. The first round of
25 screening is essentially as described in Example 5. Both cancer cells and non-cancer cells are used in the assay. The cells are contacted with a small molecule library (~300,000 compounds) and stained with Hoechst dye. Compounds that induce DNA re-replication in cancer cells but not in normal cells are identified based on the presence of >4N DNA content and/or giant nuclei. This round of screening will
30 identify compounds that inhibit geminin as well as compounds that target other

pathways to selectively induce DNA re-replication in cancer cells. All compounds identified in the first round of screening are potential anti-cancer drugs.

The primary screen selects for compounds that increase nuclear DNA content in cancer cells but not in normal cells. However, this can occur by at least
5 three ways: induction of DNA re-replication during S-phase, induction of endoreduplication during G2-phase, or endomitosis during M-phase. DNA re-replication is an aberrant event that results in DNA damage, and the DNA damage response induces apoptosis. Endoreduplication and endomitosis (multiple rounds of genome duplication without an intervening cell division) are events that occur
10 normally during animal development in specific tissues; they do not induce apoptosis. Therefore, the compounds from the initial screen are tested for their ability to induce apoptosis.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) can be used to assay for apoptosis; kits are commercially available (for
15 example, from Roche Applied Science, Indianapolis, IN). Other methods that can be used for determining apoptosis are well known to one of skill in the art. This screen will eliminate any compounds that induce excess DNA replication without inducing apoptosis.

Compounds that induce DNA re-replication and apoptosis in the first round
20 of screening are tested in the second round to identify compounds that inhibit geminin activity. Geminin activity is assessed by the interaction between geminin and Cdt1. Compounds that inhibit this interaction are geminin inhibitors. Cancer cells can be treated with an individual compound for period of time and then harvested. The harvested cells are then subjected a co-immunoprecipitation assay to
25 detect interaction between endogenous geminin and Cdt1. If one compound interferes with the interaction between geminin and Cdt1, one would expect to see a loss of interaction between Cdt1 and geminin after compound treatment.

Alternatively, geminin activity in the presence of a test compound can be assessed as follows. First, both geminin and Cdt1 are purified. Purified Cdt1 is
30 coated on each well of a 96-well plastic microtiter plate, compounds are added, followed by addition of purified geminin. After washing, anti-geminin antibody

conjugated to a detectable label (such as a fluorescent marker or enzymatic label) is added, followed by another round of washing. Anti-geminin antibody is detected using a method appropriate to the label (for example, detection of fluorescence or detection of the product of the enzyme). A reduction or absence of anti-geminin
5 antibody detection indicates a compound disrupts the interaction between geminin and Cdt1.

Active compounds that induce DNA re-replication and apoptosis can also be screened for their effect on geminin expression by Western blotting. Cdt1 protein can also be measured in order to monitor the ratio of geminin to Cdt1 in cells. This
10 ratio is a parameter that determines whether or not sufficient Cdt1 activity remains to load the MCM helicase onto chromatin. For those compounds that suppress the level of geminin, whether they do so by inhibiting geminin mRNA synthesis, or geminin translation, or geminin turnover will be determined. Geminin mRNA synthesis is measured by reverse transcriptase polymerase chain reaction (RT-PCR).
15 This assay can be done simply by measuring the relative levels of one RNA to another (e.g. geminin mRNA/tubulin mRNA ratio), or quantitatively, if necessary, to determine the actual number of mRNA molecules per cell.

If geminin RNA levels are not affected by the active compound, then cells treated with the active compound will be briefly treated with MG132, a specific
20 inhibitor of the 26S proteasome, to determine whether or not geminin protein levels can be elevated by preventing ubiquitin-dependent geminin degradation. Geminin is normally degraded as cells exit mitosis by the anaphase promoting complex (APC), which is an E3 ubiquitin ligase.

If geminin protein levels cannot be restored by inhibition of the 26S
25 proteasome, then whether or not the active compound inhibits translation of geminin mRNA will be determined by incorporating ³⁵S-methionine into cells treated with the active compound, prepare a soluble cell lysate from which geminin can be immuno-precipitated. To this cytosol, geminin protein will be added as a carrier, and then total geminin immuno-precipitated and the immuno-precipitate subjected to
30 Western immunoblotting analysis to compare the level of labeled geminin produced in untreated cells with the level produced in treated cells.

Compounds that induce DNA re-replication and apoptosis *in vitro*, regardless of whether they interfere with Cdt1 binding to geminin, can be screened for anti-tumor activity in an *in vivo* model, such as described below.

5

Example 7

Selective Killing of Cancer Cells in an *In Vivo* Model

This example describes methods to assess the efficacy of a compound to selectively kill cancer cells or prevent tumor growth or metastasis in an *in vivo* model.

10 A. Inducible geminin shRNA

In order to address whether geminin siRNA can selectively kill cancer cells *in vivo*, cancer cell lines (for example, HCT116 colon carcinoma cells, T98 glioma cells, or MCF7 breast carcinoma cells) are stably transfected with a shRNA targeted against geminin mRNA (shGem) that is expressed from a tetracycline-inducible

15 promoter. shRNA include shGem1

(TGCTGTTGACAGTGAGCGCATAGAGAGACTGAATGGTGAATAGTGAAG
CCACAGATGTATTCACCATTCAGTCTCTCTATTTGCCTACTGCCTCGGA;
SEQ ID NO: 8; underlined regions indicate target sequence), shGem2

(TGCTGTTGACAGTGAGCGAAGTCATTGTATCTTATGATTATAGTGAAGC
20 CACAGATGTATAATCATAAGATCAAATGACTCTGCCTACTGCCTCGGA;
SEQ ID NO: 9; underlined regions indicate target sequence), and control (luciferase)
shGL2

(TGCTGTTGACAGTGAGCGAACGTACGCGGAATACTTCGAATAGTGAAGC
CACAGATGTATTTCGAAGTATTCCGCGTACGTGTGCCTACTGCCTCGGA;
25 SEQ ID NO: 10; underlined regions indicate target sequence).

The transfected cells (about 10^5 to 10^7 cells embedded in 50 μ l Matrigel™ membrane matrix) are injected into SCID mice, and allowed to form a palpable tumor. Once a tumor is palpable, tetracycline is administered to the mouse. The size of the tumor is monitored for up to 20 weeks. Arrest of the tumor growth or
30 reduction in size of the tumor indicates that suppressing geminin expression is effective to kill the cancer cells.

B. Geminin siRNA expressing xenografts

Another approach to address whether geminin siRNA can selectively kill cancer cells *in vivo* is to transfect cancer cells with geminin siRNA (for example, as described in Example 1) and to then introduce the transfected cells into an immune-compromised mouse. For example, transfected cells can be injected into the tail vein to induce experimental metastases, or can be injected into a particular tissue, from which the cancer cells originally arose (for example, breast cancer cells can be injected into the mammary gland). Development of tumors (such as number or size of tumors) is monitored over time. Formation of fewer tumors or formation of smaller tumors in mice injected with cells transfected with geminin siRNA compared to mice injected with control cells indicates that suppressing geminin expression is effective to kill the cancer cells.

C. Administration of geminin siRNA

Mouse tumor models are established by injection of cancer cells into immune-compromised mice, such as SCID mice. Tumors are induced by injection of 5×10^6 MDA-MB-231 breast cancer cells in the thoracic mammary gland fat pad of mice or by intravenous injection of 1×10^6 MDA-MB-231 cells in the tail vein to induce experimental metastases. Alternatively, a glioma model can be produced by injecting human glioma-derived cancer stem cells (such as T98G glioblastoma cells) into mouse brain. Other mouse tumor models are well known to one of skill in the art.

