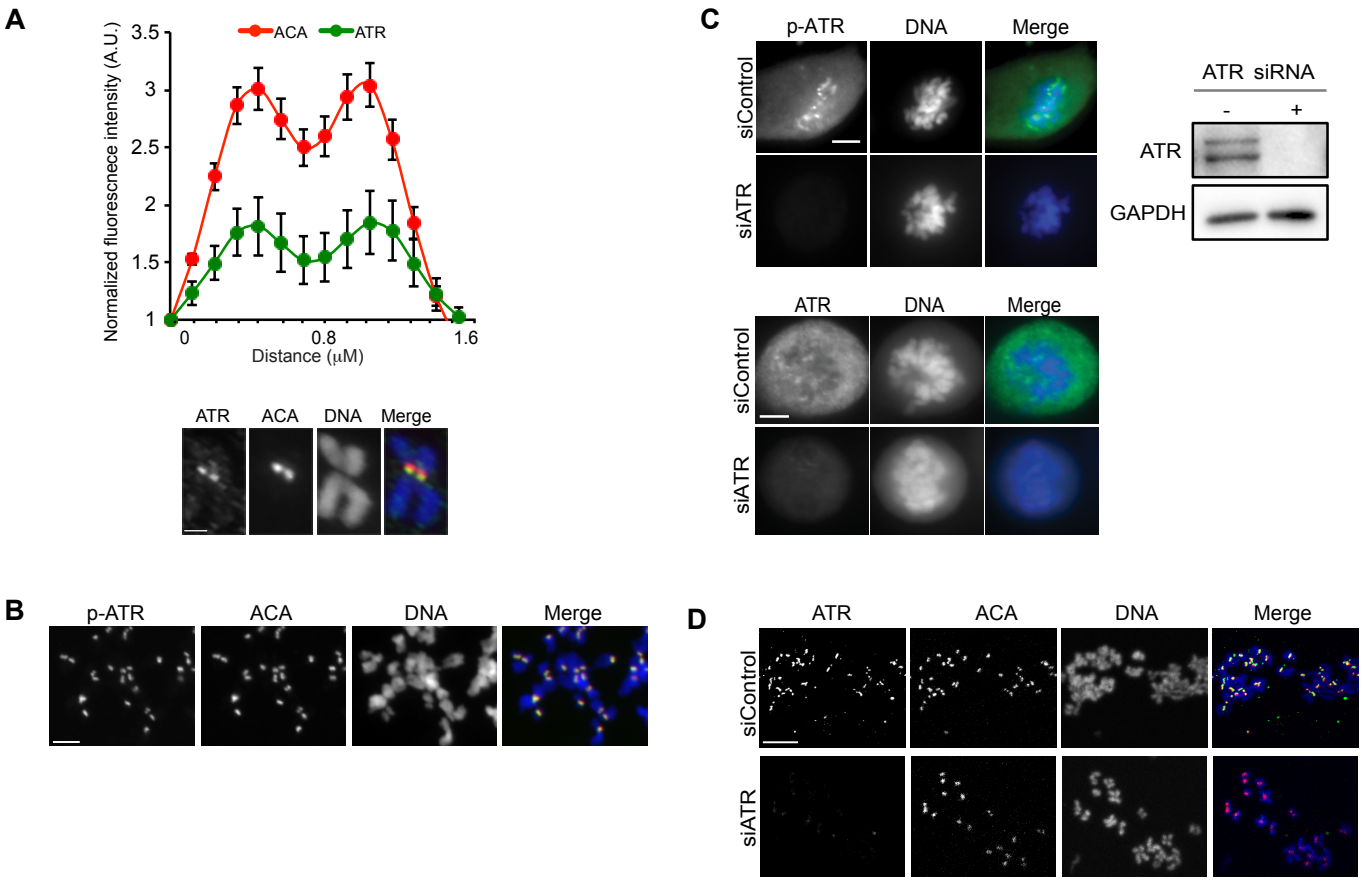
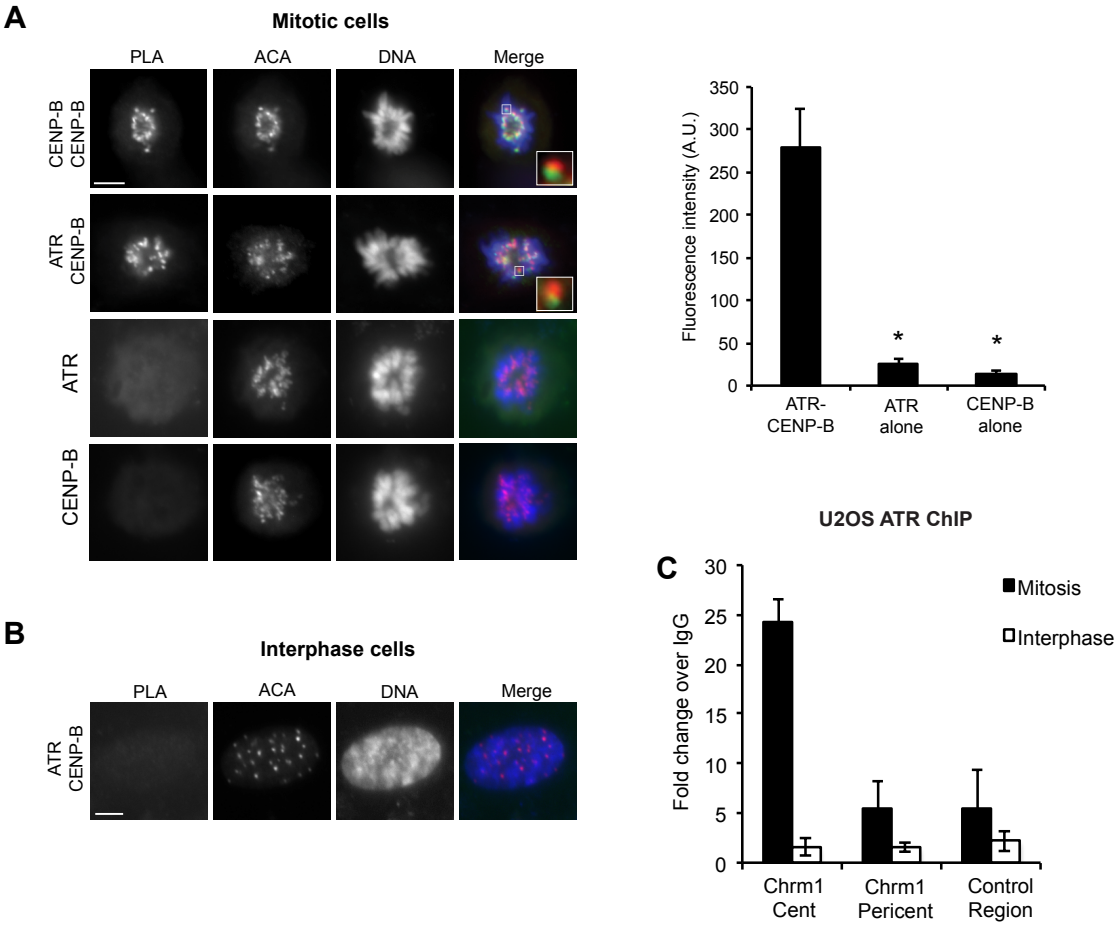
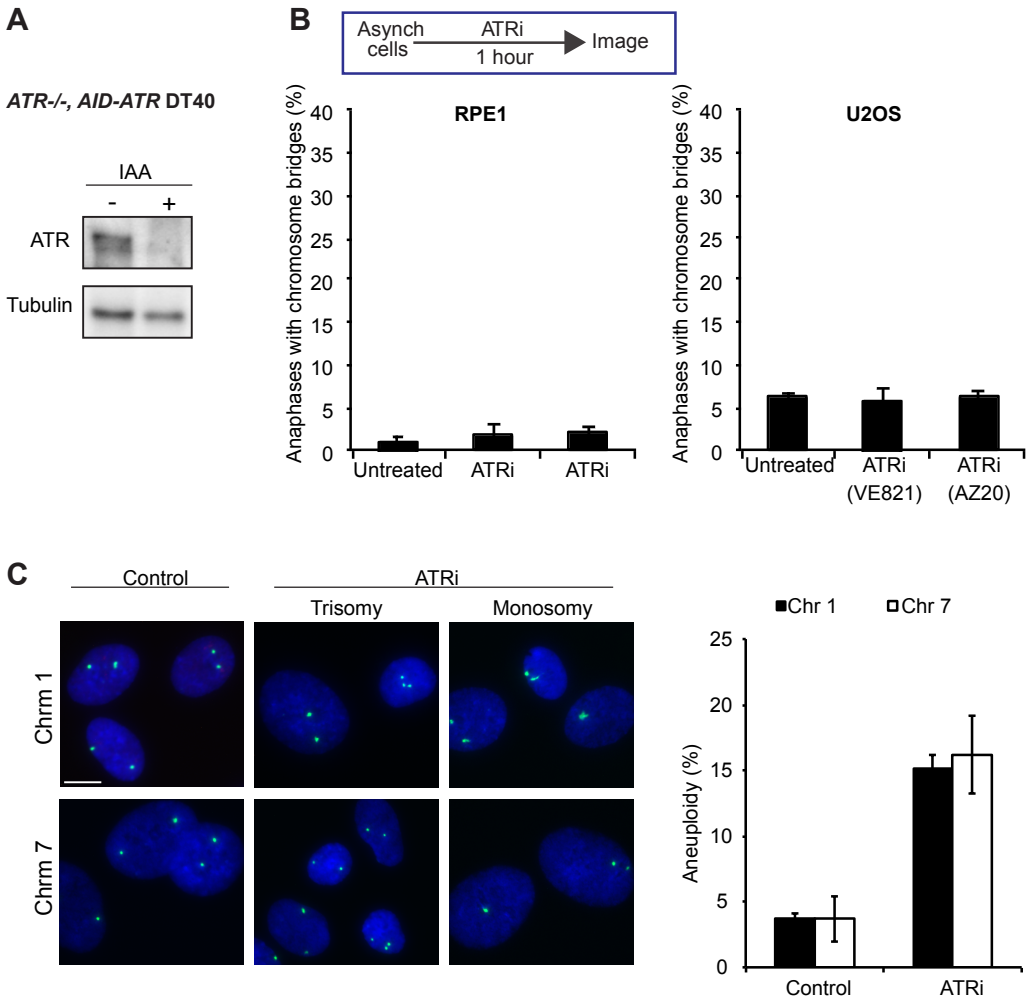
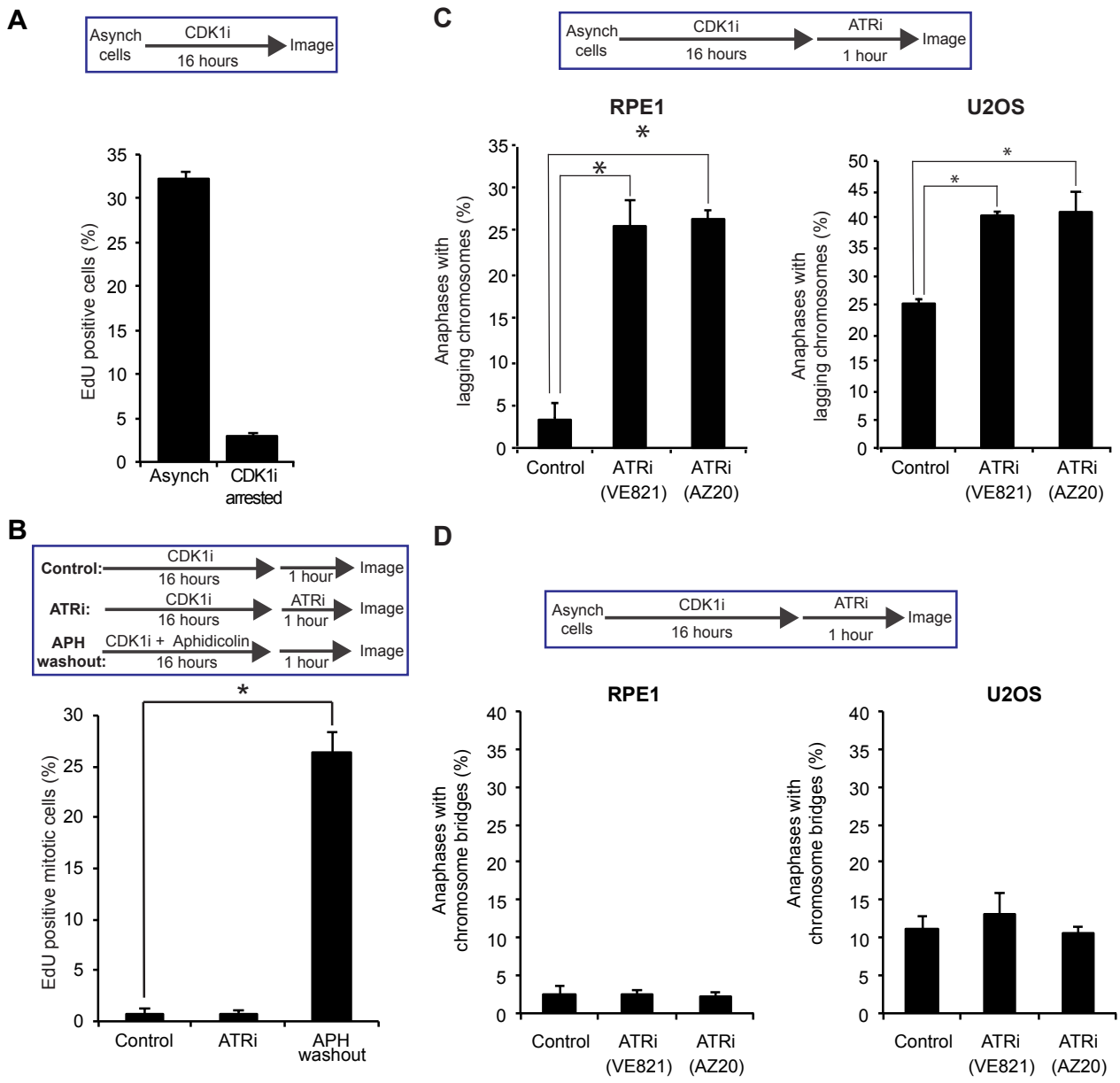
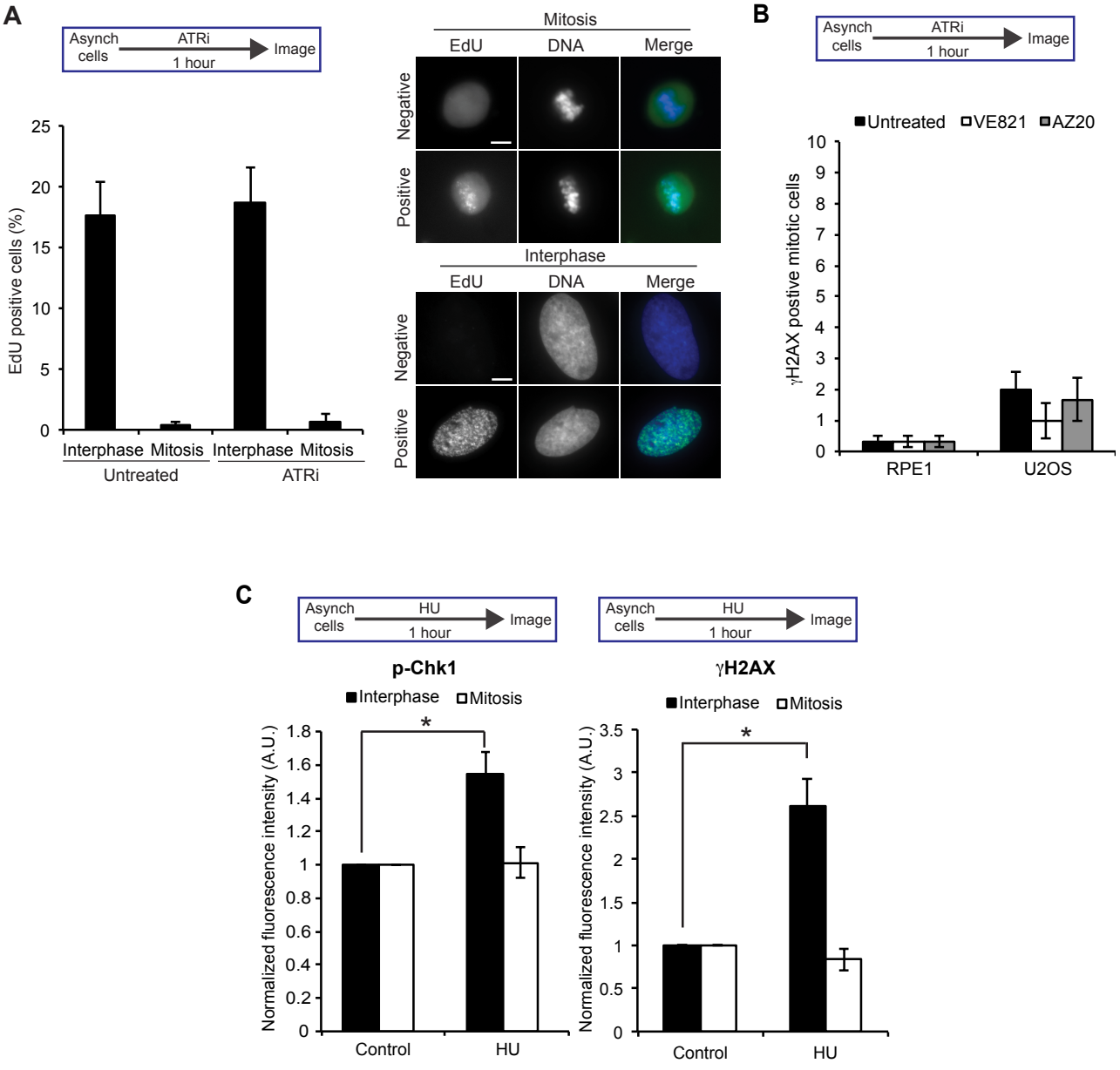
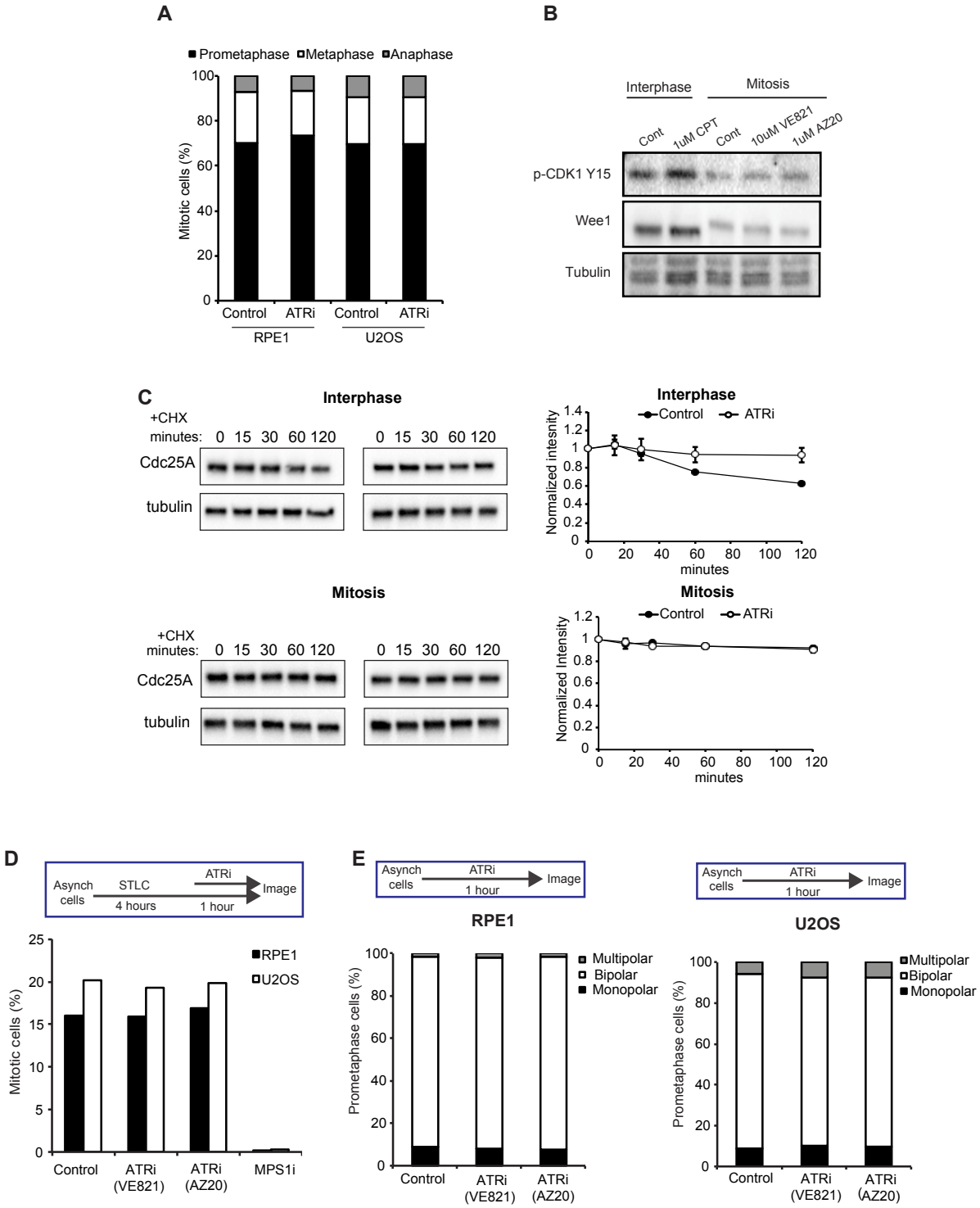

