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- (54) **Title:** TREATING CANCER USING INHIBITORS OF ATAXIA-TELANGIECTASIA MUTATED AND RAD3-RELATED (ATR)

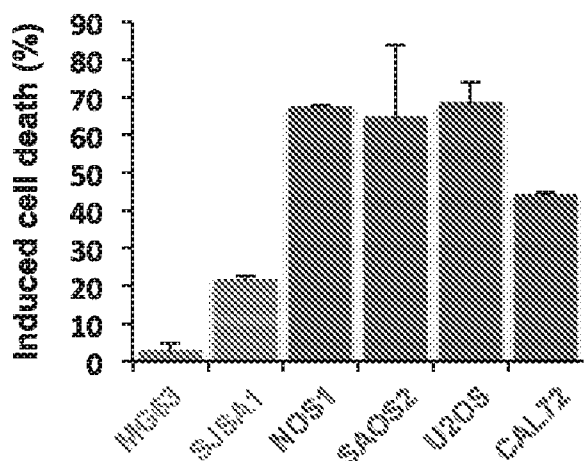


FIG. 4E

- (57) **Abstract:** Methods for identifying and treating cancers, e.g., Alternative Lengthening of Telomeres (ALT)-positive cancers or cancers associated with accumulation of ssDNA, using Ataxia-Telangiectasia mutated and Rad3-related (ATR) inhibitors.

Treating Cancer using Inhibitors of Ataxia-Telangiectasia Mutated and Rad3-Related (ATR)

CLAIM OF PRIORITY

This application claims the benefit of U.S. Provisional Application Serial No. 62/101,415, filed on January 9, 2015. The entire contents of the foregoing are incorporated herein by reference.

5 **FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT**

This invention was made with Government support under Grant Nos. GM076388 and CA166729 awarded by the National Institutes of Health. The Government has certain rights in the invention.

TECHNICAL FIELD

10 Described herein are methods for identifying and treating cancers, e.g., Alternative Lengthening of Telomeres (ALT)-positive cancers or cancers associated with accumulation of ssDNA, using Ataxia-Telangiectasia mutated and Rad3-related (ATR) inhibitors.

BACKGROUND

15 Telomeres are nucleoprotein complexes that include a hexanucleotide DNA repeat sequence (TTAGGG)_n and various telomere-associated proteins, which act to stabilize the ends of chromosomes. In normal somatic cells, significant telomere shortening leads to p53-dependent senescence or apoptosis (Heaphy and Meeker, J Cell Mol Med. 15(6):1227–1238 (2011)). Cancer cells overcome replicative
20 senescence by activating telomerase or the Alternative Lengthening of Telomeres (ALT) pathway (1-3). ALT is used in ~5% of all human cancers and is prevalent in specific cancer types, including osteosarcoma and glioblastoma (4). Currently, there are no therapies specifically targeting ALT.

SUMMARY

25 As demonstrated herein, inhibition of Ataxia-Telangiectasia mutated and Rad3-related (ATR), a critical regulator of recombination recruited by RPA, disrupts ALT and triggers chromosome fragmentation and apoptosis specifically in ALT-

positive cancer cells. Thus, described herein are methods for treating ALT positive cancers using ATR inhibitors. In addition, ssDNA accumulates in ATR-inhibitor sensitive cancers; thus, also described herein are methods for treating cancers in which levels of ssDNA above a threshold or reference level indicate that the cancer is likely to be sensitive to ATR inhibitors.

In a first aspect, provided herein are methods for treating cancer in a subject. The methods include identifying the subject as having an Alternative Lengthening of Telomeres (ALT)-positive cancer, and administering to the subject a therapeutically effective amount of an inhibitor of Ataxia-Telangiectasia mutated and Rad3-related (ATR).

In another aspect, provided herein are methods for treating cancer in a subject that include identifying the subject as having an ATR-sensitive cancer, wherein identifying the subject as having an ATR-sensitive cancer comprises obtaining a sample comprising cells from a cancer in the subject; detecting a level of ssDNA in the sample; comparing the level of ssDNA in the subject sample to a reference level of ssDNA; and identifying a subject as having an ATR-sensitive cancer if the level of ssDNA in the subject sample is above the reference level of ssDNA, and administering to the subject a therapeutically effective amount of an inhibitor of Ataxia-Telangiectasia mutated and Rad3-related (ATR).

In some embodiments of the methods described herein, the inhibitor of ATR is a small molecule. In some embodiments, the small molecule is selected from the group consisting of Schisandrin B (10.Benzo(3,4)cycloocta(1,2-f)(1,3)benzodioxole, 5,6,7,8-tetrahydro-1,2,3,13-tetramethoxy-6,7-dimethyl-, stereoisomer: NU6027 (6-(cyclohexylmethoxy)-5-nitrosopyrimidine-2,4-diamine); NVP-BEZ235 (2-methyl-2-[4-(3-methyl-2-oxo-8-quinolin-3-ylimidazo[4,5-c]quinolin-1-yl)phenyl]propanenitrile); VE-821 (2-(aminomethyl)-6-[4,6-diamino-3-[4-amino-3,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-2-hydroxycyclohexyl]oxyoxane-3,4,5-triol;sulfuric acid); VE-822 (VX-970; 3-[3-[4-(methylaminomethyl)phenyl]-1,2-oxazol-5-yl]-5-(4-propan-2-ylsulfonylphenyl)pyrazin-2-amine); AZ20 ((3R)-4-[2-(3H-indol-4-yl)-6-(1-methylsulfonylcyclopropyl)pyrimidin-4-yl]-3-methylmorpholine); AZD6738 (4-[4-[1-[[S(R)]-S-methylsulfonimidoyl]cyclopropyl]-6-[(3R)-3-methyl-4-morpholinyl]-2-pyrimidinyl]-1H-pyrrolo[2,3-b]pyridine); and

ETP-46464 (2-methyl-2-[4-(2-oxo-9-quinolin-3-yl-4H-[1,3]oxazino[5,4-c]quinolin-1-yl)phenyl]propanenitrile).

In some embodiments, the inhibitor of ATR is an inhibitory nucleic acid targeting ATR, e.g., a small interfering RNA (siRNA), small hairpin RNA (shRNA),
 5 antisense oligo, or CRISPR/Cas guide RNA.

In some embodiments, the inhibitory nucleic acid includes one or more modifications, e.g., one or more of modified bases, e.g., locked nucleic acids (LNAs), or modified backbone, e.g., peptide nucleic acids (PNAs).

Unless otherwise defined, all technical and scientific terms used herein have
 10 the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database
 15 entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and Figs., and from the claims.

20 DESCRIPTION OF DRAWINGS

Figs. 1A-F. Loss of ATRX compromises the cell-cycle regulation of TERRA. (A) RNA FISH analyses of TERRA in HeLa, SJSA1, U2OS and HUO9 cells during the cell cycle. TERRA foci colocalized with TRF2 at telomeres (Fig. S1, S3A-B). Cells were enriched in S and G2 phases with thymidine alone or thymidine block and
 25 release into the CDK1 inhibitor RO3306, respectively (Fig. S2). Scale bar: 10 μ m. (B) The percentage of cells positive for TERRA foci (> 5 foci) was quantified and graphed as the mean with error bars representing standard deviation (n=2). (C) HeLa cells were mock treated or treated with ATRX siRNA #1, and RNA FISH analysis of TERRA was performed following thymidine release. The knockdown of ATRX was
 30 confirmed by Western blot (Fig. 8A). Cells were enriched in late S and G2 phases 7 hours after thymidine release (Fig. 8B). Scale bar: 10 μ m. (D) The percentage of cells positive for TERRA foci were quantified and graphed as the mean with error bars representing the standard deviation (n=3). (E-F) HeLa cells were mock treated or

treated with ATRX siRNA #1, and were enriched in S or M phase with thymidine and nocodazole, respectively (Fig. 8B). TERRA was analyzed by RT-qPCR using the subtelomeric/telomeric primers of chromosome 15q or Xp/Yp. The results are graphed as the mean fold-change with error bars representing the standard deviation (15q n=3, Xp/Yp n=4). *: P-Value < 0.05.

Figs. 2A-G. Loss of ATRX compromises RPA release from telomeres. (A) HeLa cells were mock treated or treated with ATRX siRNA #1, and RPA and TRF2 foci were analyzed in S and G2 as in Fig. 1C. Scale bar: 10 μ m. (B) The percentage of cells positive for RPA foci was quantified and graphed as the mean with error bars representing the standard deviation (n=2) *: P-Value = 0.008 (left) and P-Value = 0.002 (right). (C) HeLa cells were either mock treated or treated with ATRX siRNA #1, and whole-cell extracts (WCE) were generated from cells in S or M phase. Biotinylated ssTEL was coated with RPA and incubated with the WCE. After the incubation, ssTEL was retrieved and the remaining RPA32 on ssTEL was analyzed by Western blot. (D) SW39TEL and SW26ALT cells were analyzed for ATRX protein expression by Western blot. (E) SW39TEL and SW26ALT cells were analyzed for TERRA transcript by dot blot using DIG-labeled anti-TERRA or 28S RNA probes. (F) Quantification of dot blots for TERRA transcript in SW39TEL and SW26ALT cells. TERRA signal was normalized to 28S signal and ratios were graphed as the mean with error bars representing the standard deviation (n=2) *: P-Value = 0.001. (G) RPA-ssTEL was incubated in WCE from SW39TEL or SW26ALT cells. The RPA32 remaining on ssTEL was analyzed by Western blot.

Figs. 3A-F. ATR inhibitor disrupts ALT activity. (A) U2OS cells were mock treated, treated with 5 μ M VE-821, 5 μ M KU-55933, or treated with siRNA for ATR, or ATM, and then immunostained for TRF2 and PML. The percentage of cells positive for APBs were graphed as the mean with error bars representing the standard deviation, experiment performed in triplicate (n=3). P-Value < 0.02. (B) Representative images from cells quantified in (A). Scale bar: 10 μ m. (C) U2OS cells were mock treated or treated with 2.5 μ M VE-821 for 4 days and analyzed for T-SCE events using G-rich (green) and C-rich (red) PNA probes. The fraction of chromosome ends with T-SCE was quantified and graphed as the mean with error bars representing the standard deviation (Mock n=1032, VE-821 n=1556). *: P-Value < 0.01. (D-E) HUO9 and U2OS cells were mock treated or treated with 5 μ M VE-821

for 24 and 48 h, respectively. C-circle amplification products were detected by dot blot in D. The levels of C-circles were quantified and graphed in E as the mean with error bars representing standard deviation (n=2). Telomerase-positive SJSA1 cells were used as a negative control. P-Value < 0.02 (F) The fraction of chromosome ends with telomere loss was quantified and graphed as the mean with error bars representing the standard deviation (Mock n=1032, VE-821 n=1556). *: P-Value < 0.01.

Figs. 4A-F. Selective killing of ALT cells by ATR inhibitor. (A-B) Stills from time-lapse live-cell imaging experiments of (A) U2OS cells stably expressing H2B-mRFP and 53BP1-GFP or (B) HeLa cells expressing H2B-mRFP following treatment with either 5 μ M VE-821 or vehicle control (DMSO). Colored arrows mark individual cells as they progress through mitosis. Time scale: hr:min. Scale bar: 30 μ m. At least 150 cells were scored for each condition over two independent experiments. (C) U2OS cells were treated with VE-821 as in A, and analyzed by immunofluorescence using 53BP1 and TRF2 antibodies. Scale bar: 10 μ m. (D) A panel of cancer cell lines were mock treated, treated with VE-821, or KU-55933 for 4-6 days. Cell viability was determined using CellTiter Glo. Dots represent IC50s calculated from experiments performed in triplicate (n=3). (E) The osteosarcoma cell lines were treated with 3 μ M VE-821 for 6 days, and cell death was quantified by Annexin V. Induced cell death is graphed as the mean with error bars representing standard deviation (n=2). (F) MGG4TEL and MGG119ALT cells were treated with increasing concentrations of VE-821 for 6 days. Cell viability was determined using CellTiter Glo. Error bars represent standard deviation, experiment performed in duplicate (n=2).

Fig. 5. Synchronization of cells in S phase and G2. HeLa, SJSA1, U2OS, and HUO9 cells were treated with thymidine alone for 18 hrs, or treated with thymidine for 18 hrs and then released into 7 mM of the CDK inhibitor RO3306 for 18 hrs. The percentage of cells in G1/S and G2/M was analyzed by FACS and quantified using FlowJo software. These synchronized cell populations were analyzed in Fig. 1A-B.

Fig. 6. TERRA persistently associates with telomeres in ALT⁺ cells. U2OS cells synchronized in S and G2 were analyzed using telomere DNA FISH and TRF2 immunofluorescence. Scale bar: 10 μ m. The percentage of cells positive for

TERRA/TRF2 colocalization was graphed as the mean of experiments performed in triplicate, with error bars representing one standard deviation.

Figs. 7A-B. U2OS cells were arrested in G2 with the CDK1 inhibitor RO3306. (A) The levels of TERRA were analyzed by dot blot. The levels of input RNA were
5 normalized to 28S RNA. (B) The relative levels of TERRA were quantified in untreated and RO3306-treated cells. Error bar: standard deviation (n=2).

Figs. 8A-B. (A) HeLa cells were either mock treated or treated with two independent ATRX siRNAs. Three days after transfection, ATRX protein levels were analyzed by Western blot with tubulin as a loading control. (B) HeLa cells were
10 either mock treated or treated with ATRX siRNA #1. Two days after transfection, cells were incubated with thymidine (Thy) or nocodazole (Noc) for 16-18 hrs. Cells in thymidine were released and collected at the indicated time points. Cell cycle analysis was performed using FACS Diva software.

Figs. 9A-B. ATRX is a cell cycle-regulated repressor of TERRA. (A) HeLa
15 cells were either mock transfected or transfected with siATRX #1. Two days after transfection, cells were treated with either thymidine or nocodazole for an additional 18 hrs. The cells were collected and RNA was extracted using the RNeasy RNA purification kit according to the manufacturer's instructions. Approximately 15 mg of total RNA was analyzed by dot blot using DIG-labeled TERRA and 28S RNA probes.
20 (B) Dots detected by probes were quantified using ImageLab software and normalized to 28S. Error bars represent the standard deviation from experiments performed in duplicate (n=2).

Figs. 10A-B. Loss of ATRX does not affect telomerase activity or induce telomere lengthening. HeLa cells were either mock transfected or transfected with
25 siATRX #1 and incubated for 4 days. (A) Telomerase activity was analyzed by the TRAPeze assay kit and (B) telomere length was assessed by TRF analysis following isolation of genomic DNA. U2OS cells were used as an ALT-positive control.

Fig. 11. ATR inhibitor disrupts APBs in ALT cells. SW26^{ALT} cells were either mock treated or treated with 5 mM VE-821 for 3 hrs and then immunostained for
30 APB using TRF2 and PML antibodies. Scale bar: 10 μ m. The percentage of cells positive for APB was graphed as the mean with error bars representing the standard deviation (n=2).

Figs. 12A-C. The effects of ATR and ATM ablation on APBs. (A, C) U2OS cells were transfected with ATR siRNA, ATM siRNA, or mock transfected. The knockdown of ATR and ATM was confirmed by Western blot. The ATR and ATM knockdown cells were analyzed in Figs. 3A-B. (B) U2OS were synchronized in G2 in methionine-free media before addition of VE-821 and KU-55933. Cells positive for APBs were graphed as the mean with error bars representing the standard deviation, experiment performed in triplicate (n=3).

Figs. 13A-B. VE821 does not affect TERRA abundance or localization. (A) U2OS cells were treated with VE-821 for 24 hrs and TERRA was analyzed by dot blot using TERRA and 28S probes. Fold change in TERRA was analyzed using ImageLab software and normalized to 28S. Error bar: standard deviation (n=2). (B) TERRA localization was analyzed using combined IF-FISH using TERRA specific probes and TRF2 antibody. Positive cells had ≥ 5 TERRA foci that colocalized with TRF2 were quantified. Error bar: standard deviation (n=2). Scale bar: 10 μ m.

Fig. 14. SW26^{ALT} cells are more sensitive to ATR inhibitor than SW39^{TEL} cells. SW26^{ALT} and SW39^{TEL} cells were treated with increasing concentrations of VE-821 for 6 days. Cell viability was analyzed using CellTiter Glo. Mean viability is representative of experiments performed in triplicate with error bars representing the standard deviation (n=3).

Figs. 15A-C. SW26^{ALT} and SW39^{TEL} cells are similarly sensitive to general DNA damage. SW26^{ALT} and SW39^{TEL} were treated with increasing concentrations of (A) doxorubicin, (B) gemcitabine, or (C) mitomycin C. All cells were treated for 4-6 days and cell viability was analyzed using Cell Titer Glo. Error bar: standard deviation (n=2).

Fig. 16. ATR inhibitor induces higher levels of genomic instability in SW26^{ALT} cells than in SW39^{TEL} cells. SW39^{TEL} and SW26^{ALT} cells were treated with 2.5 mM VE-821, collected at the indicated time points, and H2AX phosphorylation was analyzed by Western blot using the anti-gH2AX antibody

Fig. 17. VE-821 has modest effects on untransformed REP-1 cells. RPE-1 and U2OS cells were either left untreated or treated continuously with 1.5 mM VE821 for 21 days. Population doublings were calculated using the standard formula (see Methods) and results were graphed as cumulative population doubling over time in days. Error bar: standard deviation (n=2).

Fig. 18. VE-821 induces micronucleation and 53BP foci in U2OS cells. Cells expressing H2B-mRFP were either left untreated or treated with 5 mM VE-821 for 24 hr, and scored as having undergone a “normal” mitosis if no micronuclei were generated following the first anaphase; “slightly abnormal” if 1-3 micronuclei were generated; or “highly abnormal” if 4 or more micronuclei were generated. Only the first mitosis following drug addition was scored. (n=2)

Fig. 19. ATR inhibition promotes DNA damage at telomeres. U2OS cells were either mock treated, or treated with 5mM VE-821 for 24 hrs and analyzed using either IF-DNA FISH (53BP1 antibody and telomeric probe) or standard immunofluorescence using 53BP1 and ACA antibodies. The percentage of cells positive for Telomere/53BP1 or ACA/53BP1 colocalization was graphed as the mean of experiments performed in triplicate, with error bars representing one standard deviation. * P-Value = .001.

Figs. 20A-C. Knockdown of ATRX does not render HeLa and BJ cells hypersensitive to VE-821. (A) HeLa cells were either mock treated or treated with ATRX siRNA for 72 hrs and then incubated with 2.5 mM VE-821. Cells were collected at the indicated time points and analyzed by Western blot. SW26^{ALT} cells treated with VE-821 for 6 hrs were included as an ALT-positive control. (B) BJ fibroblasts stably expressing control or ATRX shRNA were analyzed for ATRX levels by Western blot. (C) BJ cells stably expressing control or ATRX shRNA were treated with increasing concentrations of VE-821 for 6 days, and cell viability was analyzed by CellTiter Glo. Mean viability is representative of experiments performed in triplicate with error bars representing the standard deviation (n=3).

Figs. 21A-D. Selective killing of ALT cells by two different ATR inhibitors, but not ATM inhibitor or gemcitabine. The indicated panel of osteosarcoma cell lines were treated with increasing concentrations of (A) VE-821 (n=3), (B) the ATR inhibitor AZ20 (n=3), (C) the ATM inhibitor KU-55933 (n=3), or (D) Gemcitabine (n=2) for 4-6 days. Cell viability was analyzed using CellTiter Glo. Error bar: standard deviation. * P-Value < .01.

Figs. 22A-B. Characterization of the ALT status of osteosarcoma cell lines. (A) MG63, SJSA1, U2OS, CAL72, and NOS1 osteosarcoma lines were analyzed for ATRX protein. (B) The indicated cell lines were analyzed for telomerase activity using the TRAPeze assay kit. + refers to the positive control included in the kit and –

refers to CHAPS lysis buffer alone, IC denotes internal control band. (C) The indicated cell lines were analyzed for APB formation by immunostaining for both TRF2 and PML proteins. Scale bar: 10 mm.

5 Figs. 23A-C. ALT-positive osteosarcoma cell lines are hypersensitive to ATR inhibition. (A) Telomerase activity was analyzed in MG63, U2OS, NY, CAL78, and HUO9 osteosarcoma cell lines using the TRAPeze assay kit. (B) ATRX protein was analyzed by Western blot in the indicated cell lines. (C) The indicated osteosarcoma cell lines were treated with increasing concentrations of VE-821 and cell viability was analyzed using Cell Titer Glo 4-6 days after treatment. Error bar: standard deviation
10 (n=3).

Fig. 24. ATRX-positive ALT lung cancer cells are hypersensitive to ATR inhibition. Cells were treated with increasing concentrations of VE-821 for 5 days. Cell viability was analyzed by CellTiter Glo. Mean viability is derived from experiments preformed in triplicate with error bars representing the standard deviation
15 (n=3).

Figs. 25A-C. Characterization of the ALT status of GSC lines. (A) MGG4^{TEL} and MGG119^{ALT} GSC lines were analyzed for telomerase activity using the TRAPeze assay kit. + refers to the positive control included in the kit and – refers to CHAPS lysis buffer alone, IC denotes internal control band. (B) ATRX protein was analyzed
20 by Western blot. (C) TERRA levels were analyzed by dot blot with the indicated probes.

Figs. 26A-C. MGG118^{ALT} and MGG4^{TEL} are similarly sensitive to general DNA damage. MGG119^{ALT} and MGG4^{TEL} cells were treated with increasing concentrations of (A) doxorubicin, (B) gemcitabine, or (C) mitomycin C. All cells
25 were treated for 4-6 days and cell viability was analyzed using Cell Titer Glo. Mean viability is representative of experiments preformed in triplicate with error bars representing the standard deviation (n=3).

Figs. 27A-E. Acute ATR inhibition exerts two distinct effects on S-phase cells. A. U2OS cells were cultured in BrdU for 36 h, treated with DMSO or ATRi (10
30 μ M VE-821), and analyzed for BrdU and γ H2AX by immunostaining. B. Quantification of the BrdU intensity of 1,000 U2OS cells treated with DMSO or ATRi. Black lines indicate median BrdU intensities of BrdU-positive cells in various cell populations. C. Quantification of the BrdU and γ H2AX intensities of 1,200 U2OS

cells treated with DMSO or ATRi. Cell subpopulation 1 displayed less ssDNA at 8 h than at 2h. Cell subpopulation 2 displayed very high levels of ssDNA and became γ H2AX-positive at 8h. D. Levels of RPA32, pRPA32, and γ H2AX in the soluble and chromatin fractions of ATRi-treated cells were analyzed by Western blot. E. The
 5 levels of chromatin-bound RPA32 and pRPA32 were analyzed in cells treated with ATRi#2 (AZ20) and ATRi#3 (EPT-46464). See also Fig. 34.

Figs. 28A-D. ATR suppresses ssDNA accumulation in early S phase. A-B. Quantification of chromatin-bound RPA, EdU incorporation, and DNA contents of 5,000 U2OS cells treated with DMSO or ATRi (10 μ M VE-821). Cells were color-
 10 coded according to the intensity of RPA staining as shown in the left panel. C. T98G cells were synchronously released from G0 and analyzed for EdU incorporation at the indicated times. D. Staining intensity of chromatin-bound RPA was analyzed at different stages of the cell cycle after ATRi or DMSO treatment. Red lines indicate mean RPA intensities in various cell populations. **, $P < 0.01$; ***, $P < 0.001$. See also
 15 Fig. 35.

Figs. 29A-H. ATRi suppresses DNA damage by promoting RRM2 accumulation and limiting origin firing. A. T98G cells were synchronously released from G0 in the presence or absence of ATRi (10 μ M VE-821). Levels of RRM2, γ H2AX, RPA70, RPA32, RPA14 and Cyclin A were analyzed during the time course.
 20 B. Asynchronously growing U2OS cells were treated with ATRi (10 μ M VE-821) or Chk1i (2 μ M MK-8776). Levels of RRM2 and E2F1 were analyzed at the indicated times. C. Levels of RRM2 and E2F1 were analyzed in U2OS cells treated with DMSO or ATRi in the presence of cycloheximide (CHX). Relative levels of RRM2 and E2F1 were quantified from 3 blots (n=3). Error bars: S.D. D. U2OS cells
 25 transfected with empty vector or E2F1-expressing plasmids were treated with ATRi for 8 h. Levels of RRM2, E2F1, and γ H2AX were analyzed. E. U2OS cells were treated with the indicated inhibitors for 8 h. Levels of RRM2, E2F1, and γ H2AX were analyzed. F. U2OS cells were treated with DMSO or ATRi for 8 h in the presence or absence of MG132 or MLN4924. G. U2OS cells infected with HA-RRM2-expressing
 30 retrovirus or control virus were treated with ATRi for 8 h. Levels of RRM2 and γ H2AX were analyzed at the indicated times. H. A model in which ATR coordinates RRM2 accumulation and origin firing in early S phase. See also Fig. 36.

Figs. 30A-G. ATRi-treated cells recover via a Chk1-mediated mechanism. A. U2OS cells were treated with DMSO, ATRi (10 μ M VE-821), or Chk1i (2 μ M MK-8776). Levels of RPA32, pRPA32, and γ H2AX were analyzed at the indicated times. B. U2OS cells were treated with increasing concentrations of ATRi or Chk1i for 24 h and then cultured in inhibitor-free media. Cell survival was analyzed 4 days after treatment. Error bar: S.D. (n=3). C. U2OS cells were treated with DMSO, ATRi, or Chk1i for 8 h. BrdU and γ H2AX intensities were quantified in 1,200 cells at the indicated times. D. U2OS cells were treated with ATRi or Chk1i. Levels of chromatin-bound RPA were analyzed at the indicated times. E. U2OS cells were treated with ATRi, and levels of pChk1 and CDC25A were analyzed at the indicated times. F. Levels of CDC25A in U2OS cells treated with ATRi or Chk1i were compared at the indicated times. G. A model in which Chk1 promotes the recovery of ATRi-treated cells with moderate ssDNA. See also Fig. 37.

Figs. 31A-F. Regulation and function of Chk1 during recovery. A. Levels of pDNA-PK, pATM, pChk1 and CDC25A were analyzed in ATRi-treated U2OS cells at the indicated times. B. U2OS cells were treated with ATRi, ATMi, DNA-PKi, or the combinations of these inhibitors. Levels of pDNA-PK, pATM, pChk1 and CDC25A were analyzed 8 h after treatment. C. The percentage of replication tracts containing fired origins was determined in RPE1 cells treated with DMSO or ATRi at the indicated times. Error bars: S.D. (n=3 experiments). **P<0.01; ***P<0.001. D. RPE1 cells were treated with DMSO or various inhibitors as indicated. The inter-origin distance was analyzed using DNA fiber assay at the indicated times. Error bars: S.E.M. (n=25 to 67 as indicated). ****, P<0.0001; n.s., not significant. E-F. RPE1 cells were treated with DMSO or various inhibitors as indicated. The length of continuous replication tracts was determined using DNA fiber assay at the indicated times (>600 forks per condition, n=3 experiments). See also Fig. 38.

Figs. 32A-G. ATRi selectively kills cells under high replication stress. A. U2OS cells were treated with ATRi for 2 h in the presence of increasing concentrations of HU. The BrdU intensity of 1,000 U2OS cells was quantified. Black lines indicate mean BrdU intensities of BrdU-positive cells in various populations. B. U2OS cells were treated with ATRi or Chk1i for 16 h in the presence of increasing concentrations of HU. Cell death was measured by the TUNEL assay. Error bars: S.D. (n=3). C-D. U2OS cells were induced to overexpress Cyclin E or left uninduced, and

treated with ATRi or Chk1i for 16h. Levels of Cyclin E and γ H2AX were analyzed by Western (C). Fractions of TUNEL-positive cells were quantified (D). Error bars: S.D. (n=3). E-F. T98G, RPE1, and MCF10A cells were treated with DMSO or ATRi. Levels of ssDNA were analyzed by native BrdU staining 2 h after ATRi treatment (E). Yellow lines indicate mean BrdU intensities of BrdU-positive cells in various populations. Levels of cell death were measured by TUNEL assay at the indicated times (F). Error bars: S.D. (n=3). G. Quantification of ssDNA, γ H2AX, and cell survival of 10 colorectal cell lines after ATRi treatment. Levels of ssDNA and γ H2AX were analyzed 2 h and 16 h after the indicated treatments, respectively. Fractions of ATRi-treated cells displaying stronger BrdU staining than untreated cells were determined (see Supplemental Methods). Cell survival was analyzed 6 days after the indicated treatments using CellTiter-Glo. See also Fig. 39.

Figs. 33A-C. Modeling the roles for ATR, DNA-PK, and Chk1 in countering replication stress. A. A fraction of early S-phase cells are particularly vulnerable to ATR inactivation. B. ATRi selectively kills cells under high replication stress, whereas Chk1i induces cell death even in cells in which replication stress is moderate. C. ATRi-induced ssDNA is an indicator of replication stress that may predict the ATRi sensitivity of cancer cells.

Figs. 34A-H. The effects of ATRi on cycling cells. A-B. U2OS cells were treated with CPT or HU in the presence of increasing concentrations of ATRi (VE-821). The phosphorylation of Chk1 at S296 and S317 was used as readout of ATR inhibition. C. U2OS cells were cultured in BrdU for 36 h, treated with DMSO or ATRi (10 μ M VE-821) for 2 h, and analyzed by native BrdU staining and PCNA immunostaining. BrdU intensity was quantified in PCNA-positive and -negative cells. D. U2OS cells were treated with DMSO or ATRi and analyzed by native BrdU staining (same data shown in Fig. 1B). Medium BrdU intensities of BrdU-positive cells in various cell populations were determined. Error bars: interquartile range (I.Q.R), n=19 to 277 in various cell populations. The fractions of ATRi-treated cells displaying high levels of ssDNA at 8 h were quantified from 3 experiments (n=3). E. U2OS cells were treated with ATRi for 8 h, and analyzed by TUNEL assay and γ H2AX immunostaining. Note that a small fraction of γ H2AX-positive cells were not strongly TUNEL-positive, indicating that γ H2AX

accumulated prior to replication catastrophe. F. Cell-cycle profiles of U2OS cells treated with DMSO or ATRi. G. Quantification of chromatin-bound RPA in 2,000 U2OS cells treated with DMSO or ATRi. Medium RPA intensities of RPA-positive cells in various cell populations were determined. Error bars: I.Q.R., n=922 to 1095 in various cell populations. H. U2OS cells were treated with ATRi for 8h, and analyzed by γ H2AX and pRPA32 immunostaining.

Fig. 35. A cell-cycle time course of synchronized cells. T98G cells were synchronized by serum starvation, released into serum containing media, and analyzed at the indicated times. Levels of the indicated cell-cycle markers were analyzed by Western.

Figs. 36A-G. Suppression of ATRi-induced DNA damage. A. RPE1 cells were treated with DMSO or the indicated inhibitors and analyzed using DNA fiber assay. Fractions of replication tracts with fired origins were determined. Error bars: S.D. (n=3). B. U2OS cells were treated with DMSO or ATRi. Levels of RRM2, E2F1, and γ H2AX were analyzed by Western. C. U2OS cells transfected with Cyclin F or control siRNA were treated with DMSO or ATRi. Levels of Cyclin F and RRM2 were analyzed by Western. D. U2OS cells were treated with DMSO or ATRi for 8 h. Relative levels of RRM2 mRNA were determined by RT-qPCR. E. U2OS cells were treated with DMSO, roscovitine, or CDK2i in the presence or absence of ATRi. Levels of pRPA32 and γ H2AX were analyzed by Western. F. U2OS cells infected with HA-RRM2-expressing retrovirus or control virus were treated with Chk1i for 8 h. Levels of RRM2 and γ H2AX were analyzed at the indicated times. G. U2OS cells transfected with CDC7 or control siRNA were treated with DMSO or ATRi for 8 h. Levels of pRPA32, γ H2AX and CDC7 were analyzed by Western.

Figs. 37A-L, related to Fig. 4. The distinct effects of ATRi and Chk1i on cycling cells. A. U2OS cells were treated with CPT for 1 h in the presence or absence of ATRi. The phosphorylation of Chk1 at S317 was used as readout of ATR inhibition. In the left panel, CPT and ATRi were added at the same time. In the right panel, CPT was added 7 h after ATRi. B. U2OS cells were treated with CPT in the presence of increasing concentrations of ATRi (VE-821) or Chk1i (MK-1775). The phosphorylation of Chk1 at S296 was used as an indicator of Chk1 activity. C. U2OS cells were treated with DMSO, 10 μ M ATRi, or increasing concentrations of Chk1i. The stabilization of CDC25A were analyzed as an indicator of Chk1 inhibition. D.

U2OS cells were treated with HU in the presence of DMSO, ATRi (10 μ M VE-821), ATRi#2 (1 μ M AZ20), ATRi#3 (10 μ M EPT-46464), Chk1i (2 μ M MK-8776), or Chk1i#2 (0.3 μ M UCN-01). E. U2OS cells were treated with DMSO and the indicated inhibitors for 8 h. Levels of pDNA-PK, pRPA32, and γ H2AX were analyzed by Western. F. U2OS cells were transfected with control siRNA, two independent ATR siRNAs, or two independent Chk1 siRNAs. Levels of the indicated proteins were analyzed by Western. G. U2OS cells transfected with control, ATR, or Chk1 siRNA were analyzed by γ H2AX immunostaining. H. U2OS cells were treated with increasing concentrations of ATRi or Chk1i. Cell survival was analyzed after 5 days of treatment. I. U2OS cells were treated with DMSO, ATRi, or Chk1i for 2 h. Levels of ssDNA were analyzed by native BrdU staining. J. U2OS cells were treated with DMSO or Chk1i at indicated. BrdU and γ H2AX intensities were quantified in 1,200 cells at the indicated times. Data of the same DMSO and Chk1i 8h samples are also shown in Fig. 4C. K-L. U2OS cells were treated with 10 μ M ATRi or the indicated concentrations of Chk1i. Levels of CDC25A were analyzed at 2 and 8 h in K. Levels of γ H2AX were analyzed at 2, 8, and 16 h in L.

Figs. 38A-K. A DNA-PK and Chk1-mediated backup pathway. A. U2OS cells were treated with ATRi and DNA-PKi as indicated. Levels of pChk1 and pDNA-PK were analyzed by Western. The sample 6+2h was treated with ATRi twice at 0 and 6 h, and analyzed at 8 h. B. U2OS cells were treated with various inhibitors as indicated. Levels of pChk1 and CDC25A were analyzed by Western. ATRi#2: AZ20. C-D. U2OS cells were treated with ATRi, ATMi, and DNA-PKi as indicated. Levels of pATM, pDNA-PK, pRPA32, γ H2AX, and pChk2 were analyzed by Western. E-F. U2OS transfected with KU70 siRNAs (E) or SLX4 and MUS81 siRNAs (F) were treated with DMSO or ATRi for 8 h. Levels of pDNA-PK, pRPA32, γ H2AX, KU70 and MUS81 were analyzed by Western. G-I, U2OS cells were transfected with Claspin (G), RAD17 (H), TopBP1 (I) siRNAs or mock treated. Transfected cells were treated with ATRi, and levels of pChk1, Chk1, and other indicated proteins were analyzed at the indicated times. J-K. U2OS cells were treated with DMSO, Chk1i, and roscovitine as indicated in J, or treated with DMSO, ATRi, and Wee1i as indicated in H. BrdU intensities of 1,000 cells were quantified. Yellow lines indicate mean BrdU intensities in various cell populations. Fractions of cells displaying high levels of ssDNA were quantified and shown on the top. ****, $P < 0.0001$.

Fig. 39A-N. ATRi but not Chk1i selectively kill cancer cells under high replication stress. A-B. U2OS cells were treated with DMSO, ATRi, or Chk1i in the absence or presence of HU. BrdU intensities of 1,000 cells were quantified at 2 h (A) or 16 h (B). ****, $P < 0.0001$; n.s., not significant. C. U2OS cells were induced to overexpress Cyclin E or left uninduced, and treated with ATRi for the indicated time. BrdU intensities of 2,000 cells were quantified. D. U2OS cells were transfected with control or two independent RB siRNAs, and treated with DMSO, ATRi or Chk1i for 16 or 40 h. RB knockdown was confirmed by Western. Fractions of γ H2AX-positive cells were quantified. Error bars: S.D. (n=3). E. T98G, RPE1, and MCF10A cells were treated with DMSO or ATRi for 8 h. Levels of ssDNA were analyzed by native BrdU staining in PCNA-positive cells. F. U2OS, RPE1, and MCF10A cells were treated with DMSO or ATRi for 8 h. Levels of ssDNA were analyzed by native BrdU staining. Fractions of cells displaying high levels of ssDNA were quantified and shown on the top. G. U2OS, RPE1, and MCF10A cells were treated with ATRi for 0, 8, and 16 h. Fractions of TUNEL-positive cells were quantified. Error bars: S.D. (n=3). H-I. Four colorectal cancer cell lines were treated with DMSO or ATRi for 2 h (H) or 16 h (I). Levels of ssDNA and γ H2AX were analyzed by native BrdU staining (H) and γ H2AX immunostaining (I), respectively. Fractions of ATRi-treated cells displaying higher levels of ssDNA or γ H2AX than control cells were labeled in red and quantified (see Supplemental Methods). J-K. Ten colorectal cancer cell lines were treated with increasing concentrations of ATRi in the absence or presence of HU. Cell survival was analyzed 6 days after the treatment (J). The S.D. of each cell survival data was determined from experimental triplicates (n=3) (K). L. The 10 colorectal cancer cell lines were cultured in BrdU-containing media for 36 h. The BrdU labeling of DNA was confirmed by denatured BrdU staining. Fractions of S-phase cells were determined by PCNA staining. M. The common mutations of colorectal cancer do not correlate with ATRi sensitivity. N. The microsatellite stability/instability (MSS/MSI), CpG island methylation phenotype (CIMP), and chromosomal instability (CIN) of colorectal cancer cell lines do not correlate with ATRi sensitivity (adapted from the reference (Ahmed et al., Epigenetic and genetic features of 24 colon cancer cell lines. *Oncogenesis* 2, e71, 2013)).