Following development of a tumor, the geminin siRNA is injected directly into the tumor, or administered to the mouse intravenously. In some examples, the siRNA is administered with a delivery reagent (for example Invivofectamine™, Invitrogen, Carlsbad, CA). Chemically modified siRNA (for example, Stealth™ RNAi, Invitrogen) can be used. The dose is 0, 1X, 2X, 4X, or 8X the amount required to kill a similar number of cells *in vitro* (for example, as determined in Example 2). The number of cells in the tumor is determined at one or more time points (for example, up to 20 weeks). Representative tumors are collected and fixed, embedded, and sectioned for histological and histochemical evaluation. A reduction in the number of cells in a tumor (for example compared to a tumor from an animal

that did not receive geminin siRNA) indicates that the geminin siRNA kills cancer cells *in vivo*. One of skill in the art will understand that similar methods may be used to evaluate candidate geminin inhibitor compounds other than geminin siRNA.

5 In view of the many possible embodiments to which the principles of the disclosure may be applied, it should be recognized that the illustrated embodiments are only preferred examples and should not be taken as limiting the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of
10 these claims.

We claim:

1. A method for selectively inducing apoptosis of cancer cells, comprising contacting a cancer cell with a compound that inhibits geminin, wherein the
5 compound is an siRNA comprising a nucleic acid sequence according to SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5, in the absence of a cell cycle checkpoint inhibitor for a period of time sufficient to induce apoptosis of the cancer cell without inducing apoptosis of non-cancer cells, thereby selectively inducing apoptosis of cancer cells.
10
2. A method for selectively inducing apoptosis of cancer cells, comprising contacting a cancer cell with a compound that inhibits geminin in the absence of a cell cycle checkpoint inhibitor for a period of time sufficient to induce apoptosis of the cancer cell without inducing apoptosis of non-cancer cells, thereby selectively
15 inducing apoptosis of the cancer cells.
3. The method of claim 1 or claim 2, wherein the cancer cell is contacted with the compound for at least about 4 days.
- 20 4. The method of any one of claims 1 to 3, wherein the compound selectively induces DNA re-replication in the cancer cell.
5. The method of any one of claims 1 to 4, wherein the cancer cell overexpresses geminin as compared to the non-cancer cell.
25
6. The method of claim 2, wherein the compound comprises an inhibitor of geminin expression.
7. The method of claim 6, wherein the inhibitor of geminin expression
30 comprises an RNAi molecule, a peptide, an antibody, a small organic molecule, an antisense molecule, or a combination of two or more thereof.

8. The method of claim 7, wherein the RNAi molecule comprises a geminin siRNA.
- 5 9. The method of claim 8, wherein the geminin siRNA comprises a nucleic acid sequence according to SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or a combination of two or more thereof.
- 10 10. The method of claim 7, wherein the RNAi molecule comprises a geminin shRNA.
11. The method of claim 10, wherein the shRNA comprises a nucleic acid sequence according to SEQ ID NO: 8, SEQ ID NO: 9, or a combination thereof.
- 15 12. The method of claim 2, wherein the compound comprises an inhibitor of geminin activity.
13. The method of claim 12, wherein the inhibitor of geminin activity comprises a peptide, an antibody, a small organic molecule, or a combination of two or more
20 thereof.
14. The method of any one of claims 1 to 13, wherein the cancer cell comprises a colon cancer cell, a breast cancer cell, a lung cancer cell, a kidney cancer cell, a bone cancer cell, or a brain cancer cell.
25
15. The method of any one of claims 1 to 14, wherein contacting the cancer cell with a compound that inhibits geminin further comprises administering a therapeutically effective amount of the compound in the absence of a cell cycle checkpoint inhibitor to a subject in need of treatment for cancer.
30

16. The method of claim 15, wherein the subject has colon cancer, breast cancer, lung cancer, kidney cancer, bone cancer, or brain cancer.
17. The method of claim 15 or claim 16, further comprising administering a
5 second anti-cancer therapeutic that does not inhibit geminin to the subject.
18. The method of claim 17, wherein the second anti-cancer therapeutic induces DNA re-replication in the cancer cells.
- 10 19. The method of any one of claims 1 to 14, further comprising:
isolating the cancer cell from a subject;
determining if the cancer cell from the subject overexpresses geminin as compared to a non-cancer cell from the subject; and
administering a therapeutically effective amount of the compound that
15 inhibits geminin to the subject if the cancer cell overexpresses geminin.
20. A method for identifying a compound that selectively induces apoptosis of cancer cells, comprising:
contacting a test compound with a cancer cell expressing geminin in the
20 absence of a cell cycle checkpoint inhibitor;
assessing cell cycle status of the cancer cell, wherein the cell cycle status is selected from the group consisting of DNA re-replication, apoptosis, cell cycle arrest, or a combination thereof; and
assessing geminin activity in the cancer cell, wherein a compound that
25 induces DNA re-replication, apoptosis, or cell cycle arrest and inhibits geminin activity in a cancer cell in the absence of a cell cycle checkpoint inhibitor indicates that the test compound is a compound that selectively induces apoptosis of cancer cells.

21. The method of claim 20, wherein the test compound is selected from the group consisting of an RNAi molecule, a peptide, an antibody, a small organic molecule, an antisense molecule, or a combination of two or more thereof.
- 5 22. The method of claim 20 or 21, wherein assessing DNA re-replication comprises measuring the number of cells with greater than about 4N DNA content or measuring the number of cells having a giant nucleus.
23. The method of claim 20 or 21, wherein assessing apoptosis comprises a
10 TUNEL assay or fluorescence activated cell sorting.
24. The method of claim 20 or 21, wherein assessing cell cycle arrest comprises determining arrest before mitosis.
- 15 25. The method of claim 24, wherein arrest before mitosis is assessed by histone H3 (Ser10) phosphorylation, wherein cells arrested before mitosis are negative for histone H3 (Ser10) phosphorylation.
26. The method of any one of claims 20 to 25, wherein assessing geminin activity
20 comprises measuring geminin mRNA or protein expression or measuring geminin binding to Cdt1.
27. The method of any one of claims 20 to 26, wherein the cancer cell is contacted with the test compound for at least about 4 days.
- 25

