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Supplemental information

ATR protects ongoing and newly assembled DNA replication forks through distinct mechanisms

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Fig. S1

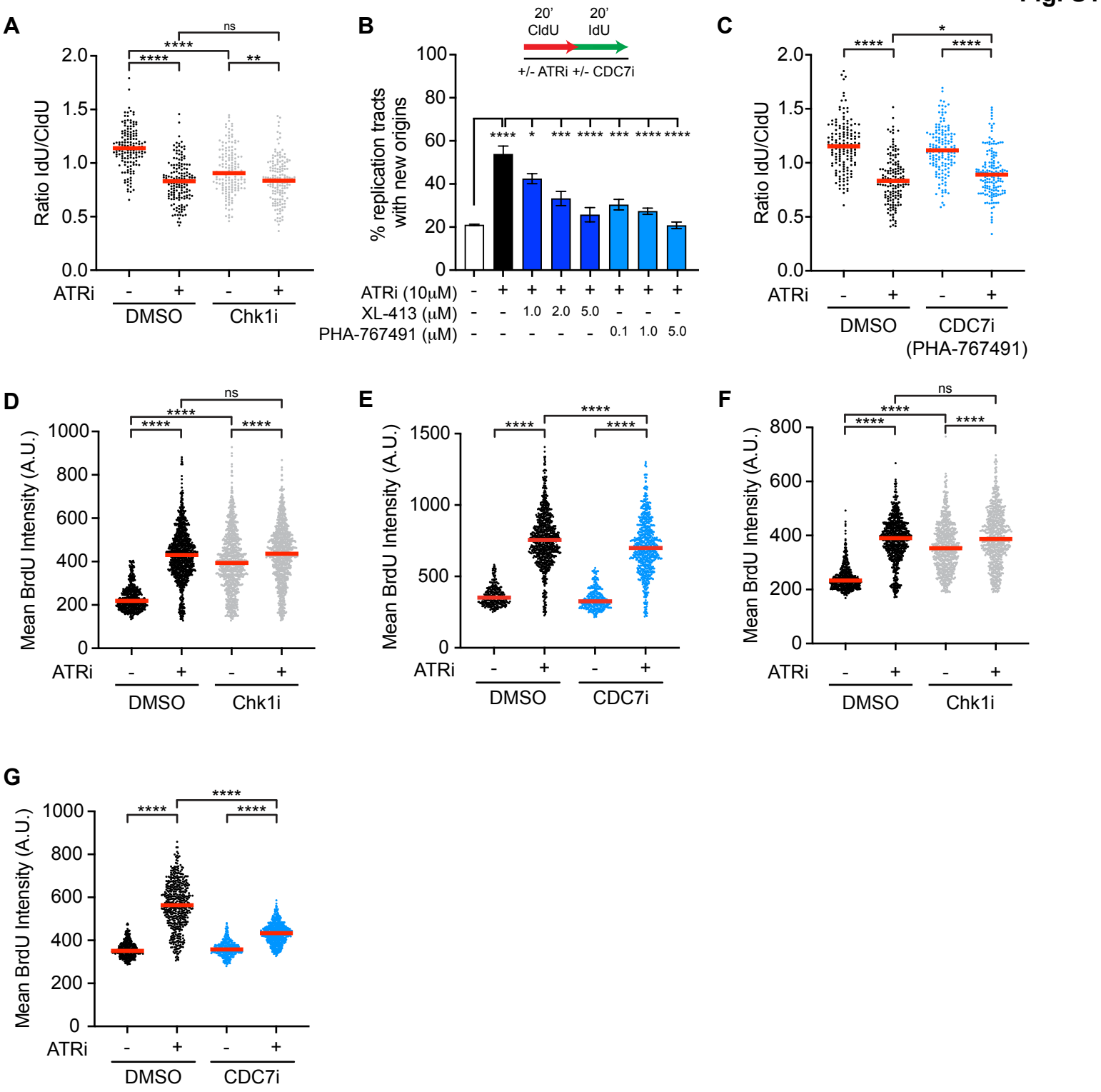
A
Ratio IdU/CldU
ATRi
DMSO
Chk1i
ns
****
**
B
20' CldU
20' IdU
+/- ATRi +/- CDC7i
% replication tracts with new origins
ATRi (10μM)
XL-413 (μM)
PHA-767491 (μM)
C
Ratio IdU/CldU
DMSO
CDC7i (PHA-767491)
*
D
Mean BrdU Intensity (A.U.)
E
F
G