DETAILED DESCRIPTION

Cancer cells rely on telomerase or the alternative lengthening of telomeres (ALT) pathway to overcome replicative mortality. ALT is mediated by recombination and prevalent in a subset of human cancers, yet whether it can be exploited therapeutically remains unknown. Loss of ATRX associates with ALT in cancers. Although it is known that ALT relies on recombination to elongate telomeres (3), how the recombinogenic state of ALT telomeres is established remains elusive. In contrast to cancer cells that are defective for homologous recombination (HR) and susceptible to PARP inhibition (5, 6), ALT-positive cells are HR-proficient (7). Thus, the reliance of ALT on recombination raises an important question as to whether recombination can be exploited in ALT-positive cancers as a means for targeted therapy.

Herein, the present inventors show that ATRX loss compromises the cell-cycle regulation of the telomeric non-coding RNA TERRA and leads to persistent association of RPA with telomeres after DNA replication, creating a recombinogenic nucleoprotein structure. Inhibition of ATR, a critical regulator of recombination recruited by RPA, disrupts ALT and triggers chromosome fragmentation and apoptosis in ALT cells. Importantly, the cell death induced by ATR inhibitors is highly selective for ALT cells across a panel of cancer cell lines, suggesting that ATR inhibition is a useful therapeutic strategy in the treatment of ALT-positive cancers. Thus, provided herein are methods for treating subjects who have ALT-positive cancers using ATR inhibitors.

In addition, as shown herein, ATRi-induced ssDNA is an indicator of replication stress. Although ATR is long known to be a master regulator of cellular responses to replication stress, whether and how replication stress can be quantified remains elusive. A quantitative understanding of replication stress is crucial for explaining the functions of ATR in specific oncogenic, developmental, aging and therapeutic contexts (Brown and Baltimore, 2000; Flynn et al., 2015; Gilad et al., 2010; Lee et al., 2012; Murga et al., 2009; Murga et al., 2011; Reaper et al., 2011; Ruzankina et al., 2007). In this study, we found that the levels of ATRi-induced ssDNA vary in individual cells and in different stages of S phase. In HU-treated cells, the levels of ATRi-induced ssDNA rise with HU concentrations, suggesting that ATRi-induced ssDNA reflects replication stress quantitatively. Furthermore, in a panel of cancer cell lines, the levels of ATRi-induced ssDNA correlate with ATRi-induced cell

death. These results suggest that ATRi-induced ssDNA is also an indicator of intrinsic replication stress, and it is predictive of ATRi sensitivity in cancer cells (Fig. 7C).

Although replication stress could arise from many different sources, induction of ssDNA may be a common effect (Flynn and Zou, 2011). Elicited by ssDNA (Zou and Elledge, 2003), ATR acts to counter replication stress by suppressing ssDNA accumulation (Toledo et al., 2013). When ATR is lost, replication stress is unleashed to drive further ssDNA formation, generating a quantifiable product reflecting its own strength. As shown herein, ATRi-induced ssDNA is a quantitative indicator of both extrinsic and intrinsic replication stress. The use of ATRi-induced ssDNA to measure replication stress can predict the outcome of ATR inhibition, providing a quantitative view of the interplay between replication stress and the ATR checkpoint and allowing selection of subjects for treatment with ATR inhibitors.

Methods of Treatment

The methods described herein include methods for the treatment of cancers associated with activation of the ALT pathway or accumulation of ssDNA. In some embodiments, the cancer is a cancer of mesenchymal origin, including those arising from bone, soft tissue, and the nervous system. Generally, the methods include administering a therapeutically effective amount of an ATR inhibitor as known in the art or described herein, to a subject who is in need of, or who has been determined to be in need of, such treatment.

As used in this context, to “treat” means to ameliorate at least one symptom of the cancer. For example, administration of a therapeutically effective amount of a compound described herein for the treatment of a cancer associated with activation of the ALT pathway can result in one or more of: decrease in tumor size; decrease or cessation in tumor growth or a reduction in tumor growth rate; a reduction in risk of metastasis; an increase in life expectancy; and/or a decrease in cancer-associated symptoms including pain.

The methods described herein include identifying a subject as having a cancer associated with activation of the ALT pathway or accumulation of ssDNA, and selecting them based on that identification (i.e., selecting them because they have a cancer associated with activation of the ALT pathway or accumulation of ssDNA).

A number of cancers have been described that are associated with activation of the ALT pathway; primarily observed in sarcomas and astrocytomas, ALT is

relatively rare in carcinomas. ALT activation has been reported to be present in about 50% of osteosarcomas, 30% of soft tissue sarcomas, 25% of the primary brain tumor, glioblastoma multiforme (GBM), and 10% of neuroblastomas (see Henson and Reddel, FEBS Lett. 584(17):3800–3811 (2010), e.g., Table 2 therein).

5 Methods known in the art can be used to identify subjects as having a cancer associated with activation of the ALT pathway (i.e., for identifying a cancer as associated with ALT activation, also referred to herein as an ALT cancer or an ALT+ cancer), as well as methods described herein. For example, detection of maintenance of telomeres in the absence of telomerase activity (Bryan et al., EMBO J., 14:4240–
10 4248 (1995)); detection of a pattern of telomere lengths, e.g., by terminal restriction fragment Southern blots, ranging from very short to extremely long, and with a modal length approximately twice that in comparable telomerase-positive or normal cells (Bryan et al., EMBO J., 14:4240–4248 (1995); Gollahon et al., Oncogene, 17:709–
15 717 (1998)); detection of rapid, unsynchronized changes in telomere length cause telomere length heterogeneity (Murnane et al., EMBO J., 13:4953–4962 (1994)); detection of ALT-associated PML bodies (APBs) (Yeager et al., Cancer Res., 59:4175–4179 (1999)); detection of copying of engineered telomeric tags from one telomere to another (Pickett et al. EMBO J., 28:799–809 (2009)); detection of tandem repeat instability at telomeres and the MS32 minisatellite (Jeyapalan et al., Hum. Mol.
20 Genet., 14:1785–1794 (2005)); detection of Telomere-sister chromatid exchange (T-SCE) (Fan et al. Nucleic Acids Res., 37:1740–1754 (2009)); detection of an increase in the level of telomeric t-circles (Cesare et al., Mol. Cell. Biol., 24:9948–9957 (2004)); detection of single stranded C-strand telomeric DNA (ss-C-strand) (Grudic et al., Nucleic Acids Res., 35:7267–7278 (2007)); detection of C circles (Henson et al.,
25 Nat. Biotechnol., 27:1181–1185 (2009)). See, e.g. Henson and Reddel, FEBS Lett. 584(17):3800–3811 (2010); and US20150247866. In some embodiments, a branched DNA assay in RNA in situ hybridization (RNA-ISH) is used, e.g., as described in WO 2015/123565.

30 In addition, methods are known in the art for detecting accumulation of ssDNA; for example; DNA can be labeled with BrdU and analyzed by native BrdU staining. Detection of chromatin-bound RPA, an ssDNA-binding protein, can also be used to measure ssDNA accumulation. A number of assays are commercially available and can also be used.

ATR Inhibitors

Once a subject has been identified as having an ALT+ cancer, a therapeutically effective amount of an ATR inhibitor can be administered. A number of ATR inhibitors are known in the art, including small molecules and inhibitory nucleic acids.

Small Molecule Inhibitors of ATR

Small molecule inhibitors of ATR useful in the present methods and compositions include, but are not limited to, Schisandrin B (10.Benzo(3,4)cycloocta(1,2-f)(1,3)benzodioxole, 5,6,7,8-tetrahydro-1,2,3,13-tetramethoxy-6,7-dimethyl-, stereoisomer: NU6027 (6-(cyclohexylmethoxy)-5-nitrosopyrimidine-2,4-diamine); NVP-BEZ235 (2-methyl-2-[4-(3-methyl-2-oxo-8-quinolin-3-ylimidazo[4,5-c]quinolin-1-yl)phenyl]propanenitrile); VE-821 (2-(aminomethyl)-6-[4,6-diamino-3-[4-amino-3,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-2-hydroxycyclohexyl]oxyoxane-3,4,5-triol;sulfuric acid); VE-822 (VX-970; 3-[3-[4-(methylaminomethyl)phenyl]-1,2-oxazol-5-yl]-5-(4-propan-2-ylsulfonylphenyl)pyrazin-2-amine); AZ20 ((3R)-4-[2-(3H-indol-4-yl)-6-(1-methylsulfonylcyclopropyl)pyrimidin-4-yl]-3-methylmorpholine); AZD6738 (4-[4-[1-[[S(R)]-S-methylsulfonimidoyl]cyclopropyl]-6-[(3R)-3-methyl-4-morpholinyl]-2-pyrimidinyl]-1H-pyrrolo[2,3-b]pyridine); and ETP-46464 (2-methyl-2-[4-(2-oxo-9-quinolin-3-yl-4H-[1,3]oxazino[5,4-c]quinolin-1-yl)phenyl]propanenitrile); see, e.g., Weber and Ryan, *Pharmacology & Therapeutics* 149:124–138 (2015); Toledo et al., *Nat Struct Mol Biol.* 2011 Jun;18(6):721-7; US20140356456; and US20100048923.

Inhibitory Nucleic Acids Targeting ATR

Inhibitory nucleic acids useful in the present methods and compositions include antisense oligonucleotides, ribozymes, external guide sequence (EGS) oligonucleotides, siRNA compounds, single- or double-stranded RNA interference (RNAi) compounds such as siRNA compounds, modified bases/locked nucleic acids (LNAs), antagomirs, peptide nucleic acids (PNAs), ribozymes, CRISPR/Cas9-guide RNAs, and other oligomeric compounds or oligonucleotide mimetics which hybridize to at least a portion of an ATR nucleic acid and inhibit its function.

ATR sequences are known in the art; for example, the human ATR sequence is in GenBank at Accession No. NM_001184.3 (GI:157266316), which is incorporated herein by reference.

In some embodiments, the inhibitory nucleic acids include antisense RNA, antisense DNA, chimeric antisense oligonucleotides, antisense oligonucleotides comprising modified linkages, interference RNA (RNAi), short interfering RNA (siRNA); a micro, interfering RNA (miRNA); a small, temporal RNA (stRNA); or a short, hairpin RNA (shRNA); small RNA-induced gene activation (RNAa); small activating RNAs (saRNAs), CRISPR/Cas9-guide RNAs, or combinations thereof. See, e.g., WO 2010040112; Burnett and Rossi (2012) Chem Biol. 19 (1):60-71.

In some embodiments, the inhibitory nucleic acids are 10 to 50, 13 to 50, or 13 to 30 nucleotides in length. One having ordinary skill in the art will appreciate that this embodies oligonucleotides having antisense portions of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides in length, or any range there within. In some embodiments, the oligonucleotides are 15 nucleotides in length. In some embodiments, the antisense or oligonucleotide compounds of the invention are 12 or 13 to 30 nucleotides in length. One having ordinary skill in the art will appreciate that this embodies inhibitory nucleic acids having antisense portions of 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 nucleotides in length, or any range there within.

In some embodiments, the inhibitory nucleic acids are chimeric oligonucleotides that contain two or more chemically distinct regions, each made up of at least one nucleotide. These oligonucleotides typically contain at least one region of modified nucleotides that confers one or more beneficial properties (such as, for example, increased nuclease resistance, increased uptake into cells, increased binding affinity for the target) and a region that is a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. Chimeric inhibitory nucleic acids of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures comprise, but are not limited to, US patent nos. 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; 5,700,922; 8,604,192; 8,697,663; 8,703,728; 8,796,437; 8,865,677; and 8,883,752 each of which is herein incorporated by reference.

In some embodiments, the inhibitory nucleic acid comprises at least one nucleotide modified at the 2' position of the sugar, most preferably a 2'-O-alkyl, 2'-O-alkyl-O-alkyl or 2'-fluoro-modified nucleotide. In other preferred embodiments, RNA modifications include 2'-fluoro, 2'-amino and 2' O-methyl modifications on the ribose of pyrimidines, abasic residues or an inverted base at the 3' end of the RNA. Such modifications are routinely incorporated into oligonucleotides and these oligonucleotides have been shown to have a higher T_m (i.e., higher target binding affinity) than; 2'-deoxyoligonucleotides against a given target.

A number of nucleotide and nucleoside modifications have been shown to make the oligonucleotide into which they are incorporated more resistant to nuclease digestion than the native oligodeoxynucleotide; these modified oligos survive intact for a longer time than unmodified oligonucleotides. Specific examples of modified oligonucleotides include those comprising modified backbones, for example, phosphorothioates, phosphotriesters, methyl phosphonates, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Most preferred are oligonucleotides with phosphorothioate backbones and those with heteroatom backbones, particularly $\text{CH}_2\text{-NH-O-CH}_2$, $\text{CH}_2\text{-N(CH}_3\text{)-O-CH}_2$ (known as a methylene(methylimino) or MMI backbone], $\text{CH}_2\text{-O-N(CH}_3\text{)-CH}_2$, $\text{CH}_2\text{-N(CH}_3\text{)-N(CH}_3\text{)-CH}_2$ and $\text{O-N(CH}_3\text{)-CH}_2\text{-CH}_2$ backbones, wherein the native phosphodiester backbone is represented as O-P-O-CH_2); amide backbones (De Mesmaeker (1995) *Ace. Chem. Res.* 28:366-374); morpholino backbone structures (Summerton and Weller, U.S. Pat. No. 5,034,506); peptide nucleic acid (PNA) backbone (wherein the phosphodiester backbone of the oligonucleotide is replaced with a polyamide backbone, the nucleotides being bound directly or indirectly to the aza nitrogen atoms of the polyamide backbone, Nielsen (1991) *Science* 254, 1497). Phosphorus-containing linkages include, but are not limited to, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates comprising 3'alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates comprising 3'-amino phosphoramidate and aminoalkylphosphoramidates, phosphonoacetate phosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and

those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'; see US patent nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050.

Morpholino-based oligomeric compounds are described in Dwaine A. Braasch and David R. Corey (2002) *Biochemistry* 41(14), 4503-4510; *Genesis*, volume 30, issue 3, 2001; Heasman, (2002) *Dev. Biol.* 243, 209-214; Nasevicius (2000) *Nat. Genet.* 26, 216-220; Lacerra (2000) *Proc. Natl. Acad. Sci.* 97, 9591-9596; and U.S. Pat. No. 5,034,506, issued Jul. 23, 1991. Cyclohexenyl nucleic acid oligonucleotide mimetics are described in Wang (2000) *Am. Chem. Soc.* 122, 8595-8602.

Modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These comprise those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts; see US patent nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,677,439; and 8,927,513 each of which is herein incorporated by reference.

One or more substituted sugar moieties can also be included, e.g., one of the following at the 2' position: OH, SH, SCH₃, F, OCN, OCH₃, OCH₃ O(CH₂)_n CH₃, O(CH₂)_n NH₂ or O(CH₂)_n CH₃ where n is from 1 to about 10; C₁ to C₁₀ lower alkyl, alkoxyalkoxy, substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN; CF₃; OCF₃; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; SOCH₃; SO₂ CH₃; ONO₂; NO₂; N₃; NH₂; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted

silyl; an RNA cleaving group; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy [2'-0-CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl)] (Martin (1995) *Helv. Chim. Acta* 78, 486). Other preferred modifications include 2'-methoxy (2'-O-CH₃), 2'-propoxy (2'-OCH₂ CH₂CH₃) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide and the 5' position of 5' terminal nucleotide.

Oligonucleotides may also have sugar mimetics such as cyclobutyls in place of the pentofuranosyl group.

Inhibitory nucleic acids can also include, additionally or alternatively, nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include adenine (A), guanine (G), thymine (T), cytosine (C) and uracil (U). Modified nucleobases include nucleobases found only infrequently or transiently in natural nucleic acids, e.g., hypoxanthine, 6-methyladenine, 5-Me pyrimidines, particularly 5-methylcytosine (also referred to as 5-methyl-2' deoxycytosine and often referred to in the art as 5-Me-C), 5-hydroxymethylcytosine (HMC), glycosyl HMC and gentobiosyl HMC, as well as synthetic nucleobases, e.g., 2-aminoadenine, 2- (methylamino)adenine, 2-(imidazolylalkyl)adenine, 2-(aminoalkylamino)adenine or other heterosubstituted alkyladenines, 2-thiouracil, 2-thiothymine, 5-bromouracil, 5- hydroxymethyluracil, 8-azaguanine, 7-deazaguanine, N6 (6-aminoethyl)adenine, 2,6- diaminopurine; 5-ribosyluracil (Carlile (2014) *Nature* 515(7525): 143-6) . Kornberg, A., *DNA Replication*, W. H. Freeman & Co., San Francisco, 1980, pp75-77; Gebeyehu (1987) *Nucl. Acids Res.* 15:4513). A "universal" base known in the art, e.g., inosine, can also be included. 5-Me-C substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2^{<0>}C. (Sanghvi, Y. S., in Crooke, S. T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions.

It is not necessary for all positions in a given oligonucleotide to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single oligonucleotide or even at within a single nucleoside within

an oligonucleotide. In some embodiments, both the nucleobase and backbone may be modified to enhance stability and activity (El-Sagheer (2014) Chem Sci 5:253-259)

In some embodiments, both a sugar and an internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, for example, an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds comprise, but are not limited to, US patent nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen (1991) Science 254, 1497-1500; and Shi (2015).

Inhibitory nucleic acids can also include one or more nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases comprise the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U).

Modified nucleobases comprise other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudo-uracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine.

Further, nucleobases comprise those disclosed in United States Patent No. 3,687,808, those disclosed in 'The Concise Encyclopedia of Polymer Science And Engineering', pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., Angewandte Chemie, International Edition', 1991, 30,

page 613, and those disclosed by Sanghvi, Y. S., Chapter 15, 'Antisense Research and Applications', pages 289-302, Crooke, S.T. and Lebleu, B. ea., CRC Press, 1993.

Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted

5 pyrimidines, 6-azapyrimidines and N-2, N-6 and 0-6 substituted purines, comprising 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds, 'Antisense Research and Applications', CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred
10 base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications. Modified nucleobases are described in US patent nos. 3,687,808, as well as 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,596,091; 5,614,617; 5,750,692, and 5,681,941, each of which is herein
15 incorporated by reference.

In some embodiments, the inhibitory nucleic acids are chemically linked to one or more moieties or conjugates that enhance the activity, cellular distribution, or cellular uptake of the oligonucleotide. Such moieties comprise but are not limited to, lipid moieties such as a cholesterol moiety (Letsinger (1989) Proc. Natl. Acad. Sci.

20 USA 86, 6553-6556), cholic acid (Manoharan (1994) Bioorg. Med. Chem. Lett. 4, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan (1992) Ann. N. Y. Acad. Sci. 660, 306-309; Manoharan (1993) Bioorg. Med. Chem. Lett. 3, 2765-2770), a thiocholesterol (Oberhauser (1992) Nucl. Acids Res. 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Kabanov (1990) FEBS Lett. 259, 327-
25 330; Svinarchuk (1993) Biochimie 75, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1, 2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan (1995) Tetrahedron Lett. 36, 3651-3654; Shea (1990) Nucl. Acids Res. 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan (1995) Nucleosides & Nucleotides 14, 969-973), or adamantane acetic acid (Manoharan
30 (1995) Tetrahedron Lett. 36, 3651-3654), a palmityl moiety (Mishra (1995) Biochim. Biophys. Acta 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-t oxycholesterol moiety (Crooke (1996) J. Pharmacol. Exp. Ther. 277, 923-937). See also US patent nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313;

5,545,730; 5,552, 538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124;
 5,118,802; 5,138,045; 5,414,077; 5,486, 603; 5,512,439; 5,578,718; 5,608,046;
 4,587,044; 4,605,735; 4,667,025; 4,762, 779; 4,789,737; 4,824,941; 4,835,263;
 4,876,335; 4,904,582; 4,958,013; 5,082, 830; 5,112,963; 5,214,136; 5,082,830;
 5,112,963; 5,214,136; 5, 245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250;
 5,292,873; 5,317,098; 5,371,241, 5,391, 723; 5,416,203, 5,451,463; 5,510,475;
 5,512,667; 5,514,785; 5, 565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371;
 5,595,726; 5,597,696; 5,599,923; 5,599, 928; 5,688,941, 8,865,677; 8,877,917 each of
 which is herein incorporated by reference.

These moieties or conjugates can include conjugate groups covalently bound
 to functional groups such as primary or secondary hydroxyl groups. Conjugate groups
 of the invention include intercalators, reporter molecules, polyamines, polyamides,
 polyethylene glycols, polyethers, groups that enhance the pharmacodynamic
 properties of oligomers, and groups that enhance the pharmacokinetic properties of
 oligomers. Typical conjugate groups include cholesterol, lipids, phospholipids,
 biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins,
 rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic
 properties, in the context of this invention, include groups that improve uptake,
 enhance resistance to degradation, and/or strengthen sequence-specific hybridization
 with the target nucleic acid. Groups that enhance the pharmacokinetic properties, in
 the context of this invention, include groups that improve uptake, distribution,
 metabolism or excretion of the compounds of the present invention. Representative
 conjugate groups are disclosed in International Patent Application No.
 PCT/US92/09196, filed Oct. 23, 1992, and U.S. Pat. No. 6,287,860, which are
 incorporated herein by reference. Conjugate moieties include, but are not limited to,
 lipid moieties such as a cholesterol moiety, cholic acid, a thioether, e.g., hexyl-5-
 tritylthiol, a thiocholesterol, an aliphatic chain, e.g., dodecandiol or undecyl residues,
 a phospholipid, e.g., di-hexadecyl-rac- glycerol or triethylammonium 1,2-di-O-
 hexadecyl-rac-glycero-3-H-phosphonate, a polyamine or a polyethylene glycol chain,
 or adamantane acetic acid, a palmityl moiety, or an octadecylamine or hexylamino-
 carbonyl-oxy cholesterol moiety. See, e.g., U.S. Pat. Nos. 4,828,979; 4,948,882;
 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731;
 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603;

5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779;
 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830;
 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469;
 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241; 5,391,723;
 5 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810;
 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and
 5,688,941.

The inhibitory nucleic acids useful in the present methods are sufficiently
 complementary to the target lncRNA, i.e., hybridize sufficiently well and with
 10 sufficient specificity, to give the desired effect. "Complementary" refers to the
 capacity for pairing, through hydrogen bonding, between two sequences comprising
 naturally or non-naturally occurring bases or analogs thereof. For example, if a base
 at one position of an inhibitory nucleic acid is capable of hydrogen bonding with a
 base at the corresponding position of a lncRNA, then the bases are considered to be
 15 complementary to each other at that position. 100% complementarity is not required.

In some embodiments, the location on a target lncRNA to which an inhibitory
 nucleic acids hybridizes is defined as a target region to which a protein binding
 partner binds. These regions can be identified by reviewing the data submitted
 herewith in Appendix I and identifying regions that are enriched in the dataset; these
 20 regions are likely to include the protein binding sequences. Routine methods can be
 used to design an inhibitory nucleic acid that binds to this sequence with sufficient
 specificity. In some embodiments, the methods include using bioinformatics methods
 known in the art to identify regions of secondary structure, e.g., one, two, or more
 stem-loop structures, or pseudoknots, and selecting those regions to target with an
 25 inhibitory nucleic acid.

While the specific sequences of certain exemplary target segments are set
 forth herein, one of skill in the art will recognize that these serve to illustrate and
 describe particular embodiments within the scope of the present invention. Additional
 target segments are readily identifiable by one having ordinary skill in the art in view
 30 of this disclosure. Target segments 5-500 nucleotides in length comprising a stretch
 of at least five (5) consecutive nucleotides within the protein binding region, or
 immediately adjacent thereto, are considered to be suitable for targeting as well.
 Target segments can include sequences that comprise at least the 5 consecutive

nucleotides from the 5'-terminus of one of the protein binding regions (the remaining nucleotides being a consecutive stretch of the same RNA beginning immediately upstream of the 5'-terminus of the binding segment and continuing until the inhibitory nucleic acid contains about 5 to about 100 nucleotides). Similarly preferred target segments are represented by RNA sequences that comprise at least the 5 consecutive nucleotides from the 3'-terminus of one of the illustrative preferred target segments (the remaining nucleotides being a consecutive stretch of the same lncRNA beginning immediately downstream of the 3'-terminus of the target segment and continuing until the inhibitory nucleic acid contains about 5 to about 100 nucleotides). One having skill in the art armed with the sequences provided herein will be able, without undue experimentation, to identify further preferred protein binding regions to target.

Once one or more target regions, segments or sites have been identified, inhibitory nucleic acid compounds are chosen that are sufficiently complementary to the target, i.e., that hybridize sufficiently well and with sufficient specificity (i.e., do not substantially bind to other non-target RNAs), to give the desired effect.

Making and Using Inhibitory Nucleic Acids

The inhibitory nucleic acids used to practice the methods described herein, whether RNA, cDNA, genomic DNA, vectors, viruses or hybrids thereof, can be isolated from a variety of sources, genetically engineered, amplified, and/or expressed, generated recombinantly or synthetically by well-known chemical synthesis techniques, as described in, e.g., Adams (1983) J. Am. Chem. Soc. 105:661; Belousov (1997) Nucleic Acids Res. 25:3440-3444; Frenkel (1995) Free Radic. Biol. Med. 19:373-380; Blommers (1994) Biochemistry 33:7886-7896; Narang (1979) Meth. Enzymol. 68:90; Brown (1979) Meth. Enzymol. 68:109; Beaucage (1981) Tetra. Lett. 22:1859; Maier (2000) Org Lett 2(13):1819-1822; Egeland (2005) Nucleic Acids Res 33(14):e125; Krotz (2005) Pharm Dev Technol 10(2):283-90 U.S. Patent No. 4,458,066. Recombinant nucleic acid sequences can be individually isolated or cloned and tested for a desired activity. Any recombinant expression system can be used, including e.g. *in vitro* bacterial, fungal, mammalian, yeast, insect or plant cell expression systems.

Nucleic acid sequences of the invention can be inserted into delivery vectors and expressed from transcription units within the vectors. The recombinant vectors can be DNA plasmids or viral vectors. Generation of the vector construct can be

accomplished using any suitable genetic engineering techniques well known in the art, including, without limitation, the standard techniques of PCR, oligonucleotide synthesis, restriction endonuclease digestion or “seamless cloning”, ligation, transformation, plasmid purification, and DNA sequencing, for example as described in Sambrook et al. “Molecular Cloning: A Laboratory Manual.” (1989)), Coffin et al. (Retroviruses. (1997)) and “RNA Viruses: A Practical Approach” (Alan J. Cann, Ed., Oxford University Press, (2000)). “Seamless cloning” allows joining of multiple fragments of nucleic acids in a single, isothermal reaction (Gibson (2009) Nat Methods 6:343-345; Werner (2012) Bioeng Bugs 3:38-43; Sanjana (2012) Nat Protoc 7:171-192). As will be apparent to one of ordinary skill in the art, a variety of suitable vectors are available for transferring nucleic acids of the invention into cells. The selection of an appropriate vector to deliver nucleic acids and optimization of the conditions for insertion of the selected expression vector into the cell, are within the scope of one of ordinary skill in the art without the need for undue experimentation. Viral vectors comprise a nucleotide sequence having sequences for the production of recombinant virus in a packaging cell. Viral vectors expressing nucleic acids of the invention can be constructed based on viral backbones including, but not limited to, a retrovirus, lentivirus, adenovirus, adeno-associated virus, pox virus or alphavirus (Warnock (2011) Methods in Molecular Biology 737:1-25). The recombinant vectors capable of expressing the nucleic acids of the invention can be delivered as described herein, and persist in target cells (e.g., stable transformants).

This can be achieved, for example, by administering an inhibitory nucleic acid, e.g., an antisense oligonucleotide that is complementary or binds to a target sequence in an ATR nucleic acid. Other inhibitory nucleic acids for use in practicing the methods described herein and that are complementary to or bind to an ATR nucleic acid can be those which inhibit post-transcriptional processing of an ATR nucleic acid, such as inhibitors of mRNA translation (antisense), agents of RNA interference (RNAi), catalytically active RNA molecules (ribozymes), and RNAs that bind proteins and other molecular ligands (aptamers). Additional methods exist to inhibit endogenous microRNA (miRNA) activity through the use of antisense-miRNA oligonucleotides (antagomirs) and RNA competitive inhibitors or decoys (miRNA sponges).

For further disclosure regarding inhibitory nucleic acids, please see
US2010/0317718 (antisense oligos); US2010/0249052 (double-stranded ribonucleic
acid (dsRNA)); US2009/0181914 and US2010/0234451 (LNAs); US2007/0191294
(siRNA analogues); US2008/0249039 (modified siRNA); WO2010/129746 and
5 WO2010/040112 (inhibitory nucleic acids); .

Antisense

In some embodiments, the inhibitory nucleic acids are antisense
oligonucleotides. Antisense oligonucleotides are typically designed to block
10 expression of a DNA or RNA target by binding to the target and halting expression at
the level of transcription, translation, or splicing. Antisense oligonucleotides of the
present invention are complementary nucleic acid sequences designed to hybridize
under stringent conditions to an ATR nucleic acid. Thus, oligonucleotides are chosen
that are sufficiently complementary to the target, i.e., that hybridize sufficiently well
15 and with sufficient specificity, to give the desired effect, while striving to avoid
significant off-target effects i.e. must not directly bind to, or directly significantly
affect expression levels of, transcripts other than the intended target. The optimal
length of the antisense oligonucleotide may vary but it should be as short as possible
while ensuring that its target sequence is unique in the transcriptome i.e. antisense
20 oligonucleotides may be as short as 12-mers (Seth (2009) J Med Chem 52:10-13) to
18-22 nucleotides in length.

In the context of this invention, hybridization means hydrogen bonding, which
may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between
complementary nucleoside or nucleotide bases. For example, adenine and thymine
25 are complementary nucleobases which pair through the formation of hydrogen bonds.
Complementary, as used herein, refers to the capacity for precise pairing between two
nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is
capable of hydrogen bonding with a nucleotide at the same position of a DNA or
RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be
30 complementary to each other at that position. The oligonucleotide and the DNA or
RNA are complementary to each other when a sufficient number of corresponding
positions in each molecule are occupied by nucleotides which can hydrogen bond
with each other. Thus, “specifically hybridizable” and “complementary” are terms

which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target.

It is understood in the art that a complementary nucleic acid sequence need not
5 be 100% complementary to that of its target nucleic acid to be specifically
hybridisable. A complementary nucleic acid sequence of the invention is specifically
hybridisable when binding of the sequence to the target DNA or RNA molecule
interferes with the normal function of the target DNA or RNA to cause a loss of
activity, and there is a sufficient degree of complementarity to avoid non-specific
10 binding of the sequence to non-target sequences under conditions in which specific
binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or
therapeutic treatment, and in the case of *in vitro* assays, under conditions in which the
assays are performed under suitable conditions of stringency. The antisense
oligonucleotides useful in the methods described herein have at least 80% sequence
15 complementarity to a target region within the target nucleic acid, e.g., 90%, 95%, or
100% sequence complementarity to the target region within an ATR nucleic acid.
Percent complementarity of an antisense compound with a region of a target nucleic
acid can be determined routinely using basic local alignment search tools (BLAST
programs) (Altschul (1990) J. Mol. Biol. 215, 403-410; Zhang and Madden (1997)
20 Genome Res. 7, 649-656). The specificity of an antisense oligonucleotide can also
be determined routinely using BLAST program against the entire genome of a given
species

For example, stringent salt concentration will ordinarily be less than about 750
mM NaCl and 75 mM trisodium citrate, preferably less than about 500 mM NaCl and
25 50 mM trisodium citrate, and more preferably less than about 250 mM NaCl and 25
mM trisodium citrate. Low stringency hybridization can be obtained in the absence of
organic solvent, e.g., formamide, while high stringency hybridization can be obtained
in the presence of at least about 35% formamide, and more preferably at least about
50% formamide. Stringent temperature conditions will ordinarily include
30 temperatures of at least about 30° C, more preferably of at least about 37° C, and most
preferably of at least about 42° C. Varying additional parameters, such as
hybridization time, the concentration of detergent, e.g., sodium dodecyl sulfate (SDS),
and the inclusion or exclusion of carrier DNA, are well known to those skilled in the

art. Various levels of stringency are accomplished by combining these various conditions as needed. In a preferred embodiment, hybridization will occur at 30° C in 750 mM NaCl, 75 mM trisodium citrate, and 1% SDS. In a more preferred embodiment, hybridization will occur at 37° C in 500 mM NaCl, 50 mM trisodium citrate, 1% SDS, 35% formamide, and 100 µg/ml denatured salmon sperm DNA (ssDNA). In a most preferred embodiment, hybridization will occur at 42° C in 250 mM NaCl, 25 mM trisodium citrate, 1% SDS, 50% formamide, and 200 µg/ml ssDNA. Useful variations on these conditions will be readily apparent to those skilled in the art. For most applications, washing steps that follow hybridization will also vary in stringency. Wash stringency conditions can be defined by salt concentration and by temperature. As above, wash stringency can be increased by decreasing salt concentration or by increasing temperature. For example, stringent salt concentration for the wash steps will preferably be less than about 30 mM NaCl and 3 mM trisodium citrate, and most preferably less than about 15 mM NaCl and 1.5 mM trisodium citrate. Stringent temperature conditions for the wash steps will ordinarily include a temperature of at least about 25° C, more preferably of at least about 42° C, and even more preferably of at least about 68° C. In a preferred embodiment, wash steps will occur at 25° C in 30 mM NaCl, 3 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 42° C. in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 68° C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. Additional variations on these conditions will be readily apparent to those skilled in the art. Hybridization techniques are well known to those skilled in the art and are described, for example, in Benton and Davis (Science 196:180, 1977); Grunstein and Hogness (Proc. Natl. Acad. Sci., USA 72:3961, 1975); Ausubel et al. (Current Protocols in Molecular Biology, Wiley Interscience, New York, 2001); Berger and Kimmel (Guide to Molecular Cloning Techniques, 1987, Academic Press, New York); and Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, Hilario (2007) Methods Mol Biol 353:27-38.

Inhibitory nucleic acids for use in the methods described herein can include one or more modifications, e.g., be stabilized against nucleolytic degradation such as by the incorporation of a modification, e.g., a nucleotide modification. For example, inhibitory nucleic acids can include a phosphorothioate at least the first, second, or

third internucleotide linkage at the 5' or 3' end of the nucleotide sequence. As another example, inhibitory nucleic acids can include a 2'-modified nucleotide, e.g., a 2'-deoxy, 2'-deoxy-2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), 2'-O-dimethylaminoethoxyethyl (2'-O-DMAEOE), or 2'-O--N-methylacetamido (2'-O--NMA). As another example, the inhibitory nucleic acids can include at least one 2'-O-methyl-modified nucleotide, and in some embodiments, all of the nucleotides include a 2'-O-methyl modification.

Modifications

Chemical modifications, particularly the use of locked nucleic acids (LNAs) (Okiba (1997) *Tetrahedron Lett* 39:5401-5404; Singh (1998) *Chem Commun* 4:455-456), 2'-O-methoxyethyl (2'-O-MOE) (Martin (1995) *Helv Chim Acta* 78:486-504; You (2006) *Nucleic Acids Res* 34(8):e60; Owczarzy (2011) *Biochem* 50(43):9352-9367), constrained ethyl BNA (cET) (Murray (2012) *Nucleic Acids Res* 40: 6135-6143), and gapmer oligonucleotides, which contain 2-5 chemically modified nucleotides (LNA, 2'-O-MOE RNA or cET) at each terminus flanking a central 5-10 base "gap" of DNA (Monia (1993) *J Biol Chem* 268:14514-14522; Wahlestedt (2000) *PNAS* 97:5633-5638), improve antisense oligonucleotide binding affinity for the target RNA, which increases the steric block efficiency. Antisense, siRNA, and other compounds of the invention, which hybridize to an ATR nucleic acid can be identified through experimentation, and representative sequences of these compounds are herein below identified as embodiments of the invention (e.g., including but not limited to the siRNA of (CCUCCGUGAUGUUGCUUGA (SEQ ID NO:3)). Techniques for the manipulation of inhibitory nucleic acids, such as, e.g., subcloning, labeling probes (e.g., random-primer labeling using Klenow polymerase, nick translation, amplification), sequencing, hybridization and the like are well described in the scientific and patent literature, see, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual* 3d ed. (2001); *Current Protocols in Molecular Biology*, Ausubel et al., eds. (John Wiley & Sons, Inc., New York 2010); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); *Laboratory Techniques In Biochemistry And Molecular Biology: Hybridization With Nucleic Acid Probes, Part I. Theory and Nucleic Acid Preparation*, Tijssen, ed. Elsevier, N.Y. (1993).