FIG. 1A

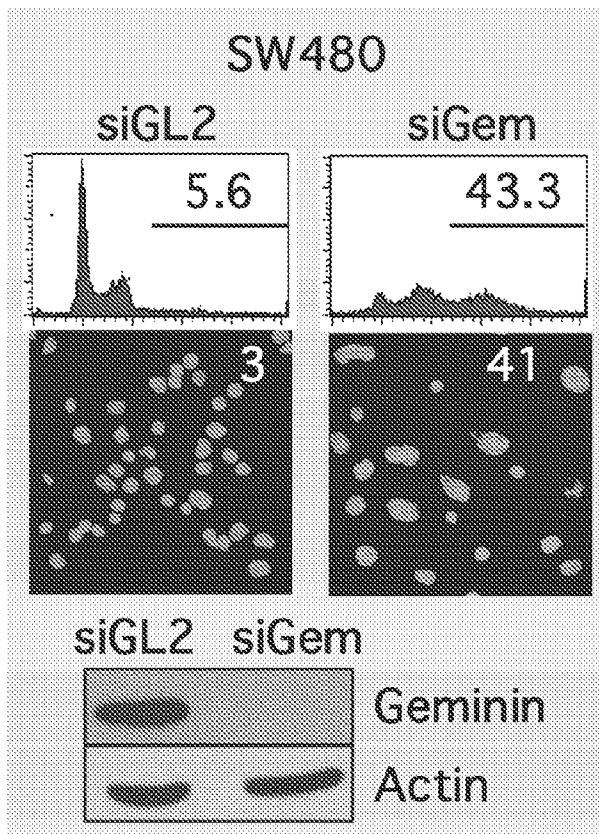


FIG. 1B

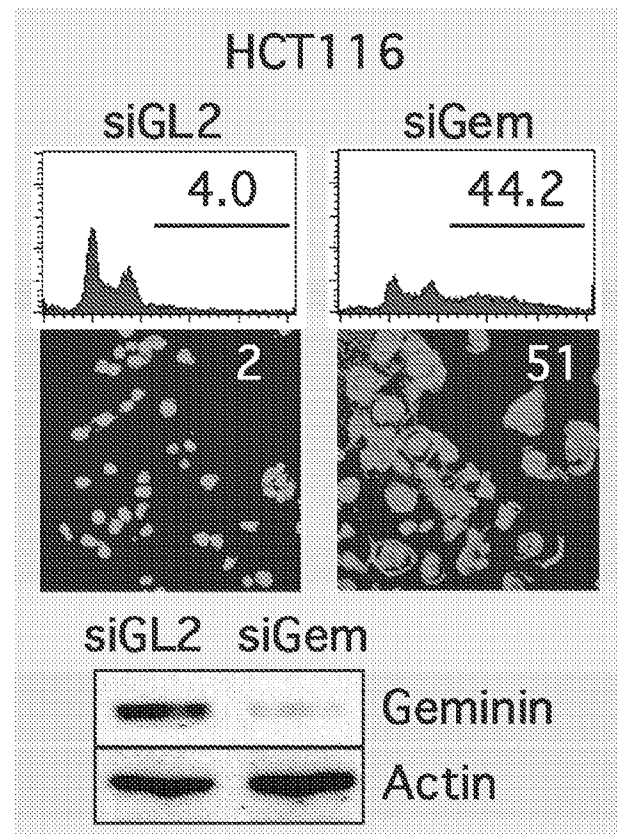


FIG. 1C

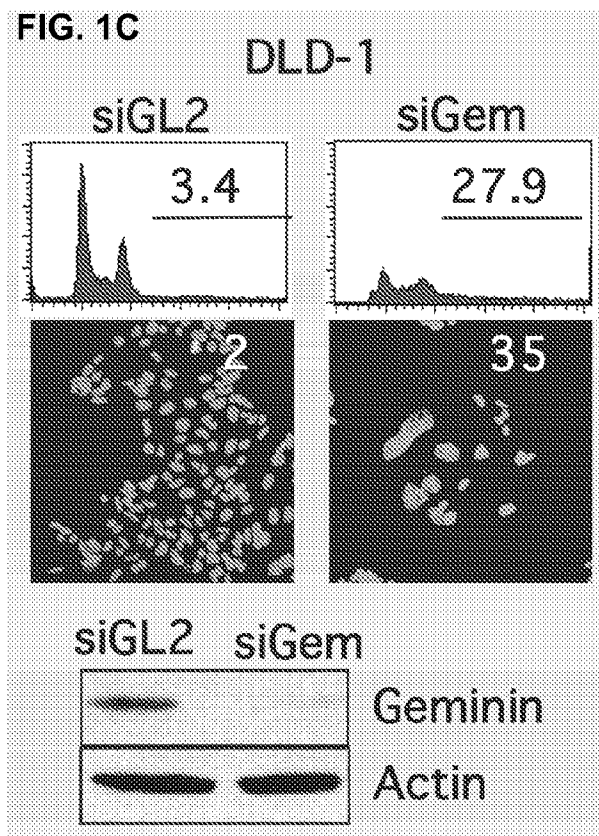


FIG. 1D

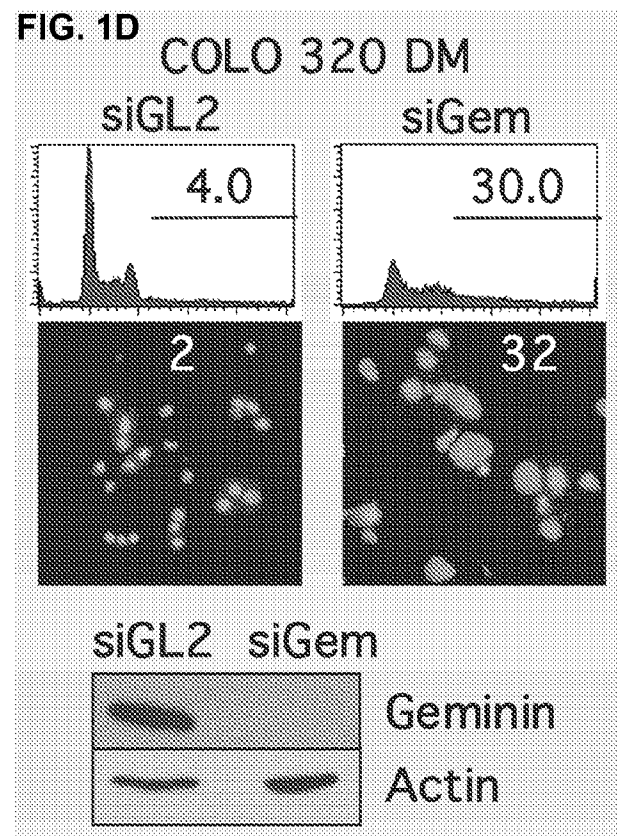


FIG. 2A