A
ACA
ATR
Normalized fluorescnece intensity (A.U.)
3.5
3
2.5
2
1.5
1
0
0.8
1.6
Distance (μM)
ATR
ACA
DNA
Merge
B
p-ATR
ACA
DNA
Merge
C
p-ATR
DNA
Merge
siControl
siATR
ATR siRNA
-
+
ATR
GAPDH
ATR
DNA
Merge
siControl
siATR
D
ATR
ACA
DNA
Merge
siControl
siATR

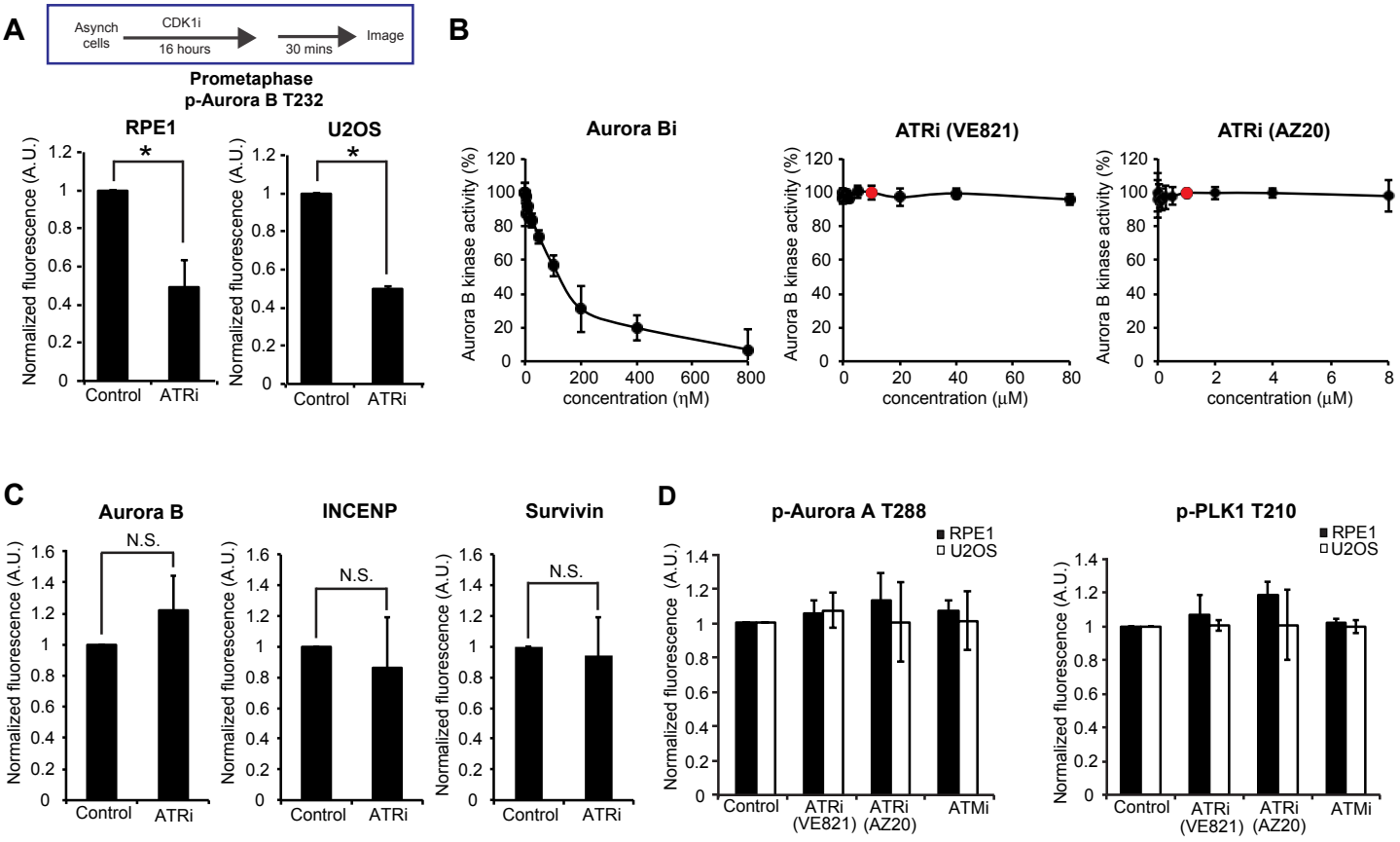

A
Mitotic cells
PLA
ACA
DNA
Merge
CENP-B
CENP-B
ATR
CENP-B
ATR
CENP-B
B
Interphase cells
PLA
ACA
DNA
Merge
ATR
CENP-B
Fluorescence intensity (A.U.)
350
300
250
200
150
100
50
0
*
*
ATR-
CENP-B
ATR
alone
CENP-B
alone
C
U2OS ATR ChIP
Mitosis
Interphase
Fold change over IgG
30
25
20
15
10
5
0
Chrm1
Cent
Chrm1
Pericent
Control
Region

