Fig. S1

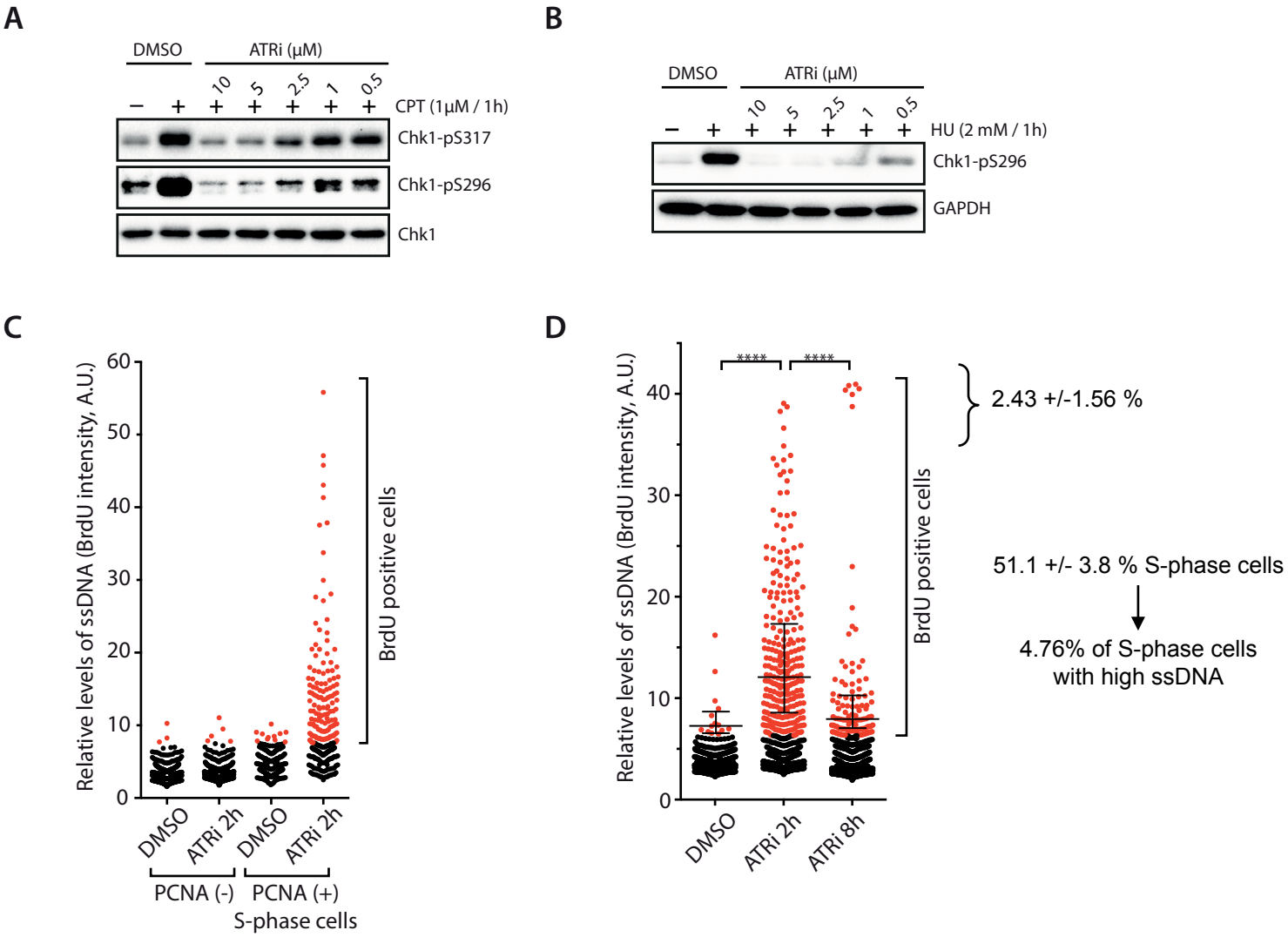
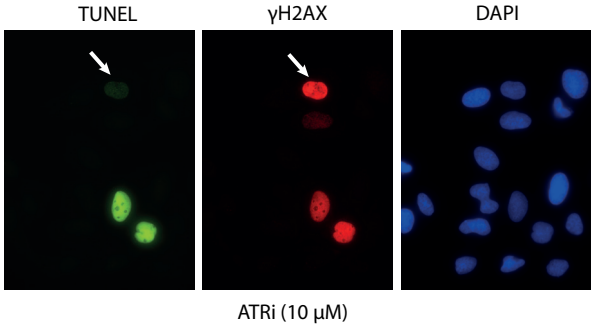
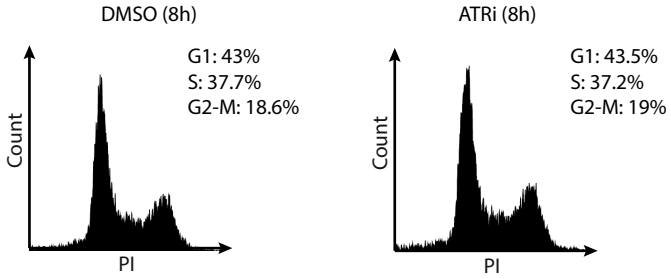

A
DMSO
ATRi (μM)
10
5
2.5
1
0.5
– + + + + + + CPT (1μM / 1h)
Chk1-pS317
Chk1-pS296
Chk1
B
DMSO
ATRi (μM)
10
5
2.5
1
0.5
– + + + + + + HU (2 mM / 1h)
Chk1-pS296
GAPDH
C
Relative levels of ssDNA (BrdU intensity, A.U.)
60
50
40
30
20
10
0
BrdU positive cells
DMSO
ATRi 2h
DMSO
ATRi 2h
PCNA (-)
PCNA (+)
S-phase cells
D
Relative levels of ssDNA (BrdU intensity, A.U.)
40
30
20
10
0
****
****
BrdU positive cells
2.43 +/-1.56 %
51.1 +/- 3.8 % S-phase cells
4.76% of S-phase cells
with high ssDNA
DMSO
ATRi 2h
ATRi 8h

Fig. S1

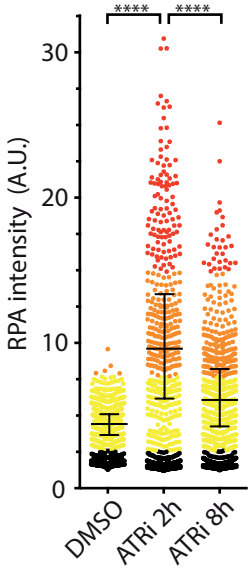
E


TUNEL
γH2AX
DAPI
ATRi (10 μM)

F


DMSO (8h)
G1: 43%
S: 37.7%
G2-M: 18.6%
Count
PI
ATRi (8h)
G1: 43.5%
S: 37.2%
G2-M: 19%
Count
PI