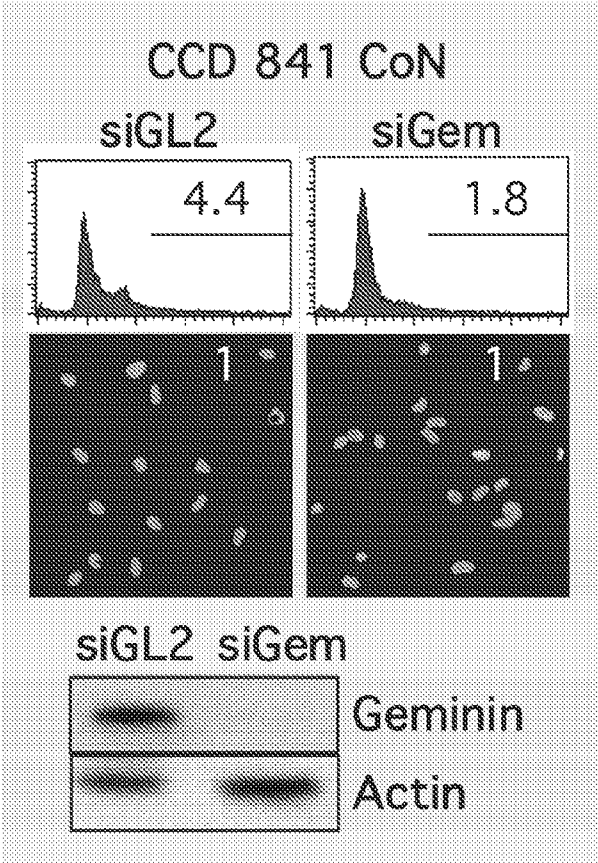


FIG. 2B

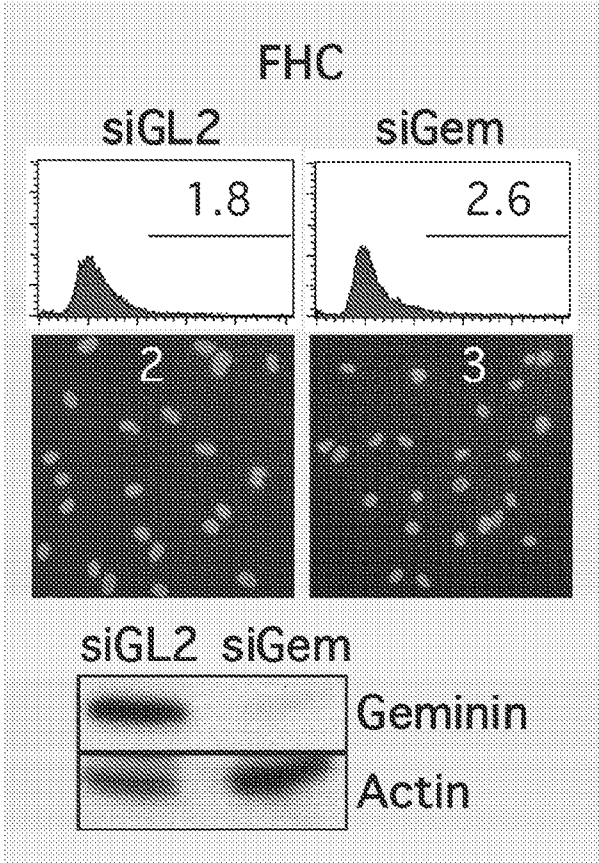


FIG. 3A

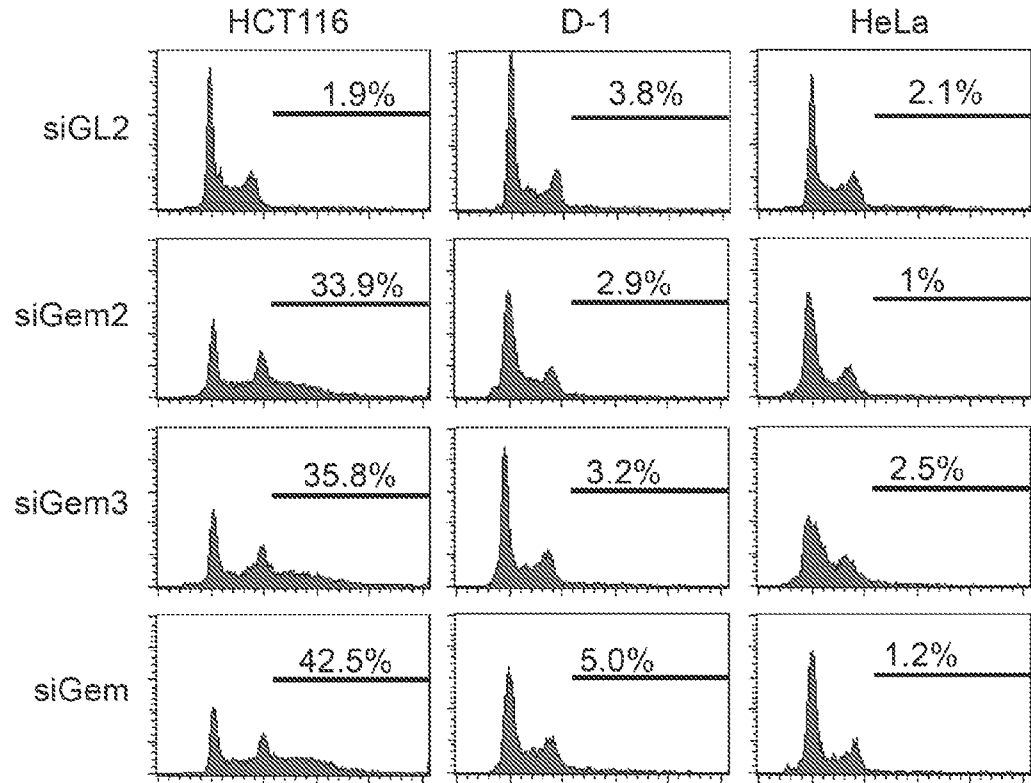
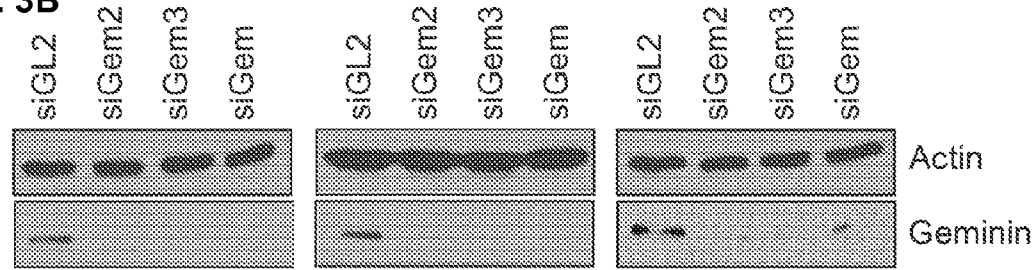
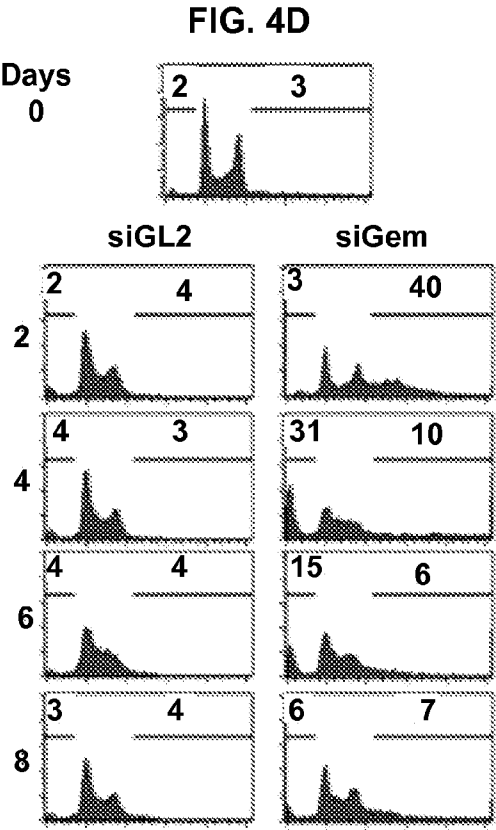