Figure S1 (related to Figure 1). CDC7 inhibitor suppresses ATRi-induced new origin firing.

(**A**) The ATR-Chk1 pathway protects against nascent strand degradation. U2OS cells were treated as in (1A) with or without ATRi (VE-821, 10 μM) and Chk1i (MK-8776, 10 μM). (**B**) Increasing concentration of CDC7i, decreases ATRi-induced new origin firing. U2OS cells were consecutively labeled with CldU (50 μM) and IdU (100 μM) for 20 min in the presence or absence of ATRi (VE-821, 10 μM) and CDC7i (XL-413, 1, 2, 5 μM; PHA-767491, 0.1, 1, 5 μM). Cells were then processed for DNA fiber analysis of the proportion of newly fired origins (IdU Only and CldU-IdU-CldU tracts). Number (n) fibers quantified >250 across two biological replicates. Significance was calculated using one-way ANOVA with Tukey's multiple comparisons test with *<.05, ***<.001, and ****<.0001. (**C**) CDC7i minimally impacts ATRi-induced nascent DNA degradation. U2OS cells were analyzed as in (1A) in the presence or absence of CDC7i (PHA-767491, 0.1 μM) and ATRi (VE-821, 10 μM). Number (n) of fibers quantified >300 across two biological replicates. Significance was calculated using Mann-Whitney Ranked Sum Test with *<0.05, **<.01 and ****<.0001 in A and C. (**D**) ATR-Chk1 prevents template ssDNA exposure. U2OS cells were treated as in (1D) with or without ATRi (VE-821, 10 μM) and Chk1i (MK-8776, 10 μM). (**E**) Template ssDNA exposure occurs mainly at ongoing forks. U2OS cells were treated as in (1D) with or without ATRi (VE-821, 10 μM) and CDC7i (PHA-767491, 0.1 μM). Number (n) of nuclei quantified >500 across three biological replicates. Significance was calculated using Mann-Whitney Ranked Sum Test with ****<.0001 in D and E. (**F**) ATR-Chk1 pathway prevents nascent ssDNA exposure. U2OS cells were treated as in (1F) with or without ATRi (VE-821, 10 μM) and Chk1i (MK-8776, 10 μM). (**G**). Nascent ssDNA exposure occurs mainly at newly fired forks. U2OS cells were treated as in (1F) with or without ATRi (VE-821, 10 μM) and CDC7i (PHA-767491, 0.1 μM). Number (n) of nuclei quantified >500 across three biological replicates. Significance was calculated using Mann-Whitney Ranked Sum Test with ****<.0001 in F and G.