G


****
****
RPA intensity (A.U.)
30
20
10
0
DMSO
ATRi 2h
ATRi 8h

H

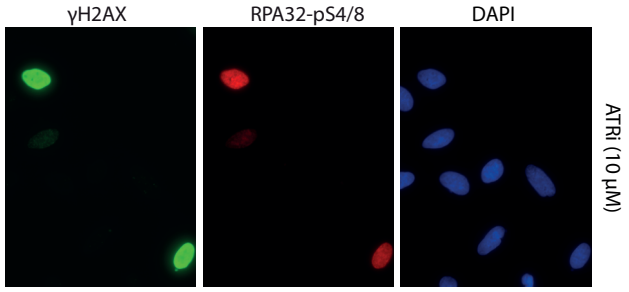

γH2AX
RPA32-pS4/8
DAPI
ATRi (10 μM)

Fig. S2

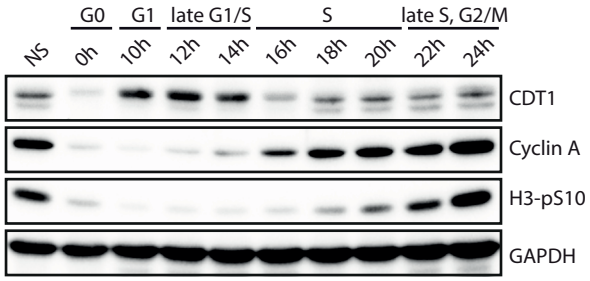
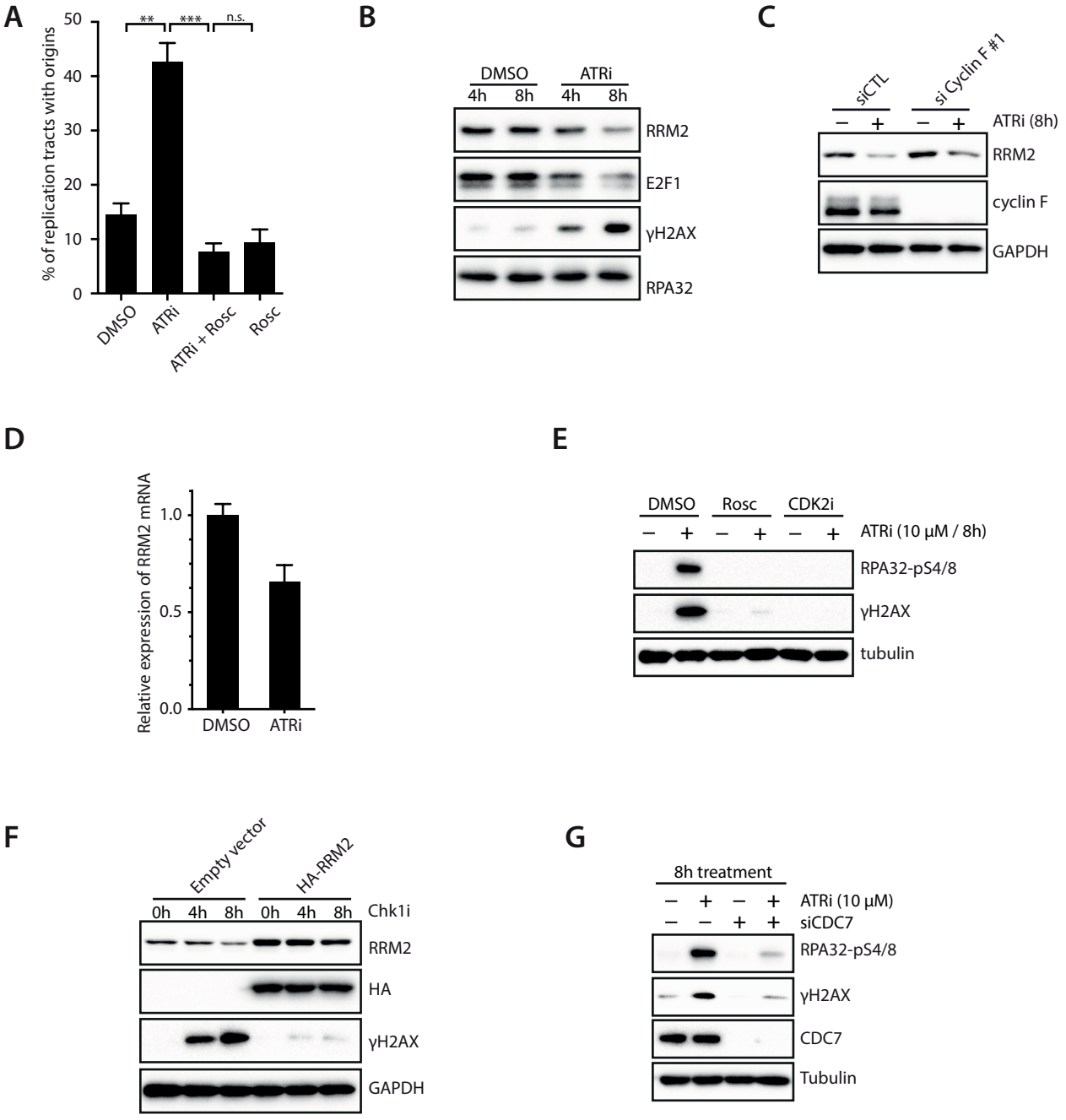
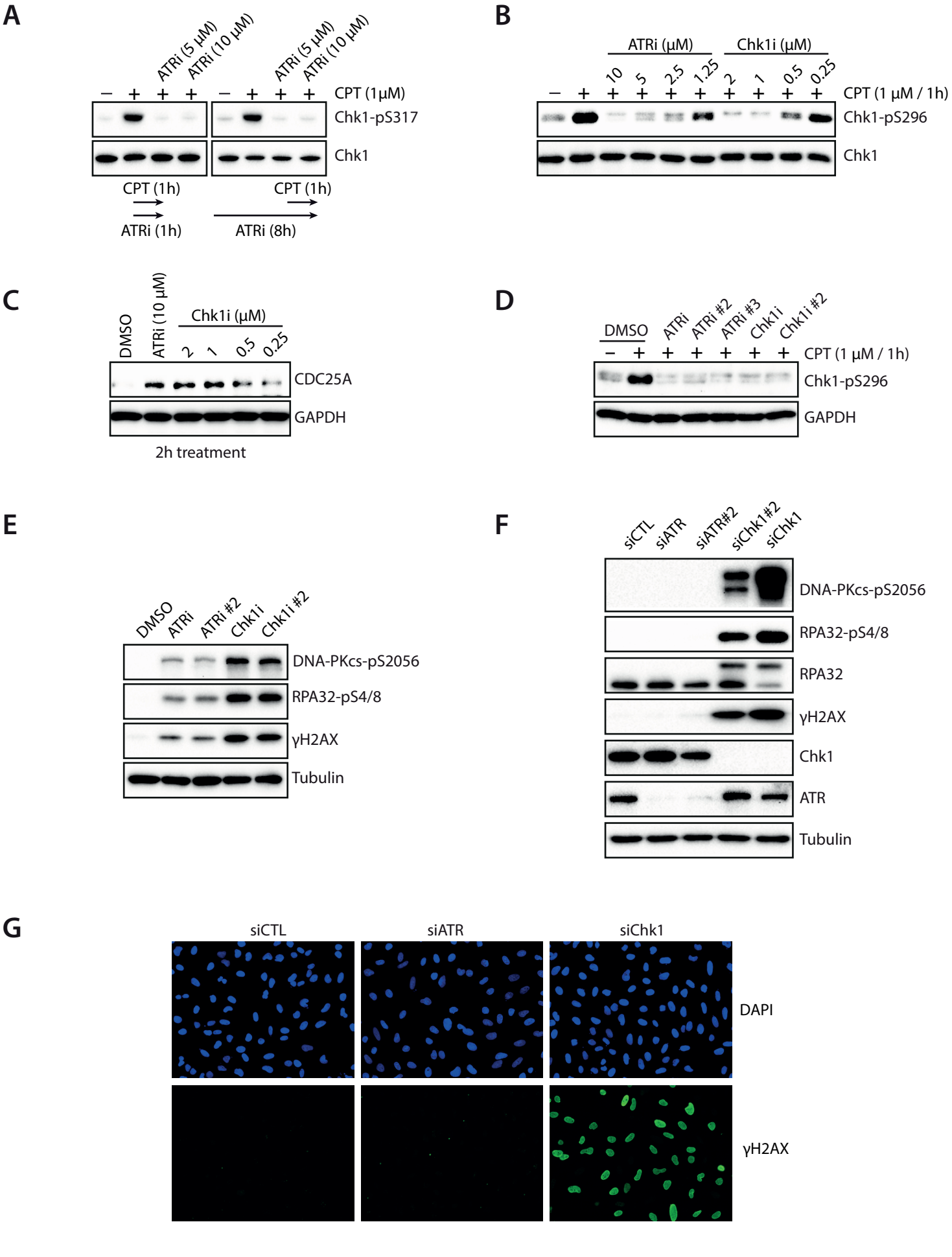
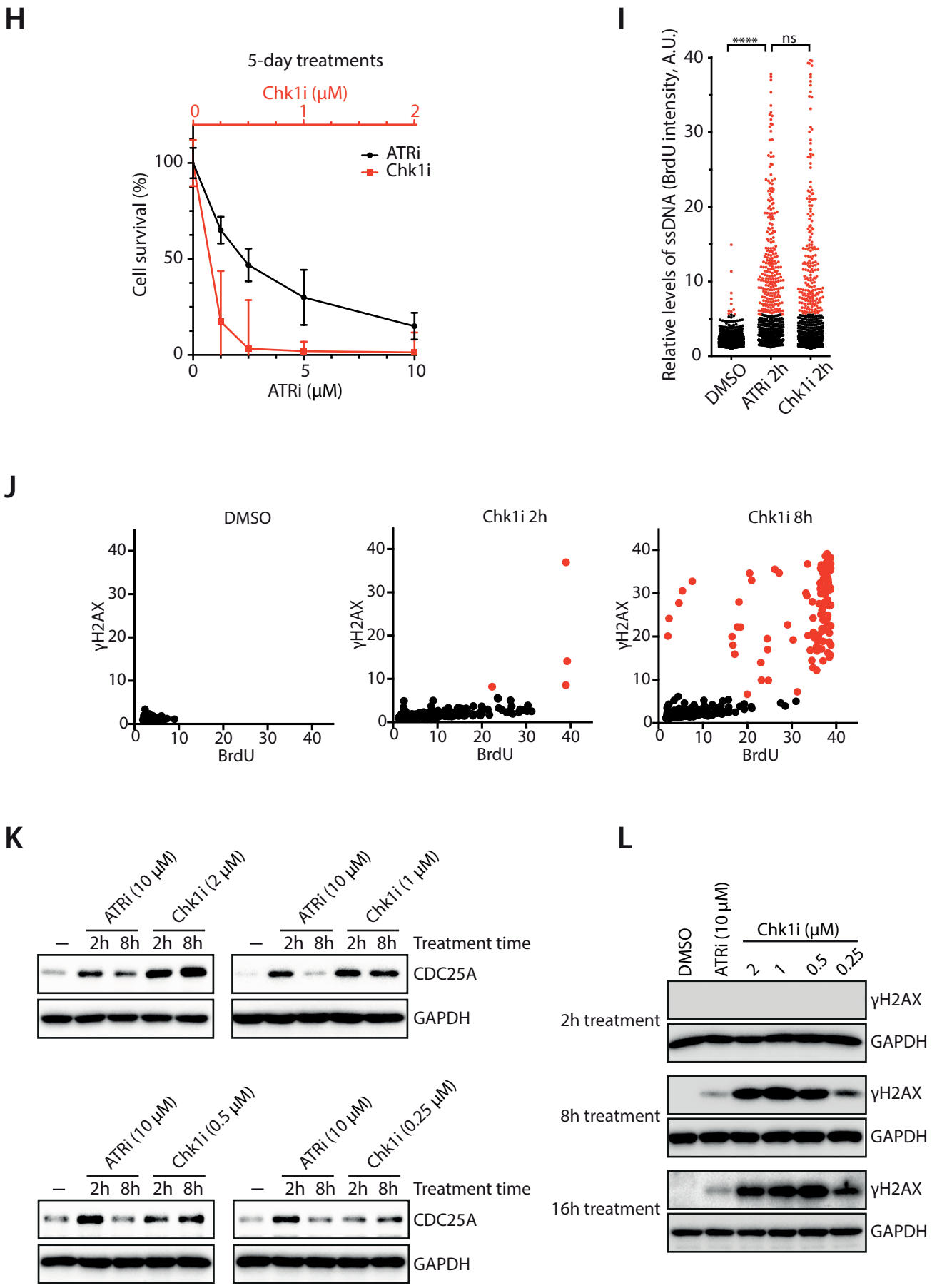