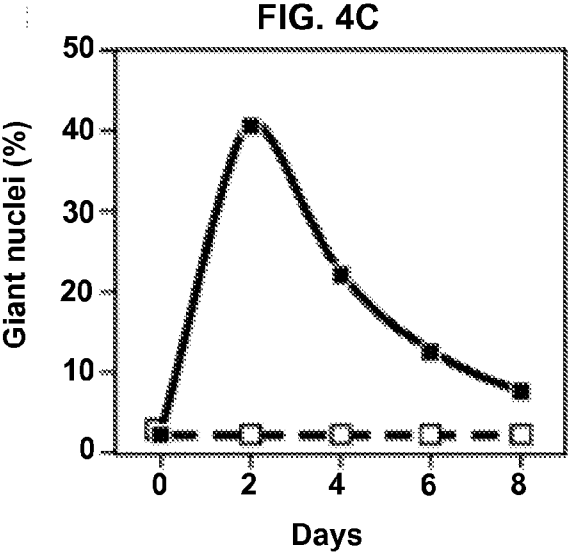
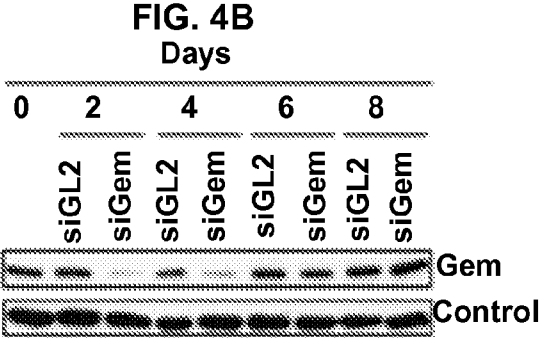
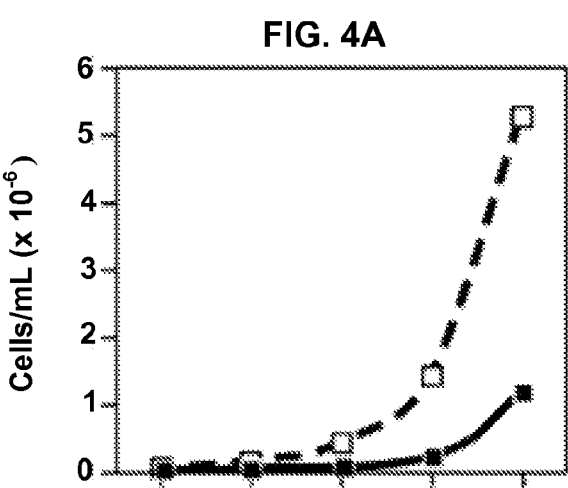
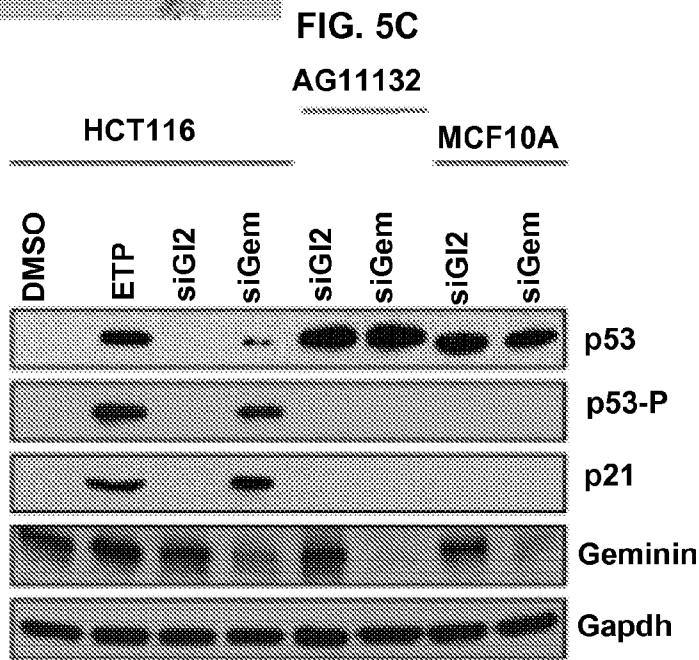
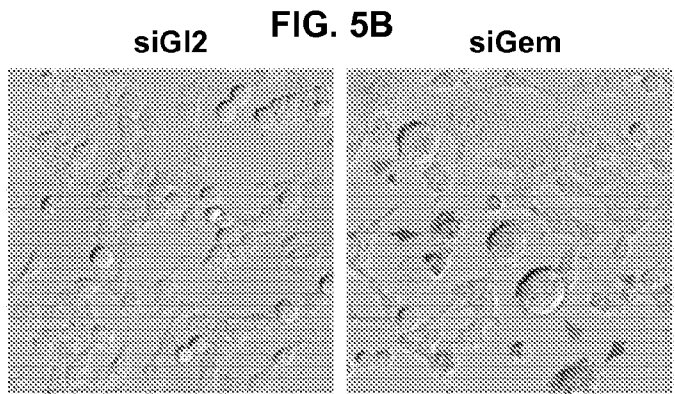
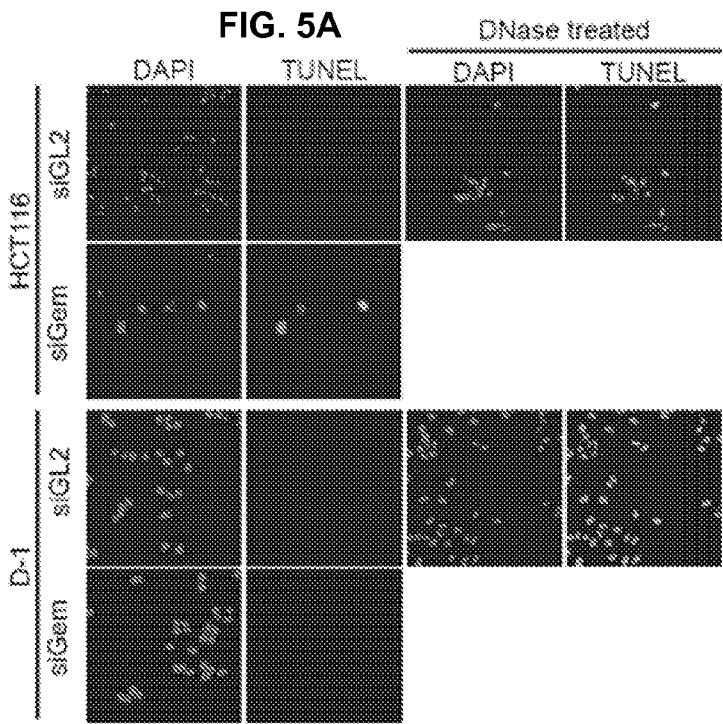