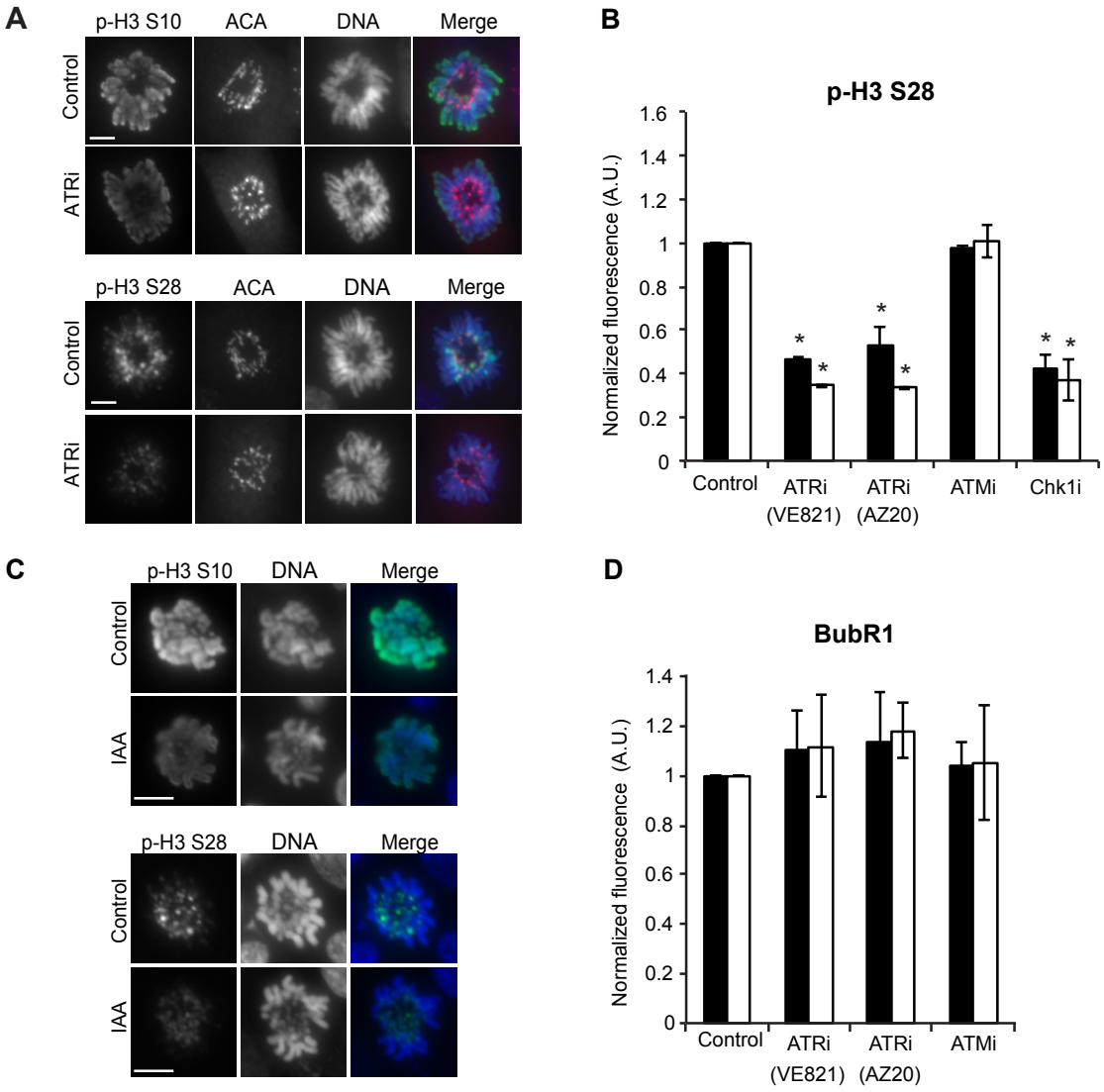

A
ATR-/-, AID-ATR DT40
IAA
-
+
ATR
Tubulin
B
Asynch cells
ATRi
1 hour
Image
RPE1
U2OS
Anaphases with chromosome bridges (%)
0
5
10
15
20
25
30
35
40
Untreated
ATRi
ATRi
(VE821)
ATRi
(AZ20)
C
Control
ATRi
Trisomy
Monosomy
Chrm 1
Chrm 7
Chr 1
Chr 7
Aneuploidy (%)
Control
ATRi


A
Asynch cells
CDK1i
16 hours
Image
EdU positive cells (%)
Asynch
CDK1i arrested
B
Control:
CDK1i
16 hours
1 hour
Image
ATRi:
CDK1i
16 hours
ATRi
1 hour
Image
APH washout:
CDK1i + Aphidicolin
16 hours
1 hour
Image
EdU positive mitotic cells (%)
Control
ATRi
APH washout
C
Asynch cells
CDK1i
16 hours
ATRi
1 hour
Image
RPE1
U2OS
Anaphases with lagging chromosomes (%)
Control
ATRi (VE821)
ATRi (AZ20)
D
Asynch cells
CDK1i
16 hours
ATRi
1 hour
Image
RPE1
U2OS
Anaphases with chromosome bridges (%)
Control
ATRi (VE821)
ATRi (AZ20)

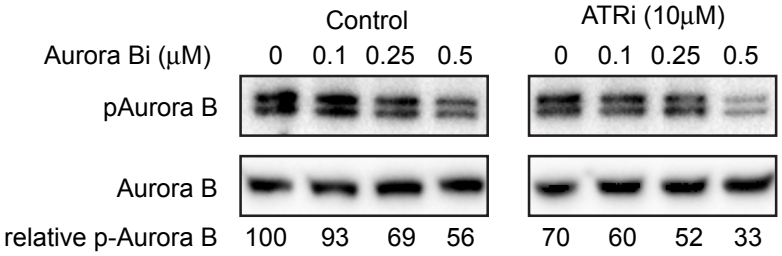

A
Asynch cells
ATRi
1 hour
Image
EdU positive cells (%)
25
20
15
10
5
0
Interphase Mitosis Interphase Mitosis
Untreated
ATRi
Mitosis
EdU
DNA
Merge
Negative
Positive
Interphase
EdU
DNA
Merge
Negative
Positive
B
Asynch cells
ATRi
1 hour
Image
Untreated
VE821
AZ20
γH2AX postive mitotic cells
10
9
8
7
6
5
4
3
2
1
0
RPE1
U2OS
C
Asynch cells
HU
1 hour
Image
p-Chk1
Interphase
Mitosis
*
Normalized fluorescence intensity (A.U.)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
HU
Asynch cells
HU
1 hour
Image
γH2AX
Interphase
Mitosis
*
Normalized fluorescence intensity (A.U.)
3.5
3
2.5
2
1.5
1
0.5
0
Control
HU


A
Prometaphase
Metaphase
Anaphase
Mitotic cells (%)
Control
ATRi
Control
ATRi
RPE1
U2OS
B
Interphase
Mitosis
Cont
1uM CPT
Cont
10uM VE821
1uM AZ20
p-CDK1 Y15
Wee1
Tubulin
C
Interphase
+CHX
minutes: 0 15 30 60 120 0 15 30 60 120
Cdc25A
tubulin
Control
ATRi
Normalized intesnity
minutes
Mitosis
+CHX
minutes: 0 15 30 60 120 0 15 30 60 120
Cdc25A
tubulin
Control
ATRi
Normalized Intensity
minutes
D
Asynch cells
STLC
4 hours
ATRi
1 hour
Image
RPE1
U2OS
Mitotic cells (%)
Control
ATRi (VE821)
ATRi (AZ20)
MPS1i
E
Asynch cells
ATRi
1 hour
Image
RPE1
Multipolar
Bipolar
Monopolar
Prometaphase cells (%)
Control
ATRi (VE821)
ATRi (AZ20)
Asynch cells
ATRi
1 hour
Image
U2OS
Multipolar
Bipolar
Monopolar
Prometaphase cells (%)
Control
ATRi (VE821)
ATRi (AZ20)

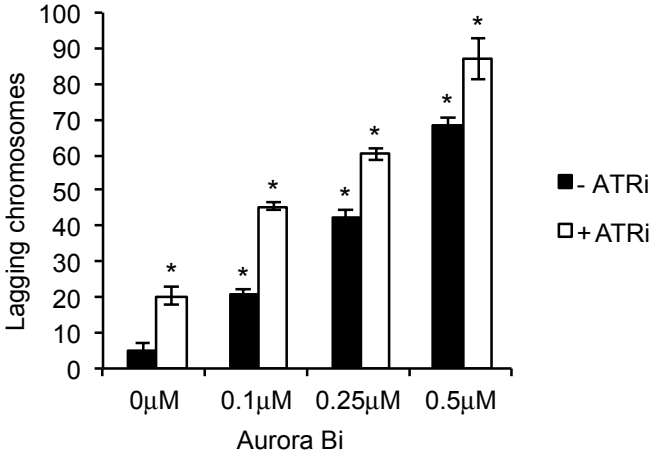

A
Asynch cells
CDK1i
16 hours
30 mins
Image
Prometaphase
p-Aurora B T232
RPE1
U2OS
Normalized fluorescence (A.U.)
Control
ATRi
B
Aurora Bi
ATRi (VE821)
ATRi (AZ20)
Aurora B kinase activity (%)
concentration (ηM)
concentration (μM)
C
Aurora B
INCENP
Survivin
N.S.
D
p-Aurora A T288
p-PLK1 T210
RPE1
U2OS
ATRi (VE821)
ATRi (AZ20)
ATMi

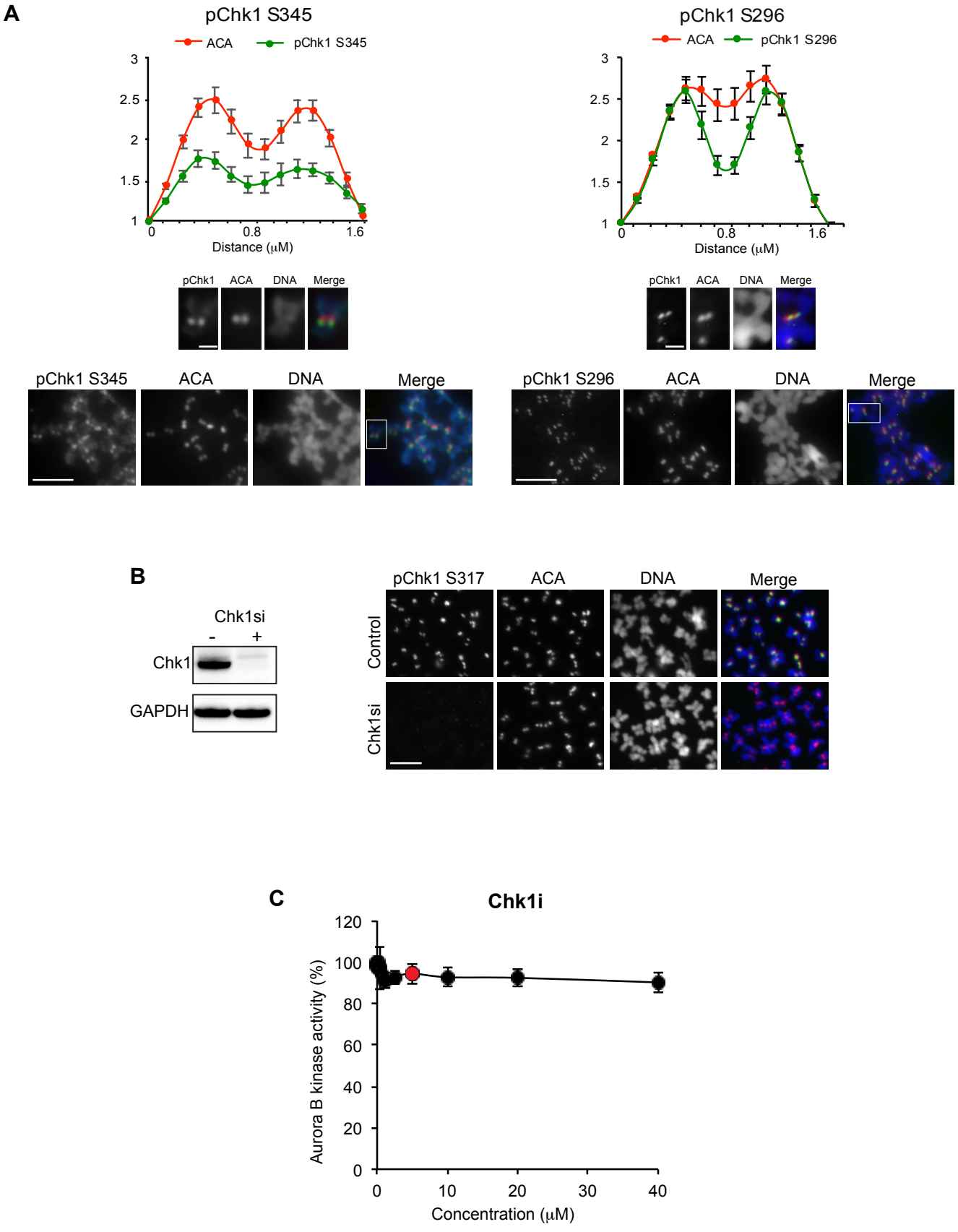

A
p-H3 S10
ACA
DNA
Merge
Control
ATRi
p-H3 S28
ACA
DNA
Merge
Control
ATRi
B
p-H3 S28
Normalized fluorescence (A.U.)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
ATRi (VE821)
ATRi (AZ20)
ATMi
Chk1i
C
p-H3 S10
DNA
Merge
Control
IAA
p-H3 S28
DNA
Merge
Control
IAA
D
BubR1
Normalized fluorescence (A.U.)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
ATRi (VE821)
ATRi (AZ20)
ATMi