G0
G1
late G1/S
S
late S, G2/M
NS
0h
10h
12h
14h
16h
18h
20h
22h
24h
CDT1
Cyclin A
H3-pS10
GAPDH

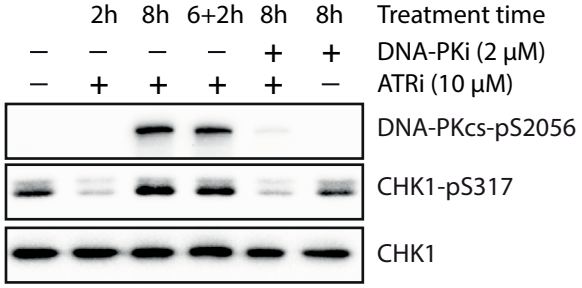
Fig. S3


A
% of replication tracts with origins
50
40
30
20
10
0
**
***
n.s.
DMSO
ATRi
ATRi + Rosc
Rosc
B
DMSO
ATRi
4h
8h
4h
8h
RRM2
E2F1
γH2AX
RPA32
C
siCTL
si Cyclin F #1
– + – +
ATRi (8h)
RRM2
cyclin F
GAPDH
D
Relative expression of RRM2 mRNA
1.0
0.5
0.0
DMSO
ATRi
E
DMSO
Rosc
CDK2i
– + – + – +
ATRi (10 μM / 8h)
RPA32-pS4/8
γH2AX
tubulin
F
Empty vector
HA-RRM2
0h 4h 8h 0h 4h 8h
Chk1i
RRM2
HA
γH2AX
GAPDH
G
8h treatment
– + – +
ATRi (10 μM)
– – + +
siCDC7
RPA32-pS4/8
γH2AX
CDC7
Tubulin

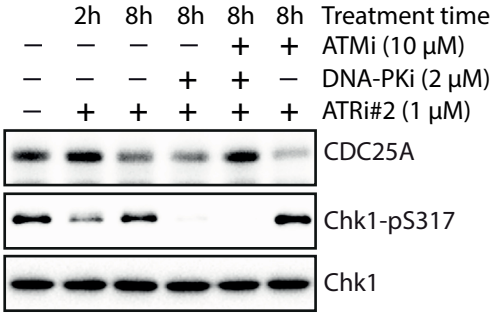

A
ATRi (5 μM)
ATRi (10 μM)
ATRi (5 μM)
ATRi (10 μM)
– + + + – + + + CPT (1μM)
Chk1-pS317
Chk1
CPT (1h)
CPT (1h)
ATRi (1h)
ATRi (8h)
B
ATRi (μM)
Chk1i (μM)
10 5 2.5 1.25 2 1 0.5 0.25
– + + + + + + + + + CPT (1 μM / 1h)
Chk1-pS296
Chk1
C
DMSO
ATRi (10 μM)
Chk1i (μM)
2 1 0.5 0.25
CDC25A
GAPDH
2h treatment
D
DMSO
ATRi
ATRi #2
ATRi #3
Chk1i
Chk1i #2
– + + + + + + CPT (1 μM / 1h)
Chk1-pS296
GAPDH
E
DMSO
ATRi
ATRi #2
Chk1i
Chk1i #2
DNA-PKcs-pS2056
RPA32-pS4/8
γH2AX
Tubulin
F
siCTL
siATR
siATR#2
siChk1#2
siChk1
DNA-PKcs-pS2056
RPA32-pS4/8
RPA32
γH2AX
Chk1
ATR
Tubulin
G
siCTL
siATR
siChk1
DAPI
γH2AX


H
5-day treatments
Chk1i (μM)
0
1
2
ATRi
Chk1i
Cell survival (%)
100
50
0
0
5
10
ATRi (μM)
I
****
ns
Relative levels of ssDNA (BrdU intensity, A.U.)
40
30
20
10
0
DMSO
ATRi 2h
Chk1i 2h
J
DMSO
Chk1i 2h
Chk1i 8h
γH2AX
BrdU
K
ATRi (10 μM)
Chk1i (2 μM)
Chk1i (1 μM)
Chk1i (0.5 μM)
Chk1i (0.25 μM)
–
2h
8h
Treatment time
CDC25A
GAPDH
L
DMSO
ATRi (10 μM)
Chk1i (μM)
2
1
0.5
0.25
2h treatment
8h treatment
16h treatment
γH2AX
GAPDH

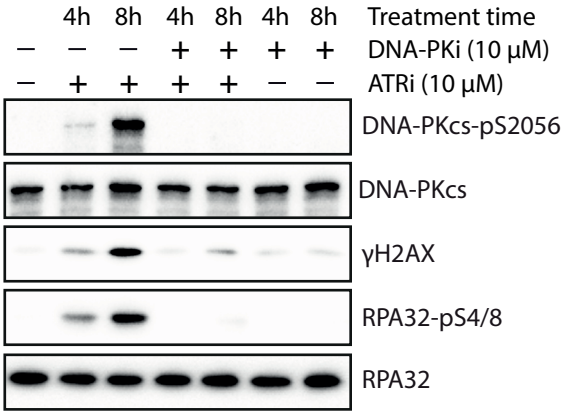
A


2h 8h 6+2h 8h 8h Treatment time
– – – – + + DNA-PKi (2 μM)
– + + + + – ATRi (10 μM)
DNA-PKcs-pS2056
CHK1-pS317
CHK1

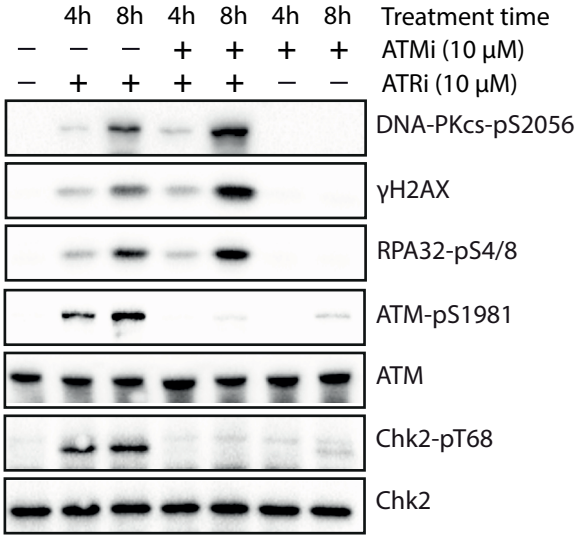
B


2h 8h 8h 8h 8h Treatment time
– – – – + + ATMi (10 μM)
– – – + + – DNA-PKi (2 μM)
– + + + + + ATRi#2 (1 μM)
CDC25A
Chk1-pS317
Chk1

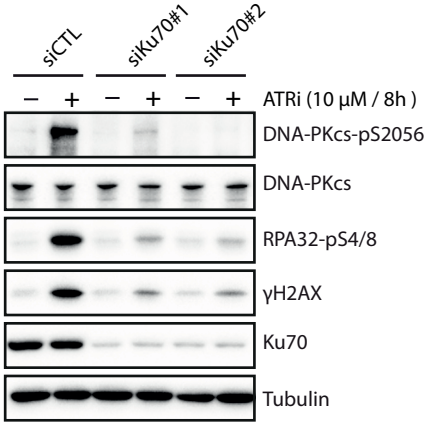
C


4h 8h 4h 8h 4h 8h Treatment time
– – – + + + + DNA-PKi (10 μM)
– + + + + – – ATRi (10 μM)
DNA-PKcs-pS2056
DNA-PKcs
γH2AX
RPA32-pS4/8
RPA32