Modified bases/Locked Nucleic Acids (LNAs)

In some embodiments, the inhibitory nucleic acids used in the methods described herein comprise one or more modified bonds or bases. Modified bases include phosphorothioate, methylphosphonate, peptide nucleic acids, or locked
5 nucleic acid (LNA) molecules. Preferably, the modified nucleotides are locked nucleic acid molecules, including [alpha]-L-LNAs. LNAs comprise ribonucleic acid analogues wherein the ribose ring is “locked” by a methylene bridge between the 2'-oxygen and the 4'-carbon – i.e., oligonucleotides containing at least one LNA monomer, that is, one 2'-O,4'-C-methylene-β-D-ribofuranosyl nucleotide. LNA bases
10 form standard Watson-Crick base pairs but the locked configuration increases the rate and stability of the basepairing reaction (Jepsen (2004) Oligonucleotides 14, 130-146). LNAs also have increased affinity to base pair with RNA as compared to DNA. These properties render LNAs especially useful as probes for fluorescence in situ hybridization (FISH) and comparative genomic hybridization, as knockdown tools for
15 miRNAs, and as antisense oligonucleotides to target mRNAs or other RNAs, e.g., lncRNAs as described herein.

The LNA molecules can include molecules comprising 10-30, e.g., 12-24, e.g., 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in each strand, wherein one of the strands is substantially identical, e.g., at least 80%
20 (or more, e.g., 85%, 90%, 95%, or 100%) identical, e.g., having 3, 2, 1, or 0 mismatched nucleotide(s), to a target region in the lncRNA. The LNA molecules can be chemically synthesized using methods known in the art.

The LNA molecules can be designed using any method known in the art; a number of algorithms are known, and are commercially available (e.g., exiqon.com).
25 You (2006) Nuc. Acids. Res. 34:e60; McTigue (2004) Biochemistry 43:5388-405; and Levin (2006) Nuc. Acids. Res. 34:e142. For example, “gene walk” methods, similar to those used to design antisense oligos, can be used to optimize the inhibitory activity of the LNA; for example, a series of oligonucleotides of 10-30 nucleotides spanning the length of a target lncRNA can be prepared, followed by testing for
30 activity. Optionally, gaps, e.g., of 5-10 nucleotides or more, can be left between the LNAs to reduce the number of oligonucleotides synthesized and tested. GC content is preferably between about 30-60%. General guidelines for designing LNAs are known in the art; for example, LNA sequences will bind very tightly to other LNA

sequences, so it is preferable to avoid significant complementarity within an LNA. Contiguous runs of three or more Gs or Cs, or more than four LNA residues, should be avoided where possible (for example, it may not be possible with very short (e.g., about 9-10 nt) oligonucleotides). In some embodiments, the LNAs are xylo-LNAs.

5 In some embodiments, the LNA molecules can be designed to target a specific region of the lncRNA. For example, a specific functional region can be targeted, e.g., a region comprising a known RNA localization motif (i.e., a region complementary to the target nucleic acid on which the lncRNA acts), or a region comprising a known protein binding region, e.g., a Polycomb (e.g., Polycomb Repressive Complex 2
10 (PRC2), comprised of H3K27 methylase EZH2, SUZ12, and EED)) or LSD1/CoREST/REST complex binding region (Tsai (2010) Science 329(5992):689-93; and Zhao (2008) Science 322(5902):750-6; Sarma (2010) PNAS 107 (51): 22196-201). Alternatively or in addition, highly conserved regions can be targeted, e.g., regions identified by aligning sequences from disparate species such as primate (e.g.,
15 human) and rodent (e.g., mouse) and looking for regions with high degrees of identity. Percent identity can be determined routinely using basic local alignment search tools (BLAST programs) (Altschul (1990) J. Mol. Biol. 215, 403-410; Zhang and Madden (1997) Genome Res. 7, 649-656), e.g., using the default parameters.

For additional information regarding LNAs see U.S. Pat. Nos. 6,268,490;
20 6,734,291; 6,770,748; 6,794,499; 7,034,133; 7,053,207; 7,060,809; 7,084,125; and 7,572,582; and U.S. Pre-Grant Pub. Nos. 20100267018; 20100261175; and 20100035968; Koshkin (1998) Tetrahedron 54, 3607-3630; Obika (1998) Tetrahedron Lett. 39, 5401-5404; Jepsen (2004) Oligonucleotides 14:130-146; Kauppinen (2005) Drug Disc. Today 2(3):287-290; and Ponting (2009) Cell
25 136(4):629-641, and references cited therein.

See also USSN 61/412,862, which is incorporated by reference herein in its entirety.

siRNA/shRNA

30 In some embodiments, the nucleic acid sequence that is complementary to an ATR nucleic acid can be an interfering RNA, including but not limited to a small interfering RNA ("siRNA") or a small hairpin RNA ("shRNA"). Methods for constructing interfering RNAs are well known in the art. For example, the interfering

RNA can be assembled from two separate oligonucleotides, where one strand is the sense strand and the other is the antisense strand, wherein the antisense and sense strands are self-complementary (i.e., each strand comprises nucleotide sequence that is complementary to nucleotide sequence in the other strand; such as where the antisense strand and sense strand form a duplex or double stranded structure); the antisense strand comprises nucleotide sequence that is complementary to a nucleotide sequence in a target nucleic acid molecule or a portion thereof (i.e., an undesired gene) and the sense strand comprises nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. Alternatively, interfering RNA is assembled from a single oligonucleotide, where the self-complementary sense and antisense regions are linked by means of nucleic acid based or non-nucleic acid-based linker(s). The interfering RNA can be a polynucleotide with a duplex, asymmetric duplex, hairpin or asymmetric hairpin secondary structure, having self-complementary sense and antisense regions, wherein the antisense region comprises a nucleotide sequence that is complementary to nucleotide sequence in a separate target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The interfering can be a circular single-stranded polynucleotide having two or more loop structures and a stem comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof, and wherein the circular polynucleotide can be processed either *in vivo* or *in vitro* to generate an active siRNA molecule capable of mediating RNA interference. RNA interference may cause translational repression and degradation of target mRNAs with imperfect complementarity or sequence-specific cleavage of perfectly complementary mRNAs.

In some embodiments, the interfering RNA coding region encodes a self-complementary RNA molecule having a sense region, an antisense region and a loop region. Such an RNA molecule when expressed desirably forms a “hairpin” structure, and is referred to herein as an “shRNA.” The loop region is generally between about 2 and about 10 nucleotides in length. In some embodiments, the loop region is from about 6 to about 9 nucleotides in length. In some embodiments, the sense region and

the antisense region are between about 15 and about 20 nucleotides in length.

Following post-transcriptional processing, the small hairpin RNA is converted into a siRNA by a cleavage event mediated by the enzyme Dicer, which is a member of the RNase III family. The siRNA is then capable of inhibiting the expression of a gene with which it shares homology. After the siRNA has cleaved its target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets (Brummelkamp (2002) *Science* 296:550-553; Lee (2002) *Nature Biotechnol.*, 20, 500-505; Miyagishi and Taira (2002) *Nature Biotechnol* 20:497-500; Paddison (2002) *Genes & Dev.* 16:948-958; Paul (2002) *Nature Biotechnol* 20, 505-508; Sui (2002) *Proc. Natl. Acad. Sci. USA* 99(6), 5515-5520; Yu (2002) *Proc Natl Acad Sci USA* 99:6047-6052; Peer and Lieberman (2011) *Gen Ther* 18, 1127-1133).

The target RNA cleavage reaction guided by siRNAs is highly sequence specific. In general, siRNA containing a nucleotide sequences identical to a portion of the target nucleic acid are preferred for inhibition. However, 100% sequence identity between the siRNA and the target gene is not required to practice the present invention. Thus the invention has the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence. For example, siRNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Alternatively, siRNA sequences with nucleotide analog substitutions or insertions can be effective for inhibition. In general the siRNAs must retain specificity for their target, i.e., must not directly bind to, or directly significantly affect expression levels of, transcripts other than the intended target. shRNAs that are constitutively expressed from promoters can ensure long-term gene silencing. Most methods commonly used for delivery of siRNAs rely on commonly used techniques for introducing an exogenous nucleic acid into a cell including calcium phosphate or calcium chloride precipitation, microinjection, DEAE-dextrin-mediated transfection, lipofection, commercially available cationic polymers and lipids and cell-penetrating peptides, electroporation or stable nucleic acid-lipid particles (SNALPs), all of which are routine in the art. siRNAs can also be conjugated to small molecules to direct binding to cell-surface receptors, such as cholesterol (Wolfrum (2007) *Nat Biotechnol* 25:1149-1157), alpha-tocopherol (Nishina (2008) *Mol Ther* 16:734-40), lithocholic acid or lauric acid (Lorenz (2004) *Bioorg Med*

Chem Lett 14:4975-4977), polyconjugates (Rozema (2007) PNAS 104:12982-12987).
A variation of conjugated siRNAs are aptamer-siRNA chimeras (McNamara (2006)
Nat Biotechnol 24:1005-1015; Dassie (2009) Nat Biotechnol 27:839-849) and
siRNA-fusion protein complexes, which is composed of a targeting peptide, such as
5 an antibody fragment that recognizes a cell-surface receptor or ligand, linked to an
RNA-binding peptide that can be complexed to siRNAs for targeted systemic siRNA
delivery (Yao (2011) Sci Transl Med 4(130):130ra48).

Ribozymes

Trans-cleaving enzymatic nucleic acid molecules can also be used; they have
10 shown promise as therapeutic agents for human disease (Usman & McSwiggen,
(1995) Ann. Rep. Med. Chem. 30, 285-294; Christoffersen and Marr (1995) J. Med.
Chem. 38, 2023-2037; Weng (2005) Mol Cancer Ther 4, 948-955; Armado (2004)
Hum Gene Ther 15, 251-262; Macpherson (2005) J Gene Med 7,552-564;
Muhlbacher (2010) Curr Opin Pharmacol 10(5):551-6). Enzymatic nucleic acid
15 molecules can be designed to cleave specific ATR nucleic acid targets within the
background of cellular RNA. Such a cleavage event renders the ATR nucleic acid
non- functional.

In general, enzymatic nucleic acids with RNA cleaving activity act by first
binding to a target RNA. Such binding occurs through the target binding portion of an
20 enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the
molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first
recognizes and then binds a target RNA through complementary base pairing, and
once bound to the correct site, acts enzymatically to cut the target RNA. Strategic
cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded
25 protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is
released from that RNA to search for another target and can repeatedly bind and
cleave new targets.

Several approaches such as *in vitro* selection (evolution) strategies (Orgel
(1979) Proc. R. Soc. London B 205, 435) have been used to evolve new nucleic acid
30 catalysts with improved properties, new functions and capable of catalyzing a variety
of reactions, such as cleavage and ligation of phosphodiester linkages and amide
linkages, (Joyce (1989) Gene 82, 83-87; Beaudry (1992) Science 257, 635-641; Joyce
(1992) Scientific American 267, 90-97; Breaker (1994) TIBTECH 12, 268; Bartel

(1993) Science 261 :1411-1418; Szostak (1993) TIBS 17, 89-93; Kumar (1995) FASEB J. 9, 1183; Breaker (1996) Curr. Op. Biotech. 1, 442; Scherer (2003) Nat Biotechnol 21, 1457-1465; Berens (2015) Curr. Op. Biotech. 31, 10-15). Ribozymes can also be engineered to be allosterically activated by effector molecules

5 (riboswitches, Liang (2011) Mol Cell 43, 915-926; Wieland (2010) Chem Biol 17, 236-242; US Patent No 8,440,810). The development of ribozymes that are optimal for catalytic activity would contribute significantly to any strategy that employs RNA-cleaving ribozymes for the purpose of regulating gene expression. The most common

10 ribozyme therapeutics are derived from either hammerhead or hairpin/paperclip motifs. The hammerhead ribozyme, for example, functions with a catalytic rate (kcat) of about 1 min⁻¹ in the presence of saturating (10 mM) concentrations of Mg²⁺ cofactor. An artificial "RNA ligase" ribozyme has been shown to catalyze the corresponding self-modification reaction with a rate of about 100 min⁻¹. In addition,

15 it is known that certain modified hammerhead ribozymes that have substrate binding arms made of DNA catalyze RNA cleavage with multiple turn-over rates that approach 100 min⁻¹. Ribozymes can be delivered to target cells in RNA form or can be transcribed from vectors. Due to poor stability of fully-RNA ribozymes, ribozymes often require chemical modification, such as, 5'-PS backbone linkage, 2'-O-Me, 2'-deoxy-2'-C-allyl uridine, and terminal inverted 3'-3' deoxyabasic nucleotides

20 (Kobayashi (2005) Cancer Chemother Pharmacol 56, 329-336).

CRISPR/Cas9

CRISPR-Cas9 nucleases enable efficient genome editing in a wide variety of organisms and cell types (Sander & Joung, Nat Biotechnol 32, 347-355 (2014); Hsu et al., Cell 157, 1262-1278 (2014); Doudna & Charpentier, Science 346, 1258096

25 (2014); Barrangou & May, Expert Opin Biol Ther 15, 311-314 (2015)). Target site recognition by Cas9 is programmed by a chimeric single guide RNA (sgRNA) that encodes a sequence complementary to a target protospacer (Jinek et al., Science 337, 816-821 (2012)), but also requires recognition of a short neighboring PAM (Mojica et al., Microbiology 155, 733-740 (2009); Shah et al., RNA Biol 10, 891-899 (2013);

30 Jiang et al., Nat Biotechnol 31, 233-239 (2013); Jinek et al., Science 337, 816-821 (2012); Sternberg et al., Nature 507, 62-67 (2014))

The CRISPR/Cas9 genome editing system can also be used to inhibit expression of ATR. A guide RNA (e.g., a single guide RNA, or a paired

crRNA/tracrRNA) that binds to an ATR nucleic acid is administered to or expressed in the cell, along with a CRISPR/Cas9 nuclease. See, e.g., Jinek et al. *Science* 337, 816–821 (2012); Jiang et al., *Nat. Biotechnol.* 31, 233–239 (2013); Hou, Z. et al. *Proc. Natl. Acad. Sci. USA* 110, 15644–15649 (2013); Mali et al., *Science* 339, 823–826 (2013); Jinek, M. et al. RNA-programmed genome editing in human cells. *Elife* 2, e00471 (2013); Horii et al., *PeerJ* 1, e230 (2013); Shalem, O. et al., *Science* 343, 84–87 (2014); Sander and Joung, *Nature Biotechnology* 32, 347–355 (2014).

Methods for selectively altering the genome of a cell are known in the art, see, e.g., US 8,993,233; US 20140186958; US 9,023,649; WO/2014/099744; WO 2014/089290; WO2014/144592; WO144288; WO2014/204578; WO2014/152432; WO2115/099850; US8,697,359; US2010/0076057; US2011/0189776; US2011/0223638; US2013/0130248; WO/2008/108989; WO/2010/054108; WO/2012/164565; WO/2013/098244; WO/2013/176772; US20150050699; US 20150071899; US20150045546; US20150031134; US20150024500; US20140377868; US20140357530; US20140349400; US20140335620; US20140335063; US20140315985; US20140310830; US20140310828; US20140309487; US20140304853; US20140298547; US20140295556; US20140294773; US20140287938; US20140273234; US20140273232; US20140273231; US20140273230; US20140271987; US20140256046; US20140248702; US20140242702; US20140242700; US20140242699; US20140242664; US20140234972; US20140227787; US20140212869; US20140201857; US20140199767; US20140189896; US20140186958; US20140186919; US20140186843; US20140179770; US20140179006; US20140170753; US 20150071899; Makarova et al., "Evolution and classification of the CRISPR-Cas systems" 9(6) *Nature Reviews Microbiology* 467-477 (1-23) (Jun. 2011); Wiedenheft et al., "RNA-guided genetic silencing systems in bacteria and archaea" 482 *Nature* 331-338 (Feb. 16, 2012); Gasiunas et al., "Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria" 109(39) *Proceedings of the National Academy of Sciences USA* E2579-E2586 (Sep. 4, 2012); Jinek et al., "A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity" 337 *Science* 816-821 (Aug. 17, 2012); Carroll, "A CRISPR Approach to Gene Targeting" 20(9) *Molecular Therapy* 1658-1660 (Sep. 2012); U.S. Appl. No. 61/652,086, filed May 25, 2012; Al-Attar et al.,

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs): The
Hallmark of an Ingenious Antiviral Defense Mechanism in Prokaryotes, Biol Chem.
(2011) vol. 392, Issue 4, pp. 277-289; Hale et al., Essential Features and Rational
Design of CRISPR RNAs That Function With the Cas RAMP Module Complex to
5 Cleave RNAs, Molecular Cell, (2012) vol. 45, Issue 3, 292-302.

Compositions and Methods of Administration

The methods described herein can include the use (e.g., for administration) of
pharmaceutical compositions comprising an ATR inhibitor as an active ingredient.
Pharmaceutical compositions typically include a pharmaceutically acceptable carrier.
10 As used herein the language “pharmaceutically acceptable carrier” includes saline,
solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and
absorption delaying agents, and the like, compatible with pharmaceutical
administration.

Pharmaceutical compositions are typically formulated to be compatible with
15 its intended route of administration. Examples of routes of administration include
parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation),
transdermal (topical), transmucosal, and rectal administration.

Methods of formulating suitable pharmaceutical compositions are known in
the art, see, e.g., Remington: The Science and Practice of Pharmacy, 21st ed., 2005;
20 and the books in the series Drugs and the Pharmaceutical Sciences: a Series of
Textbooks and Monographs (Dekker, NY). For example, solutions or suspensions
used for parenteral, intradermal, or subcutaneous application can include the
following components: a sterile diluent such as water for injection, saline solution,
fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic
25 solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants
such as ascorbic acid or sodium bisulfite; chelating agents such as
ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and
agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be
adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The
30 parenteral preparation can be enclosed in ampoules, disposable syringes or multiple
dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use can include sterile
aqueous solutions (where water soluble) or dispersions and sterile powders for the

extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle, which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying, which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules, e.g., gelatin capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules,

troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant
5 such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds can be delivered in the form of an aerosol spray from a pressured container or dispenser that contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer. Such methods include
10 those described in U.S. Patent No. 6,468,798.

Systemic administration of a therapeutic compound as described herein can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for
15 example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The pharmaceutical compositions can also be prepared in the form of
20 suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

Therapeutic compounds that are or include nucleic acids can be administered by any method suitable for administration of nucleic acid agents, such as a DNA vaccine. These methods include gene guns, bio injectors, and skin patches as well as
25 needle-free methods such as the micro-particle DNA vaccine technology disclosed in U.S. Patent No. 6,194,389, and the mammalian transdermal needle-free vaccination with powder-form vaccine as disclosed in U.S. Patent No. 6,168,587. Additionally, intranasal delivery is possible, as described in, *inter alia*, Hamajima et al., Clin. Immunol. Immunopathol., 88(2), 205-10 (1998). Liposomes (e.g., as described in
30 U.S. Patent No. 6,472,375) and microencapsulation can also be used. Biodegradable targetable microparticle delivery systems can also be used (e.g., as described in U.S. Patent No. 6,471,996).

In one embodiment, the therapeutic compounds are prepared with carriers that will protect the therapeutic compounds against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as
5 ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Such formulations can be prepared using standard techniques, or obtained commercially, e.g., from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to selected cells with
10 monoclonal antibodies to cellular antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

Typically, an effective amount of the ATR inhibitor is administered. An
15 “effective amount” is an amount sufficient to effect beneficial or desired results. For example, a therapeutic amount is one that achieves the desired therapeutic effect. An effective amount can be administered in one or more administrations, applications or dosages. A therapeutically effective amount of a therapeutic compound (i.e., an effective dosage) depends on the therapeutic compounds selected. The compositions
20 can be administered one from one or more times per day to one or more times per week; including once every other day. The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present.
25 Moreover, treatment of a subject with a therapeutically effective amount of the therapeutic compounds described herein can include a single treatment or a series of treatments.

Dosage, toxicity and therapeutic efficacy of the therapeutic compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental
30 animals, e.g., for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Compounds which exhibit high therapeutic

indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

5 The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound
10 used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more
15 accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

20 **Materials and Methods**

The following materials and methods were used in the Examples below.

Cell culture

U2OS, SAOS2, HeLa, SW26, and SW39 cells were cultured in DMEM, 10% FBS, 1% L-Glutamine, and 1% Penicillin/Streptomycin. MG63, SKLU1, CALU6,
25 and NY cells were grown in DMEM F12, 5% FBS, 1% Penicillin/Streptomycin. SJSA1, NOS1, HUUO9, and G292 cells were grown in RPMI 1640, 5% FBS, 1% Sodium Pyruvate, 1% Penicillin/Streptomycin. CAL78 was grown in RPMI 1640, 10% FBS, 1% Sodium Pyruvate, 1% Penicillin/Streptomycin. CAL72 was grown in DMEM F12, 10% FBS, and 1% Penicillin/Streptomycin. BJ fibroblasts were grown in
30 EMEM, 10% FBS, 1% penicillin/Streptomycin. MGG119 and MGG4 were grown in neurobasal medium (Invitrogen) supplemented with L-glutamine (3 mM; Mediatech), B27 supplement (Invitrogen), N2 supplement (Invitrogen), heparin (5 mg/ml; Sigma),

EGF (20 ng/ml; R and D systems), and FGF2 (20 ng/ml; Peprotec). MG63, SAOS2, SJS1, NOS1, H9, G292 and CAL78 were obtained from the Center for Molecular Therapeutics at Massachusetts General Hospital. SW26 and SW39 were a kind gift of W. Wright (UT Southwestern). BJ fibroblasts were obtained from ATCC.

5 **siRNAs, probes and antibodies**

Stealth siRNA targeting ATRX #1

(UCCCAUAGCCGUCUCAAGAUCUCA (SEQ ID NO:1)) and #2

(UAUAGAAUUCUGAUCUCA (SEQ ID NO:2)) was obtained from Invitrogen.

ATRX knockdown was analyzed by Western blot 72 hr after transfection using RNAi

10 MAX. siRNA for ATR (CCUCCGUGAUGUUGCUUGA (SEQ ID NO:3)) and ATM (GCCUCCAGGCAGAAAAAGAtt (SEQ ID NO:4)) were obtained from Invitrogen and experiments were performed 72 hr after transfection using RNAi MAX.

The following antibodies and probes were used where indicated, ATR
15 (Bethyl), ATM (Bethyl), ATRX (Santa Cruz), rabbit TRF2 (Bethyl), mouse TRF2 (Millipore), PML (Santa Cruz), RPA32 (Thermo Scientific), RAD52 (Santa Cruz), γ H2AX (Millipore), and α Tubulin (Cell Signaling), PNA-TAMRA-(CCCTAA) (Custom Biosynthesis), PNA-FITC-(TTAGGG) (Custom Biosynthesis), 28S (AACGATCAGAGTTTTCACC) (SEQ ID NO:5).

20 **Cell synchronization and FACS**

Cells were treated with 2 mM thymidine, 0.1 μ g/ml nocodazole, or 7 μ M RO3306, for 16-18 hr. Thymidine released cells were either washed three times in PBS, once in growth media, and then collected at the indicated time points or washed and released into 7 μ M RO3306 for 20 hr. For FACS, cells were collected by trypsin,
25 washed with PBS, and resuspended in PBS containing 1 mM EDTA. Cells were fixed by addition of ice-cold ethanol overnight. Fixed cells were washed in PBS, and stained with PBS, 0.1 mM EDTA, 1% BSA, 0.25% Tween 20, 10 μ g/ml propidium iodide, 0.5 mg/ml RNaseA, for 20 min at 37°C. Samples were analyzed using a FACSCalibur cytometer.

30 **Immunofluorescence analysis**

Cells were extracted with 0.25% Triton, fixed in 3% paraformaldehyde, and further permeabilized with 0.5% Triton. Cells were subsequently incubated with the primary antibodies (diluted in PBS containing 3% BSA and 0.05% Tween 20)

overnight at 4°C in a humidified chamber. Following extensive washing with PBS, cells were incubated with secondary antibodies for 45 min at room temperature, and washed again with PBS. After a 5-min incubation with DAPI, cells were mounted on slides with Vectashield. Slides were analyzed using a Nikon H600L fluorescence microscope or Zeiss LSM 710 confocal microscope. For HeLa cells with ATRX knockdown, 1×10^5 cells were reverse transfected with ATRX siRNA using Lipofectamine RNAi Max (Invitrogen), seeded onto coverslips, and incubated for 48 hr. After 48 hr, cells were treated with 2 mM thymidine for 16 hr, washed and released, and processed at the indicated time points. For ATR or ATM knockdown in U2OS cells, 0.75×10^5 cells were reverse transfected with ATR or ATM siRNA using Lipofectamine RNAi Max (Invitrogen), seeded onto coverslips, and incubated for 72 hr before APB analysis. To enhance the percentage of cells positive for APB, U2OS cells were seeded at 1.5×10^5 and allowed to incubate overnight. The following day methionine free media was added to the cells and they were incubated for an additional 84 hr.

RNA FISH

HeLa or U2OS cells adhered to coverslips were incubated for 7 min on ice, in ice-cold freshly made CSK buffer (100 mM NaCl, 300 mM Sucrose, 3 mM MgCl₂, 10 mM PIPES pH 7, 0.5% Triton X-100, 10 mM Vanadyl Ribonucleoside Complex). Cells were then rinsed in 1x PBS and fixed in 4 % Paraformaldehyde for 10 min at room temperature. The coverslips were rinsed in 70% ethanol and dehydrated in a series of ethanol washes (70%, 85%, 100%) for 5 min each at room temperature. After drying the coverslips at 37°C, they were then incubated with 10 nM PNA-TAMRA-(CCCTAA) probe in hybridization buffer (50% formamide, 2x SSC, 2 mg/ml BSA, 10% dextran sulfate, 10mM Vanadyl Ribonucleoside complex) for 16 hr at 37°C. Coverslips were washed in 2x SSC + 50% formamide 3 times at 39°C for 5 min, 3 times in 2xSSC at 39°C for 5 min each, and finally 1 time in 2x SSC + 100 ng/ml DAPI at room temperature for 10 min. Coverslips were mounted on glass microscope slides using VectaShield and sealed with nail polish.

Combined Immunofluorescence FISH

For Combined Immunofluorescence DNA FISH, cells were rinsed with PBS and treated with cytobuffer (100 mM NaCl, 300 mM sucrose, 3mM MgCl₂, 10 mM PIPES pH 7, 0.1% Triton-X 100) for 7 min at 4°C. Cells were then rinsed with PBS

and fixed in 4% paraformaldehyde for 10 min at room temperature. Cells were then permeabilized in 0.5% NP40/PBS for 10 min and blocked in PBG (0.5% BSA, 0.2% fish gelatin, PBS) for 1 hr at room temperature. Cells were then incubated with the indicated antibodies diluted in PBG and incubated overnight at 4°C. Following

5 incubation with primary antibody, the cells were washed three times with PBST (PBS containing 0.1% Triton) for 10 min each and subsequently incubated with secondary antibody diluted in PBG for 45 min at room temperature. The cells were washed three times with PBST for 10 min each and then fixed in 4% paraformaldehyde for 10 min at room temperature. Fixation was followed by digestion with RNaseA 200

10 µg/ml in 2x SSC for 30 min at 37°C. Cells were then dehydrated in a series of ethanol washes 70%, 85%, 100% for 2 min each at room temperature, and the coverslips were dried at 37°C for 20 min. 10 nM PNA-TAMRA-(CCCTAA) probe in hybridization buffer (50% formamide, 2x SSC, 2mg/ml BSA, 10% dextran sulfate) was added to coverslips and DNA was denatured at 75°C for 3 min and then place in

15 humidified chamber at 39°C overnight. The following day, the coverslips were washed in 2x SSC +50% formamide three times at 39°C for 5 min each, three times in 2xSSC at 39°C for 5 min each, and finally one time in 2x SSC at room temperature for 10 min. The coverslips were mounted on glass microscope slides with Vectashield mounting medium containing DAPI and analyzed using a Nikon H600L

20 fluorescence microscope.

Combined Immunofluorescence RNA FISH experiments were performed exactly as above except for the following modifications. The initial incubations in cytobuffer included 10 mM Vanadyl Ribonucleoside Complex. The RNaseA digestion step was omitted prior to dehydration. The denaturation step was omitted

25 during probe hybridization.

Telomere sister chromatid exchange assay

Cells were incubated with a 3:1 ratio of bromodeoxyuridine to bromodeoxycytidine (BrdU/BrdC, 10 µM/3.3 µM) for 16 hours before nocodazole was added and the cells were incubated for an additional 45 min. Cells were collected

30 by trypsinization, incubated in 75 mM KCL at 37°C for 20 min, and then fixed in ice cold 3:1 methanol/acetic acid. Cells were then centrifuged, supernatant aspirated, and resuspended in fresh fixative. This was repeated twice before fixed cells were dropped onto glass slides. Slides were treated with 0.5 mg/ml RNaseA in 2x SSC at

37°C for 10 min, incubated in 2x SSC containing 10 µg/ml Hoescht 33258 for 15 min, and then exposed to 365-nm UV light (Stratalinker 1800) for 30 min. Slides were then incubated in 10 U/µl ExoIII for 10 min at 37°C to degrade nicked DNA. Slides were dehydrated in an ethanol series (70% (v/v), 85% (v/v) and 100% ethanol) and allowed to air dry overnight. Slides were then incubated with a fluorescein isothiocyanate (FITC)-labeled G-rich telomeric peptide–nucleic acid (PNA) probe PNA-FITC-(GGGATT) in hybridization buffer (50% formamide, 2x SSC, 2 mg/ml BSA, 10% dextran sulfate) for 1 hr at room temperature and then washed in PBS containing 0.02% (v/v) Tween-20 for 10 min at room temperature. Subsequently, slides were incubated in PNA-TAMRA-(CCCTAA) probe for 1 hr at room temperature and washed in PBS containing 0.02% (v/v) Tween-20 for 20 min at 57°C. Finally, slides were incubated with 1 µg/ml DAPI in 2xSSC containing 0.02% (v/v) Tween-20 for 5 min and sealed with coverslips. Telomere sister-chromatid exchange events were imaged using a Zeiss LSM710 Confocal microscope and analyzed using Fiji software.

C-circle assay

C-circle assays were performed as previously described². Briefly, genomic DNA was purified using the Qiagen DNA Blood Mini Kit according the manufacturer's instructions. Purified DNA, was digested with AluI and MboI restriction enzymes at 37°C overnight. The digested DNA was again purified over a Qiagen PCR clean-up column and the DNA was quantified using a Nanodrop spectrophotometer. The DNA (40 ng) was diluted in 10 µl 1× Φ29 Buffer (NEB) containing BSA (NEB; 0.2 mg/ml), 0.1% Tween, 0.2 mM each dATP, dGTP, dTTP, and incubated in the presence or absence of 7.5 U ΦDNA polymerase (NEB) at 30°C for 8 hr, followed by 65°C for 20 min. C-circle amplification products were detected by dot blot using a DIG-labeled probe (CCCTAA)₄ (SEQ ID NO:6).

Live-cell imaging

HeLa and RPE-1 cells stably expressing H2B-mRFP, and U2OS cells stably expressing both H2B-mRFP and 53BP1-GFP, were grown on glass-bottom 12-well tissue culture dishes (Mattek) in phenol red-free DMEM:F12 medium (10% FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin). Cells were treated with 5 µM VE or an equivalent volume of DMSO and imaged on a Nikon Ti-E inverted microscope enclosed within a temperature and CO₂-controlled environmental chamber that

maintained an atmosphere of 37°C and 5% humidified CO₂. Fluorescent images were captured every 15 min for 36 hr with a 20X (0.75 NA) objective and 2 X 2 binning to minimize light exposure. At least 4 separate fields of view were acquired for each condition. Images were subsequently analyzed using NIS-Elements software. Cells were scored as having undergone a “normal” mitosis if no micronuclei were generated following the first anaphase; “slightly abnormal” if 1-3 micronuclei were generated; or “highly abnormal” if 4 or more micronuclei were generated. Only the first mitosis following drug addition was scored.

Quantitative Reverse Transcriptase PCR

HeLa cells were reverse transfected using Lipofectamine RNAiMax and incubated for 48 hr. Following this incubation, either 2 mM thymidine or 0.1 µg/ml nocodazole was added to cells and incubated for an additional 18 hr. The cells were collected and RNA was extracted using the RNeasy Mini kit. Following quantification, 1 µg of total RNA was reverse transcribed using the oTEL primer and SuperScript III Reverse Transcriptase for 1 hr at 55°C, followed by a 70°C incubation for 15 min. cDNA was amplified using the SYBR green master mix with the indicated primers and analyzed using the Roche Light Cycler 480 with the following PCR conditions, 95°C 10 min, (98°C 15 sec, 60°C 20 sec, 72°C 1 min) x 39, 72°C 5 min. Primer sequences for oTEL, 15q, and Xp/Yp are as follows,

oTEL 5' (CCCTAA)₅ 3' (SEQ ID NO:7)

15q Forward 5'-CAGCGAGATTCTCCCAAGCTAAG-3' (SEQ ID NO:8)

15q Reverse 5'-AACCTAACCACATGAGCAACG-3' (SEQ ID NO:9)

Xp/Yp Forward 5' GCAAAGAGTGAAAGAACGAAGCTT-3' (SEQ ID NO:10)

Xp/Yp Reverse 5'-CCCTCTGAAAGTGGACCAATCA-3' (SEQ ID NO:11)

Telomere-repeat amplification protocol

TRAP assays were performed using the TRAPeze telomerase detection kit (Millipore) according to the manufacturers recommendations. For TRAP assay on HeLa cells, HeLa cells were reverse transfected with siRNA against ATRX using Lipofectamine RNAiMax and incubated for 72 hr. HeLa, osteosarcoma, and glioblastoma cell lines were collected by trypsinization and counted to obtain 1 x 10⁶ cells. Cells were resuspended 1x CHAPS Lysis buffer and incubated on ice for 30 min. Lysates were centrifuged at 12,000x g for 20 min at 4°C and protein

concentration was determined using Bradford reagent. Approximately, 150 ng of total protein was used in each reaction and PCR amplification reactions were performed as recommended. DNA products were separated by 10% PAGE in 0.5x TBE run at 200 V for 2 hr and visualized using SYBR gold.

5 **Cellular viability**

For cell viability assays, between 250-1,500 cells were seeded per well, in triplicate, of a 96-well plate and incubated overnight. The following day cells were either left untreated, or treated with increasing concentrations of VE-821, KU-55933, AZ20, or Gemcitabine. The cells were incubated for 4-6 days and cell viability was
10 analyzed using CellTiter Glo and a SpectraMax M5 plate reader. IC50s were calculated using Excel or Prism software.

For analysis of cell death, cells were seeded in a 6-well dish at 0.5×10^5 and allowed to incubate for 8 hr. Cells were either left untreated, or treated with 3 μ M VE-821 and incubated for 6 days. Cells were collected by trypsin and stained for
15 FACS analysis using the Annexin V assay kit (Life Technologies) per the manufacturers recommendations. Cell death was analyzed using FACS Diva Software.

For analysis of population doubling, U2OS cells were seeded at 0.6×10^5 and RPE were seeded 0.3×10^5 every 3-4 days in growth media with, or without, 1.5 μ M
20 VE821. Cells were collected by trypsinization and counted using a hemacytometer for a total of 21 days. Population doubling was calculated using the standard formula $PD = \log(N_{final}/N_{initial})/\log(2)$.

DNA-protein binding assay using biotinylated ssDNA

Biotinylated ssTEL ((TTAGGG)₈ (SEQ ID NO:12)) was attached to
25 streptavidin-coated magnetic beads in 10 mM Tris-HCl (pH 8.0), 100 mM NaCl at room temperature for 30 min. The biotinylated ssTEL (0.4 pmol) was first incubated with purified RPA (1.2 pmol) for 30 min at room temperature. Recombinant RPA complex was purified from *E. coli* as previously described¹. The RPA pre-coated ssTEL was retrieved with a magnet and subsequently mixed with increasing
30 concentrations of whole cell extracts (WCE) for 30 min at room temperature. The RPA coated ssTEL was again retrieved using a magnet and the amount of RPA still bound to the ssTEL was analyzed by Western blot with the indicated antibody. To generate WCE, cells were lysed in binding buffer (10mM Tris-HCl (pH 7.5), 600 mM

NaCl, 10 µg/ml BSA, 10% glycerol, 0.05% NP-40) and sonicated for 10 sec at a power of 3, 3 times. Cell lysates were normalized with a binding buffer containing no salt and then added to the RPA-coated ssTEL reactions. For binding assays on HeLa cells, HeLa cells were either mock treated or reverse transfected with siRNA against ATRX (Lipofectamine RNAiMax) and incubated for 48 hr. Cells were then left untreated or incubated with 2 µM thymidine or 0.1 µg/ml nocodazole for 16-18 hr and then collected with trypsin prior to lysis.