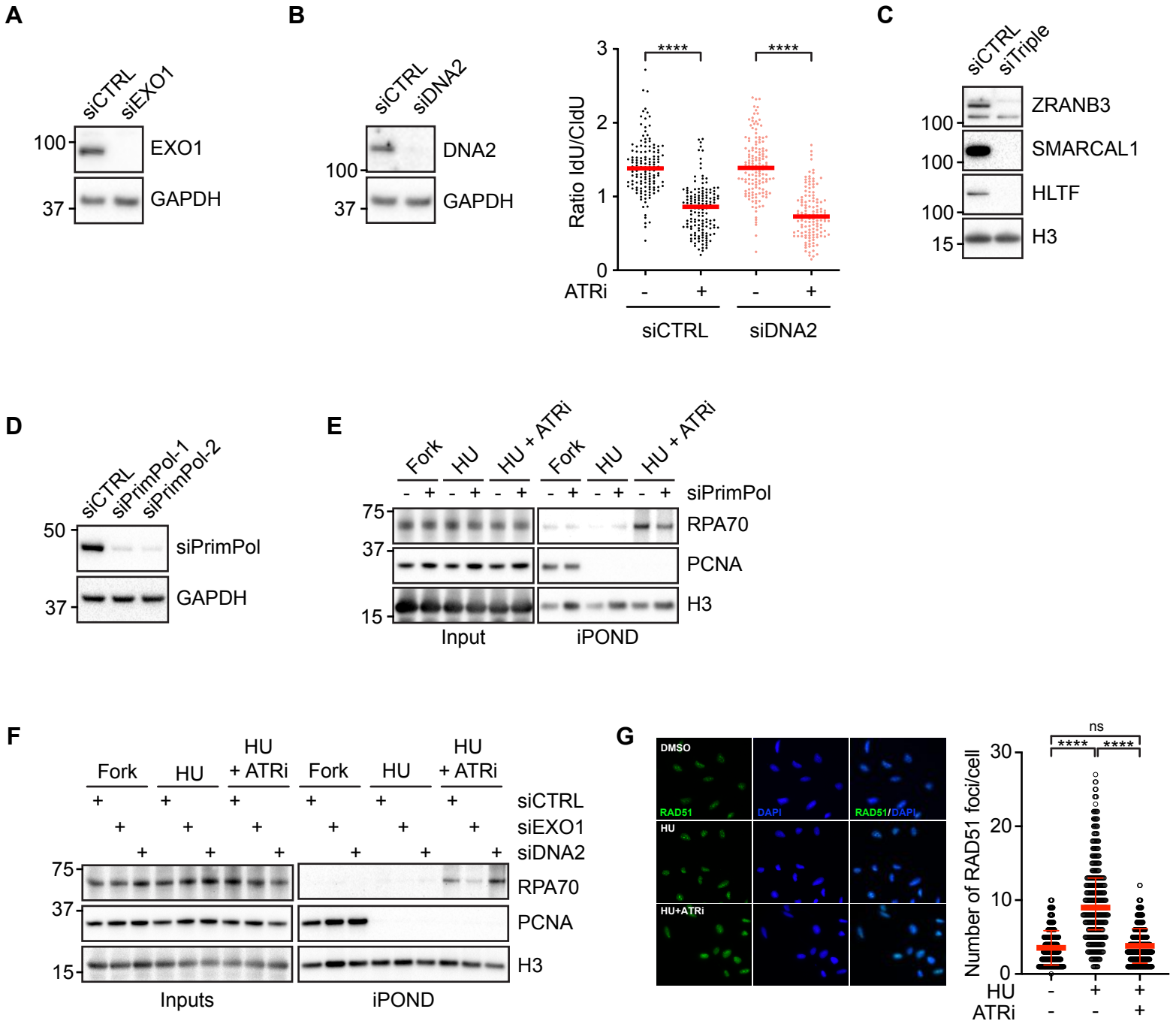
A
siCTRL
siEXO1
100
EXO1
37
GAPDH
B
siCTRL
siDNA2
100
DNA2
37
GAPDH
Ratio IdU/CldU
3
2
1
0
****
****
ATRi
-
+
-
+
siCTRL
siDNA2
C
siCTRL
siTriple
100
ZRANB3
100
SMARCAL1
100
HLTF
15
H3
D
siCTRL
siPrimPol-1
siPrimPol-2
50
siPrimPol
37
GAPDH
E
Fork
HU
HU + ATRi
Fork
HU
HU + ATRi
siPrimPol
75
RPA70
37
PCNA
15
H3
Input
iPOND
F
Fork
HU
HU + ATRi
Fork
HU
HU + ATRi
siCTRL
siEXO1
siDNA2
75
RPA70
37
PCNA
15
H3
Inputs
iPOND
G
DMSO
RAD51
DAPI
RAD51/DAPI
HU
HU+ATRi
Number of RAD51 foci/cell
30
20
10
0
ns
****
****
HU
ATRi

Figure S2 (related to Figure 2). PrimPol and EXO1 contribute to the ATRi-induced ssDNA formation.