FIG. 3B







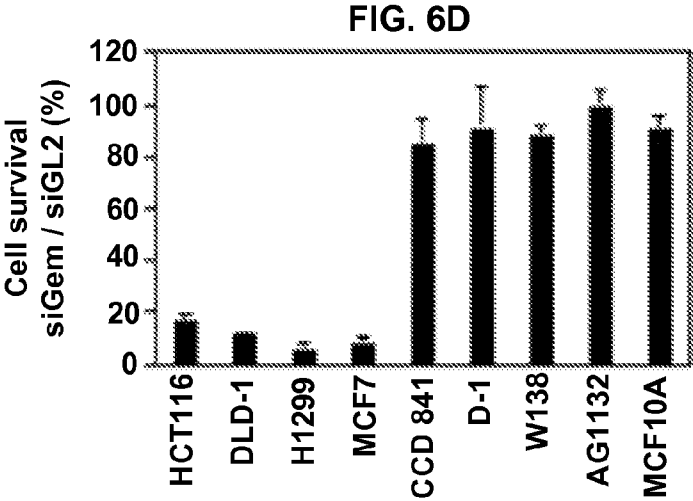
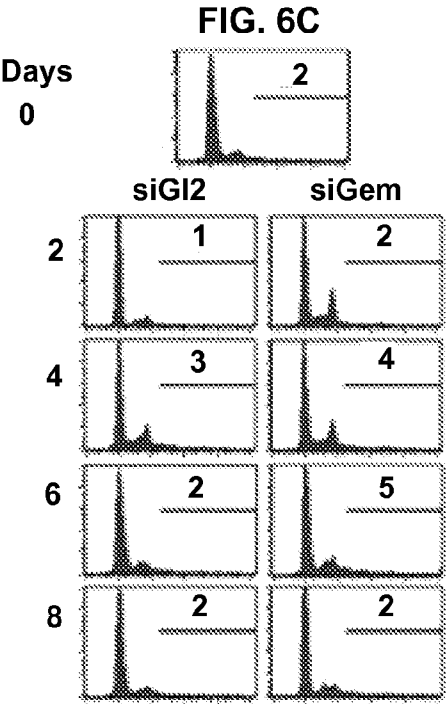
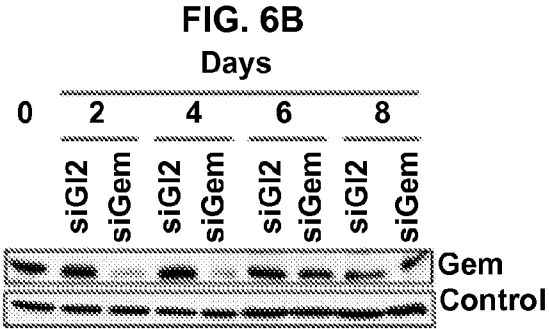
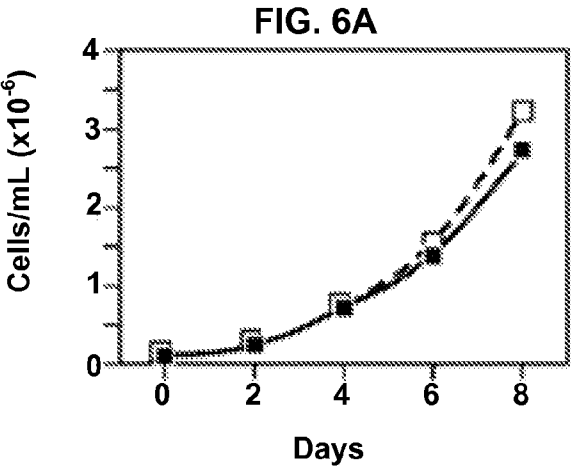