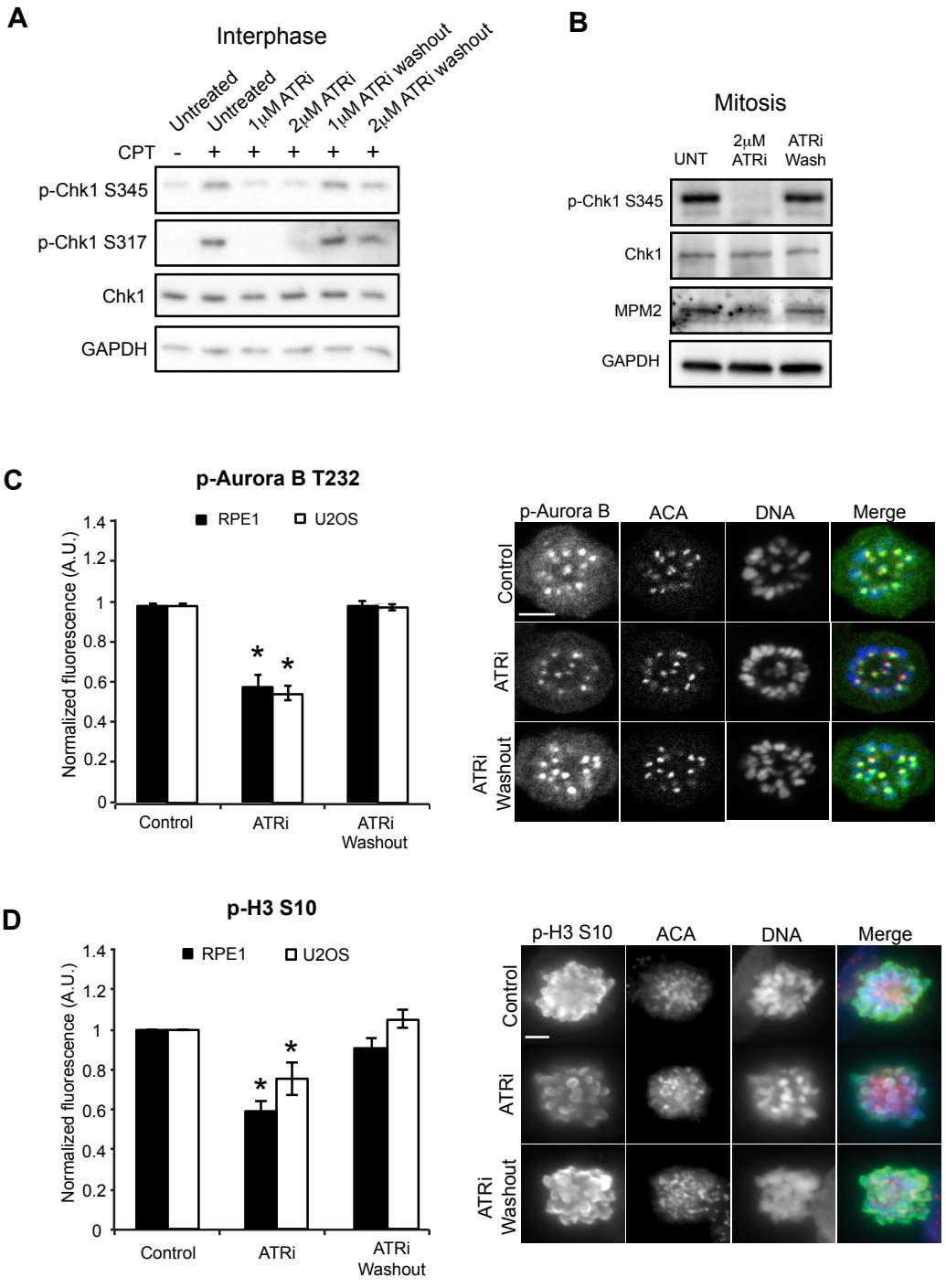
A


Control
ATRi (10μM)
Aurora Bi (μM)
0 0.1 0.25 0.5
0 0.1 0.25 0.5
pAurora B
Aurora B
relative p-Aurora B
100 93 69 56
70 60 52 33

B


Lagging chromosomes
100
90
80
70
60
50
40
30
20
10
0
0μM
0.1μM
0.25μM
0.5μM
Aurora Bi
- ATRi
+ ATRi
*


A
pChk1 S345
ACA
pChk1 S345
Distance (μM)
pChk1
ACA
DNA
Merge
pChk1 S296
ACA
pChk1 S296
Distance (μM)
pChk1
ACA
DNA
Merge
B
Chk1si
-
+
Chk1
GAPDH
pChk1 S317
ACA
DNA
Merge
Control
Chk1si
C
Chk1i
Aurora B kinase activity (%)
Concentration (μM)


A
Interphase
Untreated
Untreated
1μM ATRi
2μM ATRi
1μM ATRi washout
2μM ATRi washout
CPT - + + + + +
p-Chk1 S345
p-Chk1 S317
Chk1
GAPDH
B
Mitosis
UNT
2μM ATRi
ATRi Wash
p-Chk1 S345
Chk1
MPM2
GAPDH
C
p-Aurora B T232
RPE1
U2OS
Normalized fluorescence (A.U.)
Control
ATRi
ATRi Washout
p-Aurora B
ACA
DNA
Merge
Control
ATRi
ATRi Washout
D
p-H3 S10
RPE1
U2OS
Normalized fluorescence (A.U.)
Control
ATRi
ATRi Washout
p-H3 S10
ACA
DNA
Merge
Control
ATRi
ATRi Washout