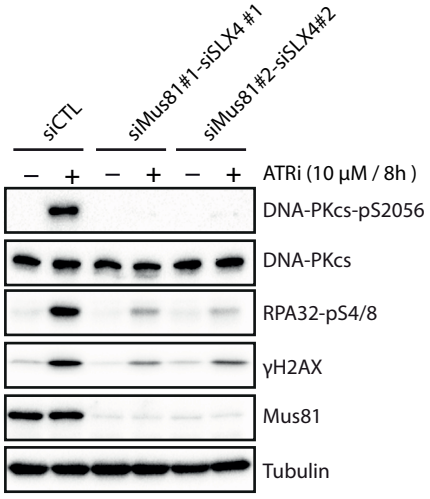
D


4h 8h 4h 8h 4h 8h Treatment time
– – – + + + + ATMi (10 μM)
– + + + + – – ATRi (10 μM)
DNA-PKcs-pS2056
γH2AX
RPA32-pS4/8
ATM-pS1981
ATM
Chk2-pT68
Chk2

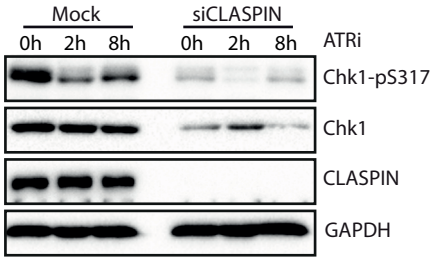
E


siCTL
siKu70#1
siKu70#2
– + – + – + ATRi (10 μM / 8h)
DNA-PKcs-pS2056
DNA-PKcs
RPA32-pS4/8
γH2AX
Ku70
Tubulin

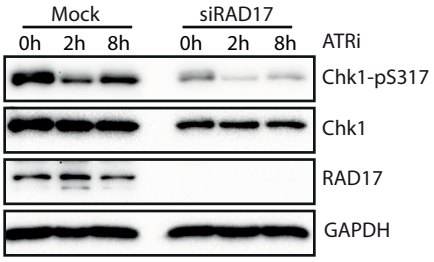
F


siCTL
siMus81#1-siSLX4 #1
siMus81#2-siSLX4#2
– + – + – + ATRi (10 μM / 8h)
DNA-PKcs-pS2056
DNA-PKcs
RPA32-pS4/8
γH2AX
Mus81
Tubulin

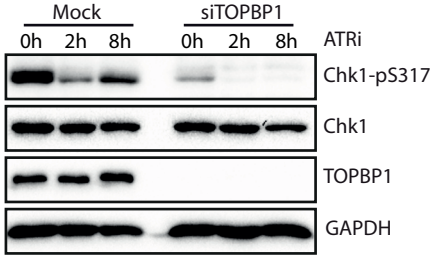
G


Mock
siCLASPIN
0h 2h 8h 0h 2h 8h
ATRi
Chk1-pS317
Chk1
CLASPIN
GAPDH

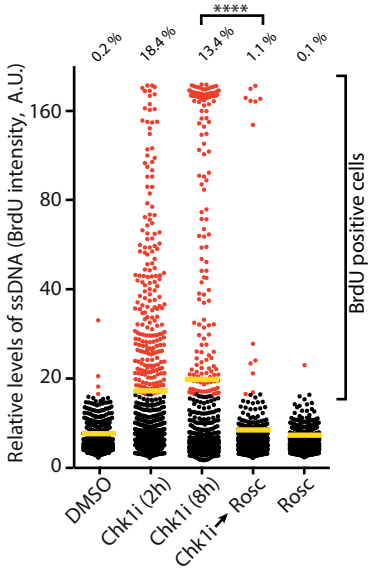
H


Mock
siRAD17
0h 2h 8h 0h 2h 8h
ATRi
Chk1-pS317
Chk1
RAD17
GAPDH

I


Mock
siTOPBP1
0h 2h 8h 0h 2h 8h
ATRi
Chk1-pS317
Chk1
TOPBP1
GAPDH

J


****
0.2 %
18.4 %
13.4 %
1.1 %
0.1 %
Relative levels of ssDNA (BrdU intensity, A.U.)
0
20
40
80
160
BrdU positive cells
DMSO
Chk1i (2h)
Chk1i (8h)
Chk1i → Rosc
Rosc

K

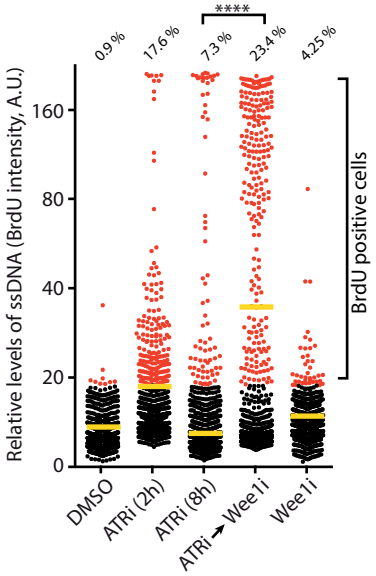
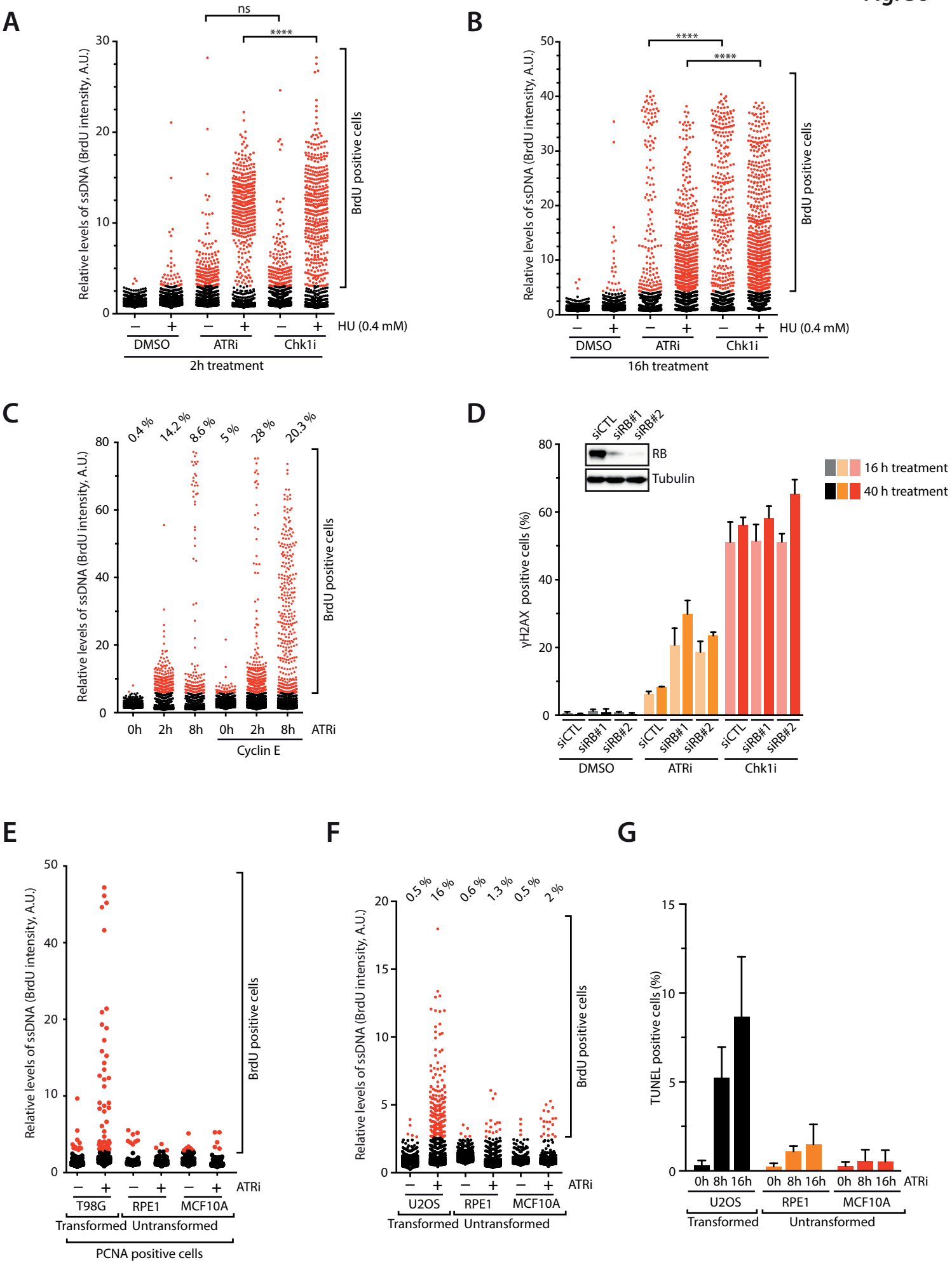
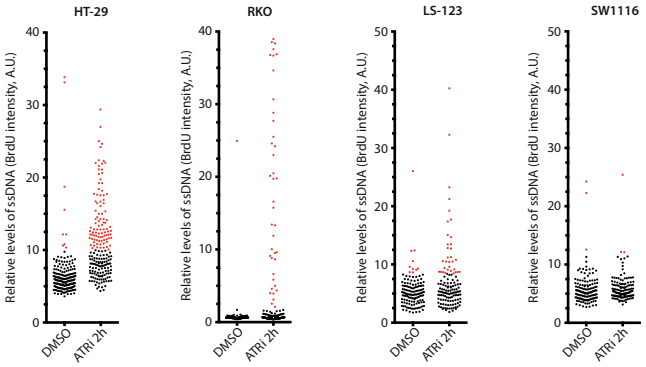