Dot blot

RNA was purified using the RNeasy purification kit and 2-10 µg was denatured in 50% formamide, 2.5 mM EDTA for 15 min at 65°C. The denatured RNA was loaded onto Hybond XL membrane using a BioRad dot blot vacuum manifold. The membrane was crosslinked for 35 sec at 125 J and washed briefly in 2x SSC. The membrane was then incubated in Ultra-Hyb hybridization buffer (Ambion) for 1 hr at 50°C. Telomeric (CCCTAA)₄ (SEQ ID NO:6) or 28S (AACGATCAGAGTTTTTCACC) (SEQ ID NO:5) probes were labeled using the DIG oligonucleotide 3'-End labeling Kit, 2nd Generation (Roche), according to the manufacturer's instructions. DIG-labeled probe (10 pmole) was added to the Ultra-Hyb hybridization buffer and incubated with the membrane overnight at 50°C. The following day, the membrane was washed twice with 2x SSC + 0.1% SDS at room temperature for 5 min each and twice with 0.5x SSC + 0.1% DS at 55 °C for 15 min. The membrane was then developed using the DIG CDP-STAR detection system (Roche) according to the manufacturer's instructions. When necessary, membranes were stripped by boiling in 0.1% SDS for 15 min at room temperature and reprobed. Fold change in TERRA was calculated after normalization to 28S using ImageLab software.

EXAMPLE 1. Alternative Lengthening of Telomeres Renders Cancer Cells Hypersensitive to ATR Inhibitors

Single-stranded DNA (ssDNA) coated by Replication Protein A (RPA) is a key intermediate in both DNA replication and homologous recombination (HR) (8). RPA transiently associates with telomeres during DNA replication, but is released from telomeres after S phase (9, 10). The release of RPA may be an important mechanism to suppress HR at telomeres. The association of RPA with telomeres in S phase is facilitated by TERRA, the telomere repeat-containing RNA, which is also

present at telomeres during this period (9, 11-13). To understand how ALT is established, we asked if the association of TERRA with telomeres is altered in ALT cells. TERRA colocalized with the telomere-binding protein TRF2 in telomerase-positive HeLa cells (9). However, in both HeLa and telomerase-positive SJS1 cells (see Fig. 22B), the levels of TERRA foci declined from S phase to G2 (Fig. 1A-B, 5) (9, 12). Although in ALT-positive U2OS cells TERRA also colocalized with the telomere marker TRF2, neither the levels of TERRA, nor the colocalization of TERRA and TRF2, declined from S to G2 (Fig. 5, 6C, 7A-B). Furthermore, in ALT-positive U2OS and HUO9 cells (see Fig. 3D, 23A), the levels of TERRA foci elevated significantly in S phase and remained high into G2 (Fig. 1A-B, 5). Thus, unlike in telomerase-positive cells, ALT cells are defective in the cell-cycle regulation of TERRA.

We next asked why TERRA persistently associates with telomeres in ALT cells. Recent studies have revealed a correlation of ALT with loss of ATRX in cancer (14-17). ATRX was detected in HeLa but not U2OS (Fig. 8A, see Fig. 23B) (14), prompting us to test if the dysregulation of TERRA in ALT cells is a result of ATRX loss. Indeed, knockdown of ATRX in HeLa cells resulted in persistent TERRA foci, and elevated TERRA levels in G2/M (Fig. 1C-D, 8A-B, 9A-B). Furthermore, the levels of TERRA derived from individual telomeres (15q and Xp/Yp) declined from S phase to mitosis in control HeLa cells but not in ATRX knockdown cells (Fig. 1E-F). These results suggest that TERRA is repressed by ATRX in G2/M.

Considering that RPA is released from telomeres in G2/M when TERRA is repressed by ATRX (9), we asked if ATRX is required for the release of RPA. In HeLa cells, numerous small replication-associated RPA foci (type-A RPA foci) were detected in S phase. As cells progressed from S to G2, type-A RPA foci became largely undetectable (Fig. 2A). However, upon ATRX knockdown, bright damage-associated RPA foci (type-B RPA foci) were detected at telomeres in a fraction of G2 cells (Fig. 2A). Knockdown of ATRX with two independent siRNAs led to a significant increase of type-B RPA foci in G2 cells (Fig. 2B). To examine the release of RPA from telomeric ssDNA biochemically, we followed this process in cell extracts using an in vitro assay that we previously established (9). A biotinylated ssDNA oligo of telomeric repeats (ssTEL) was coated with recombinant RPA and incubated in extracts from S-phase or mitotic HeLa cells. Consistent with the release

of RPA from telomeres in G2/M, RPA was released from ssTEL more efficiently in mitotic extracts than in S-phase extracts (Fig. 2C) (9). Knockdown of ATRX reduced the release of RPA from ssTEL in mitotic extracts (Fig. 2C), demonstrating that ATRX contributes to the RPA release in G2/M. To test if the loss of ATRX in ALT cells affects RPA release, we analyzed IMR90-derived SW39^{TEL} (telomerase-positive) and SW26^{ALT} (ALT-positive) cells (see also 7). ATRX was detected in SW39^{TEL} but not SW26^{ALT} (Fig. 2D). Moreover, the loss of ATRX in SW26^{ALT} associated with a 4-fold increase of TERRA compared with SW39^{TEL} (Fig. 2E-F). Importantly, RPA was released from ssTEL more efficiently in SW39^{TEL} cell extracts than in SW26^{ALT} cell extracts (Fig. 2G), showing that ALT cells lacking ATRX indeed have a reduced ability to release RPA from telomeric ssDNA.

Given that RPA-ssDNA is a key HR intermediate, we asked if ATRX loss induces ALT. Knockdown of ATRX in HeLa cells did not inactivate telomerase, nor did it induce telomere lengthening (Fig. 10A-B). These results are consistent with a previous study (14) and suggest that loss of ATRX is insufficient to establish ALT. Nevertheless, ATRX knockdown in HeLa cells promoted some features of ALT, such as the persistent association of TERRA and RPA with telomeres. Interestingly, a recent study showed that loss of ASF1 led to the acquisition of several ALT phenotypes, including accumulation of RPA at telomeres (18). We postulate that ALT is established via a multistep process in which loss of ATRX poises telomeres for ALT, but additional genetic/epigenetic changes are needed to fully activate the ALT pathway.

RPA-ssDNA is not only an HR intermediate, but also the nucleoprotein structure that recruits the key HR regulator ATR kinase (19, 20). The defective RPA release from telomeres in ATRX knockdown cells and ALT cells suggests that ATR may be recruited to telomeres during the establishment of ALT. Consistent with our hypothesis, ATR colocalizes with PML in U2OS cells but not in HeLa cells (21), suggesting its presence in APBs (ALT-associated PML bodies) (22). Furthermore, ATRIP, the regulatory partner of ATR, associates with telomeres in ALT-positive WI38-VA13 cells but not in HeLa cells (23). These findings prompted us to test if ATR is functionally required for ALT. The ATR inhibitor VE-821 (24) and ATR siRNA significantly reduced APBs in U2OS and SW26^{ALT} cells (Fig. 3A-B, 11, 12A). VE-821 also disrupted APBs in U2OS cells synchronized in G2 (Fig. 12B) (25),

ruling out cell-cycle changes as the cause of APB dispersal. In marked contrast, the ATM inhibitor KU-55933 and ATM siRNA did not affect APBs in U2OS cells (Fig. 3A-B, 12B-C), highlighting the role for ATR, but not ATM, in the maintenance of APBs in ALT cells.

5 To determine the effects of VE-821 on the recombinogenic state of ALT telomeres, we analyzed telomere sister-chromatid exchange (T-SCE) and extrachromosomal telomeric C-rich DNA (C-circles) in ALT cells. VE-821 not only decreased T-SCE in U2OS cells (Fig. 3C), but also reduced C-circle levels in U2OS and HUO9 cells (Fig. 3D-E), showing that ALT is indeed inhibited. Furthermore, VE-
10 821 elevated the frequency of telomere loss in U2OS cells (Fig. 3F), suggesting that the stability of ALT telomeres is compromised. Consistent with the idea that TERRA acts upstream of ATR to promote RPA retention at ALT telomeres, VE-821 did not affect TERRA levels and telomere association in U2OS cells (Fig. 13A-B).

In light of the effects of VE-821 on ALT telomeres, we asked if VE-821
15 selectively kills ALT cells. SW26^{ALT} was indeed more sensitive to VE-821 than SW39^{TEL} (Fig. 14). Importantly, SW26^{ALT} and SW39^{TEL} were similarly sensitive to a panel of DNA-damaging agents (Fig. 15A-C), demonstrating that the effects of VE-821 are unique to ATR inhibition but not a result of general genotoxicity. Moreover, VE-821 induced γ H2AX more efficiently in SW26^{ALT} than in SW39^{TEL} (Fig. 16),
20 suggesting that it inflicts more DNA damage in ALT cells. At a concentration that virtually eliminates U2OS cells, VE-821 only modestly reduced the proliferation of untransformed RPE-1 cells (Fig. 17). Using H2B-mRFP and live-cell imaging, we followed the chromosome segregation in U2OS, HeLa, and RPE-1 cells after VE-821 treatment. Furthermore, we used 53BP1-GFP to visualize DNA double-stranded
25 breaks (DSBs) in U2OS cells. VE-821 induced dramatic errors in anaphase chromosome segregation in U2OS but not HeLa or RPE-1 cells (Fig. 4A-B). In the following interphase, U2OS cells displayed increased micronucleation compared to HeLa or RPE-1 cells (Fig. 18). Moreover, U2OS cells exhibited numerous 53BP1 foci (Fig. 4A, 4C). A fraction of the 53BP1 foci in U2OS cells colocalized with telomeres
30 (Fig. 4C, 18). The colocalization of 53BP1 with telomeres but not centromeres was significantly induced by VE-821 (Fig. 19), suggesting that ALT telomeres are particularly fragile upon ATR inhibition. Interestingly, knockdown of ATRX in HeLa and BJ cells did not increase the induction of γ H2AX by VE-821 or VE-821

sensitivity (Fig. 20A-C), suggesting that while ATRX loss may prime cells for ALT, it is not directly responsible for the vulnerability of ALT cells to ATR inhibition.

Given the prevalence of ALT in osteosarcoma (26), we tested the effects of VE-821 on a panel of osteosarcoma cell lines. These cell lines clearly clustered into two groups (Fig. 4D). The mean IC₅₀ of VE-821 for one group (U2OS, SAOS2, CAL72, NOS1, and HUO9) was ~0.8 μ M, whereas the mean IC₅₀ for the other group (MG63 and SJSA1) was ~9 μ M (Fig. 4D, 21A). Among these lines, U2OS and SAOS2 are known ALT lines without detectable ATRX protein (Fig. 22A) (14). CAL72, NOS1, and HUO9 lacked detectable telomerase activity, ATRX protein, and displayed APBs (Fig. 22A-C, 23A-B), suggesting that they are also ALT-positive. In contrast, MG63 and SJSA1 were positive for telomerase activity, ATRX protein, and negative for APBs (Fig. 25A-B). In this panel of cell lines, VE-821 induced substantially higher levels of apoptosis in the ALT lines than in the telomerase-positive lines (Fig. 4E). The hypersensitivity of ALT cells to ATR inhibition was confirmed with a second ATR inhibitor (Fig. 21B). In contrast to ATR inhibitors, neither the ATM inhibitor KU-55933 nor the DNA replication inhibitor gemcitabine showed significant selectivity toward ALT cells (Fig. 4D, 21C-D). Notably, several ATRX-expressing ALT lines were also hypersensitive to VE-821 (Fig. 23A-C, 24) (14), again suggesting that the state of ALT telomeres but not ATRX loss *per se* renders cells hypersensitive to ATR inhibitors.

ALT is prevalent not only in osteosarcoma but also in pediatric glioblastoma (27). MGG119, a newly developed glioma stem cell (GSC) line (28), lacked detectable telomerase activity and ATRX protein, but expressed high levels of TERRA and displayed APBs (Fig. 25A-C), suggesting that it is ALT-positive. In contrast, the GSC line MGG4 was positive for telomerase activity and ATRX protein, but expressed low levels of TERRA and lacked APBs (Fig. 25A-C) (29). Importantly, although MGG119^{ALT} and MGG4^{TEL} were similarly sensitive to a panel of DNA-damaging agents (Fig. 28A-C), MGG119^{ALT} was significantly more sensitive to VE-821 than MGG4^{TEL} (Fig. 4F), suggesting that VE-821 is uniquely effective in killing ALT GSCs.

The HR defects of specific cancers have offered an opportunity for targeted therapy using PARP inhibitors (5, 6). However, in contrast to HR-defective cancers, ALT-positive cancers actively use recombination to sustain immortality. We show

that ATR inhibitors disrupt ALT and selectively kill ALT cells in vitro, suggesting the first rational strategy for the treatment of ALT-positive cancers. Furthermore, we show that functional recombination, like HR defects, can be targeted in specific contexts, presenting proof of a new therapeutic principle. Several ATR inhibitors have
 5 been recently developed and begun to enter clinical trials (24, 30-33). Our findings demonstrate that a subset of cancers reliant on recombination are hypersensitive to ATR inhibitors, offering an unexplored direction for future preclinical and clinical studies.

References for Example 1

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Example 2. Distinct but Concerted Roles of ATR, DNA-PK, and Chk1 in Countering Replication Stress during S Phase

ATR and its homologues in a number of organisms are critical for the survival of proliferating cells. In budding yeast, the ATR homologue Mec1 is essential for viability unless Sml1, a repressor of ribonucleotide reductase, is deleted (Zhao et al., 1998). In mouse and *C. elegans*, loss of ATR leads to embryonic lethality (Brown and Baltimore, 2000; Garcia-Muse and Boulton, 2005). Conditional deletion of ATR from the human colon cancer cell line HCT116 also leads to cell death (Cortez et al., 2001). However, ATR homologs in some other organisms, such as fission yeast and *Drosophila*, are not essential for viability (Enoch et al., 1992; Laurencon et al., 2003). Interestingly, in mouse, the effects of ATR loss on proliferating cells are not uniform in cell populations. For example, deletion of ATR in cells from blastocysts resulted in different levels of genomic instability, arranging from a few DNA breaks to severe chromosomal fragmentation (Brown and Baltimore, 2000). Deletion of ATR during nervous system development induced cell death only in specific progenitor cells (Lee et al., 2012). These observations raise an important question as to why some proliferating cells are more dependent on ATR than others.

How ATR functions during S phase is still poorly understood. During the response to DNA damage or replication stress, ATR phosphorylates and activates its effector kinase Chk1 (Liu et al., 2000). It has been long believed that ATR and Chk1 function as a kinase cascade. Like ATR, Chk1 is critical for genomic stability during DNA replication (Forment et al., 2011; Petermann et al., 2008; Petermann et al., 2010; Syljuasen et al., 2005). Both ATR and Chk1 have been implicated in the regulation of origin firing even in the absence of extrinsic stress (Couch et al., 2013; Eykelenboom et al., 2013; Maya-Mendoza et al., 2007; Petermann et al., 2010; Shechter et al., 2004). However, a recent study reported unexpected differences between the effects of ATR inhibitor (ATRi) and Chk1 inhibitor (Chk1i) on cycling cells (Toledo et al., 2011). Whereas Chk1i induced massive γ H2AX accumulation in a large fraction of U2OS cells, ATRi only induced γ H2AX in a small fraction of the

cells. This result raises the possibility that ATR and Chk1 may not always function as a linear pathway, and it posts a question about how ATR and Chk1 function in concert during DNA replication.

While both ATR and Chk1 are important in cycling cells, the nature of the intrinsic stress that they deal with remains enigmatic. Interestingly, certain proliferation-promoting oncogenic events, such as Ras activation and Myc overexpression, render cancer cells sensitive to ATR suppression (Gilad et al., 2010; Murga et al., 2011; Schoppy et al., 2012), leading to the hypothesis that ATR is important for countering the replication stress in cancer cells. Nevertheless, how replication stress can be measured in normal and cancer cells remains elusive. Because multiple ATRi and Chk1i are being tested in clinical trials for cancer therapy (Foote et al., 2013; Josse et al., 2014; Karp et al., 2012; Ma et al., 2013; Sausville et al., 2014; Seto et al., 2013), understanding the mechanisms of action and unique properties of these inhibitors may help to guide their applications in clinical settings.

In this study, we used multiple inhibitors and siRNAs to interrogate the functions of ATR and Chk1 during S phase. The kinase inhibitors used in this study are: ATRi (10 μ M VE-821), ATRi#2 (1 μ M AZ-20), ATRi#3 (10 μ M EPT-46464), Chk1i (2 μ M MK-8776), Chk1i#2 (0.3 μ M UCN-01), Roscovitine (25 μ M), DNA-PKi (2 μ M NU7441), ATMi (10 μ M KU-55933), and Wee1i (0.25 μ M MK-1775). When various inhibitors were used in combination with ATRi or in comparison with ATRi, they were added to cell cultures at the same time as ATRi unless indicated otherwise. Unexpectedly, we found that acute inactivation of ATR in S-phase cells led to two distinct outcomes. Upon ATRi treatment, a fraction of S-phase cells accumulated high levels of ssDNA and underwent replication catastrophe. In contrast, other S-phase cells initially acquired moderate levels of ssDNA but subsequently recovered from the “ATRi shock” through a Chk1-mediated mechanism. The critical role of ATR in suppressing replication catastrophe was traced to its functions in promoting RRM2 (ribonucleotide reductase M2) accumulation and limiting replication origin firing in early S phase. In the ATRi-treated cells escaping from replication catastrophe, ATRi triggered a DNA-PK and Chk1-mediated backup pathway to suppress origin firing. Importantly, the Chk1-mediated backup pathway in ATRi-treated cells creates a threshold of tolerable replication stress, allowing ATRi to selectively kill cells under high replication stress. In contrast to ATRi, Chk1i

disrupted the backup pathway and induced cell death even when replication stress was moderate. Notably, the levels of ATRi-induced ssDNA correlated with ATRi-induced cell death in a panel of cell lines, suggesting that ATRi-induced ssDNA is a quantitative indicator of replication stress that could be used to predict the ATRi sensitivity of cancer cells.

For the present study, U2OS, T98G and RPE1-hTERT cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin. U2OS cells expressing HA-RRM2 were generated by retroviral infection (pBabe-HA-RRM2) and puromycin selection. U2OS-derived cells carrying inducible Cyclin E were cultured in DMEM supplemented with 10% FBS and 4 µg/ml tetracycline (Bartkova et al., 2006). Cyclin E expression was induced by washing off tetracycline 24 h before drugs treatment. MCF-10A cells were cultured in DMEM/F12 supplemented with 5 % Horse Serum, 2 ng/ml EGF, 0.5 µg/ml hydrocortizone, 100 ng/ml cholera toxin, 10 µg/ml Insulin and 1% penicillin/streptomycin. HT-29, SNU-61, NCI-H747, HCT-15 and Colo-320-HSR were cultured in Roswell Park Memorial Institute 1640 Medium (RPMI 1640) GlutaMAX™-I supplemented with 10% FBS, 1% penicillin/streptomycin, 1% Glucose and 1% Sodium Pyruvate. SW1116, SW620, HT-55, RKO and LS-123 were cultured in DMEM/F12 supplemented with 10% FBS and 1% penicillin/streptomycin.

Acute ATR inactivation leads to two distinct outcomes in S-phase cells

To investigate how ATR functions in cycling cells, we acutely inactivated ATR in U2OS cells with the ATR inhibitor VE-821 and followed the effects over time (Fig. 34A-B) (Reaper et al., 2011). To visualize ssDNA, DNA was labeled with BrdU and analyzed by native BrdU staining. An increase of ssDNA was detected in S-phase cells 2 hours after ATRi treatment (Fig. 27A-C, 34C). At 8 hour after ATRi treatment, a fraction (~5%) of S-phase cells displayed very high levels of ssDNA and became strongly positive for γH2AX and TUNEL staining (Fig. 27A-C, 34D-E), indicating that they were undergoing replication catastrophe (Toledo et al., 2013). Surprisingly, however, the majority of ATRi-treated cells displayed less ssDNA at 8 hour than at 2 hour (Fig. 27A-C). This reduction in ssDNA was not due to loss of S-phase cells (Fig. 34F). Consistent with the induction of ssDNA at 2 hour, increased amounts of RPA were detected on chromatin by fractionation and immunostaining (Fig. 27D, 34G). Subsequently, the levels of RPA on chromatin gradually declined.

Similar decline of chromatin-bound RPA was also observed in cells treated with two other ATR inhibitors, AZ20 and EPT-46464 (Fig. 27E) (Foote et al., 2013; Toledo et al., 2011). Despite the overall decline of chromatin-bound RPA, phosphorylated RPA32 and γ H2AX gradually accumulated on chromatin in a fraction of cells after 2 hours (Fig. 27A-D, 34H). Thus, while a subpopulation of ATRi-treated cells acquired high levels of ssDNA and DNA damage, a distinct cell subpopulation gradually recovered. These results raise an important question as to how the distinct effects of ATRi are manifested in S-phase cells.

ATR suppresses ssDNA accumulation in early S phase

The distinct effects of ATRi on S-phase cells prompted us to investigate whether a fraction of replicating cells are particularly vulnerable to ATR inactivation. Consistent with the induction of ssDNA by ATRi, the staining of chromatin-bound RPA in individual cells gradually increased during the first 2 hours (Fig. 28A). When cells were sorted according to EdU incorporation, DNA content, and RPA staining, it was evident that the chromatin binding of RPA occurred most efficiently in a fraction of cells in early-to-mid S phase (Fig. 28B).

To test more directly if early or mid S-phase cells are most vulnerable to ATR inactivation, we treated synchronously growing T98G cells with ATRi in different stages of the cell cycle (Fig. 28C, 35). T98G cells were synchronized in G0 by serum starvation and then released into the cell cycle. Even in the absence of ATRi, low levels of ssDNA were detected in replicating cells (Fig. 28D). Interestingly, the basal levels of ssDNA in replicating cells peaked in early S phase, suggesting that cells in this cell-cycle window are facing relatively high levels of intrinsic replication stress. Furthermore, ATRi induced higher levels of ssDNA in early S-phase cells than in mid or late S-phase cells (Fig. 28D), suggesting that ATR is particularly important for the suppression of ssDNA in early S phase.

ATR suppresses DNA damage by promoting RRM2 accumulation and limiting origin firing

To understand why early S-phase cells are vulnerable to ATR inactivation, we tested if certain DNA replication factor(s) is limiting during this period. In the presence of HU, ATRi induces excessive firing of replication origins and a massive increase of stalled replication forks, which ultimately leads to exhaustion of RPA and replication catastrophe (Toledo et al., 2013). Even in the absence of HU, ATRi

induced a surge of origin firing in 2 hours (Fig. 36A). To test if RPA is limiting in early S phase, we monitored the levels of all three RPA subunits in synchronously growing T98G cells (Fig. 29A). The levels of RPA70, RPA32, and RPA14 were constant throughout the cell cycle and not affected by ATRi, ruling out RPA as the limiting factor in early S phase.

In contrast to RPA, RRM2, a cell cycle-regulated subunit of the ribonucleotide reductase, gradually accumulated in early S phase (Fig. 29A) (Chabes et al., 2003; D'Angiolella et al., 2012). Notably, ATRi attenuated the accumulation of RRM2 in S phase (Fig. 29A). Even in asynchronous U2OS cells, ATRi and Chk1i (MK-8776) reduced the levels of RRM2 (Fig. 29B, 36B), suggesting that the ATR-Chk1 pathway promotes RRM2 accumulation in cycling cells. Surprisingly, although RRM2 is an unstable protein, its degradation was not enhanced by ATRi in cells treated with cycloheximide (CHX) (Fig. 29C). Knockdown of Cyclin F, the F-box protein required for RRM2 ubiquitylation in G2 (D'Angiolella et al., 2012), did not suppress the reduction of RRM2 in ATRi-treated cells (Fig. 36C). In contrast to RRM2, E2F1, the transcription activator of the *RRM2* gene (DeGregori et al., 1995; Zhang et al., 2009), was increasingly degraded in ATRi-treated cells in the presence of CHX (Fig. 29C). Similar to RRM2, E2F1 was reduced in ATRi and Chk1i-treated cells (Fig. 29B, 36B). Concomitant with the reduction of E2F1, *RRM2* mRNA levels declined (Fig. 36D). Importantly, overexpression of E2F1 completely suppressed the reduction of RRM2 in ATRi-treated cells (Fig. 29D), suggesting that E2F1 degradation is responsible for the reduction of RRM2 by ATRi. Together, these results show that the ATR-Chk1 pathway promotes RRM2 accumulation by stabilizing E2F1.

The ATRi-induced reduction in E2F1 and RRM2 was suppressed by the CDK inhibitor roscovitine, the proteasome inhibitor MG132, and the Nedd8-activating enzyme inhibitor MLN4924 (Fig. 29E-F) (Soucy et al., 2009). In contrast, Wee1 inhibitor, which activates CDK1/2 (Beck et al., 2012; Hughes et al., 2013), drastically reduced E2F1 and RRM2 levels even in the absence of ATRi (Fig. 29E). These results suggest that the ATR-Chk1 pathway promotes RRM2 accumulation by antagonizing a CDK1/2, Cullin-RING ubiquitin ligase and proteasome-mediated mechanism that degrades E2F1 (see Fig. 29H).

In ATRi-treated cells, roscovitine not only elevated RRM2 levels but also reduced the induction of γ H2AX (Fig. 29E). Furthermore, a CDK2-specific inhibitor

also reduced γ H2AX (Fig. 29E). These results suggest that a reduction in CDK2 activity may suppress ATRi-induced DNA damage by increasing RRM2 levels. Indeed, expression of RRM2 significantly reduced the γ H2AX induced by ATRi or Chk1i (Fig. 29G, 36F). In addition to its effects on RRM2, roscovitine also decreased origin firing in ATRi-treated cells (Fig. 36A). To test if suppression of origin firing reduces ATRi-induced DNA damage, we used siRNA to knock down CDC7, a key regulator of replication initiation (Labib, 2010). Knockdown of CDC7 indeed reduced ATRi-induced γ H2AX (Fig. 36G). Thus, ATR, through restricting CDK2 activity, promotes RRM2 accumulation and limits origin firing in S phase (Fig. 29H). Both of these effects of ATR contribute to the suppression of DNA damage in replicating cells (Fig. 29H). How ATR is activated during unperturbed S phase and how E2F1 is suppressed by CDK2 and Cullin ligases remain to be investigated. The transient accumulation of ssDNA in S-phase cells may trigger limited ATR activation, thereby coordinating RRM2 accumulation and origin firing. Interestingly, the budding yeast ATR homolog Mec1 is required for priming the Mcm2-7 helicase for phosphorylation by Cdc7 (Randell et al., 2010). The limited ATR activation during S phase may promote origin firing but also restrict it to a tolerable level, preventing ssDNA from accumulating to a high level that triggers replication catastrophe (see Fig. 33A).

Recovery of ATRi-treated cells via a Chk1-mediated mechanism

The correlations of high ssDNA with replication catastrophe and moderate ssDNA with recovery suggest that the fate of ATRi-treated cells may be dictated by a threshold of ssDNA (see Fig. 33A). To investigate the underlying mechanism of recovery, we first tested if ATRi gradually loses its potency during this process. Even after 8 hours of incubation with cells, ATRi still effectively blocked the CPT-induced Chk1 phosphorylation (Fig. 37A). Although ATRi remained active at 8 hour, its effects were significantly different from those of Chk1i. Using Chk1 autophosphorylation at S296 as a functional readout (Okita et al., 2012), we carefully titrated ATRi and Chk1i to determine their respective concentrations required for Chk1 inactivation (Fig. 27A, 37B-C). We found that 10 μ M ATRi and 1-2 μ M Chk1i similarly inhibited CPT-induced Chk1 autophosphorylation (Fig. 37B). Furthermore, CDC25A, which is degraded in a Chk1-dependent manner (Busino et al., 2003; Jin et al., 2003), was similarly stabilized by 10 μ M ATRi and 1-2 μ M Chk1i in unperturbed cycling cells (Fig. 37C). At functionally equivalent concentrations, Chk1i induced

much more γ H2AX-positive cells than did ATRi (Fig. 30A). Similar observations were made using different ATRi and Chk1i (Fig. 37D-E), as well as multiple independent ATR and Chk1 siRNAs (Fig. 37F-G). Importantly, cells were significantly more sensitive to Chk1i than ATRi (Fig. 30B, 37H), showing that Chk1 is indeed more cytotoxic than ATRi. These results confirm and extend the observation by Toledo et al. (Toledo et al., 2011), prompting us to further investigate why ATRi and Chk1i exert different effects.

We next compared the effects of ATRi and Chk1i on cycling cells at different time points. ATRi and Chk1i induced similar levels of ssDNA at 2 hour (Fig. 37I).

However, at 8 hour, ssDNA was reduced in the majority of ATRi-treated cells but increased in Chk1i-treated cells (Fig. 30C, 37J). The fraction of Chk1i-treated cells displaying high levels of ssDNA was also positive for γ H2AX (Fig. 30C), suggesting that they were undergoing replication catastrophe. Consistent with the ssDNA results, the levels of chromatin-bound RPA declined from 2 to 8 hour only in ATRi-treated cells, but not in Chk1i-treated cells (Fig. 30D). These results show that the recovery observed in ATRi-treated cells does not occur in Chk1i-treated cells, raising the possibility that Chk1 is involved in recovery.

Since Chk1 is a downstream effector of ATR in the DNA damage response, it is surprising that Chk1 may function in recovery independently of ATR. To follow the function of Chk1 during recovery, we analyzed the phosphorylation of Chk1 at S317 and Chk1-mediated CDC25A destabilization in ATRi-treated cells (Busino et al., 2003; Jin et al., 2003; Zhao and Piwnicka-Worms, 2001). Before ATRi treatment, a basal level of pChk1 was detected (Fig. 30E). At 2 hour after ATRi treatment, the level of pChk1 was reduced, whereas the level of CDC25A was elevated.

Subsequently, pChk1 gradually reappeared, and CDC25A gradually declined. At 8 hour, the levels of pChk1 and CDC25A became similar to those before ATRi treatment. Importantly, CDC25A levels remained high at 8 hour in Chk1i-treated cells (Fig. 30F). Even at low concentrations that only partially stabilized CDC25A at 2 hour, Chk1i prevented the decline of CDC25A at 8 hour (Fig. 37K). Consequently, even 0.25-0.5 μ M Chk1i induced higher levels of γ H2AX than 10 μ M ATRi at late time points (Fig. 37L). Thus, during the recovery of ATRi-treated cells, Chk1 regains its basal phosphorylation on S317 and its function in destabilizing CDC25A. These

results suggest that a large fraction of S-phase cells recover from the initial ATRi response via a Chk1-mediated mechanism (Fig. 30G).

DNA-PK phosphorylates Chk1 to suppress origin firing and promote recovery in ATRi-treated cells

5 To understand the regulation of Chk1 during recovery, we investigated how Chk1 is phosphorylated at S317 at 8 hour after ATRi treatment. Even in cells that were repeatedly treated with ATRi, pChk1 still reappeared (Fig. 38A), excluding ATR as the kinase that phosphorylates Chk1. Both ATM and DNA-PK were autophosphorylated 8 hours after ATRi treatment (Fig. 31A). Surprisingly, the ATRi-
10 induced phosphorylation of Chk1, as well as the phosphorylation of H2AX and RPA32, was dependent on DNA-PK but not ATM (Fig. 31B lanes 4, 6; 38B-D). The induction of γ H2AX and pRPA32 was reduced by knockdown of KU70, a DSB (DNA double-stranded break) sensor for DNA-PK, as well as SLX4 and MUS81, two components of a nuclease complex that processes aberrant replication forks (Fig. 38E-
15 F) (Couch et al., 2013; Forment et al., 2011; Ragland et al., 2013). These results suggest that the aberrant replication forks induced by ATRi are processed by SLX4 and MUS81, leading to KU-dependent DNA-PK activation. When both DNA-PK and ATM were inhibited, the reappearance of pChk1 was eliminated and CDC25A was stabilized at 8 hour (Fig. 31B lane 5; Fig. 38B). Thus, the resurgence of Chk1
20 function during recovery is primarily driven by DNA-PK, but ATM also has a secondary role in this process.

Our finding that DNA-PK phosphorylates Chk1 in ATRi-treated cells raised a question as to whether this alternative pathway involves the known regulators of Chk1 phosphorylation in the ATR-Chk1 pathway. Knockdown of Rad17 and Clapsin, a
25 sensor and a mediator of the ATR-Chk1 pathway, did not prevent Chk1 from regaining S317 phosphorylation in ATRi-treated cells (Fig. 38G-H). In contrast, knockdown of TopBP1, an activator of the ATR kinase and a mediator of the ATR-Chk1 pathway, abolished pChk1 8 hours after ATRi treatment (Fig. 38I). Although the role of TopBP1 in the DNA-PK-Chk1 pathway is still unclear, a recent study
30 suggested that DNA-PK phosphorylates TopBP1 to promote Chk1 phosphorylation (Vidal-Eychenie et al., 2013). Our results suggest that the sensing and signaling mechanisms of the DNA-PK-Chk1 pathway are distinct from those of the ATR-Chk1 pathway.

To gain mechanistic insights into how Chk1 promotes recovery, we used DNA fiber assay to monitor origin firing and fork progression in ATRi-treated cells. At 2 hour after ATRi treatment, origin firing was elevated, reducing in the inter-origin distance and replication fork speed (Fig. 31C-E). At 8 hour, origin firing has clearly declined, whereas the inter-origin distance and fork speed have partially recovered (Fig. 31C-E). As in ATRi-treated cells, in Chk1i-treated cells the inter-origin distance was reduced and fork speed was decreased at 2 hour (Fig. 31D, 31F) (Maya-Mendoza et al., 2007; Petermann et al., 2006; Petermann et al., 2010). However, neither the inter-origin distance nor fork speed changed from 2 to 8 hour in Chk1i-treated cells (Fig. 31D, 31F), suggesting that Chk1 is needed to suppress origin firing during recovery. Consistently, when both DNA-PK and ATM were inhibited in ATRi-treated cells, the inter-origin distance did not increase and fork speed did not recover from 2 to 8 hour (Fig. 31D-E). The regain of Chk1 function in destabilizing CDC25A during recovery suggests that Chk1 suppresses origin firing by inhibiting CDK2. Indeed, inhibition of CDK in Chk1i-treated cells by roscovitine 2 hours after Chk1i treatment enabled the cells to recover (Fig. 38J). Furthermore, activation of CDK in ATRi-treated cells by Wee1i 2 hours after ATRi treatment prevented recovery (Fig. 38K).

ATRi but not Chk1i selectively kills cells under high replication stress

If the Chk1-mediated backup pathway promotes the recovery of ATRi-treated cells, it may create a threshold of tolerable replication stress. Consequently, ATRi may selectively kill cells under high replication stress, whereas Chk1i may kill cells even when replication stress is moderate (see Fig. 33B). To test these possibilities, we treated U2OS cells with ATRi in the presence of increasing concentrations of HU. As HU concentration rose, ATRi induced increasing levels of ssDNA (Fig. 32A), showing that the level of ATRi-induced ssDNA is an indicator of replication stress. Importantly, as the level of ssDNA rose, increasing fractions of ATRi-treated cells underwent replication catastrophe (Fig. 32B), confirming that ATRi selectively kills cells under high replication stress. In the presence of HU, Chk1i induced slightly more ssDNA than ATRi at 2 hour (Fig. 39A). At a later time point, Chk1i induced much more ssDNA than ATRi regardless of the presence or absence of HU (Fig. 39B). These results suggest that recovery occurs more efficiently in ATRi-treated cells than in Chk1i-treated cells independently of the levels of replication stress. In contrast to ATRi-treated cells, large fractions of Chk1i-treated cells underwent

replication catastrophe even in the absence of HU (Fig. 32B), supporting the notion that disruption of the Chk1-mediated backup pathway kills replicating cells even if they are under moderate replication stress.

Replication stress arises from not only extrinsic but also intrinsic sources, such as the oncogenic events in cancer cells (Bartkova et al., 2005; Gorgoulis et al., 2005). Cyclin E is commonly overexpressed in cancer cells, and it interferes with DNA replication (Bartkova et al., 2005; Neelsen et al., 2013). ATRi induced ssDNA, γ H2AX and replication catastrophe more efficiently in Cyclin E-overexpressing U2OS cells than in control cells (Fig. 32C-D, 39C) (Toledo et al., 2011). Loss of the tumor suppressor Rb also impairs DNA replication (Manning et al., 2014). Again, ATRi induced γ H2AX more efficiently in Rb-depleted cells than in control cells (Fig. 39D). In marked contrast to ATRi, Chk1i induced γ H2AX and replication catastrophe indiscriminately of the levels of replication stress (Fig. 32C-D, 39D). Importantly, ATRi induced much higher levels of ssDNA and replication catastrophe in two cancer cell lines, U2OS and T98G, than in two untransformed cell lines, RPE1 and MCF10A (Fig. 32E-F, 39E-G), suggesting that ATRi selectively kills cancer cells under high replication stress (see Fig. 33C). We recently showed that cancer cells dependent on the alternative telomere-lengthening (ALT) pathway are hypersensitive to ATRi (Flynn et al., 2015). Interestingly, whereas U2OS cells are ALT-positive, T98G cells express active telomerase (Sano et al., 1998). Thus, even in telomerase-positive cancer cells under high replication stress, ATRi induces massive ssDNA accumulation and replication catastrophe.