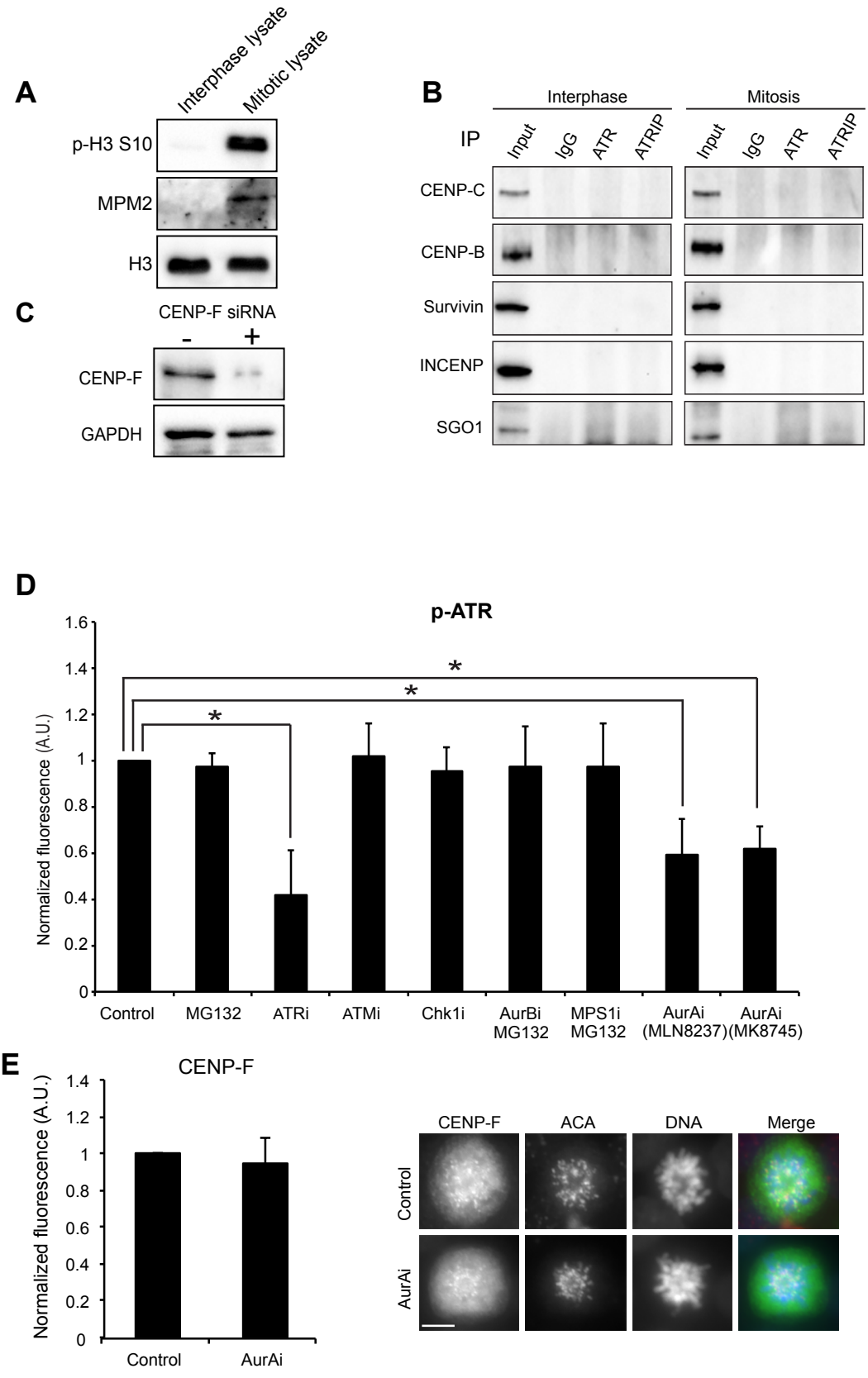
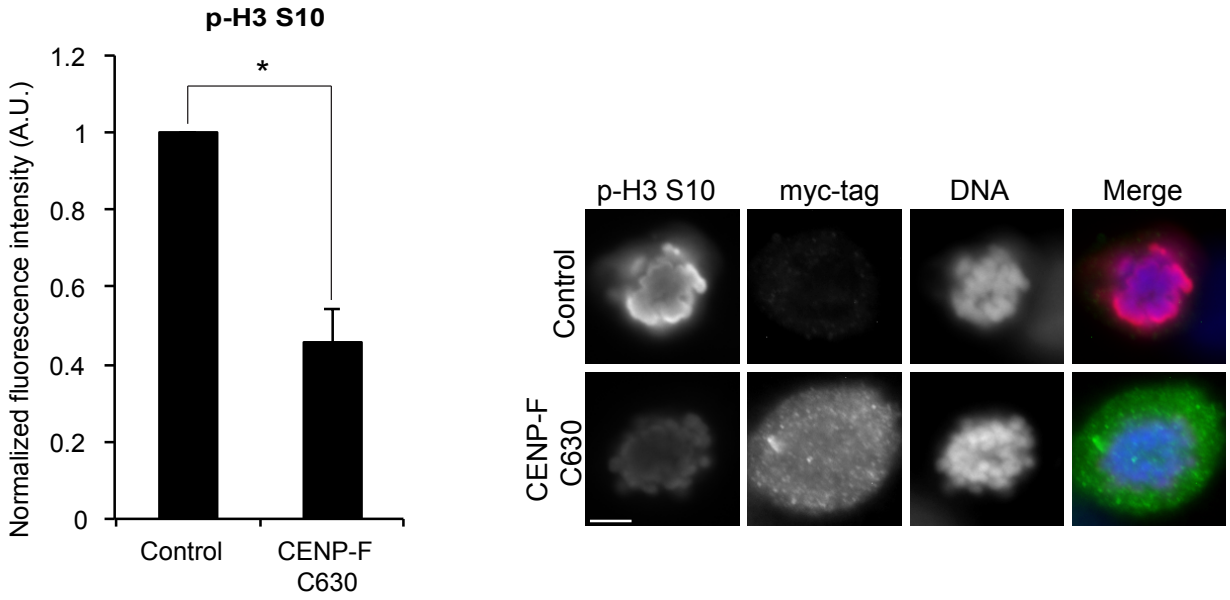
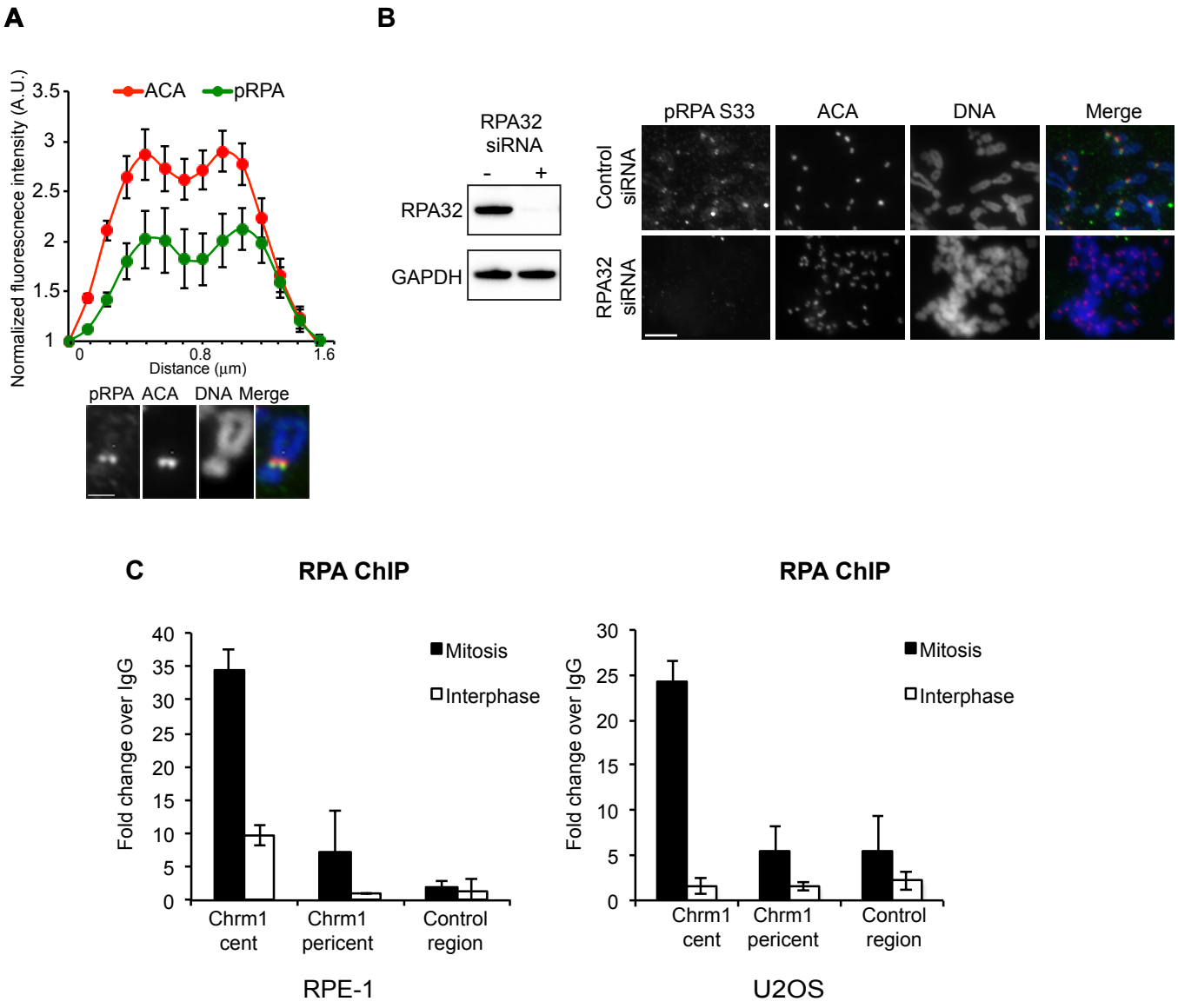
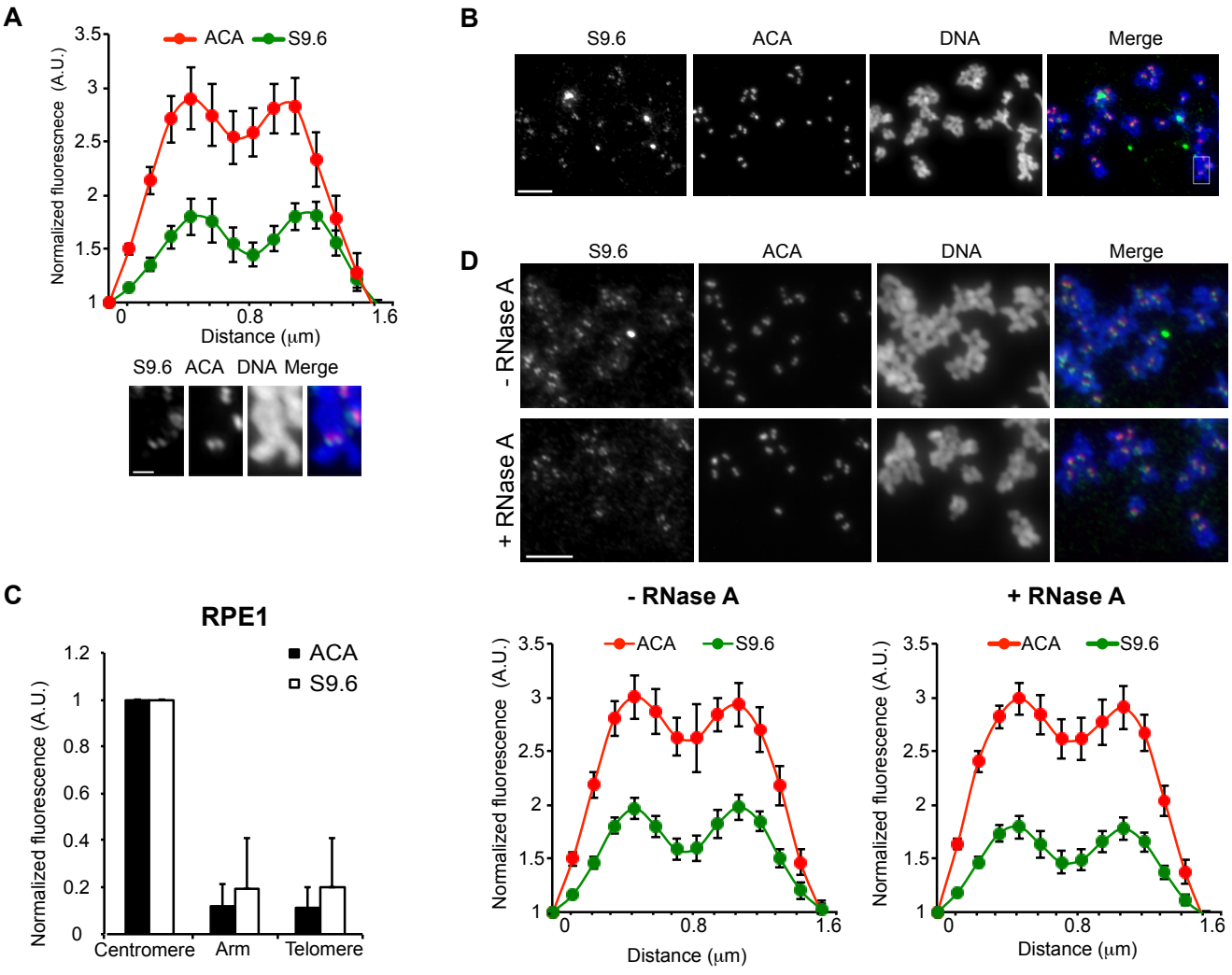
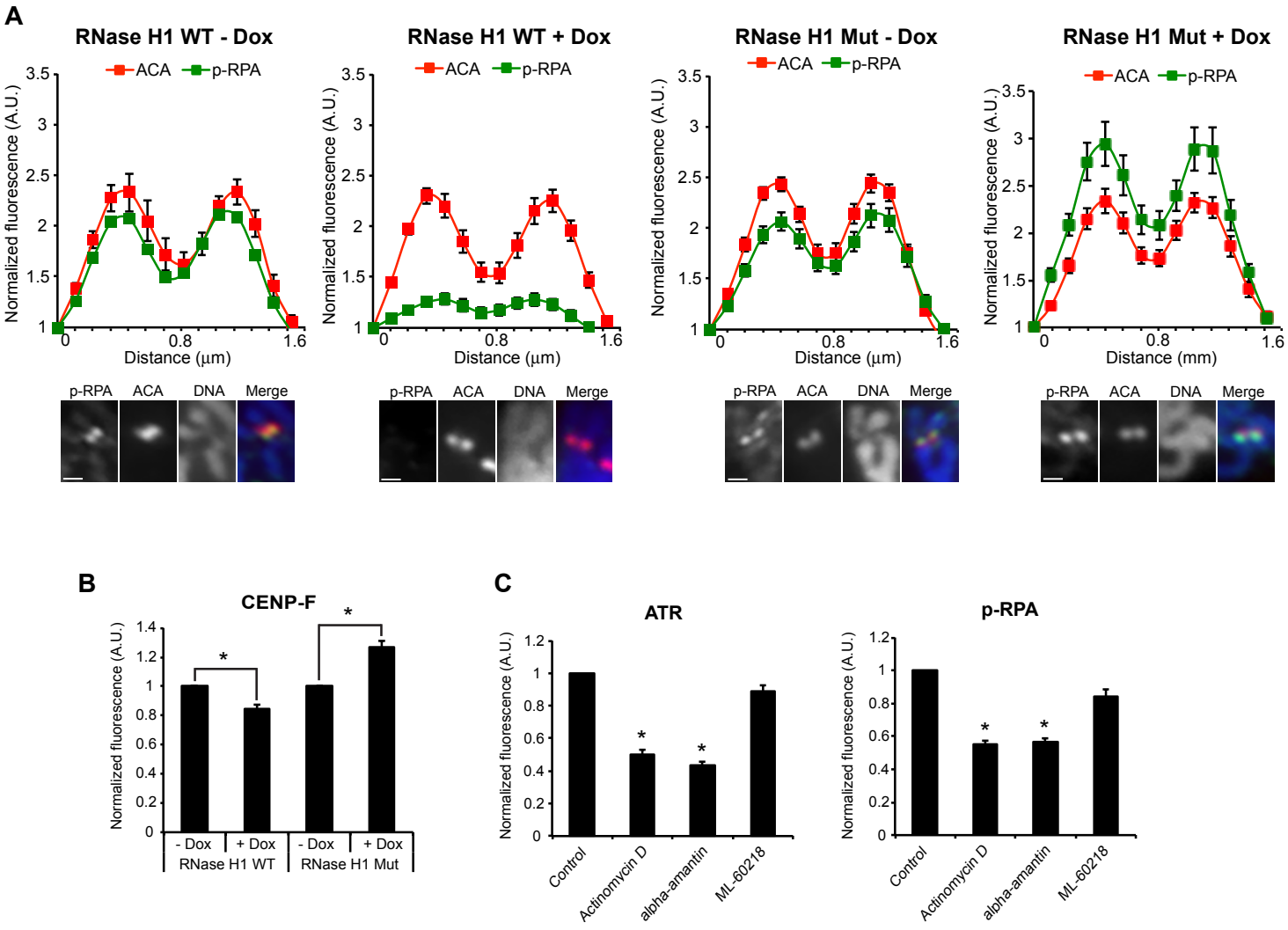

A
Interphase lysate
Mitotic lysate
p-H3 S10
MPM2
H3
B
Interphase
Mitosis
IP
Input
IgG
ATR
ATRIP
CENP-C
CENP-B
Survivin
INCENP
SGO1
C
CENP-F siRNA
-
+
CENP-F
GAPDH
D
p-ATR
Normalized fluorescence (A.U.)
Control
MG132
ATRi
ATMi
Chk1i
AurBi MG132
MPS1i MG132
AurAi (MLN8237)
AurAi (MK8745)
E
CENP-F
Normalized fluorescence (A.U.)
Control
AurAi
CENP-F
ACA
DNA
Merge
Control
AurAi