****
0.9 %
17.6 %
7.3 %
23.4 %
4.25 %
Relative levels of ssDNA (BrdU intensity, A.U.)
0
20
40
80
160
BrdU positive cells
DMSO
ATRi (2h)
ATRi (8h)
ATRi → Wee1i
Wee1i

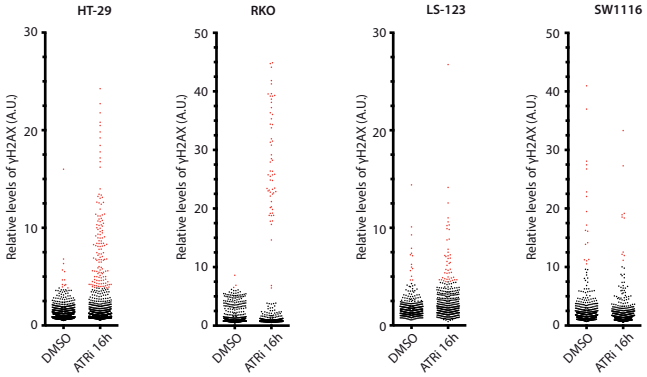
Fig. S6


A
ns
****
Relative levels of ssDNA (BrdU intensity, A.U.)
BrdU positive cells
HU (0.4 mM)
DMSO
ATRi
Chk1i
2h treatment
B
****
****
16h treatment
C
0.4 %
14.2 %
8.6 %
5 %
28 %
20.3 %
0h
2h
8h
ATRi
Cyclin E
D
siCTL
siRB#1
siRB#2
RB
Tubulin
γH2AX positive cells (%)
16 h treatment
40 h treatment
E
T98G
RPE1
MCF10A
Transformed
Untransformed
PCNA positive cells
F
0.5 %
16 %
0.6 %
1.3 %
0.5 %
2 %
U2OS
G
TUNEL positive cells (%)

H



I



J

	0	0.625	1.25	2.5	5
					ATRi (μM)
Colo-320-HSR	100%	103%	78%	41%	17%
SW620	100%	104%	97%	71%	47%
HT-29	100%	94%	97%	66%	24%
RKO	100%	108%	111%	83%	69%
HCT-15	100%	104%	100%	80%	53%
HT-55	100%	113%	113%	97%	61%
LS-123	100%	130%	134%	121%	89%
NCI-H747	100%	118%	115%	116%	103%
SNU-61	100%	106%	106%	120%	98%
SW1116	100%	129%	146%	133%	107%

	0	0.625	1.25	2.5	5
					ATRi (μM) + 0.05 mM HU
Colo-320-HSR	86%	6%	1%	1%	1%
SW620	97%	35%	7%	2%	1%
HT-29	107%	97%	80%	20%	2%
RKO	95%	95%	81%	29%	5%
HCT-15	101%	98%	93%	56%	18%
HT-55	89%	105%	87%	55%	26%
LS-123	93%	106%	85%	54%	30%
NCI-H747	89%	125%	114%	91%	32%
SNU-61	80%	84%	94%	105%	46%
SW1116	117%	123%	116%	111%	65%

100% 50% 0% Cells survival

K

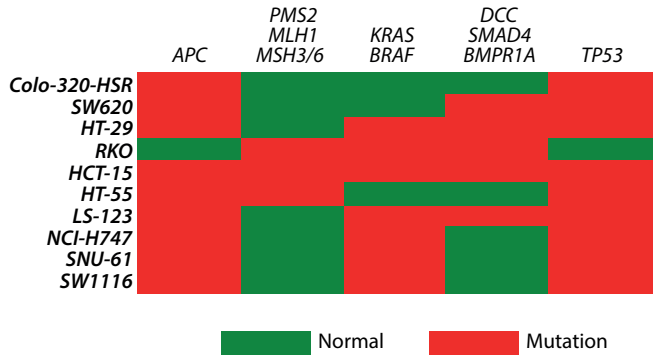
	0	0,625	1,25	2,5	5	ATRi (μM)
HT29	1%	2%	3%	1%	7%	
Colo320-HSR	3%	1%	4%	1%	5%	
SW620	1%	0%	1%	3%	4%	
RKO	3%	1%	2%	1%	4%	
HCT-15	3%	1%	3%	1%	5%	
LS-123	2%	1%	1%	2%	5%	
HT-55	3%	1%	4%	2%	1%	
NCI-H747	1%	1%	3%	6%	1%	
SNU-61	3%	4%	3%	1%	3%	
SW1116	6%	4%	3%	7%	6%	

	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%	

L

	BrdU positive cells (36 h labeling before denaturation)	S-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%

M



N

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

Supplemental Figures Legends

Fig. S1, related to Fig. 1. 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 μM 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). The fractions of S-phase cells were determined by EdU labeling or PCNA immunostaining. **E.** U2OS cells were treated with ATRi for 8 h, and analyzed by TUNEL assay and γH2AX immunostaining. Note that a small fraction of γH2AX-posistive cells were not strongly TUNEL-positive, indicating that γH2AX 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 γH2AX and pRPA32 immunostaining.