(**A)** Western blot confirmation of siRNA-mediated knockdown of EXO1. (**B**) Western blot confirmation of siRNA-mediated knockdown of DNA2 (left) and nascent DNA degradation assay indicating that DNA2 does not influence fork protection upon ATR inhibition (right). (**C**) Western blot confirmation of ZRANB3, SMARCAL1, and HLTF triple knockdown. (**D**) Western blot confirmation of PrimPol knockdown with two different siRNAs. (**E-F**) PrimPol and EXO1 are required for ATRi-induced RPA loading on ssDNA at stressed replication forks. HEK293T cells were labeled with 10 μM EdU for 15 min and either directly fixed with formaldehyde (fork) or exposed to HU (4 mM) for 5 h with or without ATRi (VE-821, 10 μM) prior to fixation. Samples were then processed for iPOND and enriched proteins were analyzed by western blotting. (**G**) ATR protects against nuclease-dependent nascent strand degradation by recruiting RAD51. U2OS cells were treated with HU (4 mM) with or without ATRi (VE-821, 10 μM) for 5 h prior to immunofluorescent staining with RAD51. Number (n) of nuclei quantified >400 across three biological replicates. Significance was calculated using Kruskal-Wallis test with Dunn's multiple comparisons test with ****<.0001.

A

% Reversed Forks (total # molecules analyzed)								
U2OS	**NT**	**+HU**	**+ATRi**	**+HU +ATRi**	**+siPrimPol**	**+HU +siPrimPol**	**+ATRi +siPrimPol**	**+HU +ATRi +siPrimPol**
Experiment #1	4 (81)	28 (78)	6 (95)	15 (89)	6 (87)	27 (81)	6 (96)	26 (77)
Experiment #2	4 (73)	23 (80)	5 (82)	13 (83)	5 (60)	21 (80)	7 (87)	21 (76)