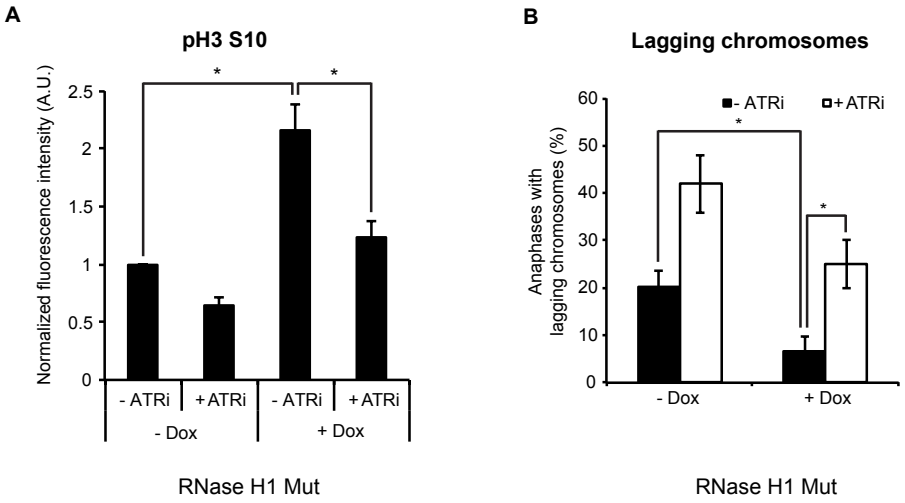
Fig S13

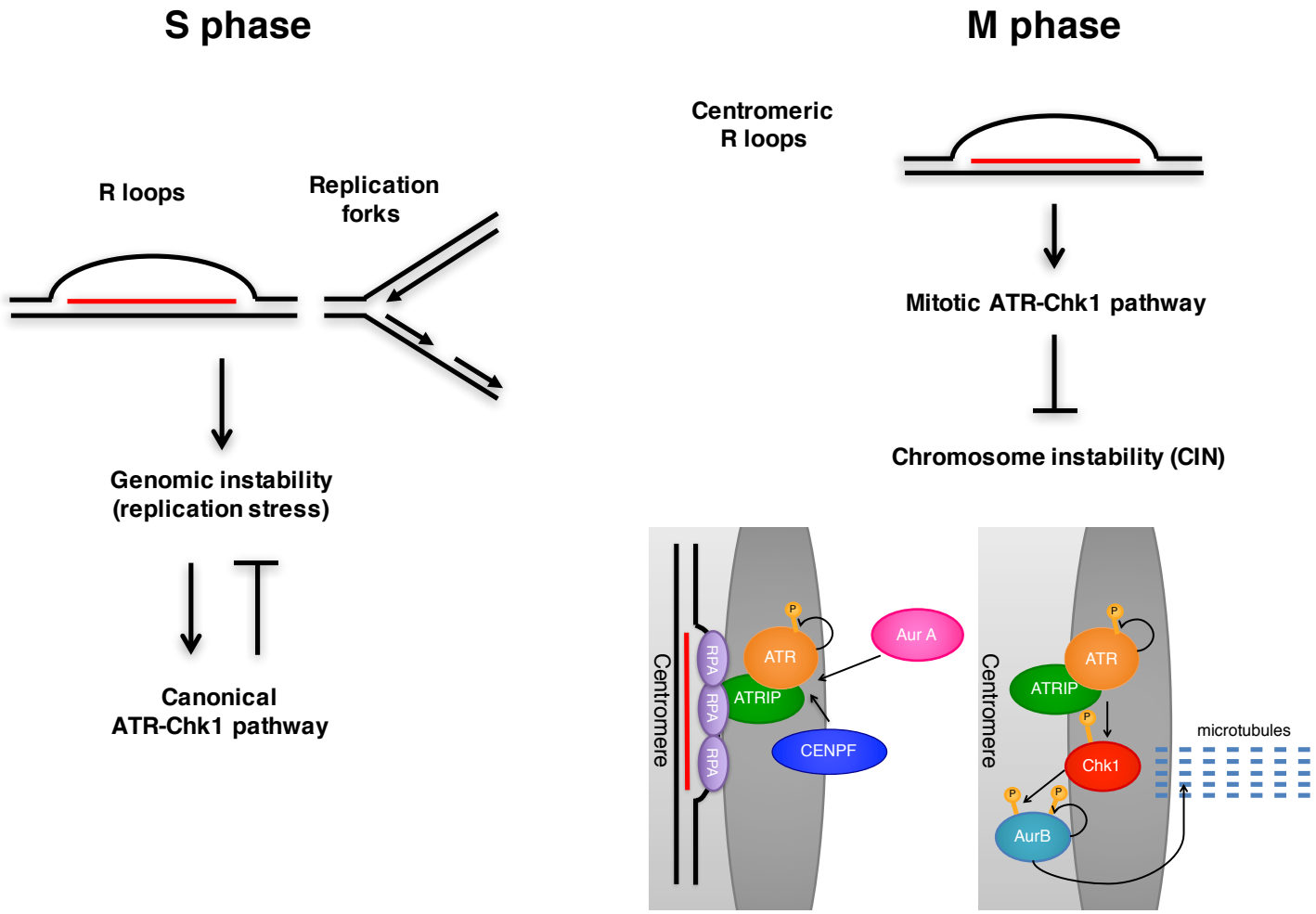

p-H3 S10
Normalized fluorescence intensity (A.U.)
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
CENP-F C630
p-H3 S10
myc-tag
DNA
Merge
Control
CENP-F C630


A
ACA
pRPA
Normalized fluorescnece intensity (A.U.)
3.5
3
2.5
2
1.5
1
0
0.8
1.6
Distance (μm)
pRPA ACA DNA Merge
B
RPA32 siRNA
- +
RPA32
GAPDH
pRPA S33
ACA
DNA
Merge
Control siRNA
RPA32 siRNA
C
RPA ChIP
Fold change over IgG
Mitosis
Interphase
Chrm1 cent
Chrm1 pericent
Control region
RPE-1
U2OS


A
ACA
S9.6
Normalized fluorescnece (A.U.)
Distance (μm)
S9.6 ACA DNA Merge
B
S9.6
ACA
DNA
Merge
D
S9.6
ACA
DNA
Merge
- RNase A
+ RNase A
C
RPE1
ACA
S9.6
Normalized fluorescence (A.U.)
Centromere
Arm
Telomere
- RNase A
+ RNase A
Normalized fluorescence (A.U.)
Distance (μm)


A
RNase H1 WT - Dox
RNase H1 WT + Dox
RNase H1 Mut - Dox
RNase H1 Mut + Dox
ACA
p-RPA
Normalized fluorescence (A.U.)
Distance (μm)
Distance (mm)
p-RPA
ACA
DNA
Merge
B
CENP-F
Normalized fluorescence (A.U.)
- Dox
+ Dox
RNase H1 WT
RNase H1 Mut
C
ATR
p-RPA
Normalized fluorescence (A.U.)
Control
Actinomycin D
alpha-amantin
ML-60218


A
pH3 S10
Normalized fluorescence intensity (A.U.)
2.5
2
1.5
1
0.5
0
*
*
- ATRi
+ ATRi
- ATRi
+ ATRi
- Dox
+ Dox
RNase H1 Mut
B
Lagging chromosomes
Anaphases with lagging chromosomes (%)
60
50
40
30
20
10
0
- ATRi
+ ATRi
*
*
- Dox
+ Dox
RNase H1 Mut


S phase
R loops
Replication forks
Genomic instability (replication stress)
Canonical ATR-Chk1 pathway
M phase
Centromeric R loops
Mitotic ATR-Chk1 pathway
Chromosome instability (CIN)
Centromere
RPA
RPA
RPA
ATR
ATRIP
Aur A
CENPF
P
Centromere
ATR
ATRIP
Chk1
AurB
microtubules

Legends for Supplemental Figures

Figure S1 (related to Figure 1). ATR is present and active at centromeres during mitosis. (**A**) Line scan analysis (top) and representative images (bottom) of ATR and ACA in centromeric regions of chromosome spreads of unsynchronized mitotic RPE1 cells. Asynchronously growing cells were treated with nocodazole for 15 min to facilitate spreading of chromosomes. (**B**) p-ATR and ACA staining in chromosome spreads of unsynchronized mitotic RPE1 cells. Scale bar, 10 μm. (**C**) Representative whole-cell images of p-ATR (top left) and ATR (bottom left) staining in RPE1 cells transfected with control or ATR siRNA. Western blot (right) confirms the knockdown of ATR in RPE1 cells. Scale bar, 5 μm. (**D**) Representative images of ATR staining on chromosome spreads of RPE1 cells transfected with control or ATR siRNA. Scale bar, 10 μm.

Figure S2 (related to Figure 1). ATR is present at centromeres in unperturbed mitotic cells. (**A**) Representative images (left) and quantification (right) of Proximity ligation assay (PLA) signals of mitotic RPE1 cells. Mouse anti-CENP-B and rabbit anti-CENP-B antibodies were paired as a positive control for PLA. Mouse anti-CENP-B antibody alone and rabbit anti-ATR antibody alone were used as negative controls for PLA. Specific PLA signals were detected using mouse anti-CENP-B and rabbit anti-ATR antibodies. Representative images of RPE1 cells showing PLA (green), ACA (red) and DNA (blue) signals . Scale bar, 5 μm. (**B**) No PLA signal was detected in interphase RPE1 cells using mouse anti-CENP-B and rabbit anti-ATR antibodies. (**C**) Quantitative PCR of ATR ChIP in mitotic and interphase U2OS cells.

Figure S3 (related to Figure 1). Acute ATR ablation does not lead to chromosome bridges. (**A**) Western blot of ATR in *ATR*$^{-/-}$*, AID-ATR* DT40 cells untreated or treated with 0.5 mM IAA for 30 min. (**B**) Percentage of anaphase cells with chromosome bridges

in asynchronous RPE1 and U2OS cell populations untreated or treated with ATRi for 1 h. (**C**) RPE1 cells were untreated or treated with ATRi in mitosis, and analyzed using chromosome 1 and 7 alpha satellite FISH probes (green) in the following G1 (see Methods). Representative images of diploid and aneuploid RPE1 cells (left) and quantification of aneuploid RPE1 cells (right) are shown. Scale bar, 5 μm. Error bars in all panels represent SEM. *P ≤ 0.01, two-tailed *t*-test.

Figure S4 (related to Figure 1): Post-replication ATRi treatment does not induce DNA synthesis in mitosis. (**A**) Percentage of EdU-positive cells in asynchronous or CDK1i-arrested RPE1 cell populations. (**B**) RPE1 cells were synchronized in G2 with CDK1i in the presence or absence of aphidicolin, and then released into mitosis in the presence or absence of ATRi. EdU-positive mitotic cells were quantified. (**C**) RPE1 and U2OS cells were synchronized in G2 with CDK1i, and then released into mitosis in the presence or absence of ATRi. Anaphase cells with lagging chromosomes were quantified. (**D**) RPE1 and U2OS cells were synchronized in G2 with CDK1i and then released into mitosis in the presence or absence of ATRi. Anaphase cells with chromosome bridges were quantified. Error bars in all panels represent SEM. *P ≤ 0.01, two-tailed *t*-test.

Figure S5 (related to Figure 2). The canonical ATR pathway is not active in mitosis. (**A**) Percentage of EdU-positive interphase or mitotic RPE1 cells after mock treatment (control) or ATRi treatment (ATRi) (left). Representative images of EdU-negative and -positive RPE1 cells in interphase or mitosis (right). Scale bar, 5 μm. (**B**) Percentage of γH2AX-positive mitotic cells (≥ 2 foci) after mock or ATRi treatments. (**C**) Fluorescence intensities of p-Chk1 (left) and γH2AX (right) in interphase or mitotic RPE1

cells after mock treatment (control) or hydroxyurea (HU) treatment. Error bars in all panels represent SEM. *P ≤ 0.01, two-tailed *t*-test.

Figure S6 (related to figure 2). ATRi does not affect mitotic progression, CDK1 Y15 phosphorylation, CDC25A levels, mitotic exit, and multipolar spindles. (**A**) Percentage of RPE1 and U2OS cells in various mitotic phases after mock or ATRi treatments. (**B**) Western blot of phospho-CDK1 Y15 and Wee1 in interphase and mitotic RPE1 cells. Tubulin serves as a loading control. (**C**) Analysis of CDC25A stability in cycloheximide (CHX) treated RPE1 cells in the presence or absence of ATRi. Cells were first synchronized at G1/S using double thymidine block. Interphase cells were analyzed immediately after release from the thymidine block. Mitotic cells were obtained by releasing cells from the thymidine block and synchronizing them in mitosis with nocodazole (see Methods). (**D**) Percentage of mitotic RPE1 cells after STLC treatment for 4 h followed by mock treatment (control) or treatments with the indicated inhibitors for 1 h. MPS1 inhibitor (MPS1i) was used as a control that induces premature mitotic exit. (**E**) Percentage of mitotic RPE1 cells containing monopolar, bipolar, or multipolar spindles after mock or ATRi treatments. Error bars in all panels represent SEM.

Figure S7 (related to Figure 2): ATR promotes Aurora B activation in mitosis. (**A**) Fluorescence intensity of centromeric p-Aurora B in prometaphase RPE1 and U2OS cells. Cells were synchronized in G2 with CDK1i, and released into mitosis in the presence of absence of ATRi for 30 min. (**B**) AurBi but not ATRi inhibits Aurora B kinase activity in vitro. Aurora B activity was measured using the ADP-Glo kit (see Methods). Red dots signify the highest doses of the indicated inhibitors used the other experiments in this study. (**C**) Fluorescence intensities of centromeric Aurora B, INCENP, and Survivin in mitotic RPE1 cells. Cells were treated with STLC for 4 h followed by mock or ATRi treatment for 1 hr. (**D**) Fluorescence intensities of p-Aurora A T288 and p-PLK1

T210 in mitotic RPE1 (black bars) or U2OS (open bars) cells. Cells were treated as in (C). Error bars in all panels represent SEM. *P ≤ 0.01, two-tailed *t*-test.