Fig. S2, related to Fig. 2. 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.

Fig. S3, related to Fig. 3. 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 γH2AX 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 γH2AX 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 γH2AX 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, γH2AX and CDC7 were analyzed by Western.

Fig. S4, 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 indictor of Chk1 activity. **C.** U2OS cells were treated with DMSO, 10 μM ATRi, or increasing concentrations of Chk1i. The stabilization of CDC25A was analyzed as an indicator of Chk1 inhibition. **D.** U2OS cells were treated with HU in the presence of DMSO, ATRi (10 μM VE-821), ATRi#2 (1 μM AZ20), ATRi#3 (10 μM EPT-46464), Chk1i (2 μM MK-8776), or Chk1i#2 (0.3 μM UCN-01). **E.** U2OS

cells were treated with DMSO and the indicated inhibitors for 8 h. Levels of pDNA-PK, pRPA32, and γH2AX 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 γH2AX 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 as indicated. BrdU and γH2AX 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 μM ATRi or the indicated concentrations of Chk1i. Levels of CDC25A were analyzed at 2 and 8 h in (K). Levels of γH2AX were analyzed at 2, 8, and 16 h in (L).

Fig. S5, related to Fig. 5. 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, γH2AX, 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, γH2AX, 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-**

H. 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. S6, related to Fig. 6. 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 γH2AX-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 γH2AX were analyzed by native BrdU staining (H) and γH2AX immunostaining (I), respectively. Fractions of ATRi-treated cells displaying higher levels of ssDNA or γH2AX 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., 2013)].

Supplemental Methods

Antibodies

The antibodies used in this study include: γH2AX monoclonal antibody (Cell Signaling or EMD Millipore), RPA32 monoclonal antibody (Thermos), CDC7 monoclonal antibody (Abcam), CDC25A monoclonal antibody (Santa Cruz), DNA-PKcs monoclonal antibody (Cell Signaling), MUS81 monoclonal antibody (Abcam), KU70 monoclonal antibody (GeneTex), Chk1 monoclonal antibody (Santa Cruz), Chk2 monoclonal antibody (Upstate), BrdU monoclonal antibody (BD Biosciences), H3 polyclonal antibody (Abcam), PCNA polyclonal antibody (Abcam), RPA32 pS4/8 polyclonal antibody (Bethyl), Tubulin polyclonal antibody (Cell Signaling), DNA-PKcs pS2056 polyclonal antibody (Abcam), ATM polyclonal antibody (Bethyl), ATM pS1981 polyclonal antibody (Abcam), Chk2 pT68 polyclonal antibody (Cell Signaling), Chk1 pS296 polyclonal antibody (Cell Signaling), Chk1 pS317 polyclonal antibody (Cell Signaling), RB monoclonal antibody (Cell Signaling), Cyclin E monoclonal antibody (Santa Cruz), Cyclin A polyclonal antibody (Santa Cruz), CDT1 polyclonal l antibody (Santa Cruz), H3 pS10 polyclonal antibody (EMD Millipore), GAPDH polyclonal antibody (EMD Millipore),

RRM2 monoclonal antibody (Abnova), ATR polyclonal antibody (Bethyl), E2F1 polyclonal antibody (Santa Cruz), Cyclin F polyclonal antibody (Santa Cruz), RAD17 polyclonal antibody (Abcam), Claspin polyclonal antibody (Bethyl), TopBP1 polyclonal antibody (Bethyl), and HA monoclonal antibody (Covance).

RNA interference

siRNA transfections were done by reverse transfection with Lipofectamine RNAiMax (Invitrogen). siATR#1-2, siChk1#1-2, siRPA70, siMUS81#1-2, siSLX4#1-2, siKU70#2, siClaspin, siTopBP1, siRAD17, and siCyclin F (Dharmacon or Invitrogen) were transfected at 20 nM. siDNA-PK, siRB#1-2 and siKU70#1 (Silencer® Select siRNAs from Ambion) were transfected at 5 nM. Cells were treated with various drugs 48 h after transfection. The sequences of the siRNAs used in this study are:

siATR#1: CCUCCGUGAUGUUGCUUGA
siATR#2: CCCGCGUUGGCGUGGUUGA
siChk1#1: GCAACAGUUAUUUCGGUAUA
siChk1#2: GCGUGCCGUAGACUGUCCA
siKU70#1: GACAUAUCCUUGUUCUACA
siKU70#2: GGCCUUGGAUUUGAUGGAGCCGGAA
siMUS81#1: CGCGCUUCGUAUUUCAGAA
siMUS81#2: CAGCCCUGGUGGAUCGAUA
siSLX4#1: GCUACCCGGACACUUGUCAUUGUUA
siSLX4#2: UCUGCUUUCUAGGGUGGCAUUUGGA
siDNA-PKcs: GCGUUGGAGUGCUACAACA
siRB#1: GAACAGGAGUGCACGGAUA
siRB#2: CAACCCAGCAGUUCGAUAU
siCDC7: GCAGUCAAAGACUGUGGAU
siClaspin: GGAAAGAAAGGCAGCCAGA
siTopBP1: GUGGUUGUAACAGCGCAUCUU
siRAD17: AACAGACUGGGUUGACCCAUC
siCyclin F: GGAUAAACCUAUGCAUACA

Use of inhibitors and chemicals

The kinase inhibitors used in this study are listed in the Method section. 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. Other chemicals used in this study include: Camptothecin (1 μM), BrdU (10 μM), 5-chlorodeoxyuridine (CldU), 5-iododeoxyuridine (IdU), tetracycline (4 μg/ml), MG132 (10 μM), Cycloheximide (50 μg/ml), and hydroxyurea (as indicated).

Cells synchronization

T98G were cultured in DMEM supplemented with 0.02 % FBS and 1 % penicillin/streptomycin for 48 h. Subsequently, cells were released into fresh DMEM media containing 10 % FBS and analyzed at the indicated times.