FIG. 7A

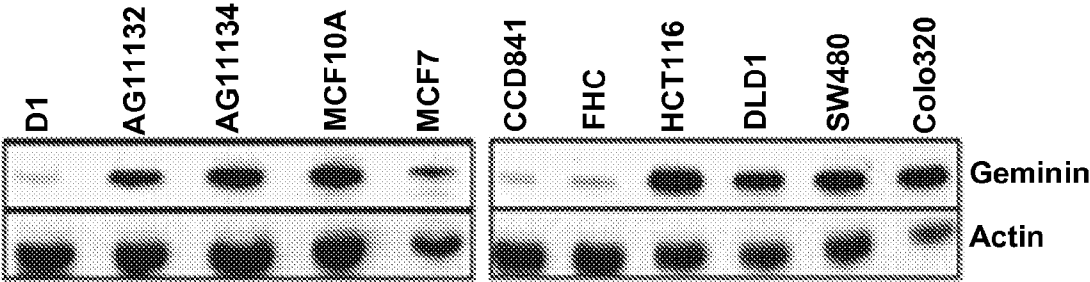
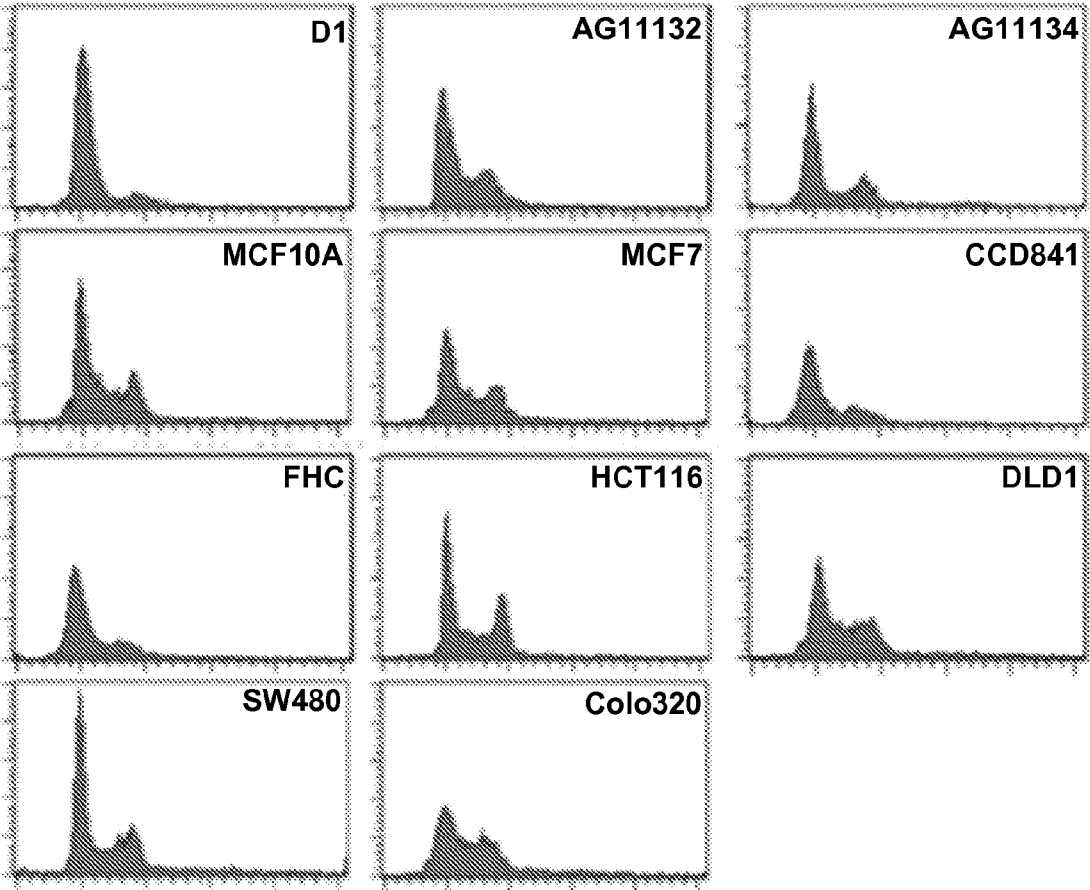
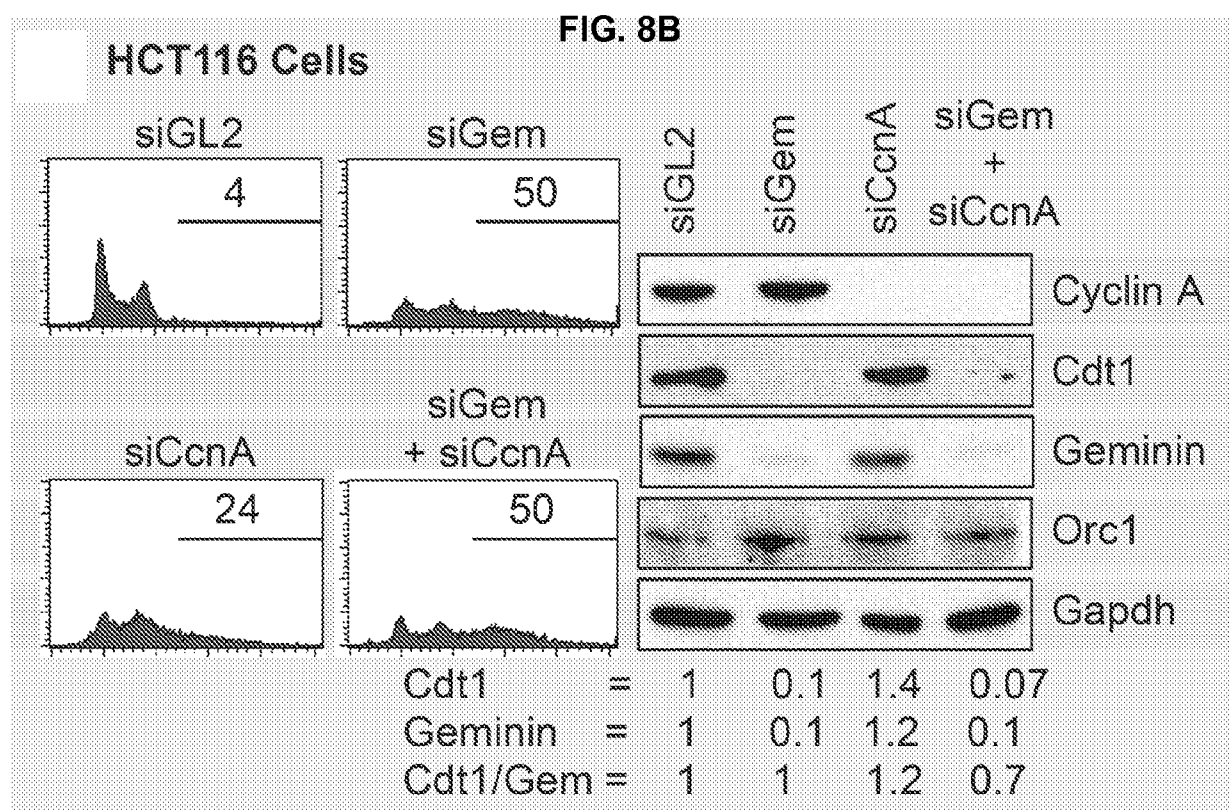
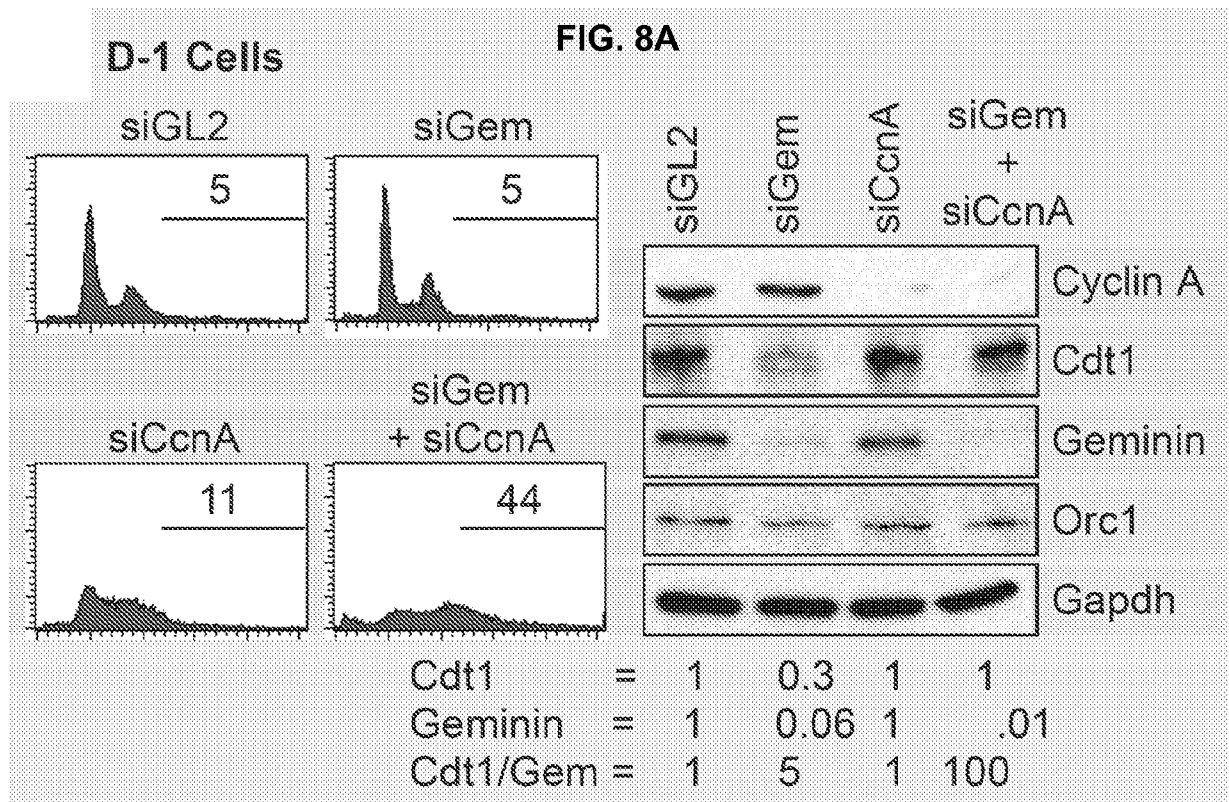
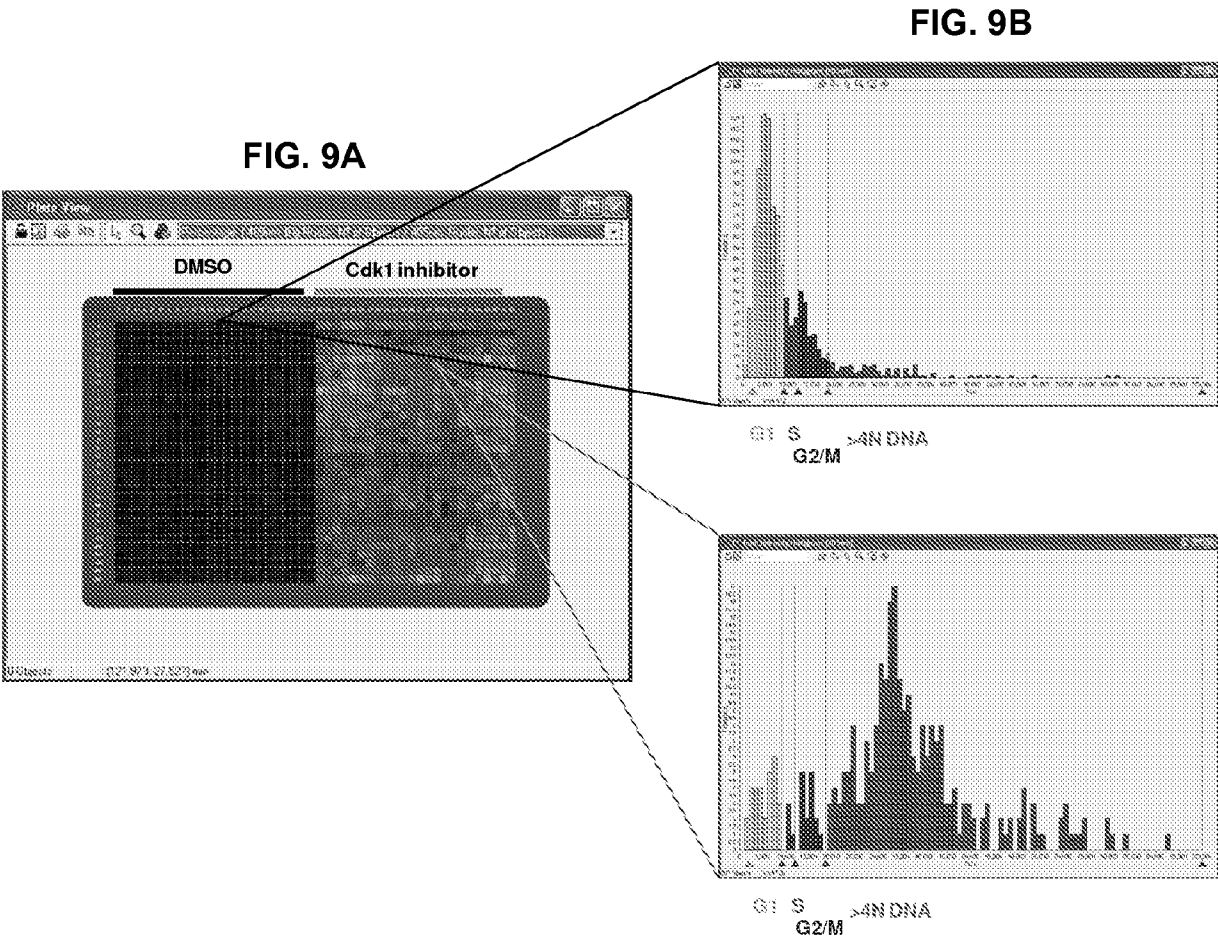