ATRi-induced ssDNA is an indicator of replication stress in cancer cells

ATRi induced higher levels of ssDNA and replication catastrophe in cancer cells than in non-transformed cells, suggesting that ATRi-induced ssDNA may be an indicator of replication stress in cancer cells. To test this possibility, we treated a panel of 10 colorectal cancer cell lines with ATRi and analyzed its effects on ssDNA (at 2 hour), γ H2AX (at 16 hour), and cell survival (in 6 days) (Fig. S6H-K). Cell lines of colorectal cancer were selected for this experiment because the oncogenic drivers in this context have been well characterized (Kinzler and Vogelstein, 1996). Furthermore, colorectal cancer is not associated with ALT (Heaphy et al., 2011), giving us an opportunity to test if ATRi-induced ssDNA predicts ATRi sensitivity in non-ALT cells. All the cell lines replicated DNA efficiently (Fig. 39L), and they

displayed a range of ATRi sensitivity (Fig. 39J-K). Remarkably, when the cell lines were ranked according to the ATRi-induced ssDNA accumulation, a correlation of ATRi-induced ssDNA and ATRi sensitivity was clearly discernable (Fig. 32G). This correlation was even more evident when a low concentration of HU was used to sensitize the ATRi response (Fig. 32G, 39J-K). These results suggest that the levels of ATRi-induced ssDNA in the colorectal cancer cells are indeed reflective of intrinsic replication stress and predictive of ATRi sensitivity. In contrast to ATRi-induced ssDNA, none of the common mutations of colorectal cancer, either individually or in combinations, are predictive of ATRi sensitivity (Fig. 39M) (Kinzler and Vogelstein, 1996). Furthermore, the microsatellite instability, CpG island methylation phenotype, and chromosomal instability of these cell lines did not correlate with ATRi sensitivity (Fig. 39N) (Ahmed et al., 2013). Therefore, ATRi-induced ssDNA in cancer cells is a unique indicator of replication stress that predicts ATRi sensitivity.

Although ATR is long known to be a master regulator of cellular responses to replication stress, whether and how replication stress can be quantified remains elusive. A quantitative understanding of replication stress is crucial for explaining the functions of ATR in specific oncogenic, developmental, aging and therapeutic contexts (Brown and Baltimore, 2000; Flynn et al., 2015; Gilad et al., 2010; Lee et al., 2012; Murga et al., 2009; Murga et al., 2011; Reaper et al., 2011; Ruzankina et al., 2007). In this study, we found that the levels of ATRi-induced ssDNA vary in individual cells and in different stages of S phase. In HU-treated cells, the levels of ATRi-induced ssDNA rise with HU concentrations, suggesting that ATRi-induced ssDNA reflects replication stress quantitatively. Furthermore, in a panel of cancer cell lines, the levels of ATRi-induced ssDNA correlate with ATRi-induced cell death. These results suggest that ATRi-induced ssDNA is also an indicator of intrinsic replication stress, and it is predictive of ATRi sensitivity in cancer cells (Fig. 33C). Although replication stress could arise from many different sources, induction of ssDNA may be a common effect (Flynn and Zou, 2011). Elicited by ssDNA (Zou and Elledge, 2003), ATR acts to counter replication stress by suppressing ssDNA accumulation (Toledo et al., 2013). When ATR is lost, replication stress is unleashed to drive further ssDNA formation, generating a quantifiable product reflecting its own strength. We propose that ATRi-induced ssDNA is a quantitative indicator of both extrinsic and intrinsic replication stress. The use of ATRi-induced ssDNA to measure

replication stress may help to explain the roles of ATR in different functional contexts and predict the outcomes of ATR inhibition, providing a quantitative view of the interplay between replication stress and the ATR checkpoint.

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OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

20

WHAT IS CLAIMED IS:

1. A method of treating cancer in a subject, the method comprising identifying the subject as having an Alternative Lengthening of Telomeres (ALT)-positive cancer, and administering to the subject a therapeutically effective amount of an inhibitor of Ataxia-Telangiectasia mutated and Rad3-related (ATR).
2. The method of claim 1, wherein the inhibitor of ATR is a small molecule.
3. The method of claim 2, wherein the small molecule is selected from the group consisting of Schisandrin B (10.Benzo(3,4)cycloocta(1,2-f)(1,3)benzodioxole, 5,6,7,8-tetrahydro-1,2,3,13-tetramethoxy-6,7-dimethyl-, stereoisomer: NU6027 (6-(cyclohexylmethoxy)-5-nitrosopyrimidine-2,4-diamine); NVP-BEZ235 (2-methyl-2-[4-(3-methyl-2-oxo-8-quinolin-3-ylimidazo[4,5-c]quinolin-1-yl)phenyl]propanenitrile); VE-821 (2-(aminomethyl)-6-[4,6-diamino-3-[4-amino-3,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-2-hydroxycyclohexyl]oxyoxane-3,4,5-triol;sulfuric acid); VE-822 (VX-970; 3-[3-[4-(methylaminomethyl)phenyl]-1,2-oxazol-5-yl]-5-(4-propan-2-ylsulfonylphenyl)pyrazin-2-amine); AZ20 ((3R)-4-[2-(3H-indol-4-yl)-6-(1-methylsulfonylcyclopropyl)pyrimidin-4-yl]-3-methylmorpholine); AZD6738 (4-[4-[1-[[S(R)]-S-methylsulfonylimidoyl]cyclopropyl]-6-[(3R)-3-methyl-4-morpholinyl]-2-pyrimidinyl]-1H-pyrrolo[2,3-b]pyridine); and ETP-46464 (2-methyl-2-[4-(2-oxo-9-quinolin-3-yl-4H-[1,3]oxazino[5,4-c]quinolin-1-yl)phenyl]propanenitrile).
4. The method of claim 1, wherein the inhibitor of ATR is an inhibitory nucleic acid targeting ATR.
5. The method of claim 4, wherein the inhibitory nucleic acid is a small interfering RNA (siRNA), small hairpin RNA (shRNA), antisense oligo, or CRISPR/Cas guide RNA.
6. The method of claim 4 or 5, wherein the inhibitory nucleic acid includes one or more modifications.

7. The method of claim 6, wherein the modifications comprise modified bases, e.g., locked nucleic acids (LNAs), or modified backbone, e.g., peptide nucleic acids (PNAs).
8. A method of treating cancer in a subject, the method comprising identifying the subject as having an ATR-sensitive cancer, wherein identifying the subject as having an ATR-sensitive cancer comprises obtaining a sample comprising cells from a cancer in the subject; detecting a level of ssDNA in the sample; comparing the level of ssDNA in the subject sample to a reference level of ssDNA; and identifying a subject as having an ATR-sensitive cancer if the level of ssDNA in the subject sample is above the reference level of ssDNA, and administering to the subject a therapeutically effective amount of an inhibitor of Ataxia-Telangiectasia mutated and Rad3-related (ATR).
9. The method of claim 8, wherein the inhibitor of ATR is a small molecule.
10. The method of claim 9, wherein the small molecule is selected from the group consisting of Schisandrin B (10.Benzo(3,4)cycloocta(1,2-f)(1,3)benzodioxole, 5,6,7,8-tetrahydro-1,2,3,13-tetramethoxy-6,7-dimethyl-, stereoisomer: NU6027 (6-(cyclohexylmethoxy)-5-nitrosopyrimidine-2,4-diamine); NVP-BEZ235 (2-methyl-2-[4-(3-methyl-2-oxo-8-quinolin-3-ylimidazo[4,5-c]quinolin-1-yl)phenyl]propanenitrile); VE-821 (2-(aminomethyl)-6-[4,6-diamino-3-[4-amino-3,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-2-hydroxycyclohexyl]oxyoxane-3,4,5-triol;sulfuric acid); VE-822 (VX-970; 3-[3-[4-(methylaminomethyl)phenyl]-1,2-oxazol-5-yl]-5-(4-propan-2-ylsulfonylphenyl)pyrazin-2-amine); AZ20 ((3R)-4-[2-(3H-indol-4-yl)-6-(1-methylsulfonylcyclopropyl)pyrimidin-4-yl]-3-methylmorpholine); AZD6738 (4-[4-[1-[[S(R)]-S-methylsulfonimidoyl]cyclopropyl]-6-[(3R)-3-methyl-4-morpholinyl]-2-pyrimidinyl]-1H-pyrrolo[2,3-b]pyridine); and ETP-46464 (2-methyl-2-[4-(2-oxo-9-quinolin-3-yl-4H-[1,3]oxazino[5,4-c]quinolin-1-yl)phenyl]propanenitrile).
11. The method of claim 8, wherein the inhibitor of ATR is an inhibitory nucleic acid targeting ATR.

12. The method of claim 11, wherein the inhibitory nucleic acid is a small interfering RNA (siRNA), small hairpin RNA (shRNA), antisense oligo, or CRISPR/Cas guide RNA.
13. The method of claim 11 or 12, wherein the inhibitory nucleic acid includes one or more modifications.
14. The method of claim 13, wherein the modifications comprise modified bases, e.g., locked nucleic acids (LNAs), or modified backbone, e.g., peptide nucleic acids (PNAs).

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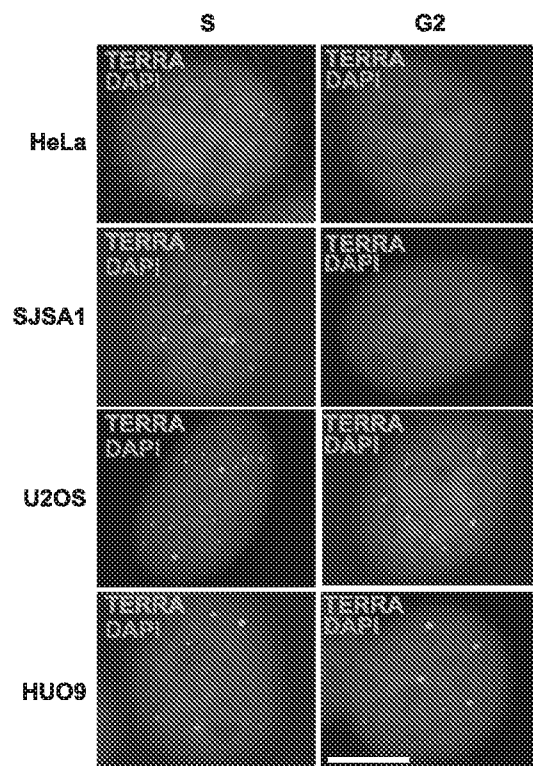


FIG. 1A

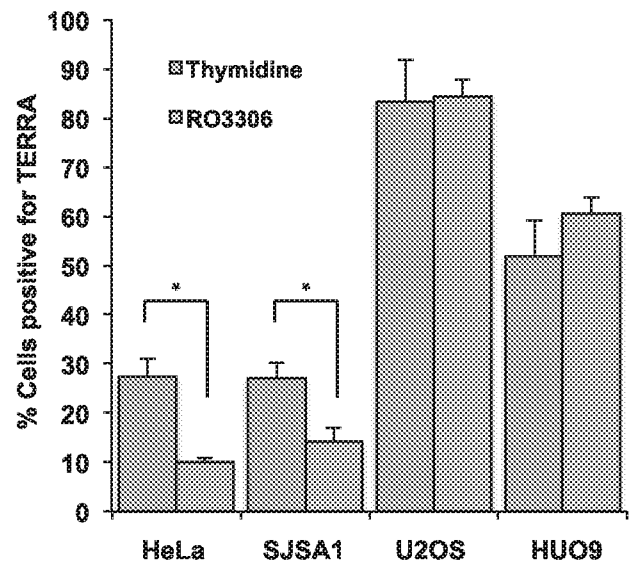
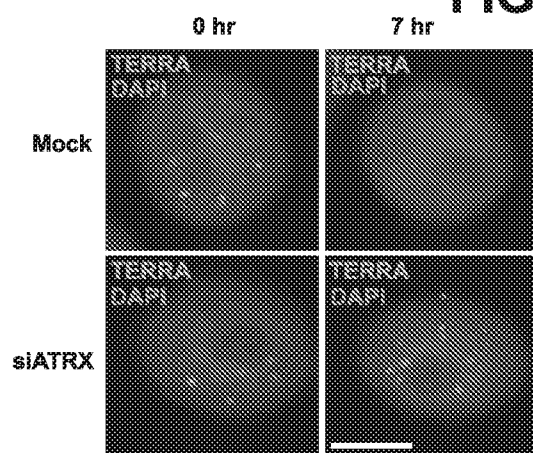


FIG. 1B



Thymidine release

FIG. 1C

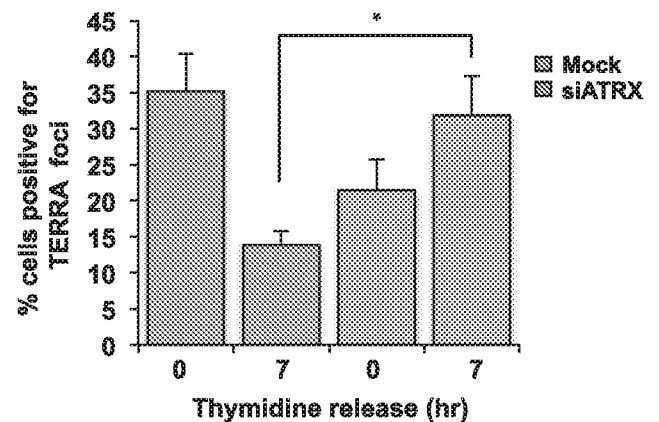


FIG. 1D

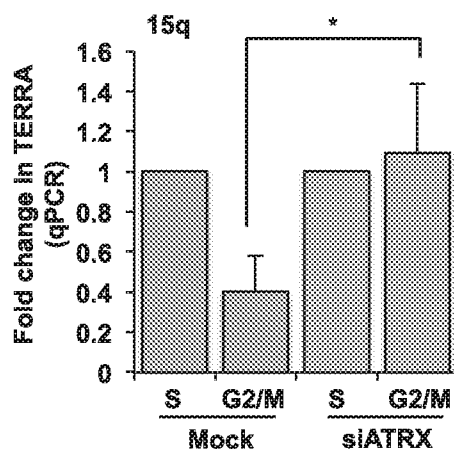


FIG. 1E

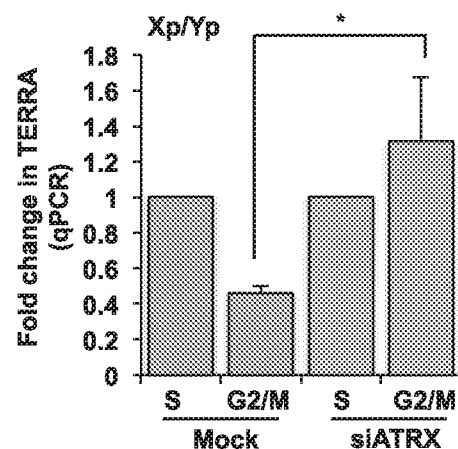


FIG. 1F

2/35

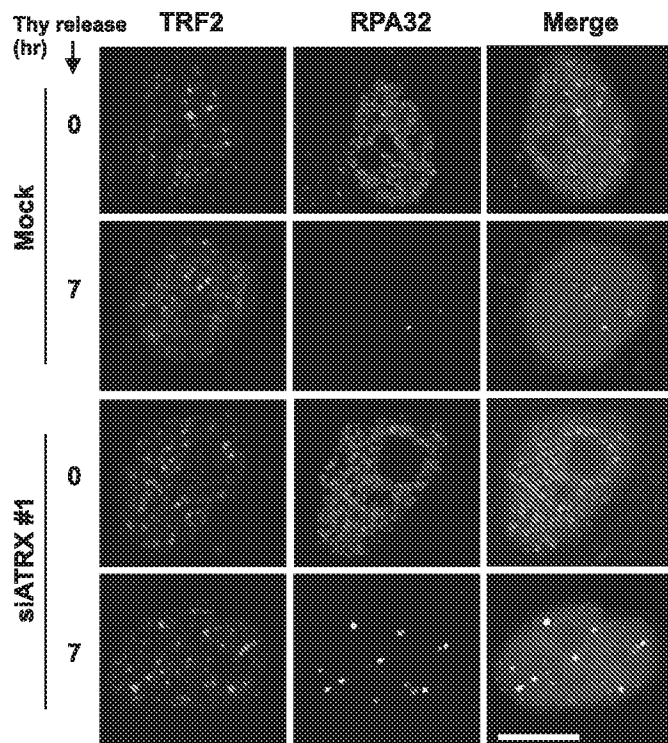


FIG. 2A

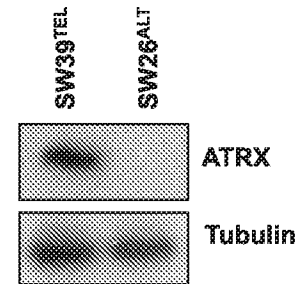


FIG. 2D

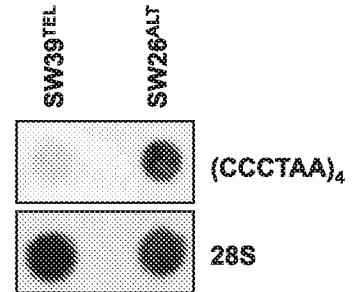


FIG. 2E

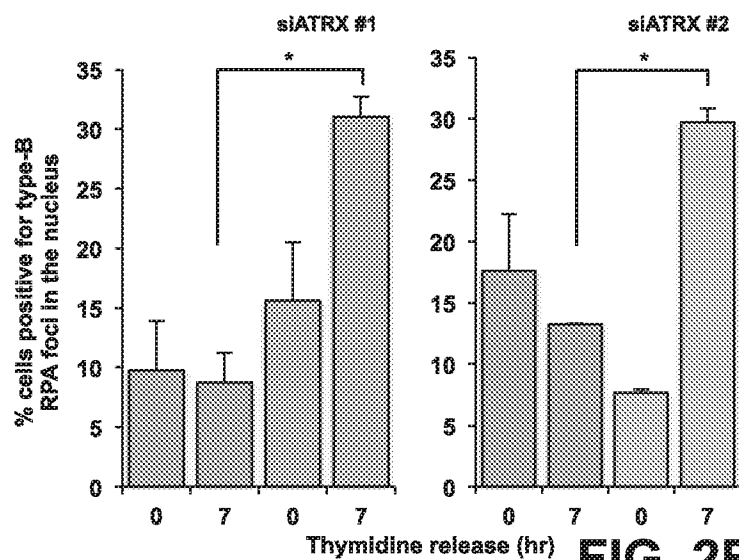


FIG. 2B

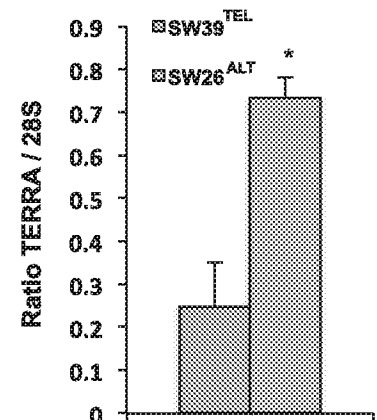


FIG. 2F

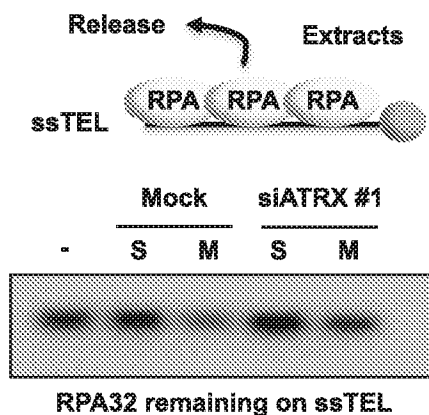


FIG. 2C

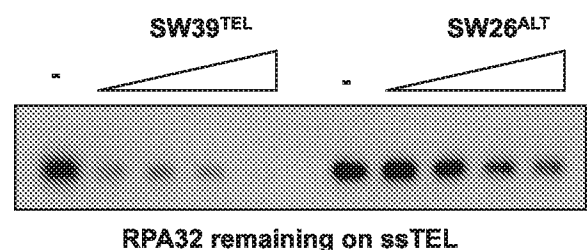


FIG. 2G

3/35

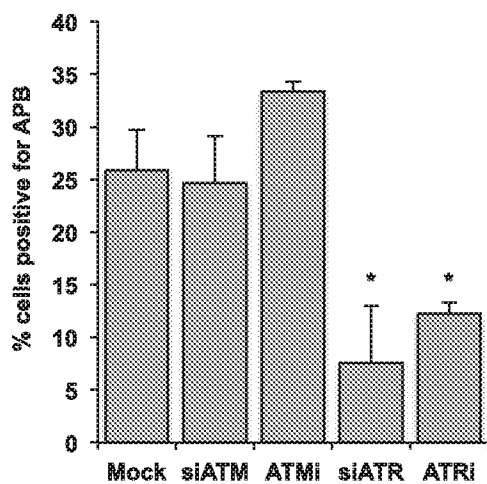


FIG. 3A

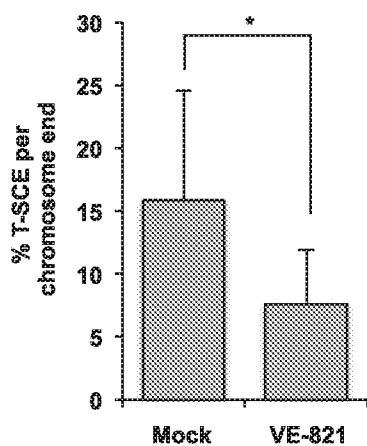


FIG. 3C

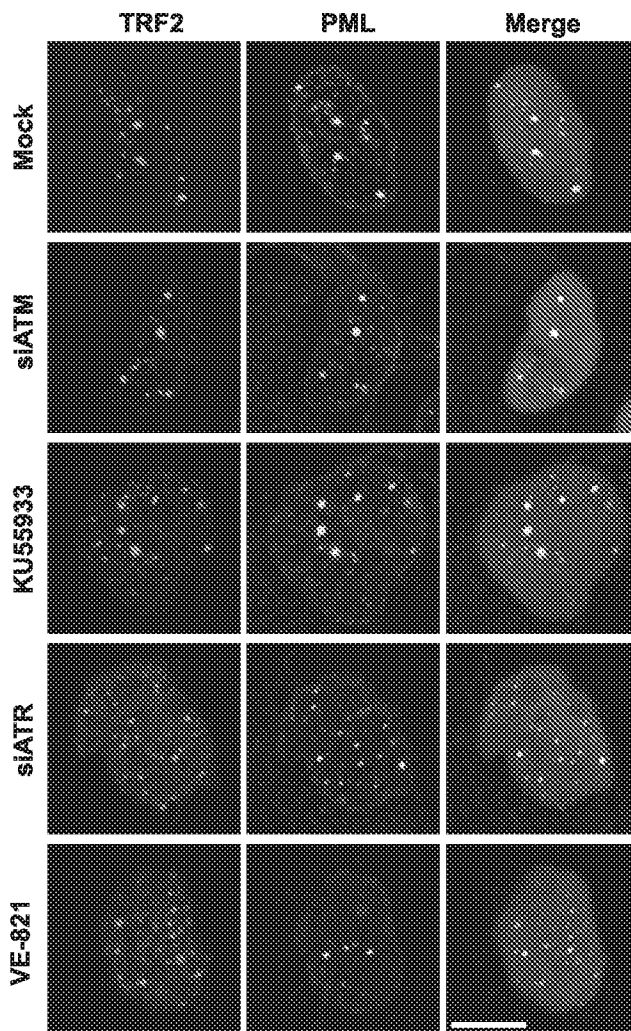


FIG. 3B

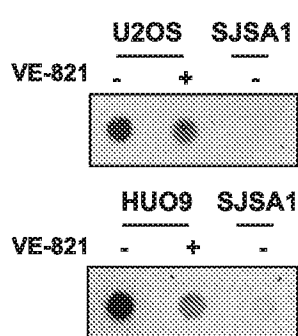


FIG. 3D

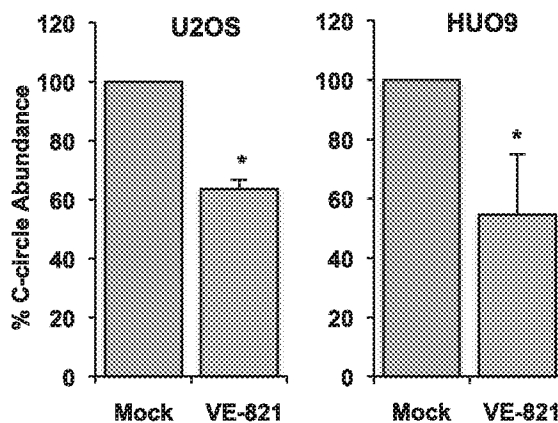


FIG. 3E

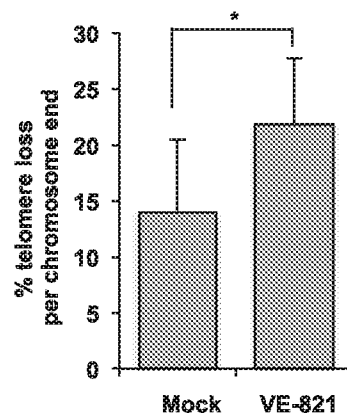
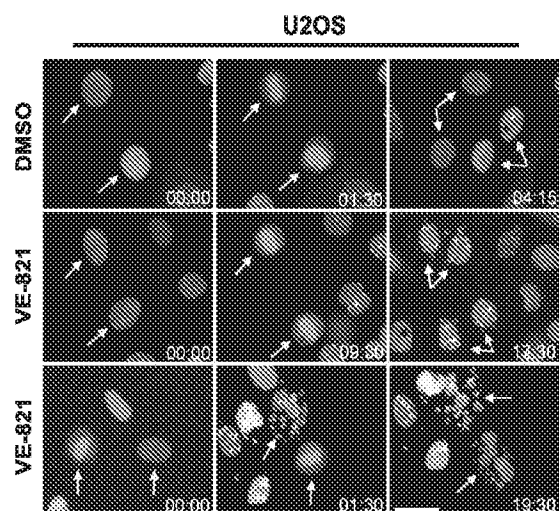
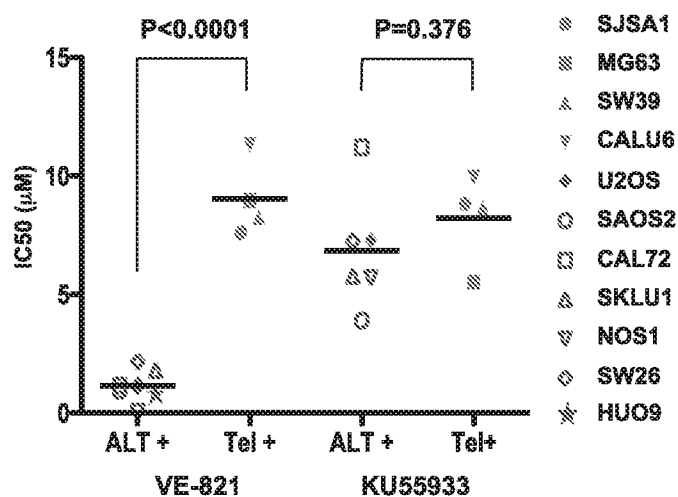
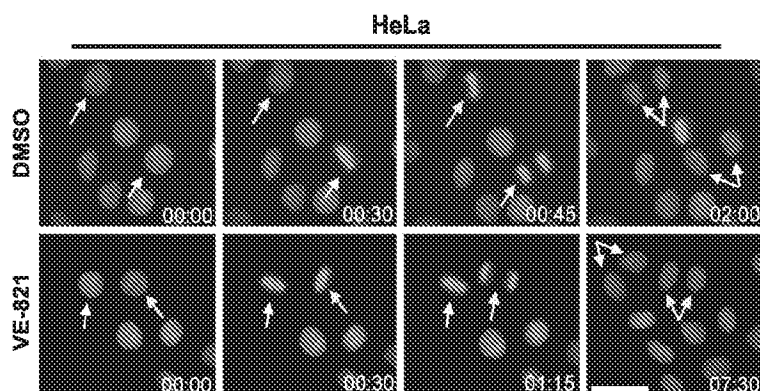
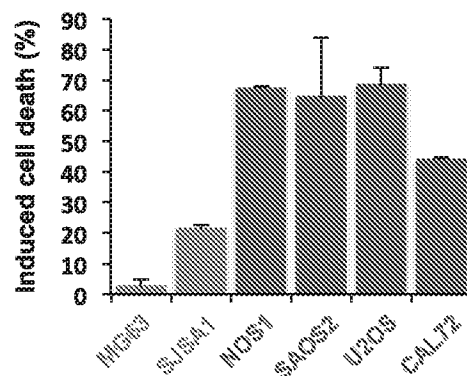
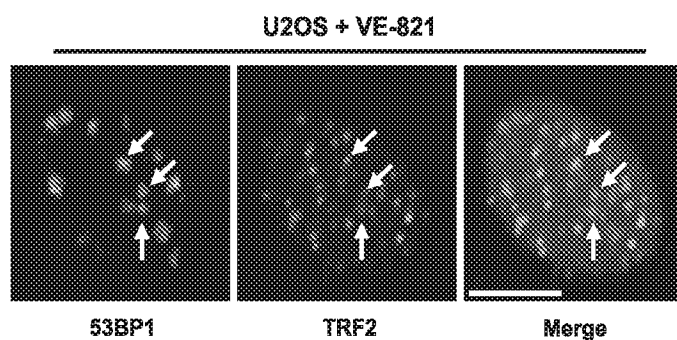
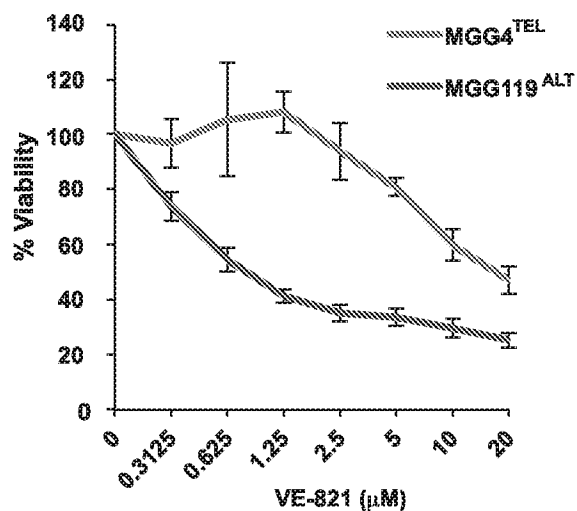


FIG. 3F

4/35

**FIG. 4A****FIG. 4D****FIG. 4B****FIG. 4E****FIG. 4C****FIG. 4F**

5/35

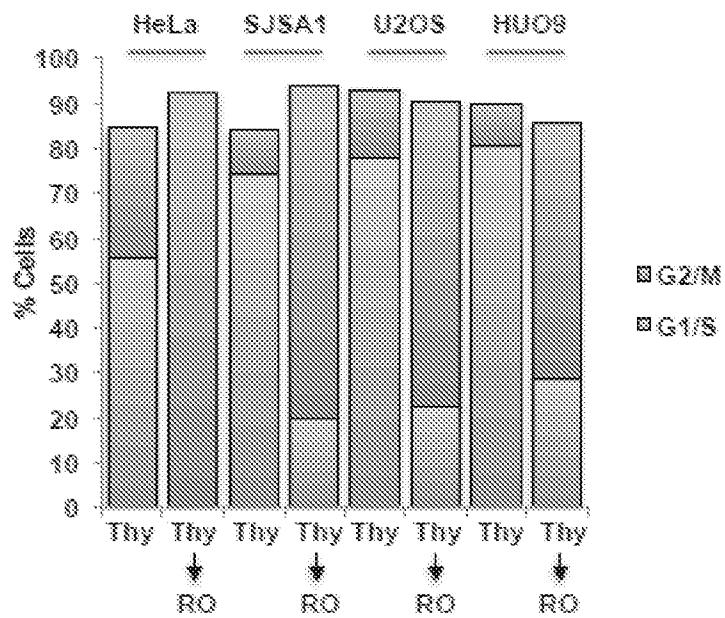


FIG. 5

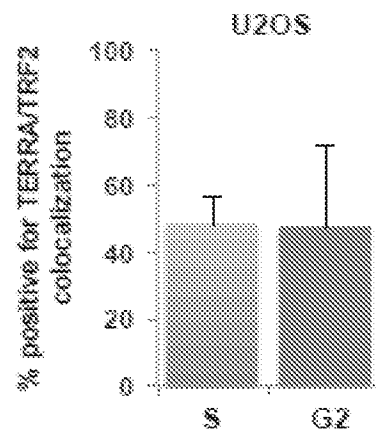


FIG. 6

6/35

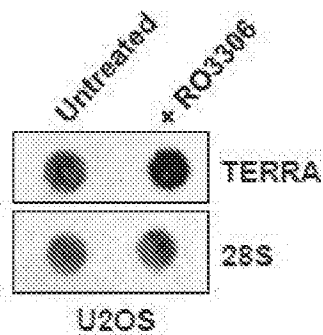


FIG. 7A

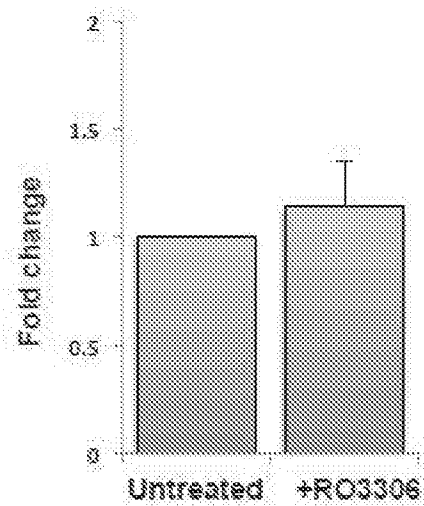


FIG. 7B



FIG. 8A

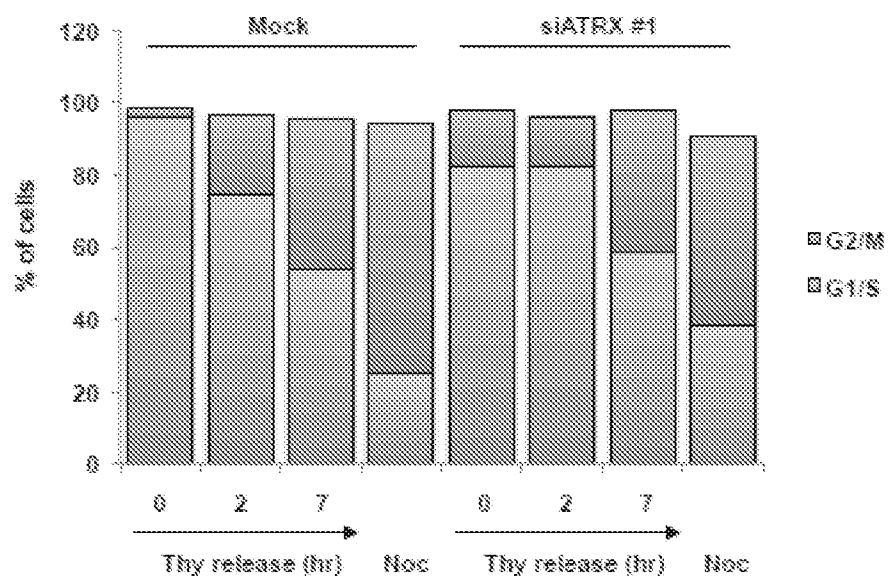


FIG. 8B

7/35

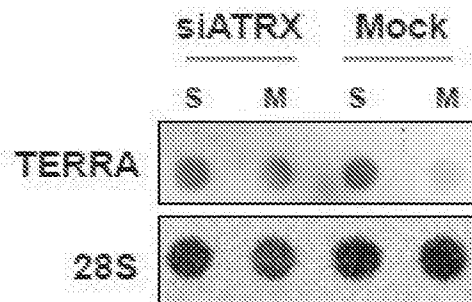


FIG. 9A

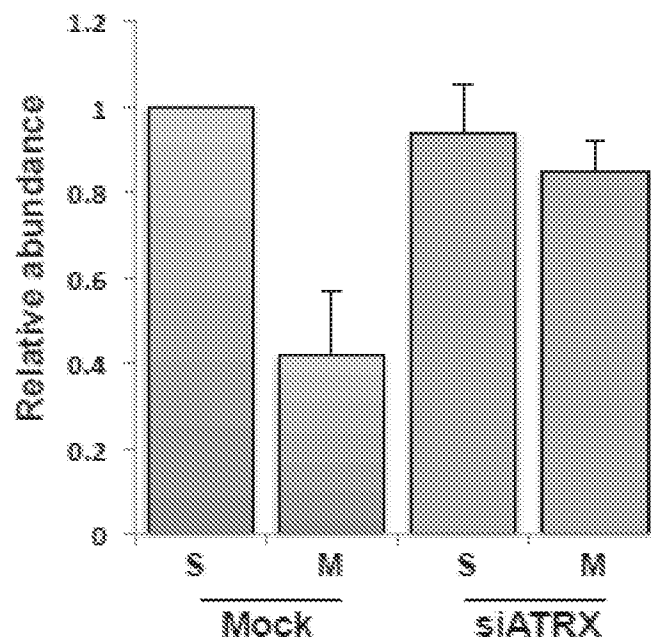


FIG. 9B

8/35

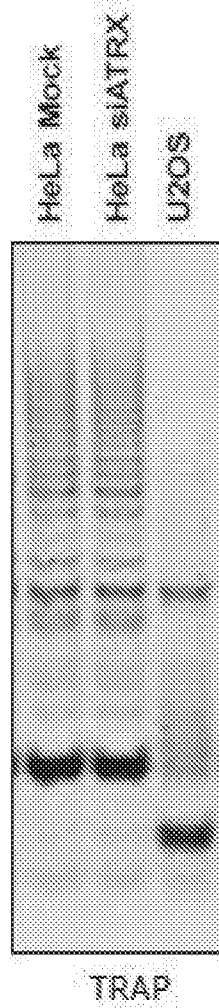


FIG. 10A

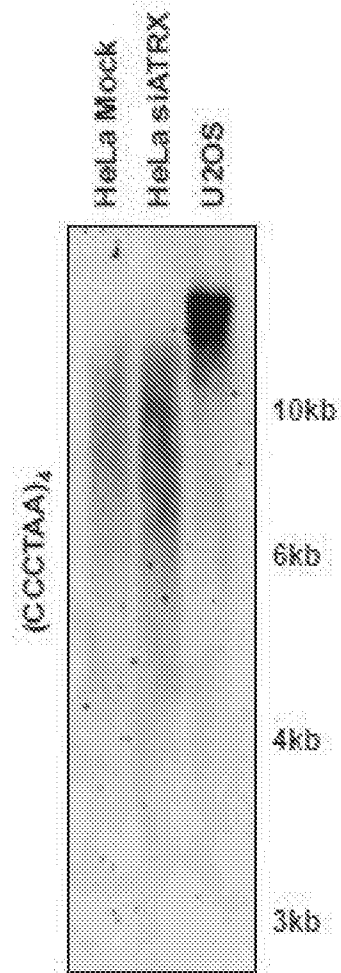
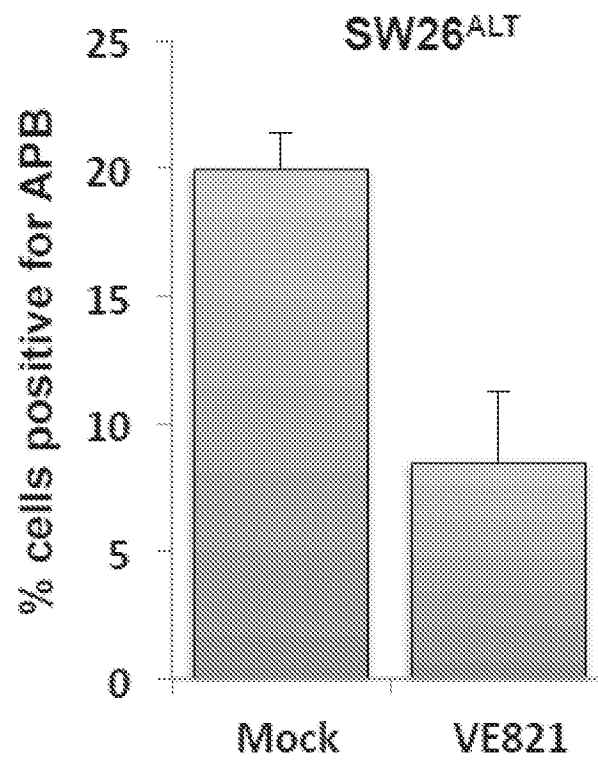
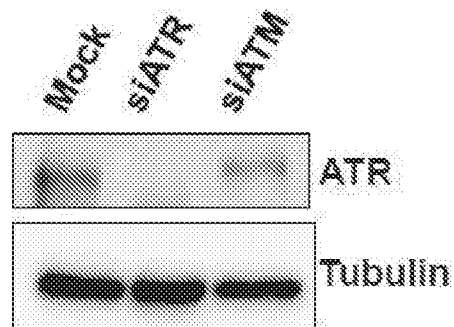