Figure S8 (related to Figure 2) ATR promotes phosphorylation of Aurora B targets. (**A**) Representative images of p-H3 S10 and p-H3 S28 in mitotic RPE1 cells untreated or treated with ATRi. Cells were treated with STLC for 4 h followed by mock or ATRi treatment for 1 hr. Scale bar, 5 μm. (**B**) Fluorescence intensities of p-H3 S28 in mitotic RPE1 (black bars) or U2OS (open bars) cells. Cells were treated with STLC for 4 h, and then mock treated (control) or treated with the indicated inhibitors for 1 h. (**C**) Representative images of p-H3 S28 and p-H3 S10 in *ATR*$^{-/-}$, *AID-ATR* DT40 cells untreated or treated with IAA for 30 min. Scale bar, 5 μm. (**D**) Fluorescence intensity of centromeric BubR1 in mitotic RPE1 (black bars) or U2OS (open bars) cells. Cells were treated as in (A). Error bars in all panels represent SEM. *P ≤ 0.01, two-tailed *t*-test.

Figure S9 (related to Figure 2) Partial inhibition of Aurora B leads to an increase of lagging chromosomes. (**A-B**) RPE1 cells were treated with increasing concentrations of AurBi (ZM 044739) in the presence or absence of ATRi (10 μM VE-821). Western blots of Aurora B, p-Aurora B (A), and quantification of the rate of lagging chromosomes (B) are shown. Error bars in all panels represent SEM.

Figure S10 (related to Figure 2) Specificity of p-Chk1 antibody and inhibitor. (**A**) Line scan analysis (top) and representative images (bottom) of p-Chk1 S345 and ACA (left), or p-Chk1 S296 and ACA (right), in unsynchronized mitotic RPE1 cells. Cells were treated with nocodazole for 15 min to facilitate spreading of chromosomes. Scale bar, 2 μm for sister chromatids and 10 μm for multiple chromosomes. (**B**) Western blot (left) and chromosome spreads (right) of RPE1 cells treated with control or Chk1 siRNA. Chk1 and p-Chk1 S317 antibodies were used for Western blot and staining of chromosome

spreads as indicated. Asynchronously growing cells were treated with nocodazole for 15 min to facilitate spreading of chromosomes. (**C**) Chk1i does not affect Aurora B activity in vitro. Aurora B activity was measured with the ADP-Glo kit in the presence of increasing concentrations of Chk1i. The red dot signifies the highest dosage of Chk1i used in other experiments of this study. Scale bar, 10 μm.

Figure S11 (related to Figure 3). Reversible inhibition of ATR by ATRi. (**A**) Western blot of p-Chk1 in interphase cells treated with 1 μM or 2 μM VE-821 (ATRi), and with or without ATRi washout. (**B**) Western blot of p-Chk1 in mitotic cells treated with 2μM VE-821 (ATRi), and with or without ATRi washout. Mitotic cells were isolated by shake off after 4 h of nocodazole treatment. Cells were then mock treated (control) or treated with 2 μM VE-821 for 1 h in the presence of nocodazole. The ATRi washout sample was released from VE-821 for 1 h in the presence of nocodazole. (**C**) Quantification of fluorescence intensity (left) and representative images (right) of centromeric p-Aurora B in STLC-arrested prometaphase RPE1 cells. (**D**) Quantification of fluorescence intensity (left) and representative images (right) of p-H3 S10 in STLC-arrested prometaphase RPE1 cells. Error bars in all panels represent SEM. *P ≤ 0.01, two-tailed *t*-test. Scale bar, 5 μm.

Figure S12 (related to Figure 3). Aurora A and CENP-F are specifically required for ATR activation at centromeres. (**A**) Western blot of interphase and mitotic lysates used for immunoprecipitation. MPM2 and p-H3 S10 are specific markers for mitosis. (**B**) Immunoprecipitates of endogenous ATR and ATRIP from interphase or mitotic RPE1 cells. Input = 5%. (**C**) Western blot confirms the knockdown of CENP-F by siRNA. (**D**) Fluorescence intensity of p-ATR in STLC-arrested prometaphase RPE1 cells after mock treatment (control) or treatments with the indicated inhibitors. (**E**) Fluorescence intensity

(left) and representative images (right) of centromeric CENP-F in STLC-arrested prometaphase RPE1 cells after mock or ATRi treatment for 1 h. Error bars in all panels represent SEM. *P ≤ 0.01, two-tailed *t*-test. Scale bar, 5 μm.

Figure S13 (related to Figure 3). CENP-F C630 overexpression decreases phosphorylation of Aurora B targets. Quantification (left) and representative images (right) of U2OS cells infected with control or CENP-F C630 expressing retrovirus. Error bars in all panels represent SEM. *P ≤ 0.01, two-tailed *t*-test. Scale bar, 5 μm.

Figure S14 (related to Figure 4). RPA is present at centromeres in mitosis. (**A**) Line scan analysis (top) and representative images (bottom) of p-RPA and ACA in unsynchronized mitotic RPE1 cells. Cells were treated with nocodazole for 15 min to facilitate spreading of chromosomes. Scale bar, 2 μm. (**B**) Western blot (left) and chromosome spreads (right) of RPE1 cells treated with control and RPA32 siRNA. RPA32 and p-RPA32 antibodies were used for Western blot and staining of chromosome spreads as indicated. (**C**) Quantitative PCR analysis of RPA ChIP in interphase and mitotic RPE1 (left) and U2OS (right) cells. Error bars in all panels represent SEM. Scale bar, 10 μm.

Figure S15 (related to Figure 4). R loops are present at centromeres in mitosis. (**A**) Line scan analysis (top) and representative images (bottom) of S9.6 staining in unsynchronized mitotic RPE1 cells. Cells were treated with nocodazole for 15 min to facilitate spreading of chromosomes. Scale bar, 2 μm. (**B**) Chromosome spreads were stained as in (A). S9.6 staining was detected at centromeres on all chromosomes. Scale bar, 10 μm. (**C**) Quantification of S9.6 and ACA fluorescence intensities at centromeres, telomeres, and chromosome arms. Fluorescence was normalized to DAPI staining. (**D**) Line scan analysis (bottom) and representative images (top) of S9.6 staining in

chromosome spreads of RPE1 cells. Chromosome spreads were untreated (-RNaseA) or treated with RNaseA (+RNaseA). Error bars in all panels represent SEM. Scale bar, 10 μm.

Figure S16 (related to Figure 4). R loops promote ATR activation at centromeres and accurate chromosome segregation. (**A**) Line scan analysis (top) and representative images (bottom) of centromeric p-RPA and ACA staining in HeLa-derived RNaseH1 WT/MUT inducible cell lines. Cells were synchronized in G2 with CDK1i, uninduced (-DOX) or induced (+DOX) to express RNaseH1 WT/MUT, and then released into mitosis. Scale bar, 2 μm. (**B**) Fluorescence intensity of centromeric CENP-F in RNaseH1 WT/MUT inducible cell lines. Cells were uninduced (-DOX) or induced (+DOX) to express RNaseH1 WT/MUT as in (B). (**C**) Fluorescence intensities of ATR (left) and p-RPA (right) in STLC arrested mitotic RPE1 cells untreated (control) or treated with various RNA polymerase inhibitors for 1 h. Error bars in all panels represent SEM. *P ≤ 0.01, two-tailed *t*-test.

Figure S17 (related to figure 4). R loops promote accurate chromosome segregation through ATR. (**A**) Fluorescence intensity of p-H3 S10 in HeLa cells uninduced (-DOX) or induced (+DOX) to express RNaseH1 MUT. Cells were synchronized in G2 with CDK1i, uninduced or induced to express RNaseH1 MUT, and released into mitosis in the presence or absence of ATRi. **(B)** Percentage of anaphase cells with lagging chromosomes. Cells that inducibly express RNaseH1 MUT were treated as in (A).

Figure S18 (related to figure 4). A model of cell cycle-regulated interplays between R loops and ATR. A model depicting the distinct functional relationships between R loops and ATR in S and M phases of the cell cycle. In S phase, R loops induce genomic

instability by interfering with DNA replication. The R loop-associated genomic instability activates the canonical ATR-Chk1 pathway, triggering a feedback loop to prevent further genomic instability. In M phase, centromeric R loops activate the mitotic specific ATR-Chk1 pathway. This pathway promotes faithful chromosome segregation, thereby suppressing chromosome instability.

Supplemental Methods

Cell culture.

RPE-1 (ATCC, CRL-4000) and U2OS (ATCC, HTB-96) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin. Media for H2B-GFP-RPE1 cells, and RNaseH1 WT and RNaseH1 Mut HeLa cells was supplemented with G418 (Mediatech). RNaseH1 WT and RNaseH1 Mut HeLa cells were treated with 200ng/ml doxycycline for 4 hours after 16 hours of CDK1 inhibition, and subsequently allowed into mitosis. Cells were then arrested with nocodazole (for chromosome spreads) at 100ng/ml for 1 hour, or fixed after 30 minutes (for p-Aurora B, p-H3) or 1 hour (for quantification of lagging chromosomes). Cells were incubated with 10mM S-Trityl-L-cysteine (STLC) (Tocris, CAS 2799-07-7) for a total of 5 hours and either fixed for imaging or collected through a mitotic shake off for western blot and immunoprecipitation analysis.

DT40 cells were cultured in RPMI medium supplemented with 10% FBS, 1% Chicken Serum (Invitrogen) and 1% penicillin/streptomycin. DT40 cells were treated with 3-Indoleacetic acid (Auxin or IAA) (Sigma-Aldrich) for 30 minutes at a final concentration of 0.5mM as described (4). Auxin was prepared fresh for each experiment at 0.5M in ethanol. All cell lines were validated as mycoplasma free.

Antibodies

Antibodies used for this study were: human anti-centromere antisera (ACA) (Antibodies Inc., 15-234-0001), ATR (Bethyl Laboratories, A300-138A), ATR N-19 (used for DT40 cell western blot) (Santa Cruz Biotechnology, sc-1887), Aurora B (Novus Biologicals, NB100-294), BubR1 (Novus Biologicals, NB100-353), CENP-B (Santa Cruz Biotechnology, sc-32285), CENP-B (Abcam, ab25734), CENP-F (Abcam, ab90), Chk1 (Santa Cruz Biotechnology, sc-8408), INCENP (Novus Biologicals, NB100-2286), γH2AX

(Cell Signaling Technology, 9718), H3 (Abcam, ab1791), myc tag (Cell Signaling Technology, 2278), phospho-Aurora A T288 (Cell Signaling Technology, 3079), phospho-Plk1 T210 (Cell Signaling Technology, 9062), phospho-ATR T1989 (GTX128145, GeneTex), phospho-Aurora B (Rockland, 600-401-677), phospho-CHK1 S317 (Cell Signaling Technology,12302), phospho-Chk1 S345 (Cell Signaling Technology, 2348), phospho-H3 S10 (Millipore, 06.570), phospho-H3 S10 (used for DT40 cells, and CENP-F C630 OX), (Abcam, ab14955), phosho-H3 S28 (Cell Signaling Technology, 9713), Survivin (Novus Biologicals, NB500-201), S9.6 (Antibodies Inc.,), tubulin (Sigma-Aldrich, T9026), phospho-RPA S33 (Bethyl, A-300-246A), RNaseH1 (Abcam, ab56560), RPA32/RPA2 (Abcam, ab2175), Alexa Fluor 647 (Jackson Immuno Research; anti-human 109-605-088), FITC (Jackson Immuno Research, anti-mouse 715-545-150; anti-rabbit, 711-545-152)

Cell transfection

siRNA transfections were conducted using Oligofectamine (Invitrogen), and cells analyzed 48 hours later. RNA duplexes for ATR (CCUCCGUGAUGUUGCUUGA) CENP-F (GCUACAACUUUUAUCCGAA), Chk1 (GCAACAGUUAUUUCGGUAUAUU) and RPA32 (GCACCUUCUCAAGCCGAAA), were purchased from ThermoFisher Scientific. U2OS cells were infected using CENP-F C630 expressing retrovirus or control virus and fixed 24 hours after infection.

Inhibitors

The kinase inhibitors that were used in this study are as follows: ATRi (2 μM or 10 μM VE-821 as indicated), ATRi (1 μM AZ20), CDK1i (10 μM RO-3306), Chk1i (2 μM MK-8776), ATMi (10 μM KU 55933), Aurora Bi (0.3 μM or as indicated ZM 447439), cycloheximide (20 ug/ml), MG132 (5 μM, 20 μM as indicated), Aurora Ai (5 μM MLN

8237), Aurora Bi (1 μM MK 0457), MPS1i (1 μM Reversine), RNA polymerase inhibitors: Actinomycin D (2 μg/ml), α-amantin (2 μg/ml) , ML-60218, and thymidine (2 mM).

Immunofluorescence microscopy

Cells were fixed with 3.5% paraformaldehyde for 15 minutes, incubated with ice-cold methanol for 10 minutes, washed with PBS + 0.5% Triton X-100 for 5 minutes, and washed with PBS + 3% Bovine serum albumin (BSA) for 10 minutes. Antibodies were diluted in PBS + 1% BSA, and cells on coverslips were incubated with antibodies for 12 hours at 4^{o}C. Cells were then washed with PBS for 5 minutes. Secondary antibodies were diluted in PBS + 1% BSA, and cells on coverslips were incubated with secondary antibodies for 1hour at room temperature (~25^{o}C). Images were acquired on a Nikon Eclipse 90i with a Retiga 200R camera and processed in Photoshop CS4 (Adobe). Representative undeconvoluted single stack images are shown.