FIG. 7B







INTERNATIONAL SEARCH REPORT

International application No
PCT/US2009/060949

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N15/11

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)
C12N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 757 306 A1 (GENECARE RES INST CO LTD [JP]) 28 February 2007 (2007-02-28)	1-21, 23-24, 26-27 22, 25
Y	paragraph [0004] paragraph [0006] paragraph [0038] paragraph [0080] paragraph [0338]	
X	WO 2008/073922 A2 (ASURAGEN INC [US]; JOHNSON CHARLES D [US]; BYROM MIKE [US]; BADER ANDR) 19 June 2008 (2008-06-19) claims 1,5,6 ----- -/--	2-7, 12-19

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

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" 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)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" 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

"X" 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

"Y" 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

Date of the actual completion of the international search

1 December 2009

Date of mailing of the international search report

11/12/2009

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Rutz, Berthold

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2009/060949

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

a. type of material

☒

a sequence listing

☐

table(s) related to the sequence listing

b. format of material

☐

on paper

☒

in electronic form

c. time of filing/furnishing

☒

contained in the international application as filed

☐

filed together with the international application in electronic form

☐

furnished subsequently to this Authority for the purpose of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2009/060949

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SALABAT MOHAMMAD R ET AL: "Geminin is overexpressed in human pancreatic cancer and downregulated by the bioflavanoid apigenin in pancreatic cancer cell lines." MOLECULAR CARCINOGENESIS NOV 2008, vol. 47, no. 11, 10 April 2008 (2008-04-10), pages 835-844, XP002558193 ISSN: 1098-2744 the whole document	2-7, 12-21
X	MIZUSHINA YOSHIYUKI ET AL: "Coenzyme Q10 as a potent compound that inhibits Cdt1-geminin interaction." BIOCHIMICA ET BIOPHYSICA ACTA FEB 2008, vol. 1780, no. 2, February 2008 (2008-02), pages 203-213, XP002557418 ISSN: 0006-3002 the whole document	2,4-5, 12-14,20
Y	ZHU WENGE ET AL: "Rereplication by depletion of geminin is seen regardless of p53 status and activates a G2/M checkpoint." MOLECULAR AND CELLULAR BIOLOGY AUG 2004, vol. 24, no. 16, August 2004 (2004-08), pages 7140-7150, XP002557315 ISSN: 0270-7306 the whole document	22,25
Y	MELIXETIAN MARINA ET AL: "Loss of Geminin induces rereplication in the presence of functional p53." THE JOURNAL OF CELL BIOLOGY 24 MAY 2004, vol. 165, no. 4, 24 May 2004 (2004-05-24), pages 473-482, XP002557314 ISSN: 0021-9525 the whole document	22,25
A	ZHU WENGE ET AL: "An ATR- and BRCA1-mediated Fanconi anemia pathway is required for activating the G2/M checkpoint and DNA damage repair upon rereplication." MOLECULAR AND CELLULAR BIOLOGY JUN 2006, vol. 26, no. 12, June 2006 (2006-06), pages 4601-4611, XP002557312 ISSN: 0270-7306 the whole document	1-27

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2009/060949

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LIN JIE JESSIE ET AL: "ATR pathway is the primary pathway for activating G2/M checkpoint induction after re-replication." THE JOURNAL OF BIOLOGICAL CHEMISTRY 19 OCT 2007, vol. 282, no. 42, 19 October 2007 (2007-10-19), pages 30357-30362, XP002557313 ISSN: 0021-9258 the whole document	1-27
A	TACHIBANA KIKU-E K ET AL: "Depletion of licensing inhibitor geminin causes centrosome overduplication and mitotic defects." EMBO REPORTS NOV 2005, vol. 6, no. 11, November 2005 (2005-11), pages 1052-1057, XP002557316 ISSN: 1469-221X the whole document	1-27
A	HOOK ET AL: "Mechanisms to control rereplication and implications for cancer" CURRENT OPINION IN CELL BIOLOGY, CURRENT SCIENCE, LONDON, GB, vol. 19, no. 6, 28 November 2007 (2007-11-28), pages 663-671, XP022382274 ISSN: 0955-0674 the whole document	1-27
A	PETROPOULOU CHARIKLIA ET AL: "Cdt1 and Geminin in cancer: markers or triggers of malignant transformation?" FRONTIERS IN BIOSCIENCE : A JOURNAL AND VIRTUAL LIBRARY 2008, vol. 13, 1 May 2008 (2008-05-01), pages 4485-4494, XP002557419 ISSN: 1093-4715 the whole document	1-27
X,P	ZHU WENGE ET AL: "Selective killing of cancer cells by suppression of geminin activity." CANCER RESEARCH 1 JUN 2009, vol. 69, no. 11, 1 June 2009 (2009-06-01), pages 4870-4877, XP002557317 ISSN: 1538-7445 the whole document	1-27

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 2-7, 12-19(all partially)

Present claim 2-7 and 12-19 encompass methods using compounds defined only by their desired function, contrary to the requirements of clarity of Article 6 PCT, because the result-to-be-achieved type of definition does not allow the scope of the claim to be ascertained. The fact that any compound could be screened does not overcome this objection, as the skilled person would not have knowledge beforehand as to whether it would fall within the scope claimed, except for the compounds disclosed in the description, see Examples 1 (siRNA) and 7 (shRNA) . Undue experimentation would be required to screen compounds randomly. This non-compliance with the substantive provisions is to such an extent, that the search was performed taking into consideration the non-compliance in determining the extent of the search for claims 2-7 and 12-19. The search of claims 2-7 and 12-19 was consequently restricted to RNAi molecules, antibodies and antisense molecules.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.2), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2009/060949

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 2-7, 12-19(all partially)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2009/060949

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 1757306	A1	28-02-2007	WO 2005097189 A1	20-10-2005
			KR 20060135945 A	29-12-2006
			US 2009028861 A1	29-01-2009
WO 2008073922	A2	19-06-2008	AU 2007333109 A1	19-06-2008
			CA 2671299 A1	19-06-2008
			EP 2104737 A2	30-09-2009
			US 2009163430 A1	25-06-2009