B

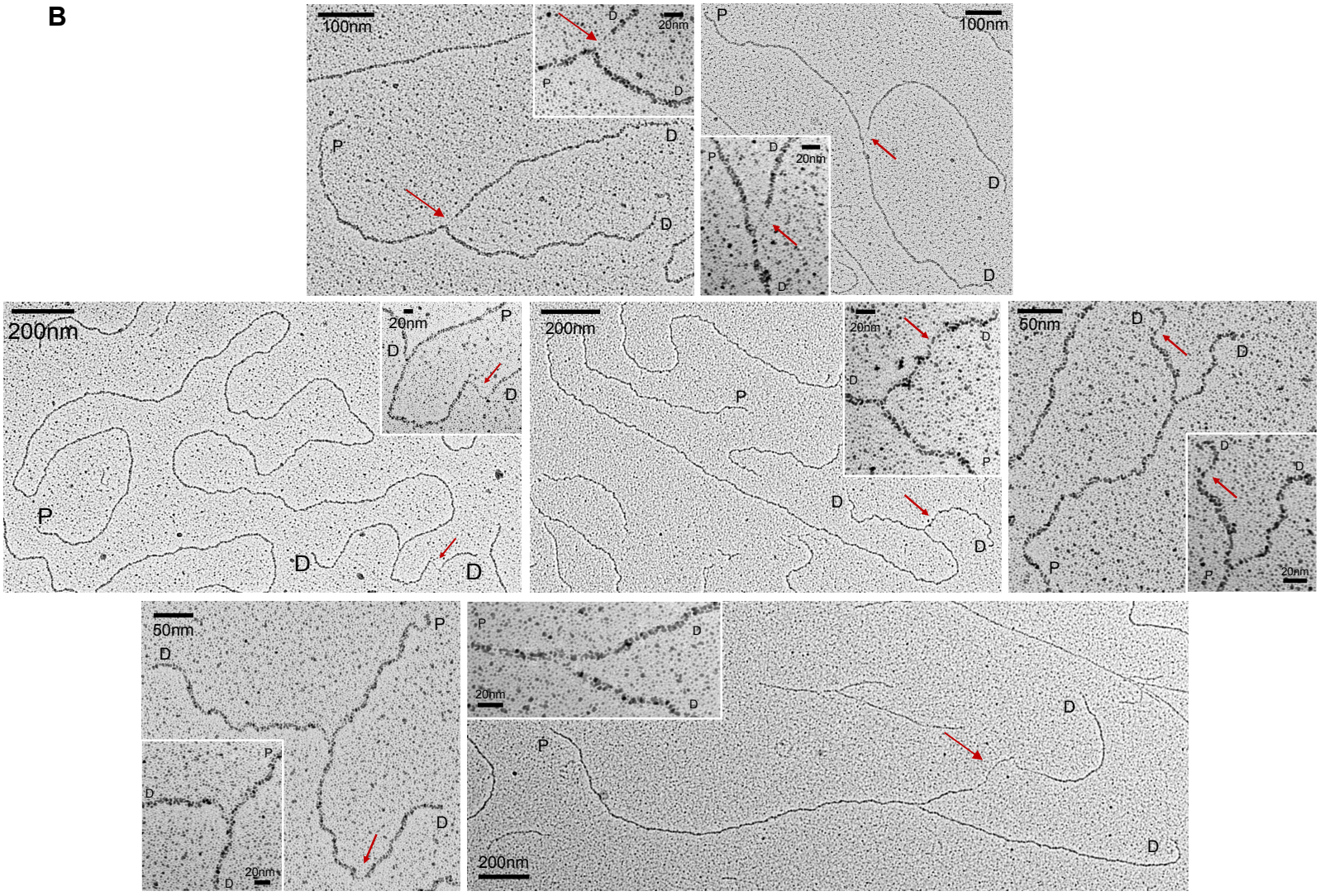

Figure S3 (related to Figure. 3). Electron microscopy (EM) data

(**A**) Percentage of reversed replication forks observed in two independent EM experiments for samples in Figure 3C. (**B**) Seven additional EM images of replication forks with ssDNA gaps in cells treated with HU and ATRi. Insets show the magnified fork junctions. Red arrows show the location of the ssDNA gaps. P, parental strand; D, daughter strand; Red arrow, ssDNA. Junction gaps (Top); Daughter strand gaps (Middle and bottom).