FIG. 10B

9/35

**FIG. 11****FIG. 12**

10/35

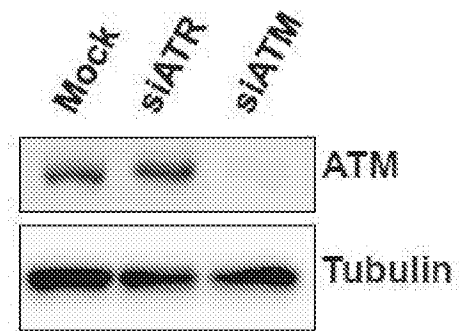
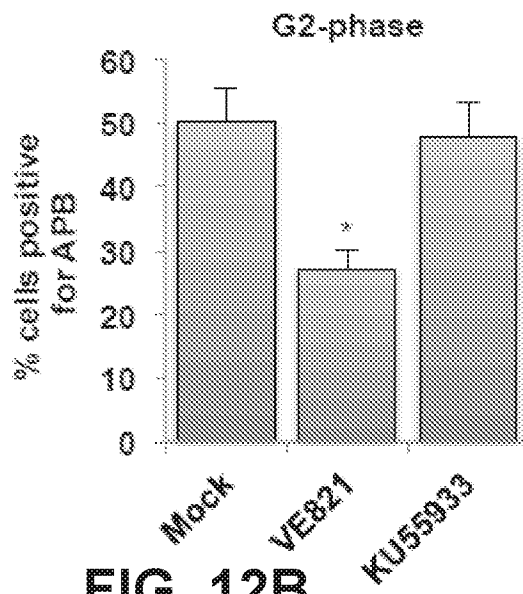


FIG. 12C

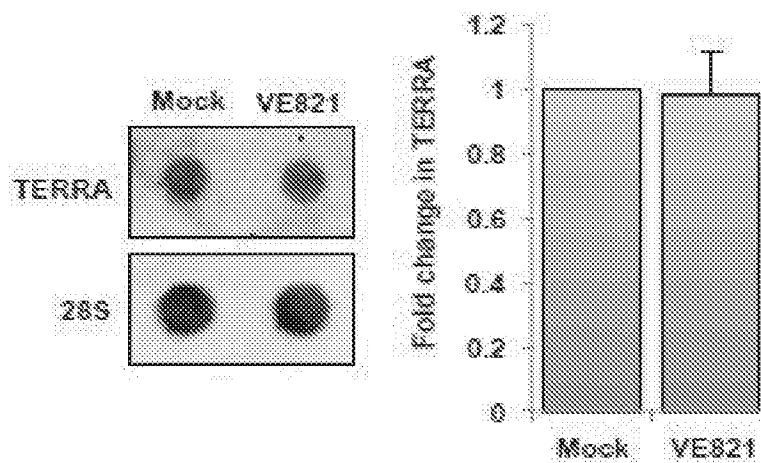


FIG. 13A

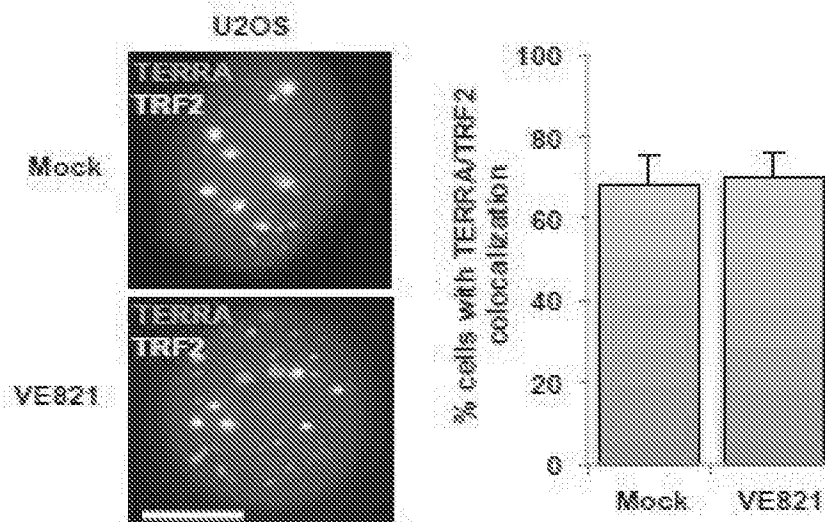


FIG. 13B

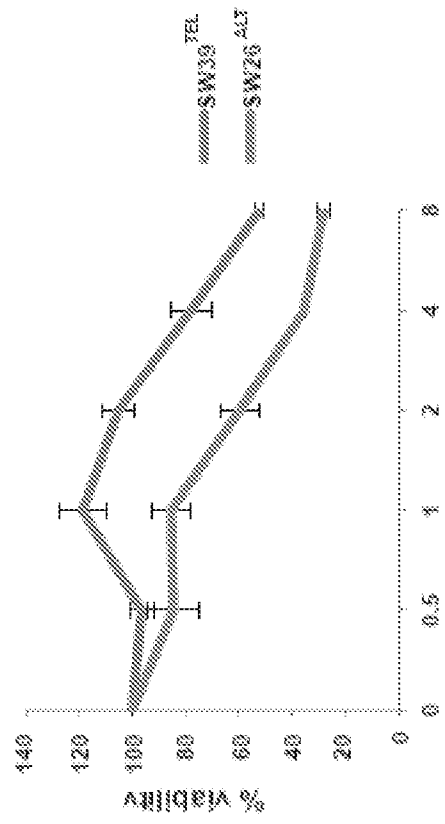


FIG. 14

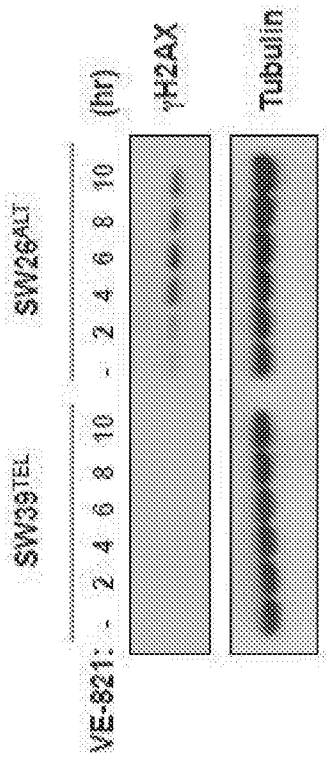


FIG. 15

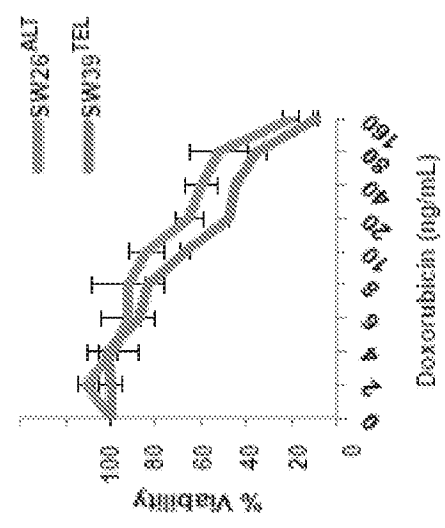


FIG. 15A

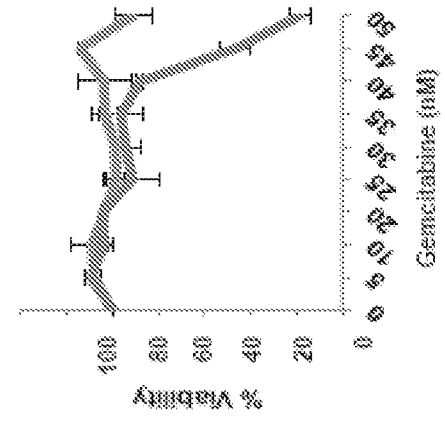


FIG. 15B

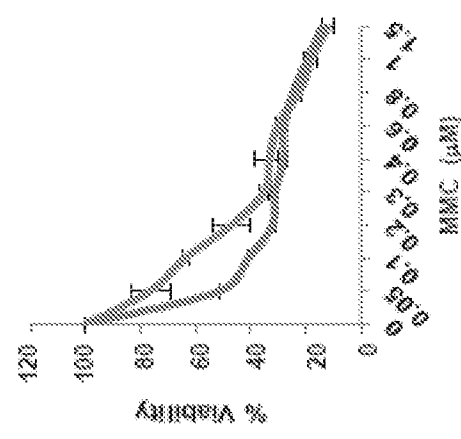


FIG. 15C

12/35

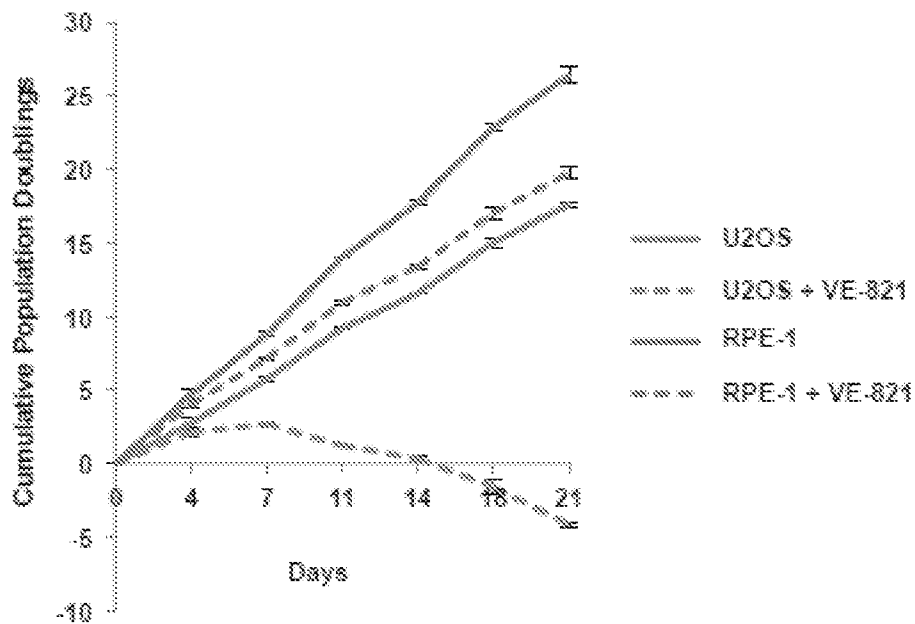


FIG. 17

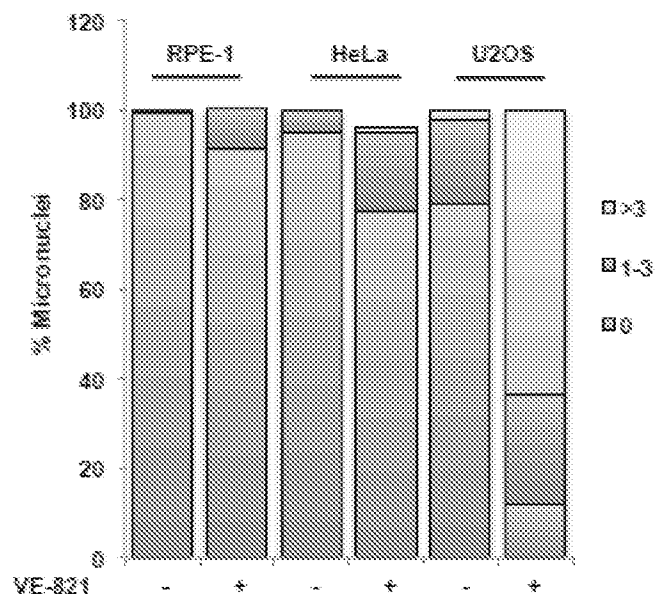


FIG. 18

13/35

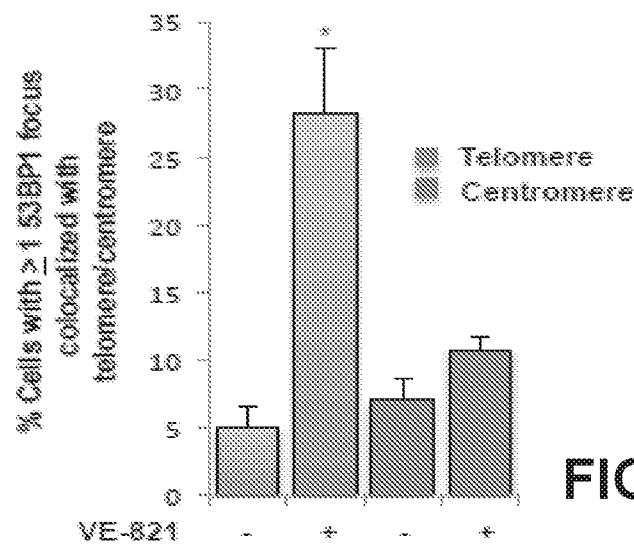


FIG. 19

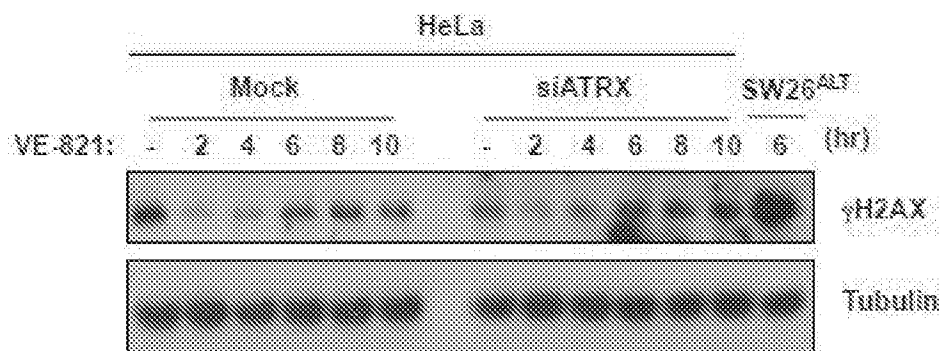


FIG. 20A

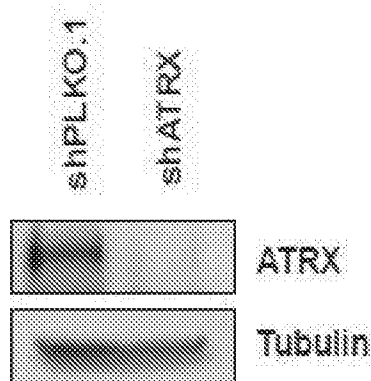


FIG. 20B

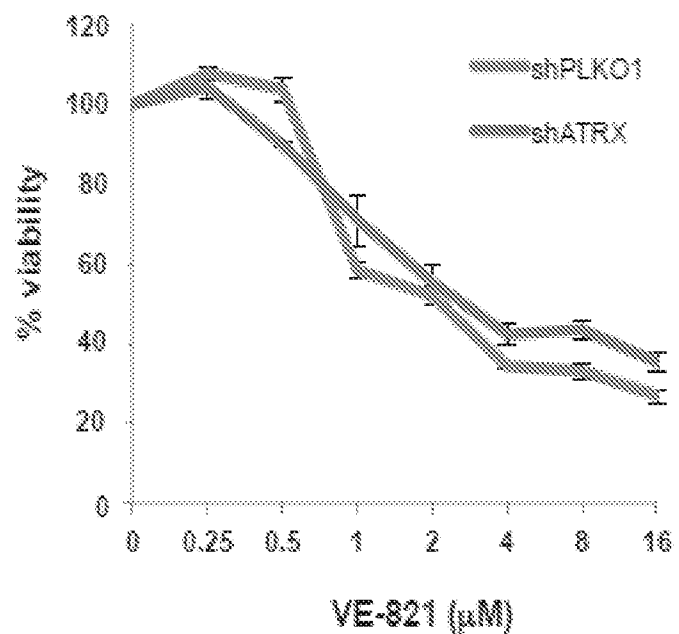
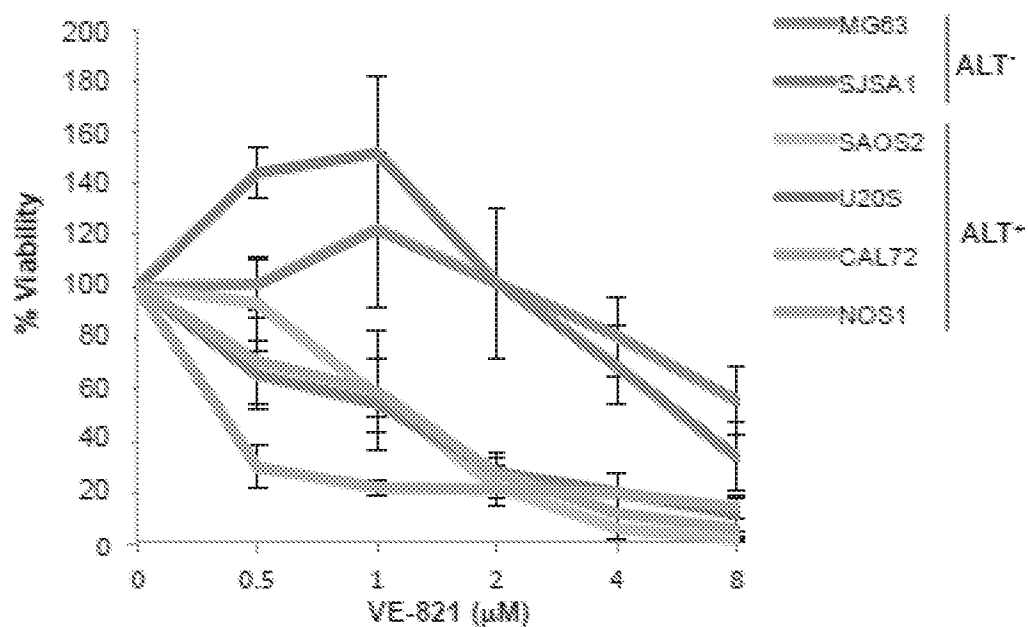
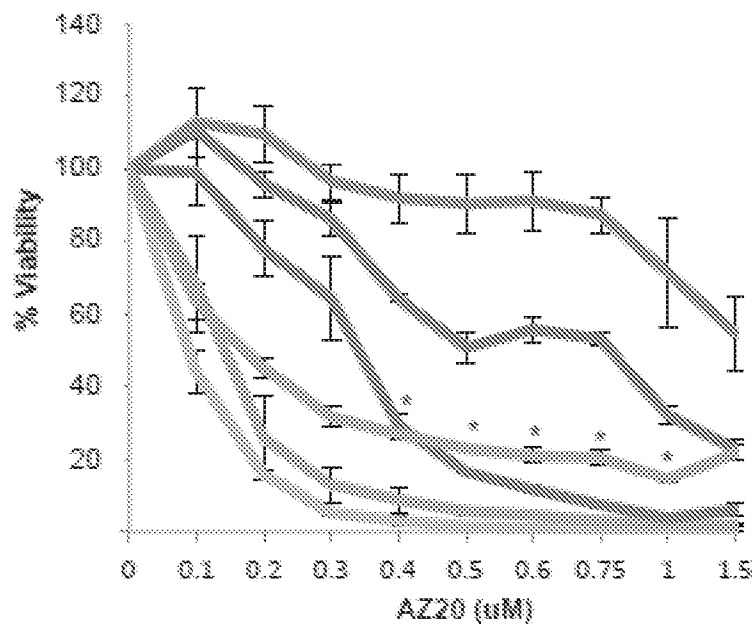


FIG. 20C

14/35

**FIG. 21A****FIG. 21B**

15/35

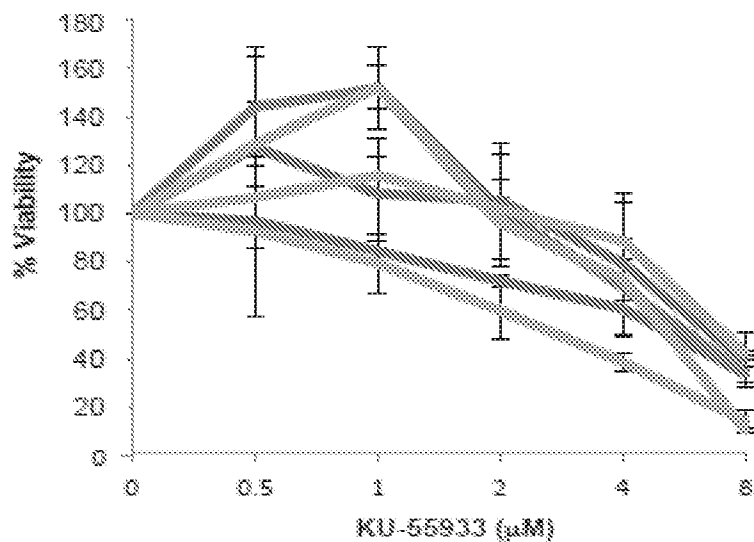


FIG. 21C

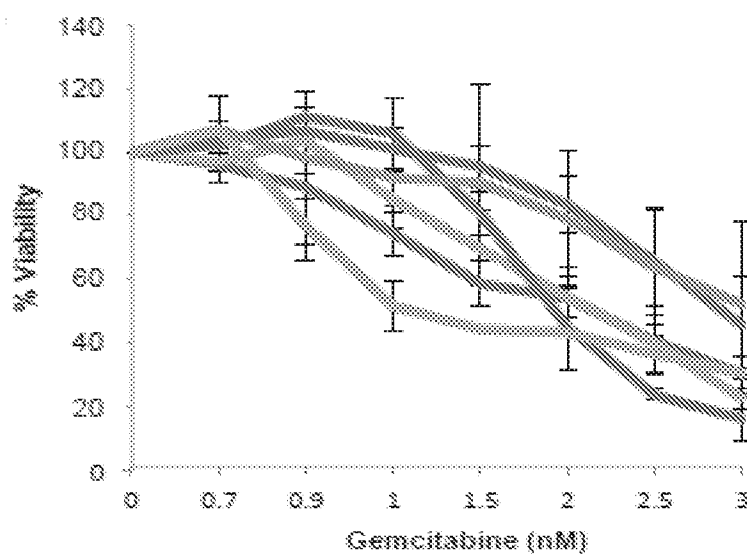


FIG. 21D

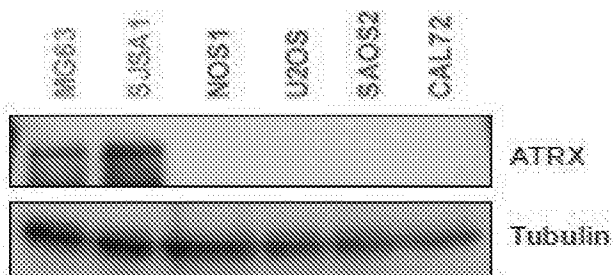


FIG. 22A

16/35

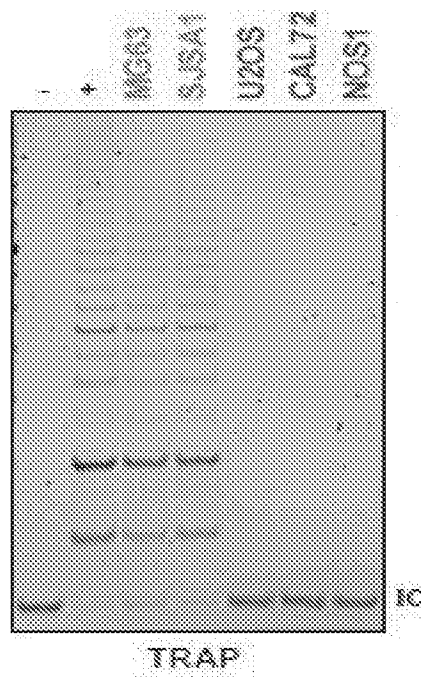


FIG. 22B

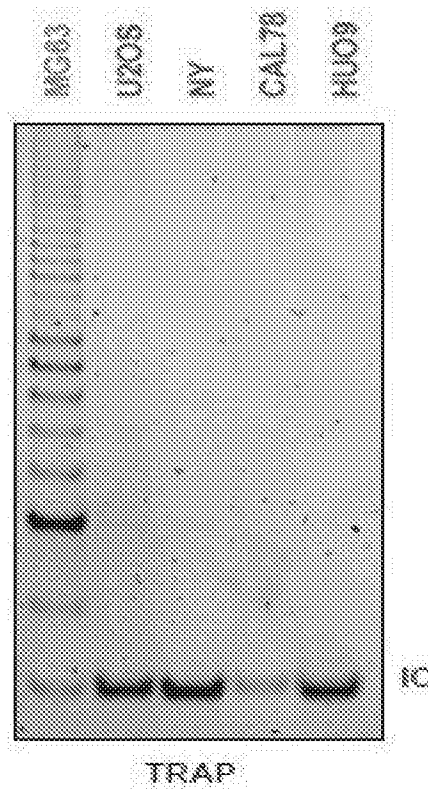
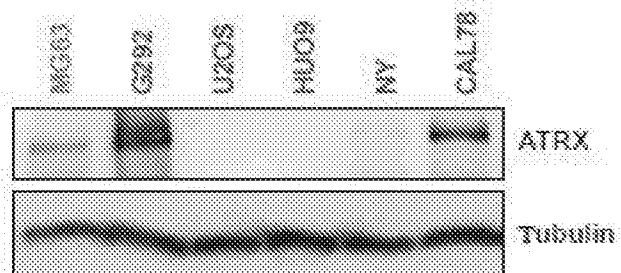
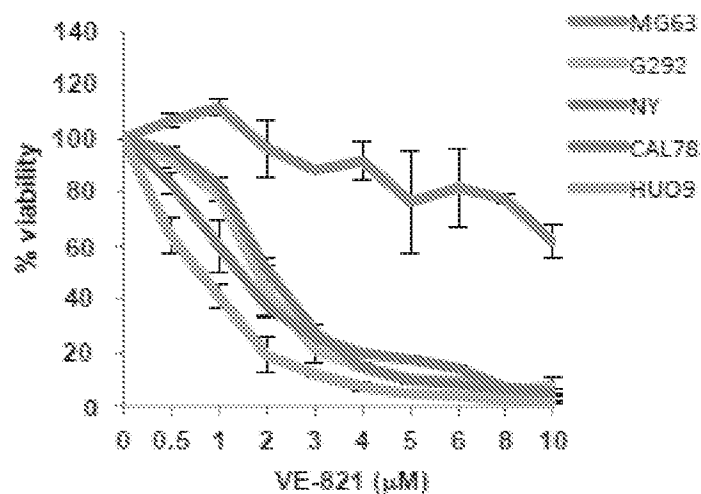
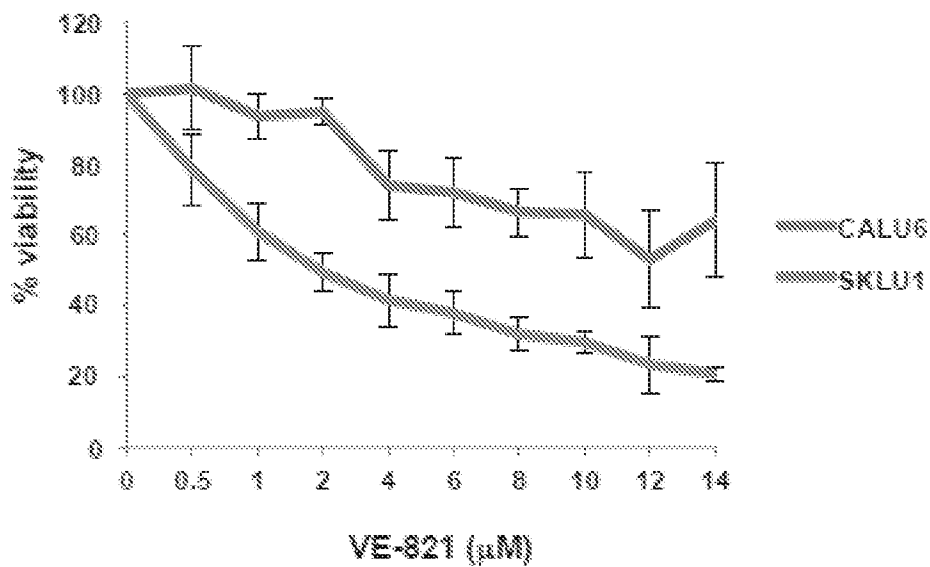
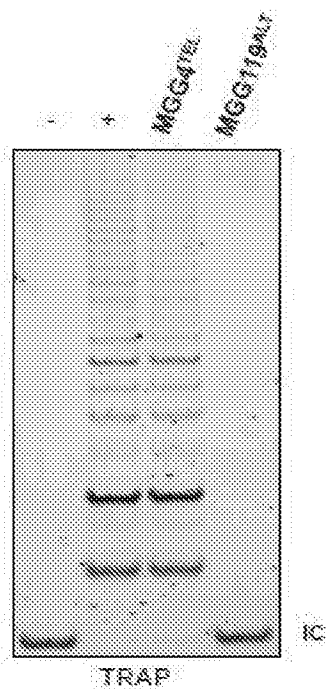
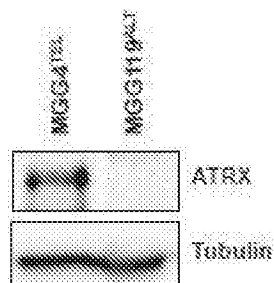
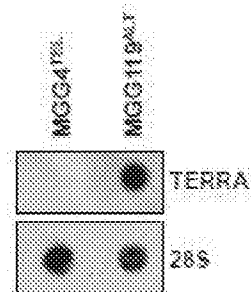
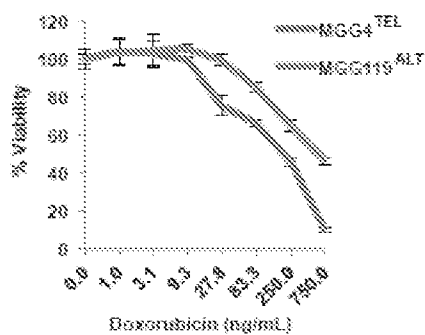
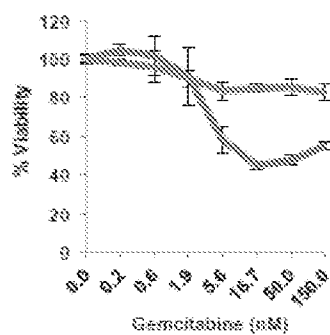
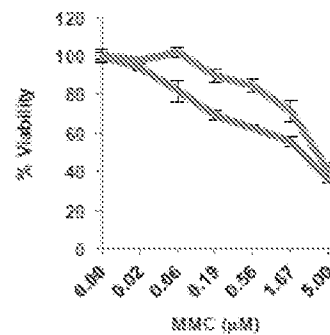