For quantitative assessments of unsynchronized and STLC arrested cells, cells were untreated (control) or treated with various inhibitors for 1 hour, and stained with centromeric and kinetochore proteins (including: p-Aurora B, p-ATR, p-RPA, BubR1, p-PLK1, Aurora B, INCENP, Survivin, and CENPF), ACA and DAPI. Average pixel intensities of p-Aurora B and ACA were measured in approximately 15 centromeric regions over the entire cell. Background fluorescence was subtracted from both p-Aurora B and ACA measurements, and the ratios of intensities (p-Aurora B/ACA) were calculated and averaged over multiple centromeres from multiple mitotic cells ($n \geq 30$ cells). The relative intensity under the control condition was defined as 1, and the intensity under other conditions was normalized to the control condition in the same experiment. Values for each independent experiment were averaged and the standard error was calculated. Three independent experiments were conducted with minimum

total of 300 centromeres calculated. Mitotic stage was identified using chromosome alignment.

For prometaphase arrested cells, cells were arrested with 10mM STLC for a total of 5 hours. At hour 4, cells were either untreated (control) or treated with various inhibitors.

For quantitative analysis of cells stained with p-Aurora A and α-tubulin, or p-H3 S10/p-H3 S28 and DAPI, integrated pixel intensities were measured over the entire cell. Background fluorescence was subtracted from both measurements and the ratio of intensities (e.g. p-Aurora A/Tubulin) was calculated. The relative intensities of the proteins analyzed (e.g. p-Aurora) under the control condition were defined as 1, and the intensities under other conditions were normalized to the control condition in the same experiment. Values for each independent experiment were averaged and the standard error was calculated. Three independent experiments were conducted with minimum total of 450 cells measured.

Lagging chromosomes measured in RPE1, U2OS and HeLa cells were measured using ACA to mark centromeres and DAPI to mark the chromosomes. The following criteria was used to identify anaphases with lagging chromosomes: the minimum length between the two ACA planes that demarcate the chromosomes separating had to be a minimum of 8μm, with a single ACA foci in between the planes, and a minimum distance between the single lagging ACA foci and the rest of the ACA plane being 0.5 μm. Furthermore, the ACA foci must be localized to a chromosome, by use of DAPI staining. For DT40 cells, only DAPI was used to identify lagging chromosomes. A minimum total of 300 anaphases were scored per condition over 3 replicates for all cell lines and experiments.

Chromosome spreads and line-scan analysis

Line scan analyses of ATR/p-ATR/p-Chk1/p-RPA/S9.6 and ACA were done on chromosome spreads. For chromosome spreads, asynchronous cells were treated with 100 ng/mL of nocodazole for 5 hours and mitotic cells collected through mitotic shake-off. Alternatively, asynchronous cells were treated acutely with 100 ng/ml of nocodazole for 15 minutes to allow for microtubule depolymerization and proper spreading of chromosomes, and mitotic cells collected through mitotic shake-off.

Cells were subsequently washed with 1X PBS. Cells were then resuspended in 7 mM KCL for 10 minutes and spun onto glass slides. Slides were incubated in KCM buffer (10 mM KCl, 20 mM NaCl, 10 mM Tris-HCl pH 8, 0.5 mM EDTA, 0.1% Triton X-100) for 10 minutes. Primary antibodies were diluted in KCM buffer + 1% BSA. Cells were incubated with primary antibodies for 2 hours in a humidified chamber, washed with KCM buffer, and incubated with secondary antibodies in KCM buffer + 1% BSA for 1 hour. Cells were subsequently washed with KCM buffer and fixed with 3.5% paraformaldehyde and imaged. All buffers used were supplemented with 100 nM okadaic Acid (Tocris, 1136) to prevent dephosphorylation of proteins. Analysis of chromosomes was conducted in ImageJ using the line scanning function. A line was drawn through the centromeres using ACA as reference. Fluorescence intensity was normalized to the first time distance point immediately outside the ACA signal. Fluorescence intensities were averaged across distance and standard error calculated per distance point. 5 chromosome pairs were used per cell with a minimum total of 30 chromosome pairs used per condition.

S9.6 staining was measured across chromosome arms by measuring the average pixel intensity at 3 regions of approximately 0.8 mM diameter: the centromere (as demarcated by ACA staining), the telomere (at the end of the chromosome arm), and the chromosome arm (a point approximately equidistant from the centromere and

telomere measurements). All points were normalized to DAPI staining to ensure that proper chromosome location was made.

Proximity Ligation Assay (PLA)

PLA was conducted using the Duolink® PLA Fluorescence Protocol (Sigma Aldrich). Briefly, asynchronous RPE1 cells were pre-treated with microtubule-stabilizing buffer [MTSB (pH 6.8); 4 M glycerol, 100 mM PIPES, 1 mM EGTA and 5 mM $MgCl_2$]. Cells were then treated with MTSB plus 0.05% Triton X-100 and subsequently incubated with MTSB again for 2 min. Cells were then fixed with paraformaldehyde and methanol as above. After primary antibody incubation overnight at 4^{o}C, and secondary antibody against human ACA was added and washed, coverslips were blocked using the Duolink® Blocking solution for 60 minutes at room temperature. Duolink PLA probes were added at a 1:5 concentration in the Duolink® Antibody Dilutant for 1 hour at 37^{o}C. The ligation, amplification and wash steps were conducted as state in the protocol. Lastly Wash buffer B was supplemented with DAPI and cells washed for 5 minutes and subsequently mounted onto slides.

Live cell imaging

H2B-GFP expressing RPE-1 cells were plated onto glass bottom dishes and imaged using a Zeiss LSM 710 inverted confocal microscope. Cells were maintained at 37^{o}C with 5% CO_2. Bright field was used to identify prometaphase cells. After the first image was acquired, cells were either treated with DMSO (control) or 10 μM ATRi. Cells were imaged every 3 minutes with a 63X, 1.4 numerical aperture objective until telophase onset. Twenty ATRi treated cells and 12 mock treated cells were analyzed.

Immunoprecipitation

Asynchronous cells were treated with 10 μM STLC for a total of 5 hours. At hour 4, cells were either untreated (control) or treated with various inhibitors, and mitotic cells collected through mitotic shake-off. For interphase cells, asynchronous cells were treated for 1 hour with various inhibitors, and mitotic shake-off was done to remove mitotic cells in the population. Subsequently, cells were trypsinized and collected. Cells were then washed twice with ice-cold 1X PBS, and lysed with lysis buffer containing 20 mM HEPES-KOH (pH 7.4), 150 mM KCl, 0.5 mM DTT, 10% glycerol, protease inhibitors, 100mM Okadaic (Torcis 1136) Acid, 0.01% NP-40 and 10U/μL benzonase (Sigma Aldrich). Lysates were then thoroughly sonicated. Antibody-coupled Protein G Dynalbeads ® (ThermoFisher) were added to lysates and incubated for 16 hours with rocking at 4^{o}C. Lysates were washed 3 times with lysis buffer (without benzonase) at 4^{o}C, and resuspended in SDS sample buffer.

Kinase assay

ADP-Glo and Aurora B kinase assay kit (Promega) was used as directed. Briefly, white optical 96 well plates were used. 7 ng Aurora B, 25 μM ATP and 0.1 μg/uL MBP were diluted in kinase buffer. ATRi, Chk1i and Aurora Bi were used at titrating conditions and incubated for 60 minutes at room temperature. ADP-Glo^{TM} was added and incubated for a subsequent 40 minutes to the reaction. After, Kinase Detection reagent was added and the reaction was incubate for a subsequent 30 minutes. Luminescence was record on a luminescence microplate reader, with an integration time of 0.5 seconds.

Fluorescence in situ hybridization (FISH)

RPE1 cells were synchronized using CDK1i, then allowed to enter mitosis in the presence or absence of ATRi. ATRi was subsequently washed off after 2 hours and cells allowed to continue into G1 for 2 more hours. After, cells were washed with PBS and

treated with 75mM potassium chloride for 20 minutes, then fixed and washed twice with methanol-acetic acid (3:1). Dual-colored FISH was performed using α-satellite and p-arm subtelomere probes specific for chromosomes 1 and 7 (Cytocell) according to manufacturer's protocol. A minimum of 500 nuclei were scored according to the criteria of Cimini et al., for each condition (12). FISH images were acquired at a single focal plane with a 20x 0.8 NA objective.

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation was carried out as previously described (20). Briefly, 5-6 million RPE1 and U2OS mitotic cells (in 10 cm plates) were fixed by addition of 1% Formaldehyde (Sigma) final concentration to the culture medium. Crosslinking was performed at room temperature for 10 min followed by quenching with 0.125 M glycine. Samples were washed twice with ice-cold PBS. First extraction step (10 min at 4°C) was with Buffer 1 (50 mM HEPES/KOH pH 7.5; 140 mM NaCl; 1 mM EDTA; 10% Glycerol; 0.5% NP-40; 0.25% Triton) followed by the second extraction step with Buffer 2 (200 mM NaCl; 1 mM EDTA; 0.5 mM EGTA; 10 mM Tris pH 8). Nuclei were pelleted by centrifugation, resuspended in SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris pH 8.0) and subjected to sonication with Q800R2 sonicator (Qsonica) generating genomic DNA fragments with an average size of 200-600 bp. Chromatin (100 µg) was pre-cleared with protein G Dynabeads (Invitrogen) for 1 hour at 4°C followed by immunoprecipitation (overnight at 4°C) with 5 µg of either GFP or RPA antibody. This was followed by pulldown with BSA-pretreated protein G Dynabeads (Invitrogen) and incubated for 2 hours at 4°C. Beads were washed once with Low salt buffer (0.1% SDS; 1% Triton; 2 mM EDTA; 20 mM Tris pH 8; 150 mM NaCl), once with High salt buffer (0.1% SDS; 1% Triton; 2 mM EDTA; 20 mM Tris pH 8; 500 mM NaCl), once with LiCl wash buffer (10 mM Tris pH 8.0; 1% sodium deoxycholate; 1% NP- 40, 250 mM LiCl; 1 mM EDTA)

and twice with TE. Elution of the beads (to recover immune complexes) was then carried out twice (10 min each) in TE + 1% SDS + 0.1% $NaHCO_3$ at 65°C and followed by reverse crosslinking overnight at 65°C. The eluted DNA was then subjected to RNaseA treatment (10 µg/ml) for 1 hour at 37°C followed by Proteinase K treatment (4 µL 0.5M EDTA, 8 µL 1M Tris pH 6.9, 2 µL Proteinase K 20 mg/ml) for 1 hr at 45°C. DNA isolation was then carried out by using QIAquick PCR purification kit (QIAGEN), resuspended in elution buffer and q-PCR performed using Faststart Universal SYBR Green mix - Rox added (Roche) and analyzed on a LightCycler 480II machine (Roche). ChIP-qPCR results were analyzed and plotted as either fold change over IgG or percentage (%) of IP/input signal (% input). Significance (p value) was calculated using Student's t test.

Primers used for this study:

Site	Forward	Reverse
Chr 1 centromere	TCATTCCCACAAACTGCGTTG	TCCAACGAAGGCCACAAGA
Chr 1 pericentromere	CATCGAATGGAAATGAAAGGAGTC	ACCATTGGATGATTGCAGTCAA
Control region	CAGTGGTGTGGTGTGATCTTG	GGCAAAACCCTGTATCTGTGA

DNA-RNA hybrid immunoprecipitation

DRIP was performed as previously described (30). Briefly, mitotic DNA was extracted with phenol/chloroform in MaXtract High Density phase lock tubes (Qiagen), precipitated with EtOH/sodium acetate, washed with 70% EtOH, and resuspended in TE. DNA was digested with HindIII, BsrGI, XbaI, EcoRI, and SspI at 37°C overnight. For RNaseH-treated samples, 4.4 µg of DNA was treated with RNaseH overnight at 37°C overnight. DNA was purified by phenol/chloroform and precipitated with EtOH/sodium acetate, followed by 70% EtOH washes. 4 µg of precipitated DNA was bound with 10 µg of S9.6 antibody in 1x binding buffer (10mM $NaPO_4$ pH 7.0, 140 mM NaCl, 0.05% Triton X-100)

overnight at 4°C. Protein A/G agarose beads were added for an additional 2h. Antibody-DNA complex were immunoprecipitated and washed 3 times in 1x binding buffer, followed by 1 h incubation in Elution Buffer (50mM Tris pH8.0, 10 mM EDTA, 0.5% SDS, Proteinase K) at 55°C. DNA was precipitated by EtOH/sodium acetate as described above. Quantitative PCR of immunoprecipitated DNA fragments and input DNA was performed on a Roche LightCycler 480 Instrument using FastStart Universal SYBR-Green Master mix (Roche).

Primers used for this study:

Chrm 1 centromere	same as ChIP primer above
Chrm1 pericentromere	same as ChIP primer above
Control region (SNRPN Negative)	Fwd: GCCAAATGAGTGAGGATGGT Rvs: TCCTCTCTGCCTGACTCCAT