Fig. S4

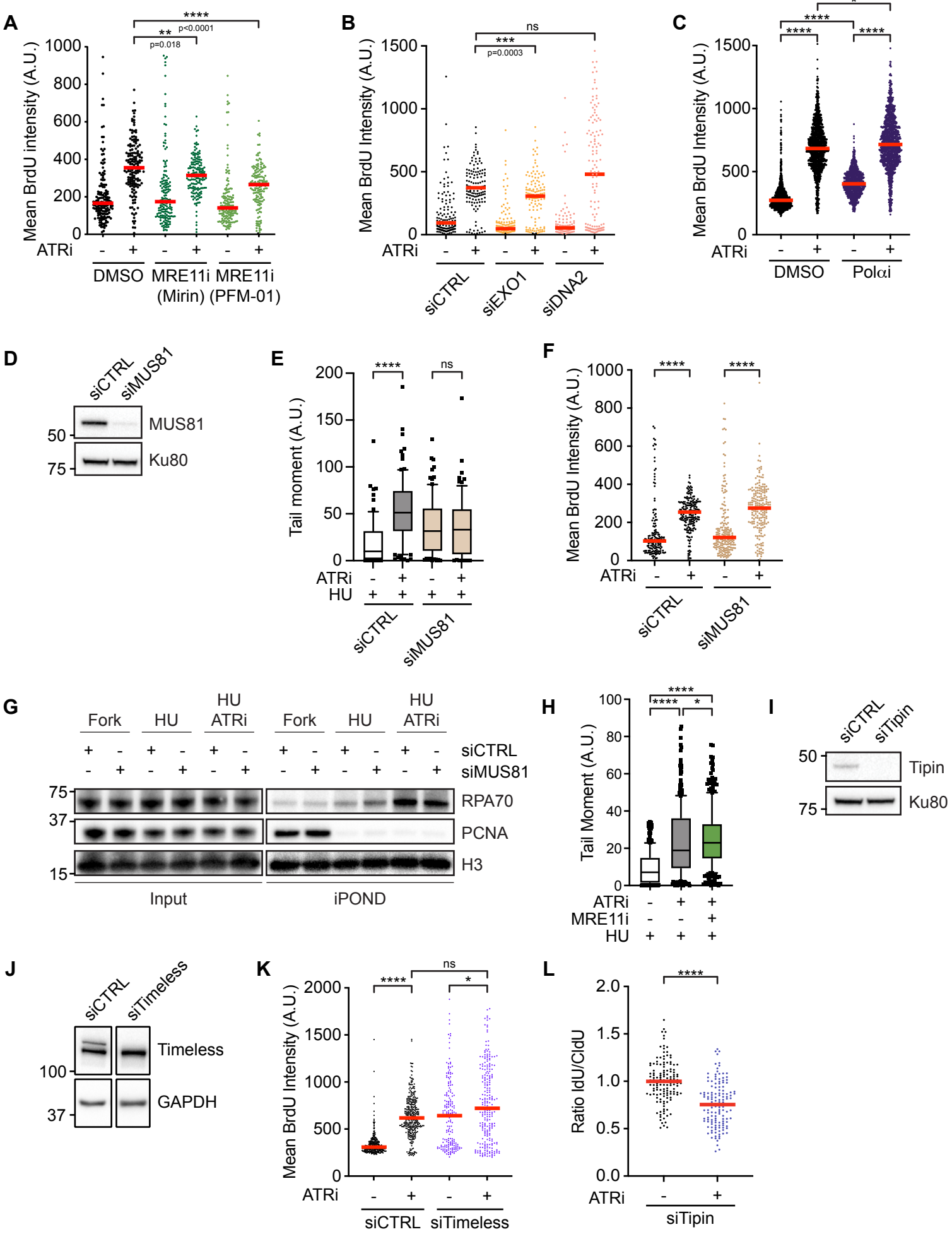
A
Mean BrdU intensity (A.U.)
****
**
p<0.0001
p=0.018
ATRi
DMSO
MRE11i (Mirin)
MRE11i (PFM-01)
B
Mean BrdU Intensity (A.U.)
ns
***
p=0.0003
siCTRL
siEXO1
siDNA2
C
Mean BrdU Intensity (A.U.)
*
DMSO
Polαi
D
siCTRL
siMUS81
MUS81
Ku80
E
Tail moment (A.U.)
ns
HU
F
Mean BrdU Intensity (A.U.)
G
Fork
HU
HU ATRi
siCTRL
siMUS81
RPA70
PCNA
H3
Input
iPOND
H
Tail Moment (A.U.)
MRE11i
I
siTipin
Tipin
J
siTimeless
Timeless
GAPDH
K
L
Ratio IdU/CldU

Figure S4 (related to Figure 4). Testing the effects of MUS81 and Timeless depletion on the ATRi-induced template ssDNA exposure.