FIG. 23A

17/35

**FIG. 23B****FIG. 23C****FIG. 24**

18/35

**FIG. 25A****FIG. 25B****FIG. 25C****FIG. 26A****FIG. 26B****FIG. 26C**

19/35

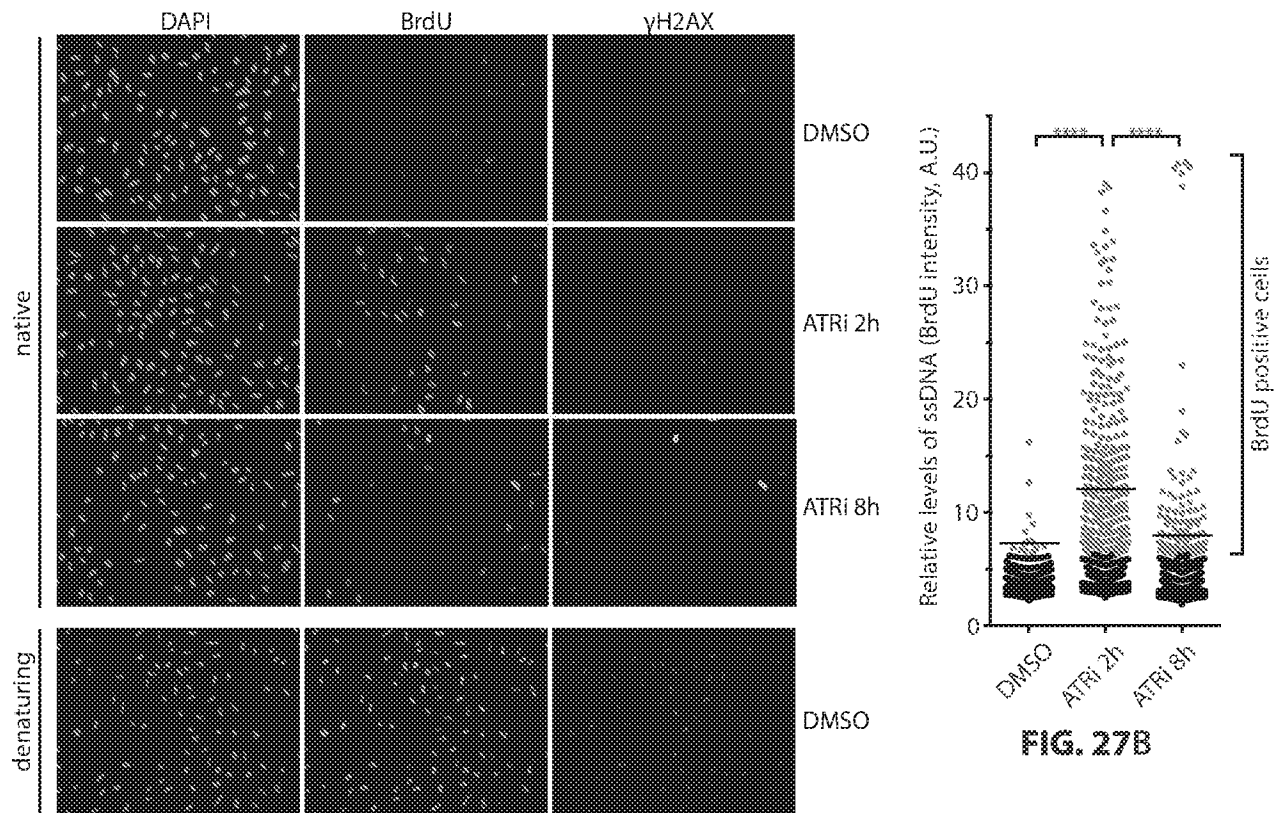


FIG. 27A

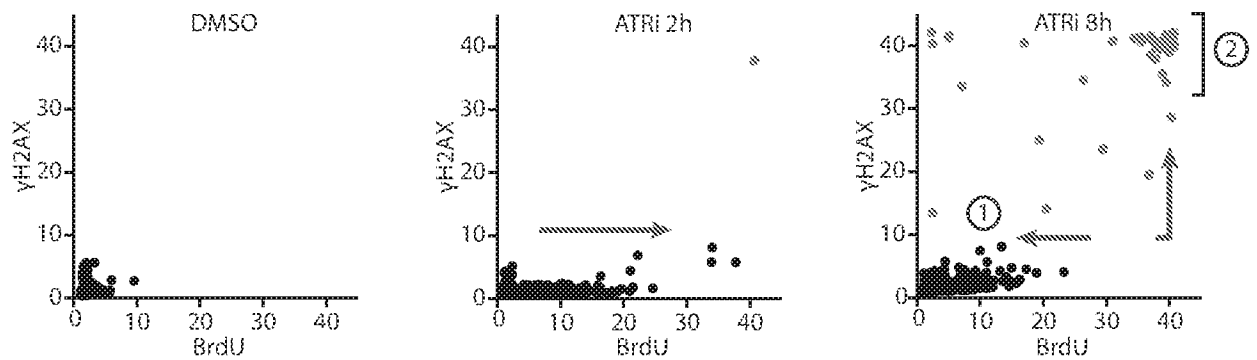


FIG. 27C

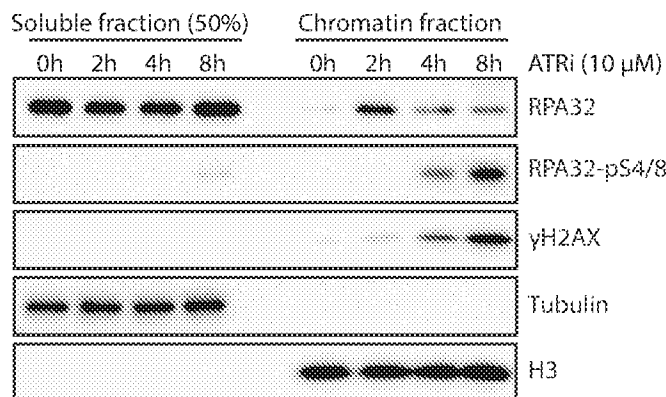


FIG. 27D

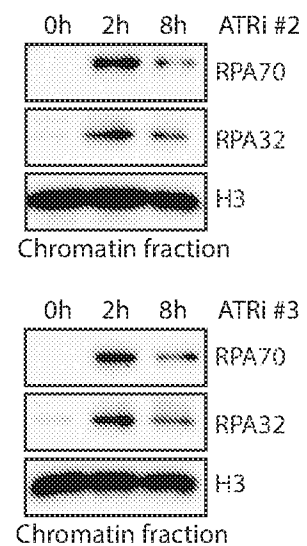


FIG. 27E

20/35

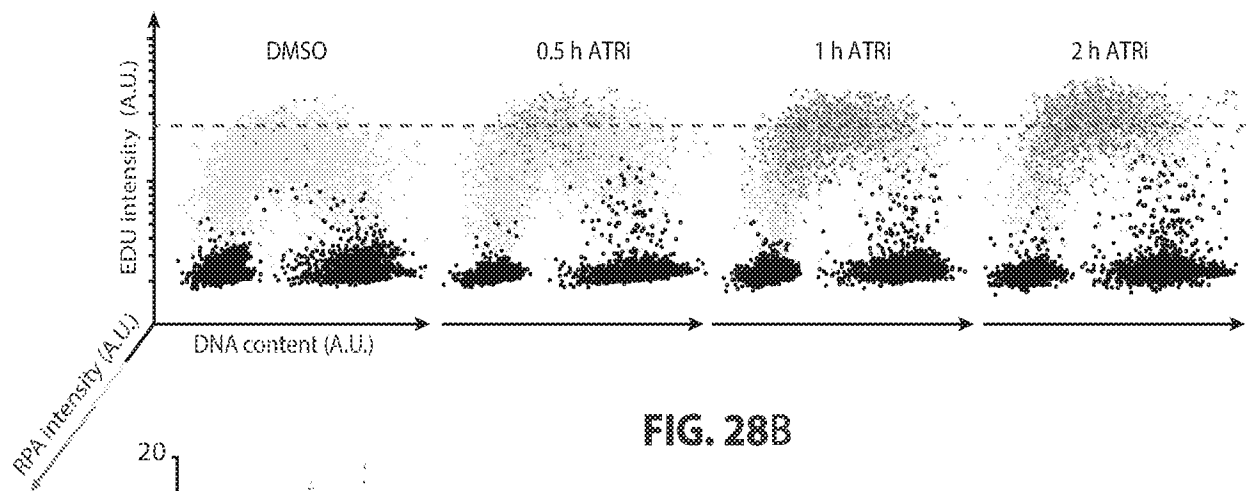


FIG. 28B

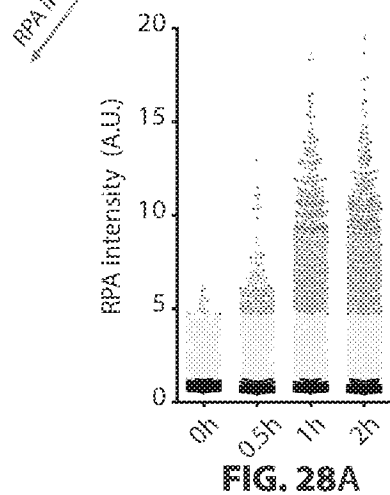


FIG. 28A

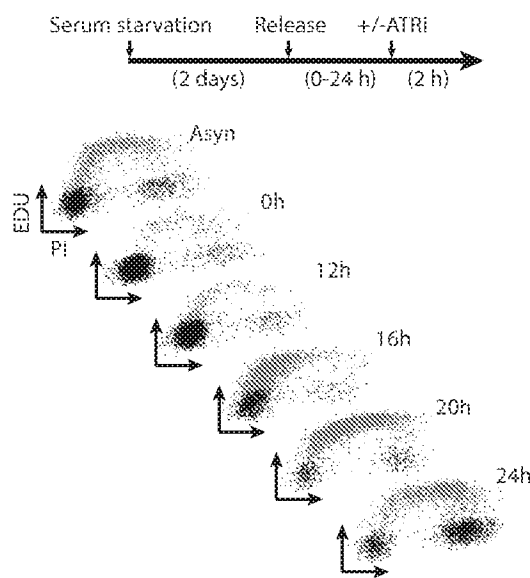


FIG. 28C

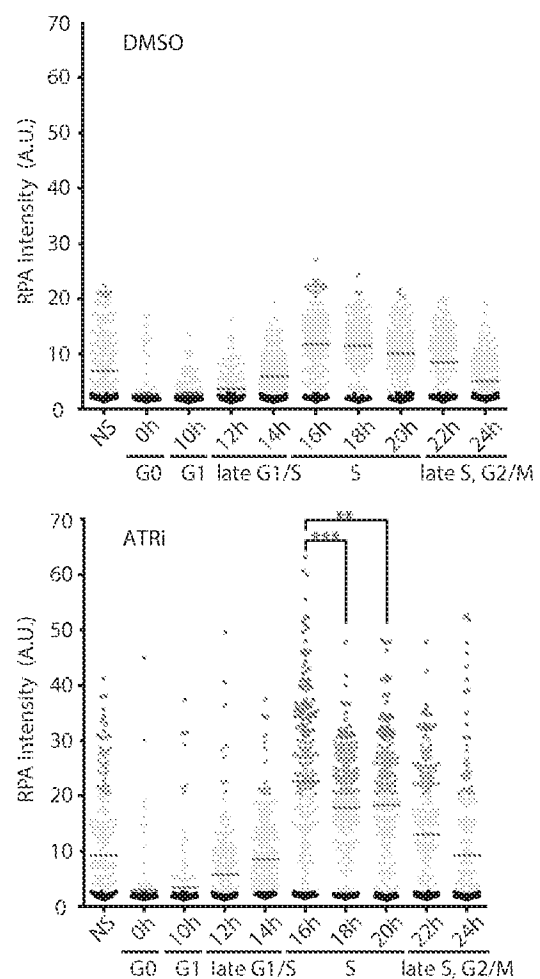


FIG. 28D

21/35

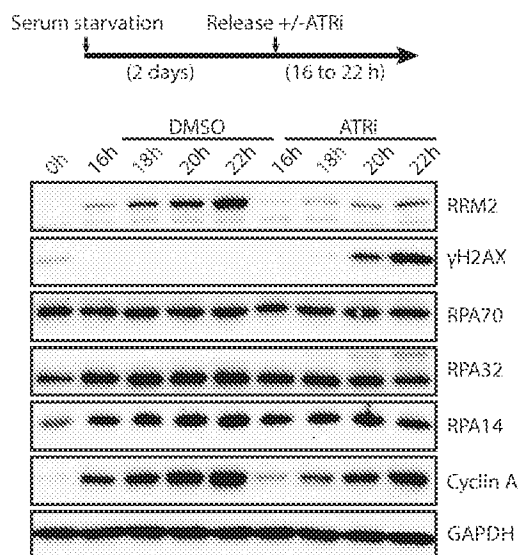


FIG. 29A

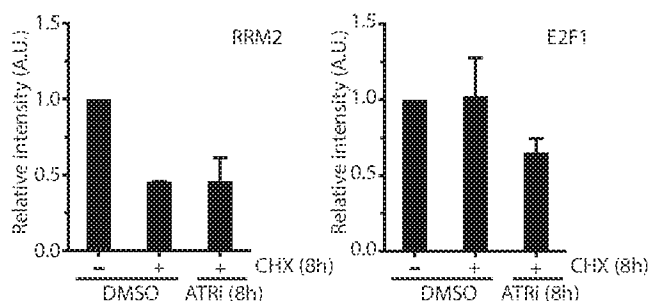
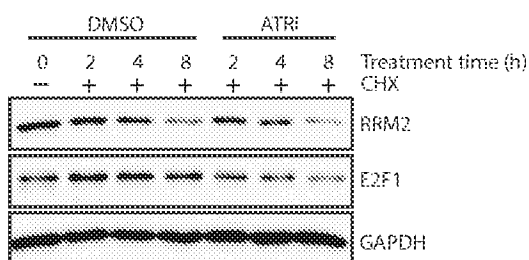


FIG. 29C

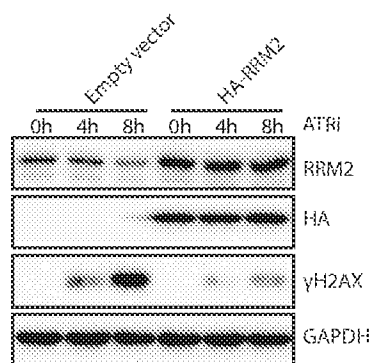


FIG. 29G

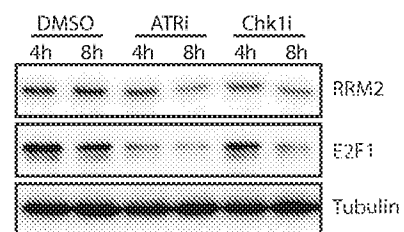


FIG. 29B

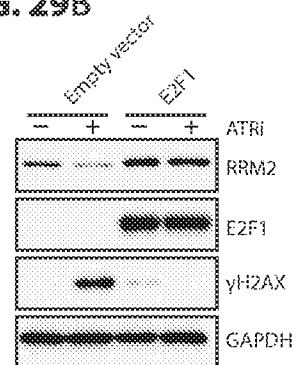


FIG. 29D

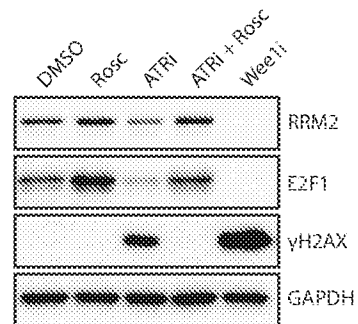


FIG. 29E

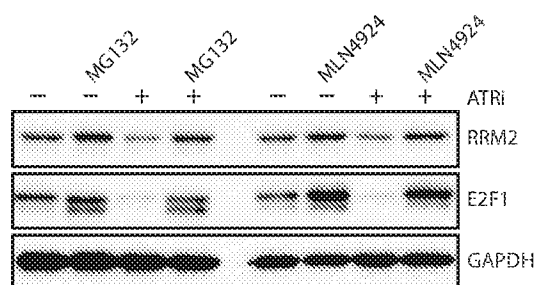


FIG. 29F

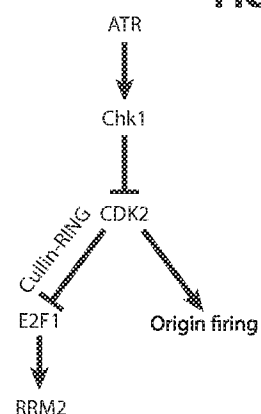


FIG. 29H

22/35

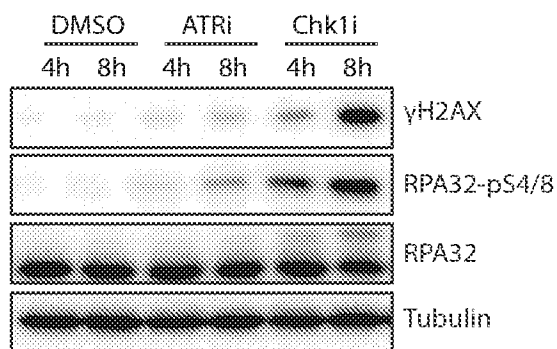


FIG. 30A

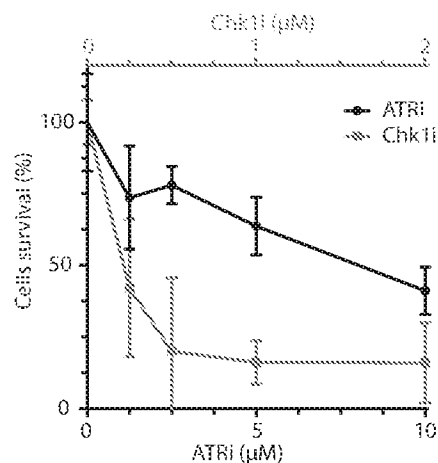


FIG. 30B

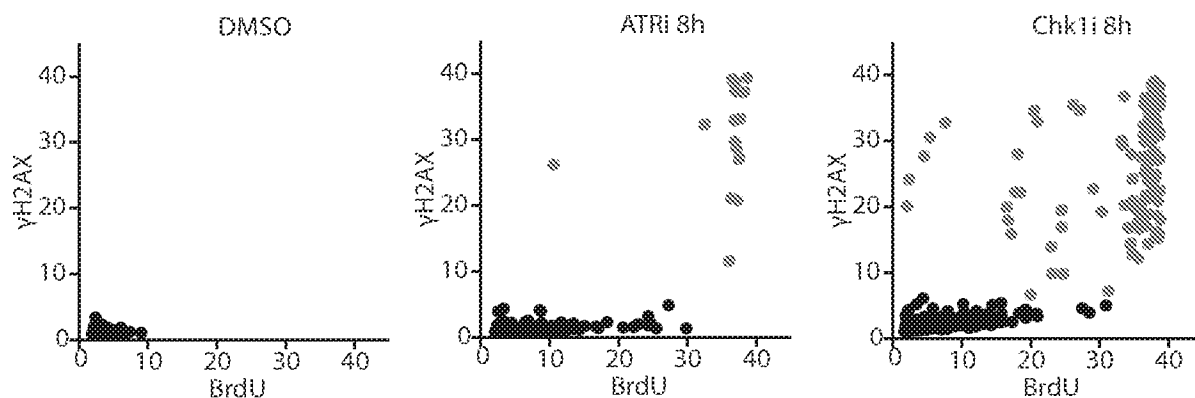


FIG. 30C

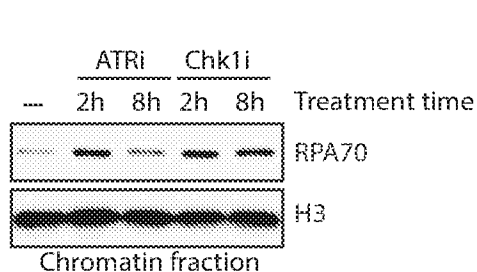


FIG. 30D

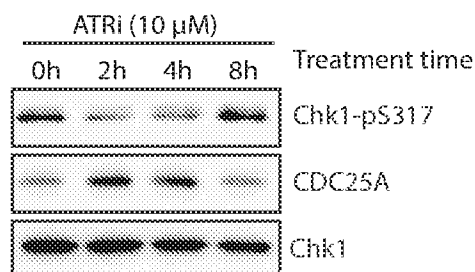


FIG. 30E

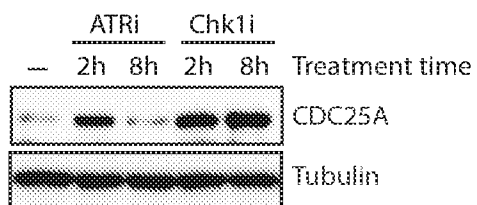


FIG. 30F

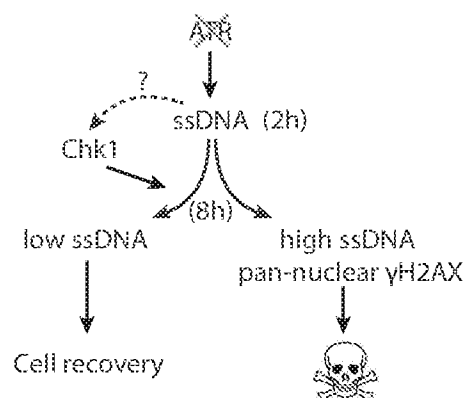


FIG. 30G

23/35

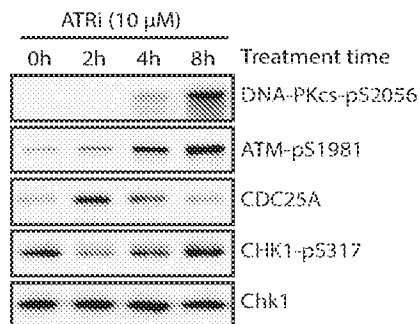


FIG. 31A

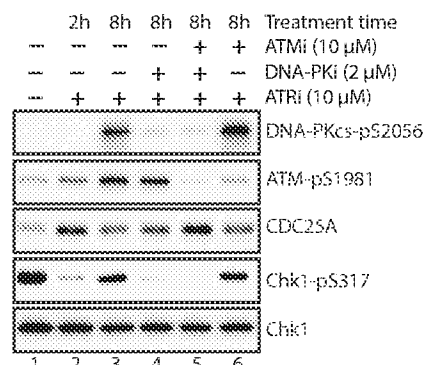


FIG. 31B

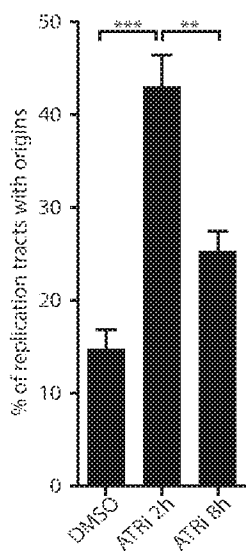


FIG. 31C

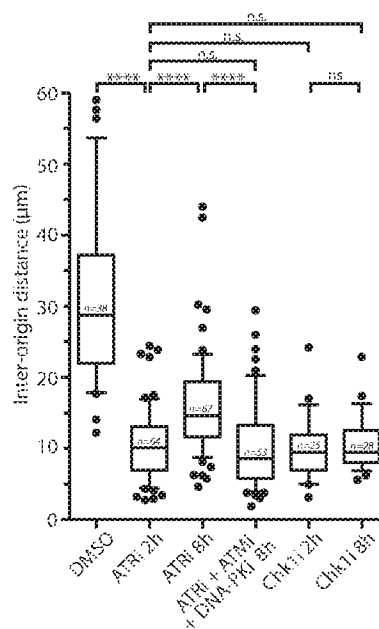


FIG. 31D

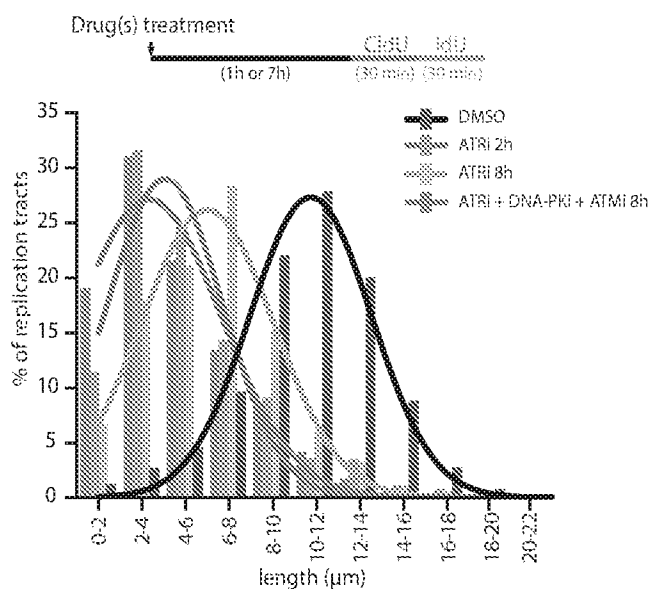


FIG. 31E

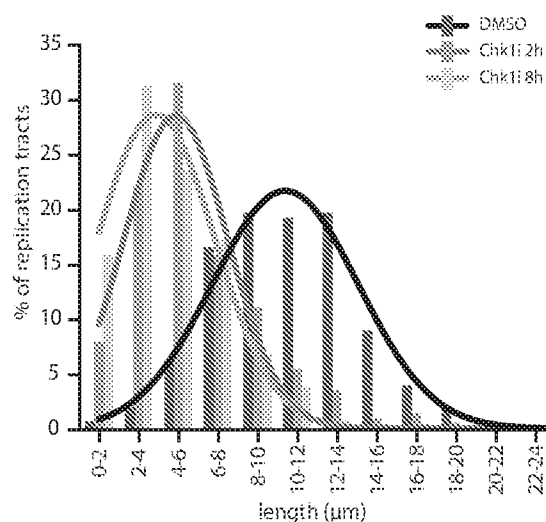


FIG. 31F

24/35

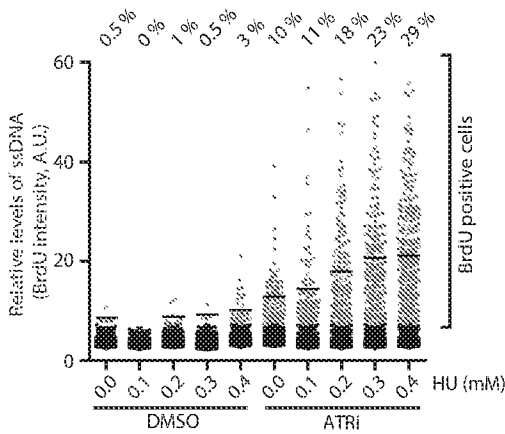


FIG. 32A

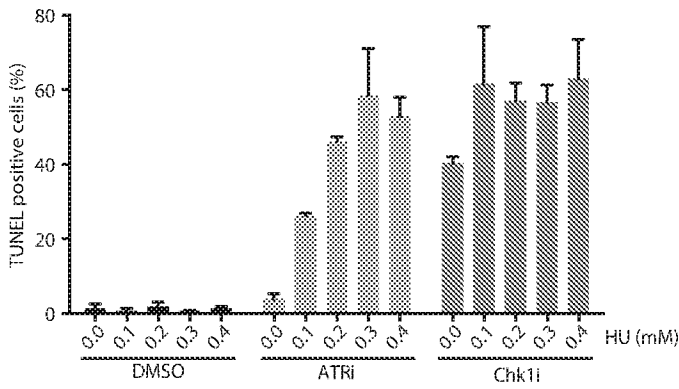


FIG. 32B

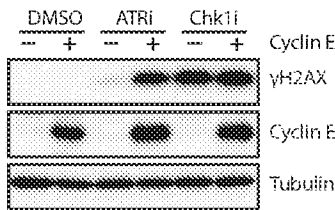


FIG. 32C

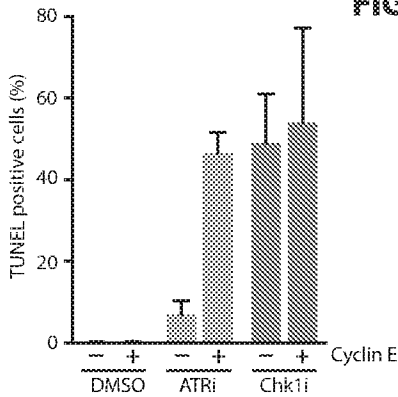


FIG. 32D

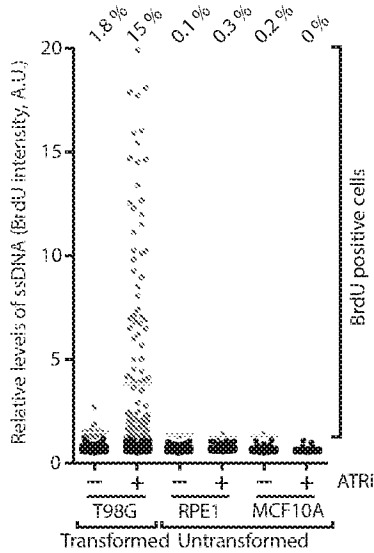


FIG. 32E

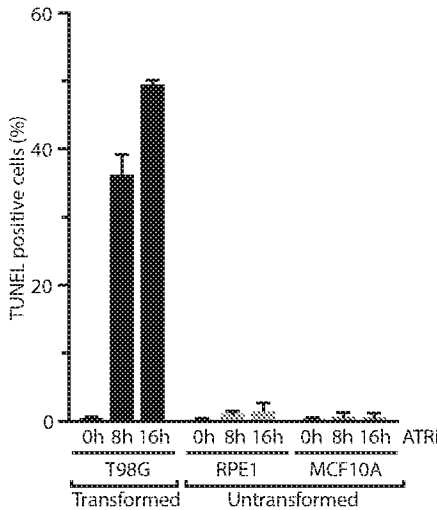


FIG. 32F

	High ssDNA (in PCNA-positive cells)	γH2AX positive cells	Cells survival (5 μM ATRi)	Cells survival (2.5 μM ATRi + 0.05 mM HU)
HT-29	56.7%	3.2%	24%	20%
Colo-320-HSR	18.9%	7.9%	17%	1%
SW620	15.5%	6.2%	42%	4%
RKO	15.4%	8.9%	69%	29%
HCT-15	9.0%	1.5%	83%	56%
LS-123	6.6%	3.0%	89%	54%
HT-55	2.2%	1.4%	61%	55%
NCI-H747	2.0%	1.3%	100%	91%
SNU-61	1.9%	1.2%	100%	100%
SW1116	1.4%	1.0%	100%	100%

FIG. 32G

25/35

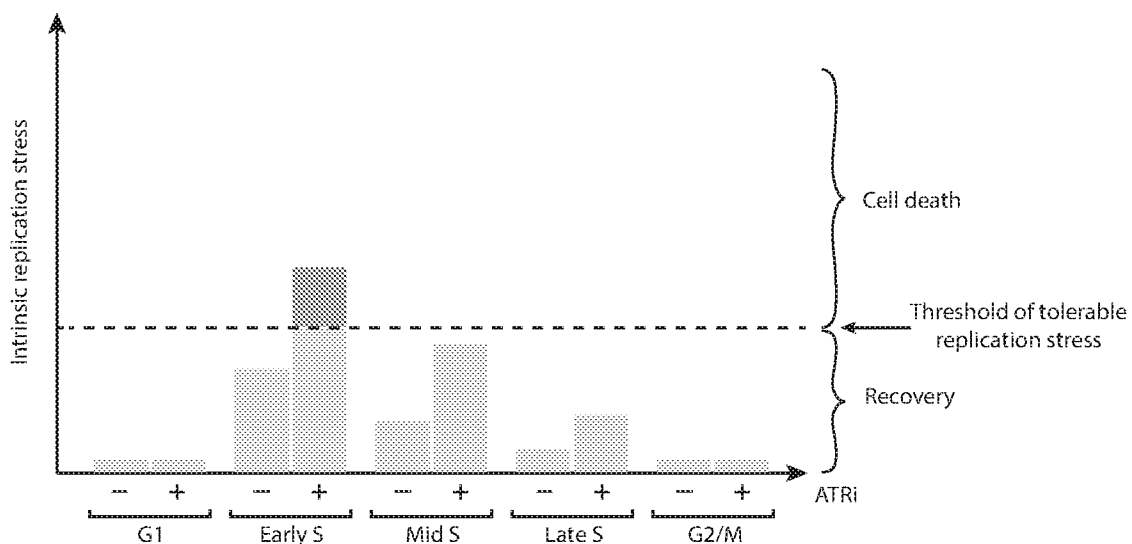


FIG. 33A

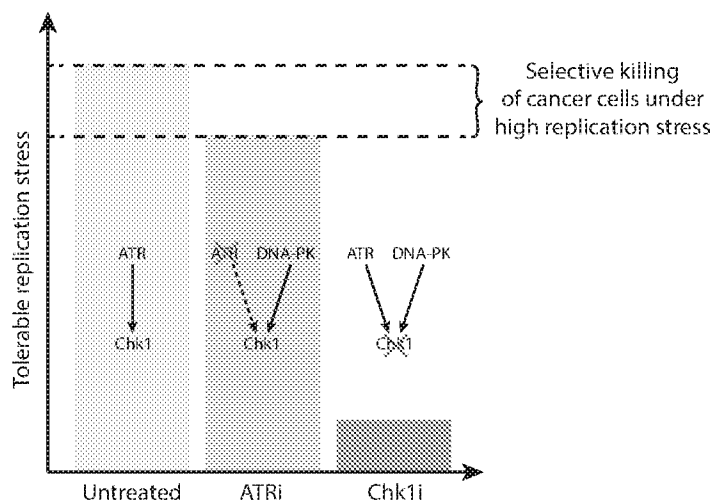


FIG. 33B

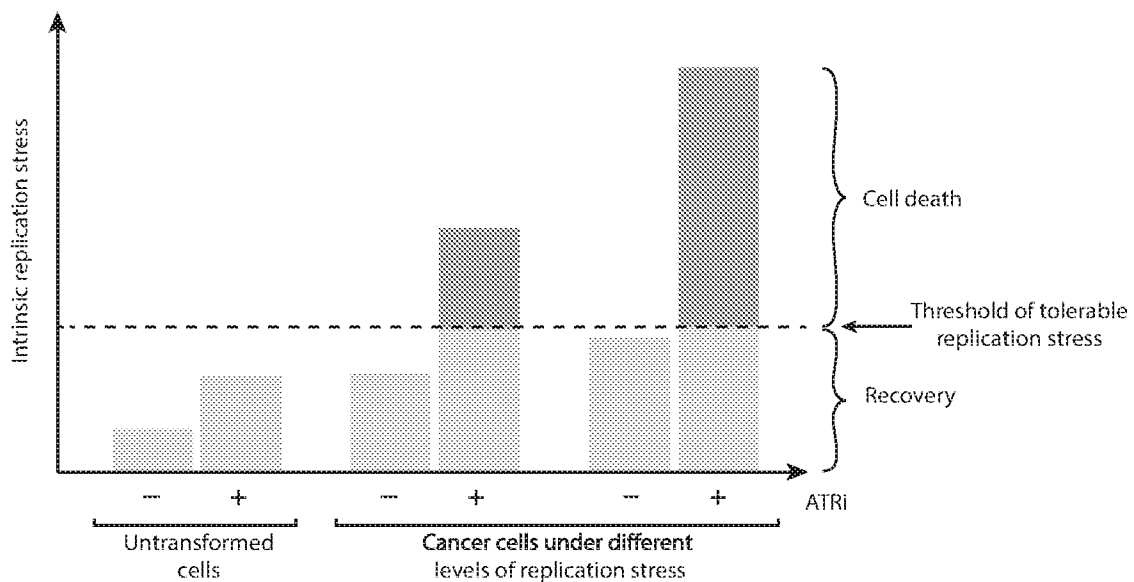
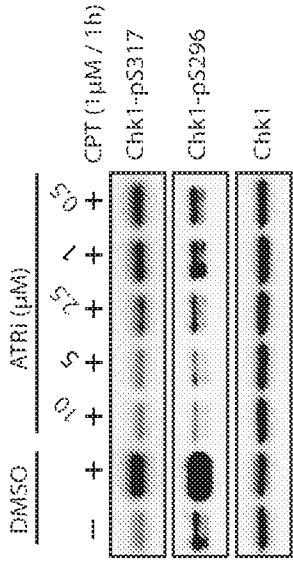