Immunofluorescence

To visualize ssDNA, cells were cultured in 10 μM BrdU for 36 h before the treatments with various inhibitors. To measure DNA synthesis, cells were pulse-labeled with 10 μM EdU for 15 min and processed with the Click-iT EdU Alexa Fluor 488 Imaging Kit according to manufacturer's instructions (Life Technologies). For immunostaining, cells were incubated in pre-extraction buffer (10 mM PIPES pH 6.8, 100 mM $NaCl_2$, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA and 0.2% Triton X-100) for 5 min, fixed with 3% paraformaldehyde in 1xPBS for 20 min, washed with 1xPBS, and fixed with cold methanol (-20°C) for 20 min. Subsequently, cells were washed with 1xPBS, treated with cold acetone (4°C) for 30 sec, washed a second time with 1xPBS, and blocked in PBS-T (1xPBS containing 0.05 % Tween-20) containing 2% BSA for 1 h. Cells were then incubated with the primary antibody diluted in 1xPBS containing 2% BSA at room temperature for 2 h. Coverslips were washed three times with PBS-T before the 1-hr incubation with the appropriate secondary antibodies conjugated to fluorophores (Cy3 or Alexa-488). After three washes with 1xPBS, cells were stained with DAPI. The images were captured using a Nikon 90i microscope.

Quantitative fluorescence analysis of individual cells

Images of 1,000 to 5,000 randomly selected cells were acquired using a Nikon Eclipse 90i microscope with a Nikon Plan Apo Lambda 10X objective. Signals of BrdU, EdU, RPA32, γH2AX, and DAPI of individual cells were quantified using the Fiji software. The staining signals of each nucleus were measured in DAPI-stained area. To measure DNA content, the mean intensity of DAPI staining of each cell was multiplied to the size of DAPI-stained area.

To quantify the induction of ssDNA and γH2AX by ATRi in various colorectal cancer cell lines, we manually set a threshold for each cell line above the majority (98-98.5%) of DMSO-treated control cells. The ATRi-treated cells that displayed ssDNA or γH2AX above these thresholds were scored as positive.

Chromatin fractionation

Fractionation of cell extracts was performed essentially as described by Mendez and Stillman (Mendez and Stillman, 2000). A total of $\sim 3 \times 10^6$ U2OS cells were washed with 1xPBS and resuspended in 200 µl of solution A (10 mM HEPES at pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 10 mM NaF, 1 mM Na_2VO_3 and protease inhibitors). Triton X-100 was added to a final concentration of 0.1%, and the cells were left on ice for 5 min. Cytoplasmic proteins (S1) were separated from nuclei by low-speed centrifugation (1400xg for 4 min). Isolated nuclei were washed three times with solution A and lysed in 200 µl of solution B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT and protease inhibitors). After a 30 min incubation at 4°C, soluble nuclear proteins (S2) were separated from chromatin by centrifugation (1700xg for 4 min) and combined with S1 (soluble fraction). Isolated chromatin was washed three times with solution B and collected by centrifugation (1700xg for 4 min). Finally, chromatin was

resuspended in 200 µl of Laemmli buffer and sheared by sonication. The soluble and chromatin fractions were analyzed by Western using various antibodies.

DNA fiber assay

Cells were labeled with CldU (100 µM) for 30 min and then with IdU (250 µM) for 30 min in the presence or absence of various inhibitors. DNA fibers were spread as described (Jackson and Pombo, 1998). Briefly, cells suspended in 1xPBS (2.5 µl of suspension at ~10^6 cell/ml) were spotted on to a glass slide and allowed to dry. Cells were subsequently treated with 7.5 µl of spreading buffer (0.5% SDS, 200 mM Tris-HCl pH7.4, 50 mM EDTA) for 3-5 min. Slides were tilted (~15°) to allow cell lysates to slowly run down the slide. The DNA on slides was air dried, fixed in methanol-acetic acid (3:1) for 2 min, and dried overnight at room temperature. Fixed DNA was denatured with 2.5 N HCl for 30 min at room temperature. Then DNA was washed 3 times for 5 min with PBS-T (PBS + 0.05% Tween-20) and blocked in PBS-T containing 2% BSA for 30 min at 37°C. DNA fibers were incubated with rat anti-BrdU (OBT0030, AbDSerotec) (1:200) and mouse anti-BrdU (BD Biosciences) (1:20) antibodies for 1 h at 37°C, followed by the incubation with Alexa-488-conjugated anti-mouse (1:100) and Cy3-conjugated anti-rat (Jackson ImmunoResearch) (1:100) secondary antibodies for 30 min 37°C. Slides were washed 3 times with PBS-T containing 1% BSA and mounted using VectaShield (Vector Labs). DNA fibers were imaged at 60X with a Nikon 90i microscope.

TUNEL assay

Cells were incubated in pre-extraction buffer (10 mM PIPES pH 6.8, 100 mM $NaCl_2$, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA and 0.2% Triton X-100) for 5 min. The TUNEL-positive cells were detected by using the ApopTag® Fluorescein Direct *In situ* Apoptosis Detection Kit as described by the supplier (EMD Millipore).

Cell viability assay

Cells were seeded in 96-well plates at a density of 500 cells per well. On the following day, cells were treated with the indicated concentration of ATRi in the presence or absence of HU (0.05 mM). Cell viability was measured 6 days after the treatment using CellTiter-Glo Luminescent reagent (Promega).

Flow cytometry analysis

To analyze the cell-cycle distribution of cells, cells were pulse-labeled with 10 μM EdU for 30 min and then processed using the Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay Kit according to manufacturer's instructions (Life Technologies). An additional incubation with γH2AX-FITC antibody (EMD Millipore) was performed according to manufacturer's instructions when indicated. Data acquisition was performed on a FACS LSRII apparatus equipped with the FACS Diva software (BD Biosciences). Further analysis was carried out using the Kaluza software (Beckman Coulter).

Quantitative RT-PCR

Total RNA was extracted from U2OS cells using RNeasy Mini kit (Qiagen) according to the manufacturer's instruction. Following extraction, total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). RT products were analyzed by real time qPCR using SYBR Green in an ABI PRISM 7500 sequence detection system (Applied Bio-system). For each sample tested, the levels of *RRM2* mRNA were normalized to the levels of *GAPDH* mRNA. When relative levels of *RRM2* mRNA were compared between DMSO and ATRi treated cells, the levels in DMSO treated cells were defined as 1. The sequences of the PCR primers are: *GAPDH*-forward, AGGTCGGTGTGAACGGATTTG ; *GAPDH*-reverse,

TGTAGACCATGTAGTTGAGGTCA; *RRM2*-forward, TTCTTTGCAGCA-AGCGATGG ; *RRM2*-reverse, TTGGCTAAATCGCTCCACCA.

Supplemental reference:

Ahmed, D., Eide, P.W., Eilertsen, I.A., Danielsen, S.A., Eknaes, M., Hektoen, M., Lind, G.E., and Lothe, R.A. (2013). Epigenetic and genetic features of 24 colon cancer cell lines. Oncogenesis *2*, e71.

Jackson, D.A., and Pombo, A. (1998). Replicon clusters are stable units of chromosome structure: evidence that nuclear organization contributes to the efficient activation and propagation of S phase in human cells. J Cell Biol *140*, 1285-1295.

Mendez, J., and Stillman, B. (2000). Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis. Mol Cell Biol *20*, 8602-8612.