(**A**) The exonuclease and endonuclease activity of MRE11 partially contribute to the exposure of template ssDNA in the presence of HU and ATRi. Cells were treated as in (4A) with MRE11i (Mirin, 50 μM or PFM-01, 100 μM) with or without ATRi (VE-821, 10 μM). Number (n) of nuclei quantified >150 in each sample across two biological replicates. Significance was calculated using Mann-Whitney Ranked Sum Test with **<.01, ****<.0001. (**B**) EXO1 but not DNA2 partially contribute to template DNA exposure at stressed replication forks. Cells were treated as in (4A) with or without ATRi (VE-821, 10 μM) following 48 h siRNA knockdown of EXO1 or DNA2. Number (n) of nuclei quantified >100 in each sample across two biological replicates. Significance was calculated using Mann-Whitney Ranked Sum Test with ***<.001. (**C**) Loss of DNA polymerase α partially contributes to the exposure of template ssDNA. Cells were treated as in (4A) with CD437 (2.5 μM) with or without ATRi (VE-821, 10 μM). Number (n) of nuclei quantified >800 in each sample across three biological replicates. Significance was calculated using Mann-Whitney Ranked Sum Test with *<.05, ****<.0001. (**D**) Western blot confirmation of siRNA-mediated knockdown of MUS81. (**E-G**) MUS81-mediated double-strand breaks are not responsible for excessive template DNA exposure and ssDNA generated at stressed replication forks upon ATR inhibition. Cells were exposed to HU (4 mM) and ATRi (VE-821, 10 μM) for 5 h and processed for neutral comet assay in (E). Box plots represent the tail moment of comets. Number (n) of cells quantified >100 in each sample across two biological replicates. Significance was calculated using Mann-Whitney Ranked Sum Test with ****<.0001. Cells were treated with or without ATRi (VE-821, 10 μM) following 48 h siRNA knockdown of MUS81 and processed for the template strand exposure assay in (F). Number (n) of nuclei quantified >150 in each sample across two biological replicates. Significance was calculated using Mann-Whitney Ranked Sum Test with ****<.0001. HEK293T cells were labeled with 10 μM EdU for 15 min and either directly fixed with formaldehyde (fork) or exposed to HU for 5 h with or without ATRi (VE-821, 10 μM) prior to fixation. Samples were then process for iPOND and enriched proteins were analyzed by western blotting in (G). (**H**) The endonuclease activity of MRE11 does not contribute to template ssDNA exposure through

generating DSBs. Cells were exposed to HU (4 mM) with or without ATRi (VE-821, 10 μM) and MRE11i (PFM-01, 100 μM) for 5 h and processed for neutral comet assay. Box plots represent the tail moment of comets. Number (n) of cells quantified >250 in each sample across two biological replicates. Significance was calculated using Mann-Whitney Ranked Sum Test with *<.05, ****<.0001. (**I**) Western blot confirmation of siRNA-mediated knockdown of Tipin. (**J**) Western blot confirmation of siRNA-mediated knockdown of Timeless. (**K**) ATRi and Timeless depletion promote template ssDNA exposure through the same mechanism. U2OS cells were treated with or without ATRi (VE-821, 10 μM) following 48 h siRNA knockdown of Timeless (0.5 nM) and processed for the template strand exposure assay. Number (n) of nuclei quantified >250 in each sample across two biological replicates. Significance was calculated using Mann-Whitney Ranked Sum Test with *<.05, ****<.0001. (**L**) Loss of Tipin increases nascent strand degradation. Cells were treated as in (2A) with or without ATRi (VE-821, 10 μM) following 48 h siRNA knockdown of Tipin. Number (n) of fibers quantified >250 in each sample across three biological replicates. Significance was calculated using Mann-Whitney Ranked Sum Test with ****<.0001.