FIG. 33C



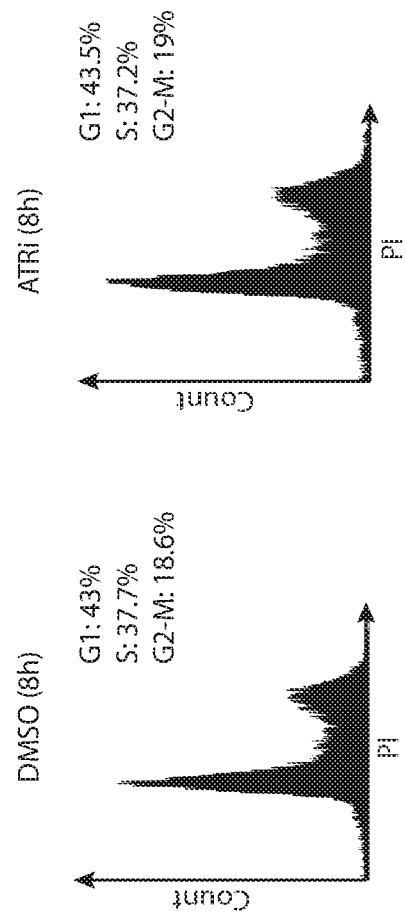


FIG. 34F

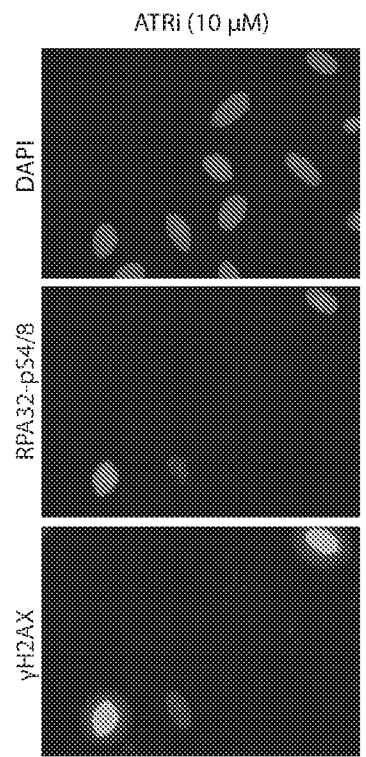


FIG. 34H

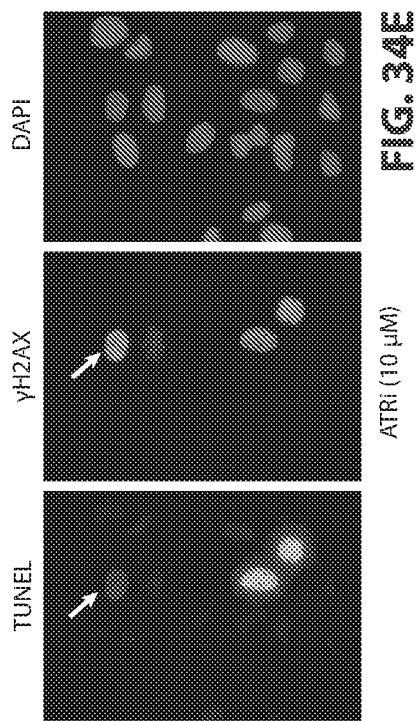


FIG. 34E

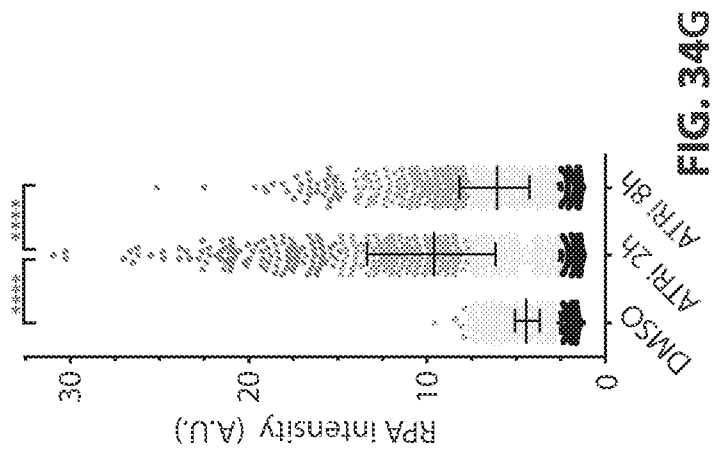


FIG. 34G

28/35

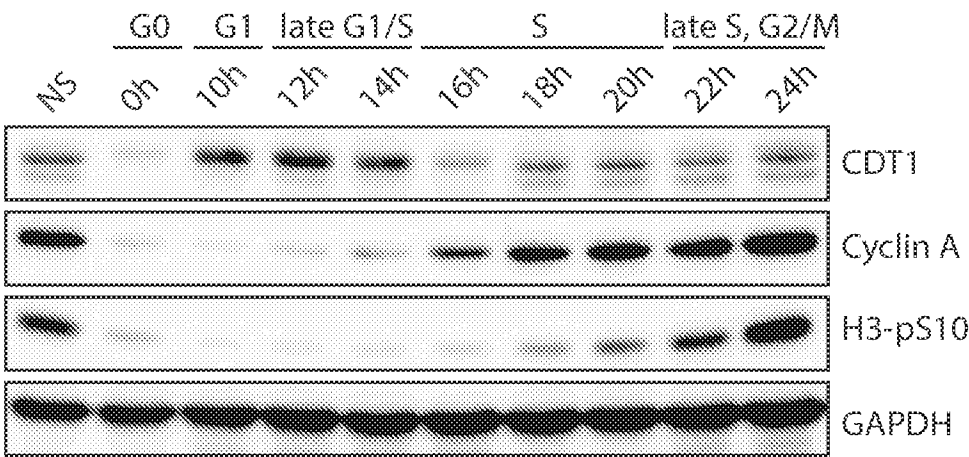


FIG. 35

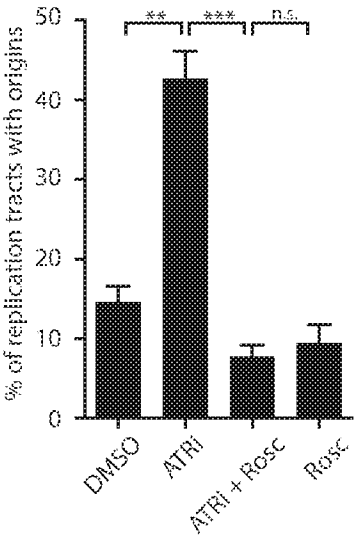


FIG. 36A

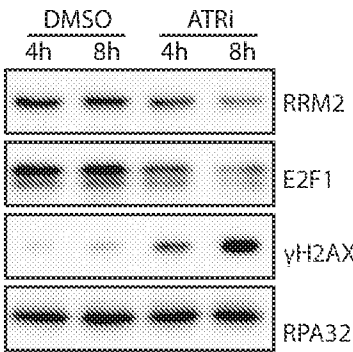


FIG. 36B

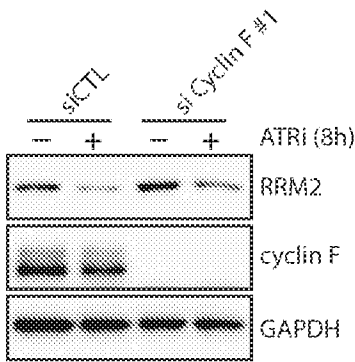


FIG. 36C

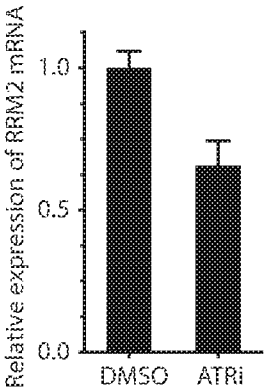


FIG. 36D

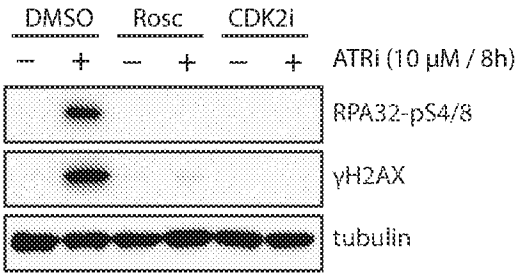


FIG. 36E

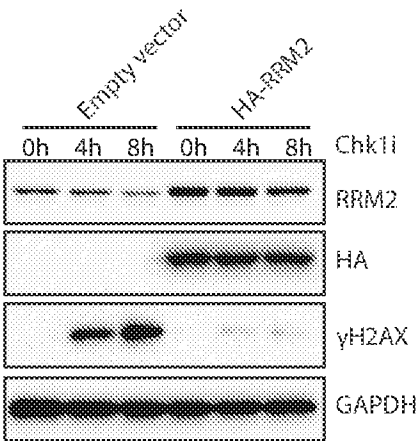


FIG. 36F

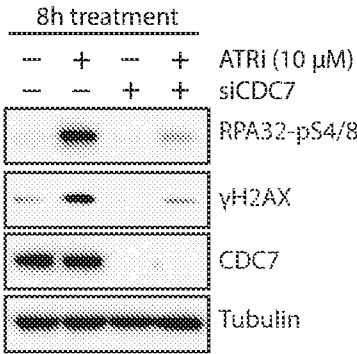
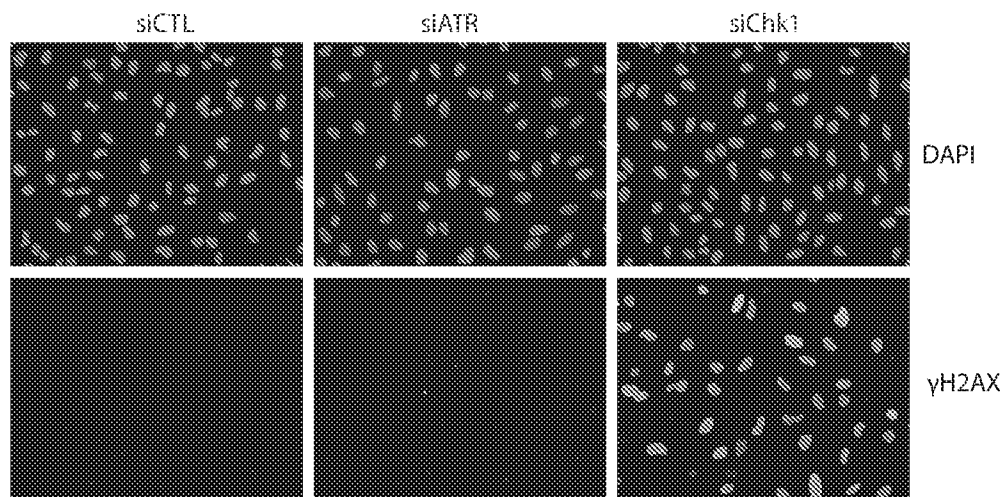
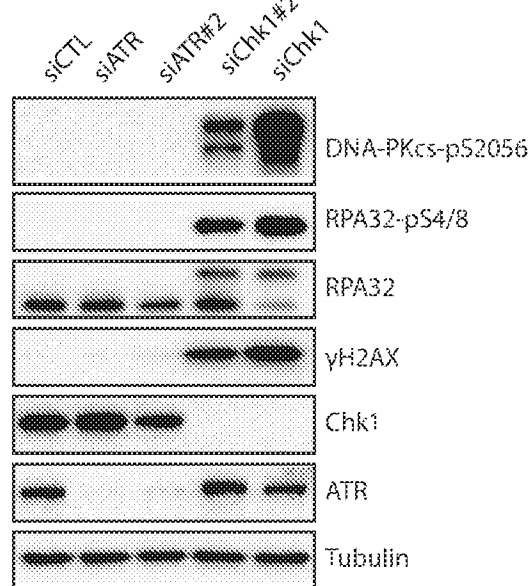
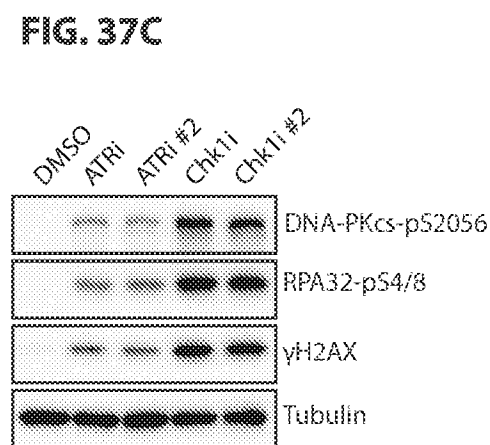
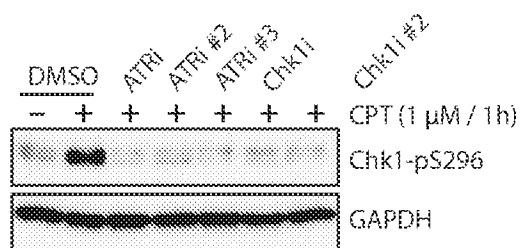
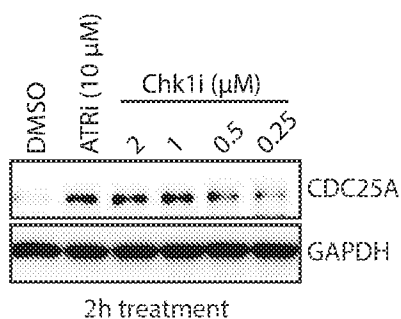
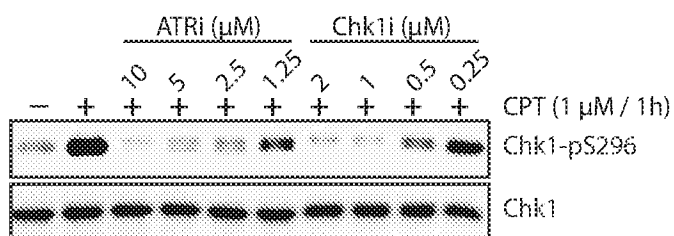
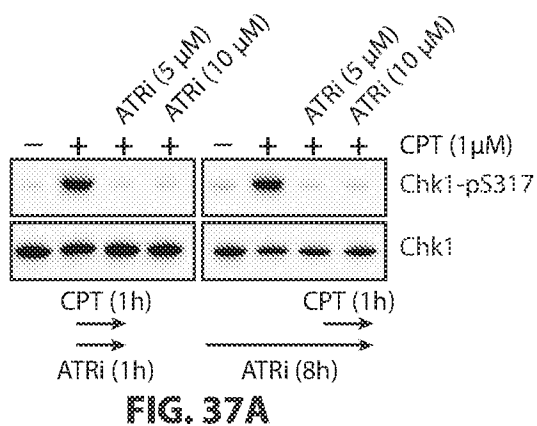


FIG. 36G

30/35



31/35

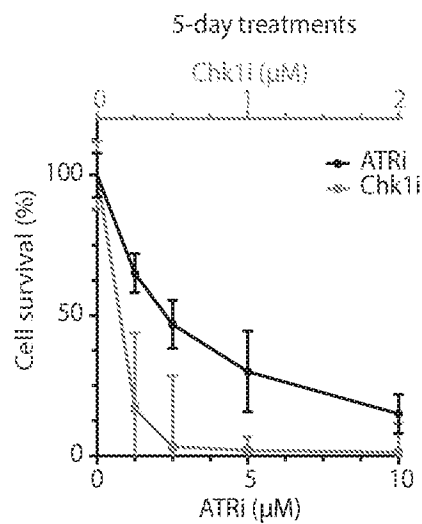


FIG. 37H

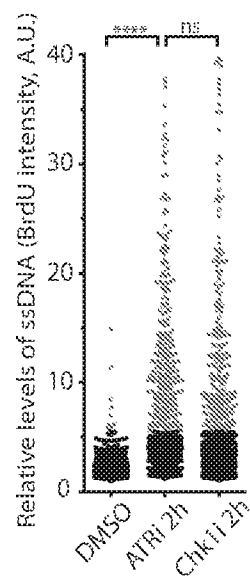


FIG. 37I

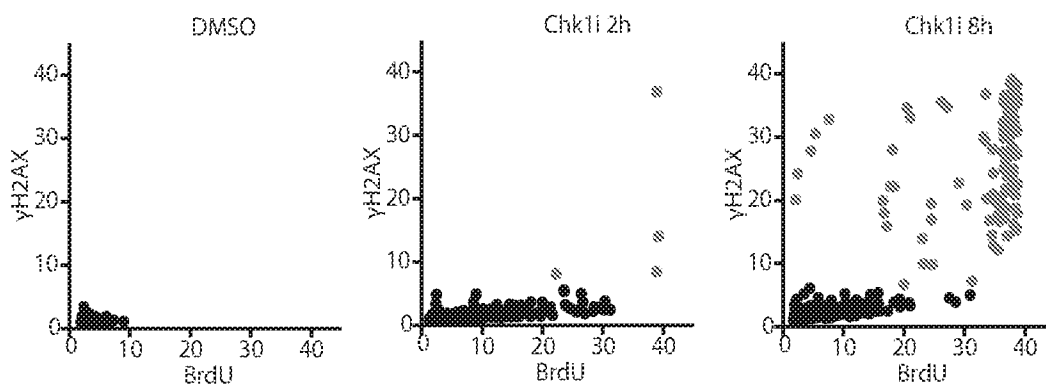


FIG. 37J

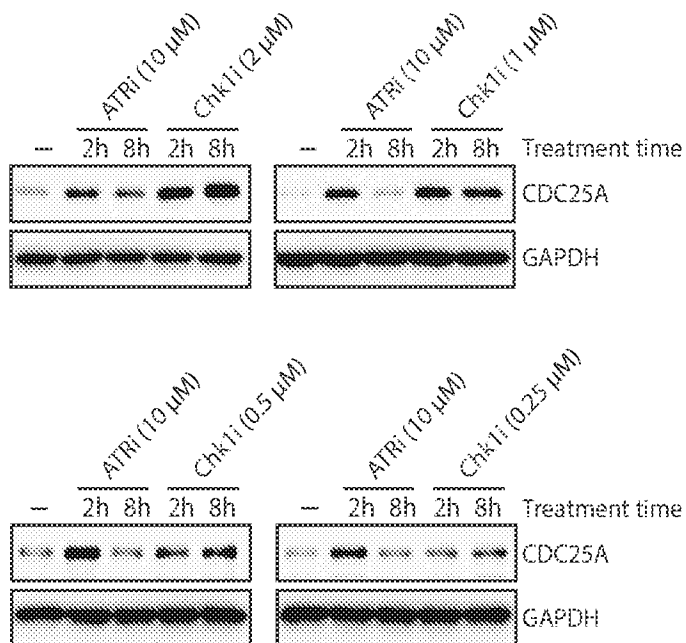


FIG. 37K

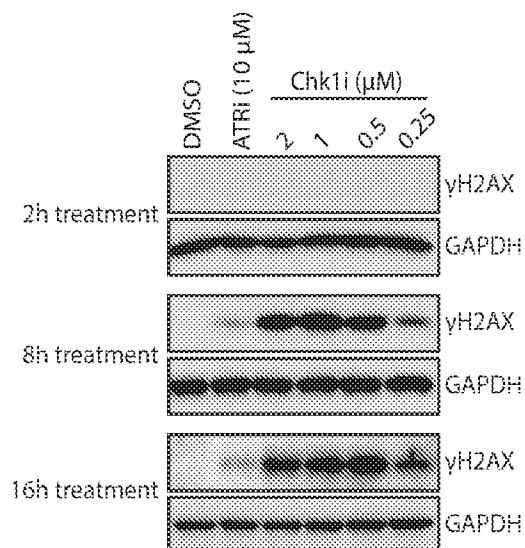


FIG. 37L

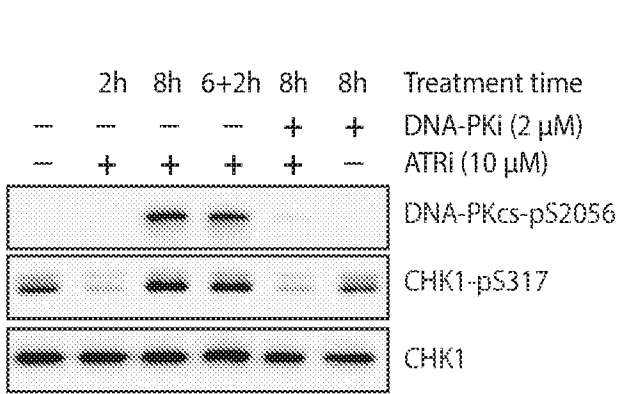


FIG. 38A

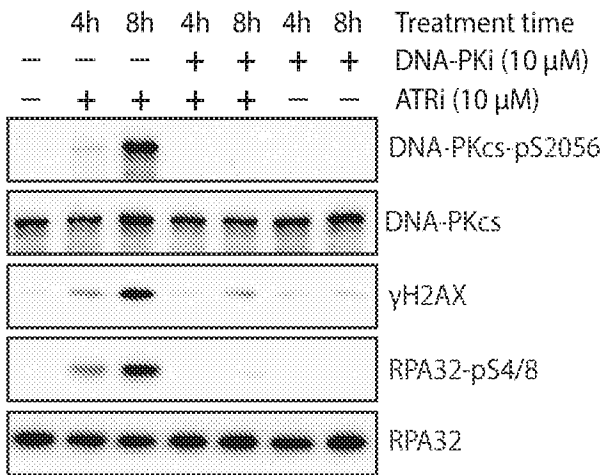


FIG. 38C

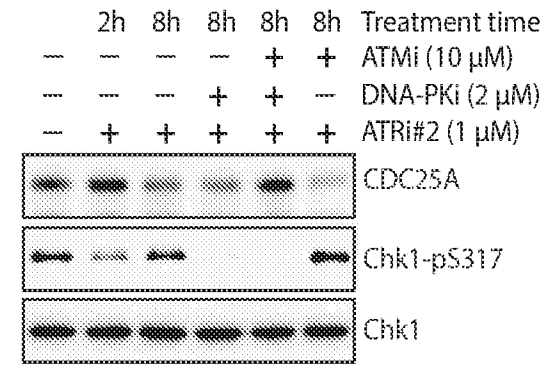


FIG. 38B

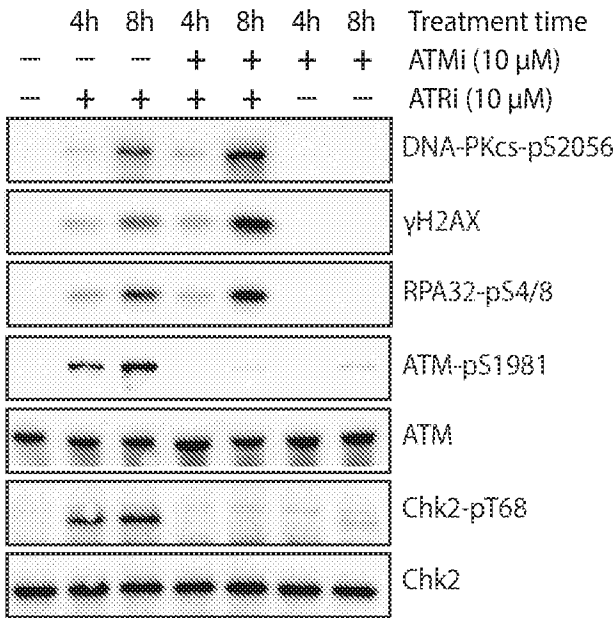


FIG. 38D

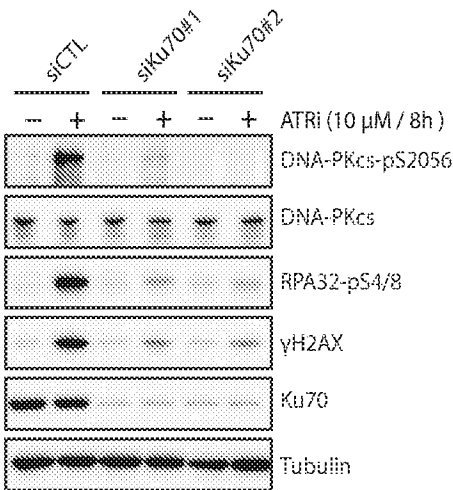


FIG. 38E

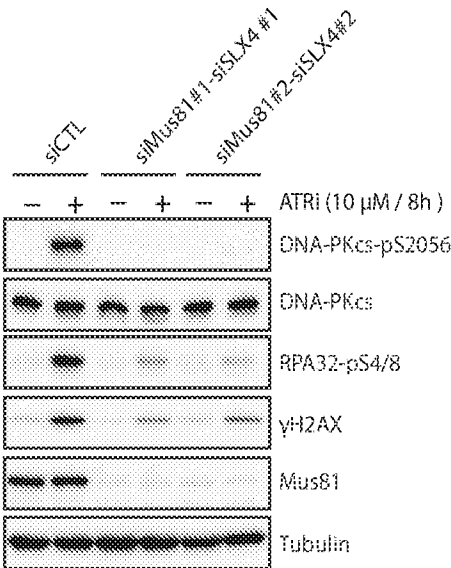


FIG. 38F

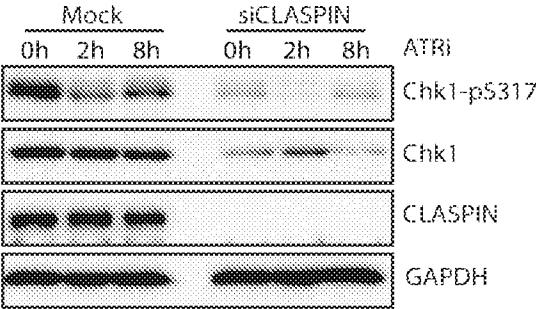


FIG. 38G

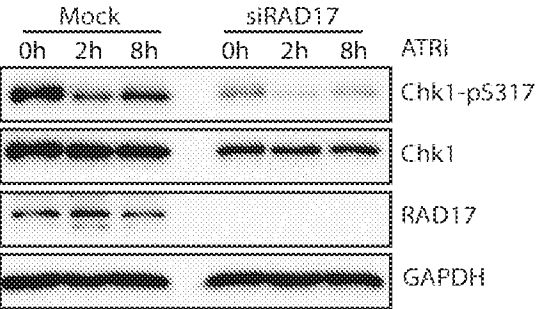


FIG. 38H

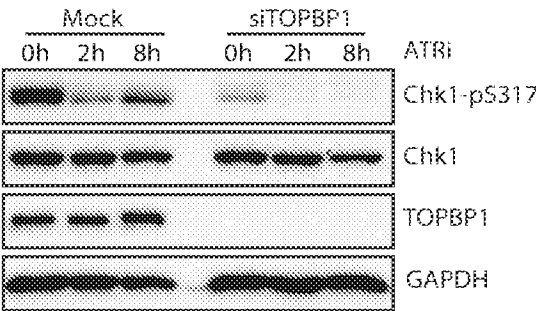


FIG. 38I

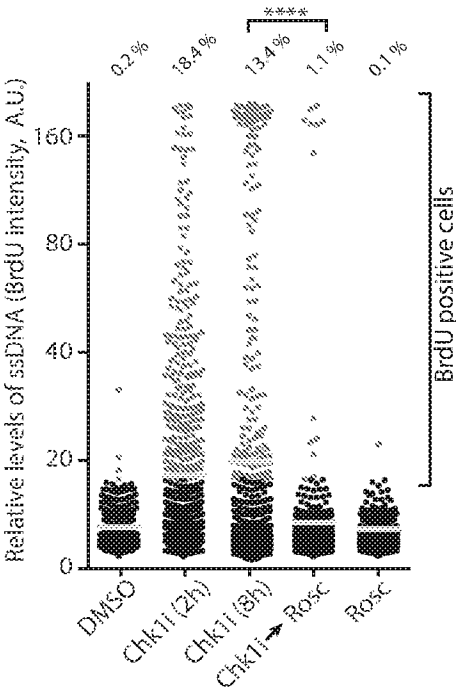


FIG. 38J

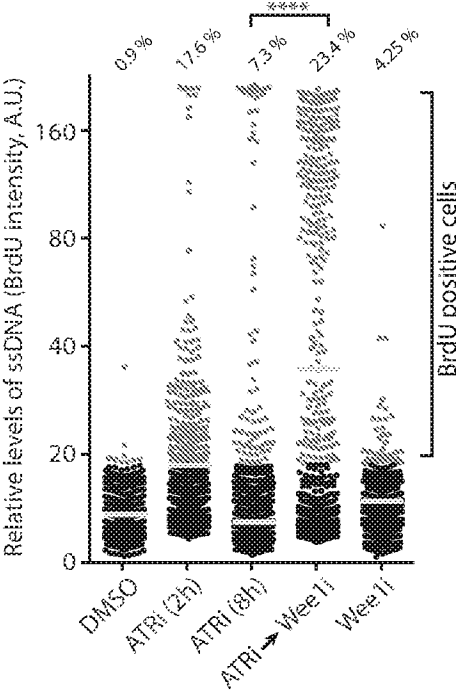


FIG. 38K

34/35

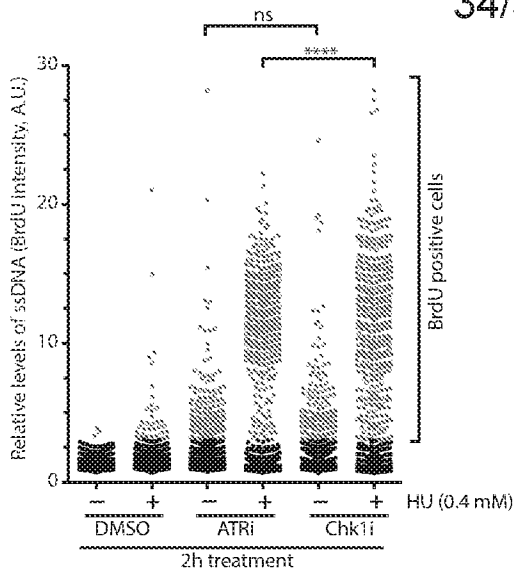


FIG. 39A

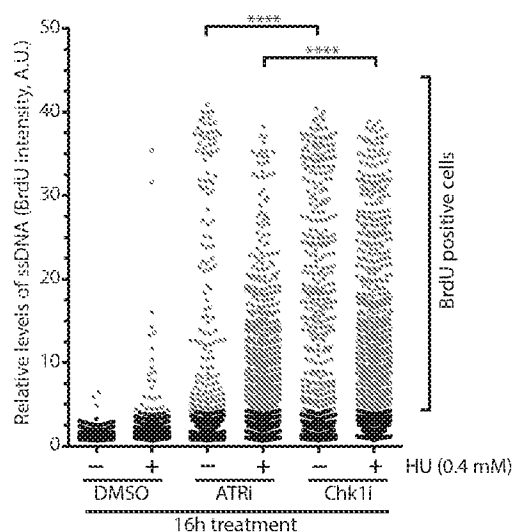


FIG. 39B

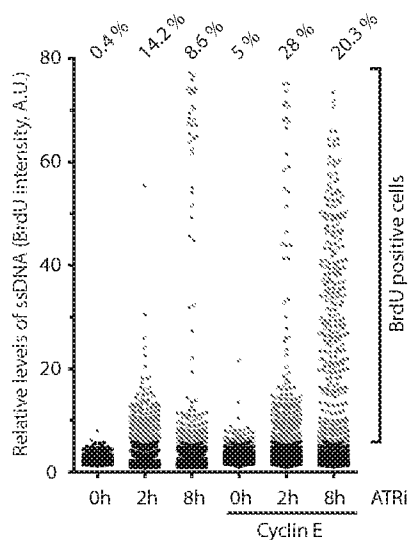


FIG. 39C

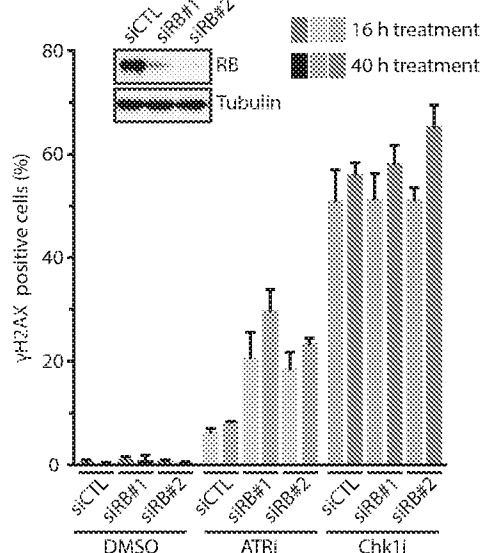


FIG. 39D

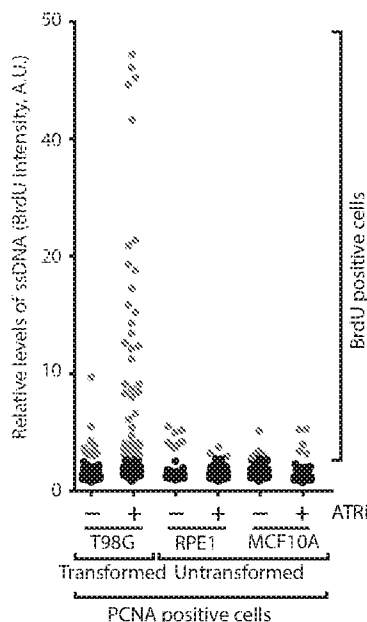


FIG. 39E

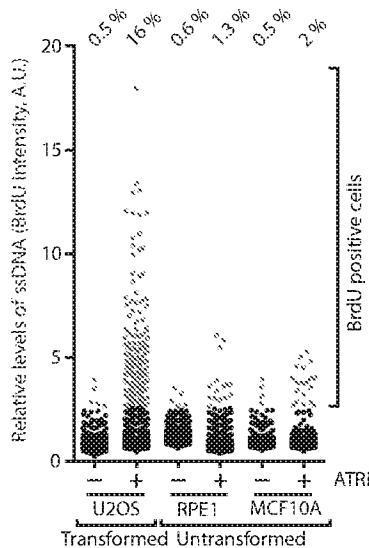


FIG. 39F

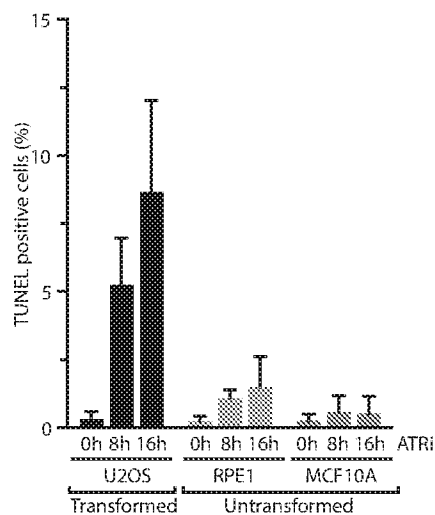


FIG. 39G

35/35

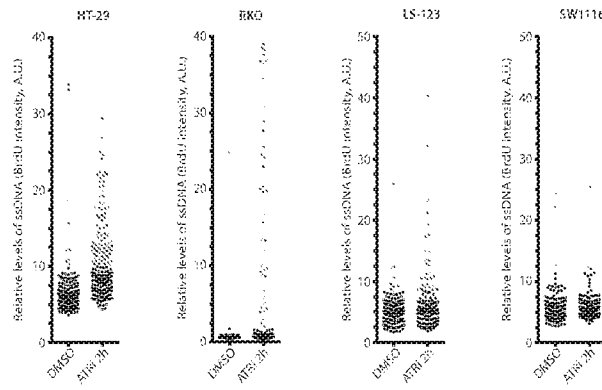


FIG. 39H

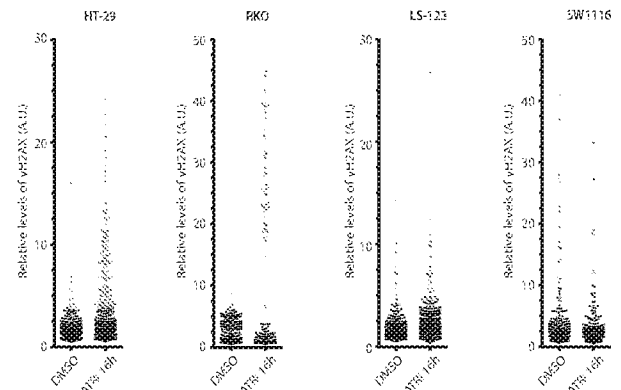


FIG. 39I

	0	0.625	1.25	2.5	5	ATRi (μM)
Colo-320-HSR	100%	102%	72%	41%	17%	
SW620	100%	104%	97%	71%	47%	
HT-29	100%	91%	37%	66%	24%	
RKO	100%	108%	111%	83%	89%	
HCT-15	100%	104%	106%	60%	52%	
HT-55	100%	112%	113%	97%	61%	
LS-123	100%	130%	133%	127%	82%	
NCI-H747	100%	112%	115%	112%	123%	
SNU-61	100%	105%	109%	120%	95%	
SW1116	100%	120%	146%	132%	107%	

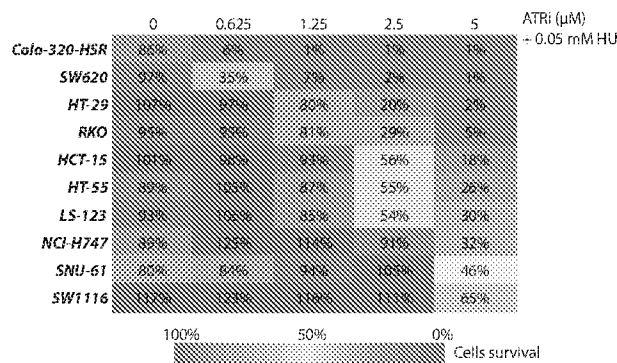


FIG. 39J

	0	0.625	1.25	2.5	5	ATRi (μM)
HT29	1%	2%	3%	1%	7%	
Colo320-HSR	3%	1%	4%	1%	5%	
SW620	1%	0%	1%	3%	4%	
RKO	3%	1%	2%	1%	4%	
HCT-15	3%	1%	3%	1%	5%	
LS-123	2%	1%	1%	2%	5%	
HT-55	3%	1%	4%	2%	1%	
NCI-H747	1%	1%	3%	6%	1%	
SNU-61	3%	4%	3%	1%	3%	
SW1116	6%	4%	3%	7%	6%	

	0	0.625	1.25	2.5	5	ATRi (μM) + 0.05 mM HU
HT29	1%	2%	2%	2%	3%	
Colo320-HSR	3%	10%	9%	3%	8%	
SW620	1%	6%	10%	9%	1%	
RKO	3%	2%	3%	4%	4%	
HCT-15	3%	2%	2%	5%	2%	
LS-123	2%	2%	4%	7%	4%	
HT-55	3%	3%	2%	4%	1%	
NCI-H747	1%	2%	5%	7%	2%	
SNU-61	3%	5%	3%	5%	2%	
SW1116	6%	2%	1%	2%	4%	

FIG. 39K

BrdU positive cells
(36 h labeling before denaturation)
5-phase cells
(PCNA-positive cells)

Colo-320-HSR	86 %	43 %
SW620	99 %	47 %
HT-29	94 %	38 %
RKO	99 %	32 %
HCT-15	97 %	42 %
LS-123	84 %	33 %
HT-55	97 %	40 %
NCI-H747	93 %	34 %
SNU-61	84 %	38 %
SW1116	84 %	31 %

FIG. 39L

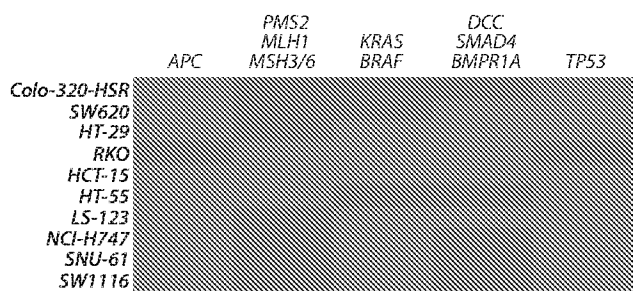


FIG. 39M

Cell line	MSI	CIMP	CIN
Colo-320-HSR	MSS	-	+
SW620	MSS	-/+	+
HT-29	MSS	+	+
RKO	MSI	+	-
HCT-15	MSI	+	-
SW1116	MSS	+	+

FIG. 39N