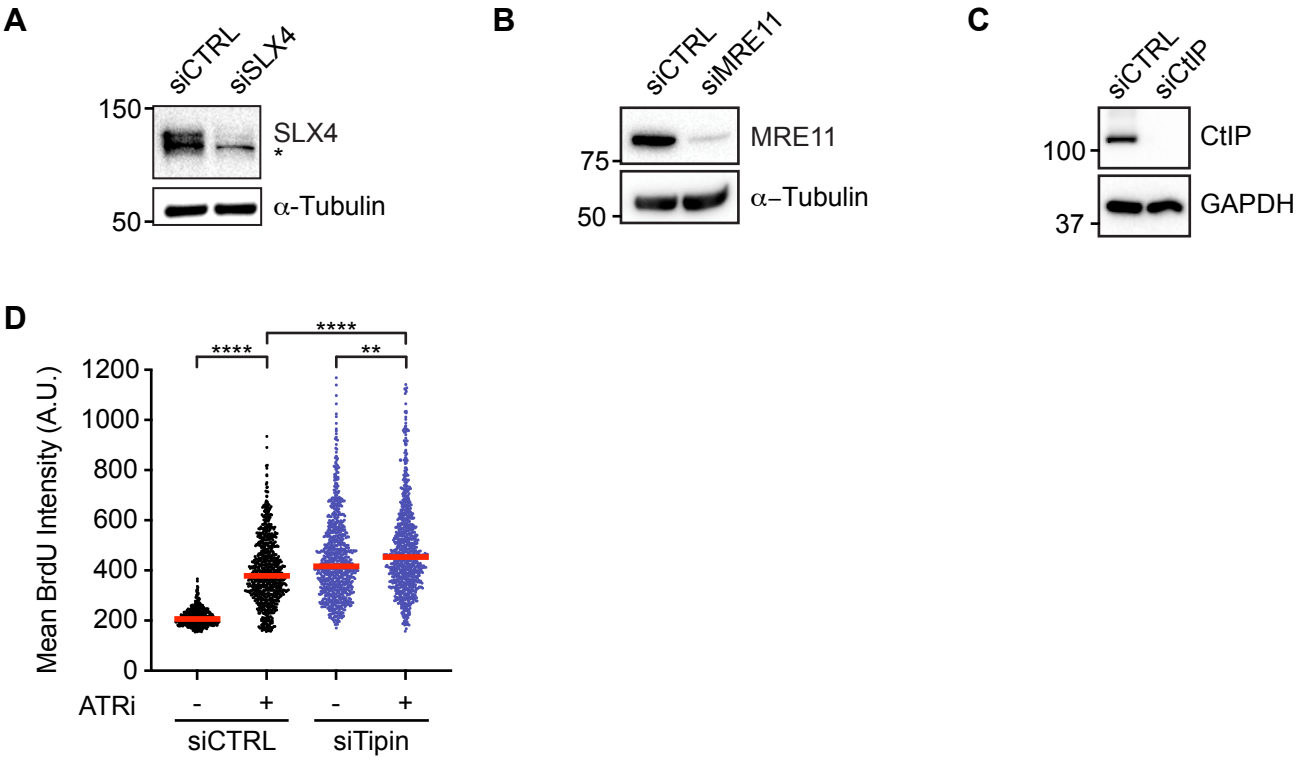
A
siCTRL
siSLX4
150
SLX4
*
50
α-Tubulin
B
siCTRL
siMRE11
75
MRE11
50
α–Tubulin
C
siCTRL
siCtIP
100
CtIP
37
GAPDH
D
Mean BrdU Intensity (A.U.)
1200
1000
800
600
400
200
0
****
****
**
ATRi
-
+
-
+
siCTRL
siTipin

Figure S5 (related to Figure 5). Confirmation of SLX4, MRE11, and CtIP knockdown.

(**A-C**) Western blot confirmation of siRNA-mediated knockdown of SLX4 (A), MRE11 (B), and CtIP (C). (**D**) Fork uncoupling due to loss of Tipin contributes to nascent ssDNA exposure. Cells were treated as in (5A) with or without ATRi (VE-821, 10 μM) following 48 h siRNA knockdown of Tipin. Number (n) of nuclei quantified >500 across three biological replicates. Significance was calculated using Mann-Whitney Ranked Sum Test with **<.01, ****<.0001.

Fig. S6

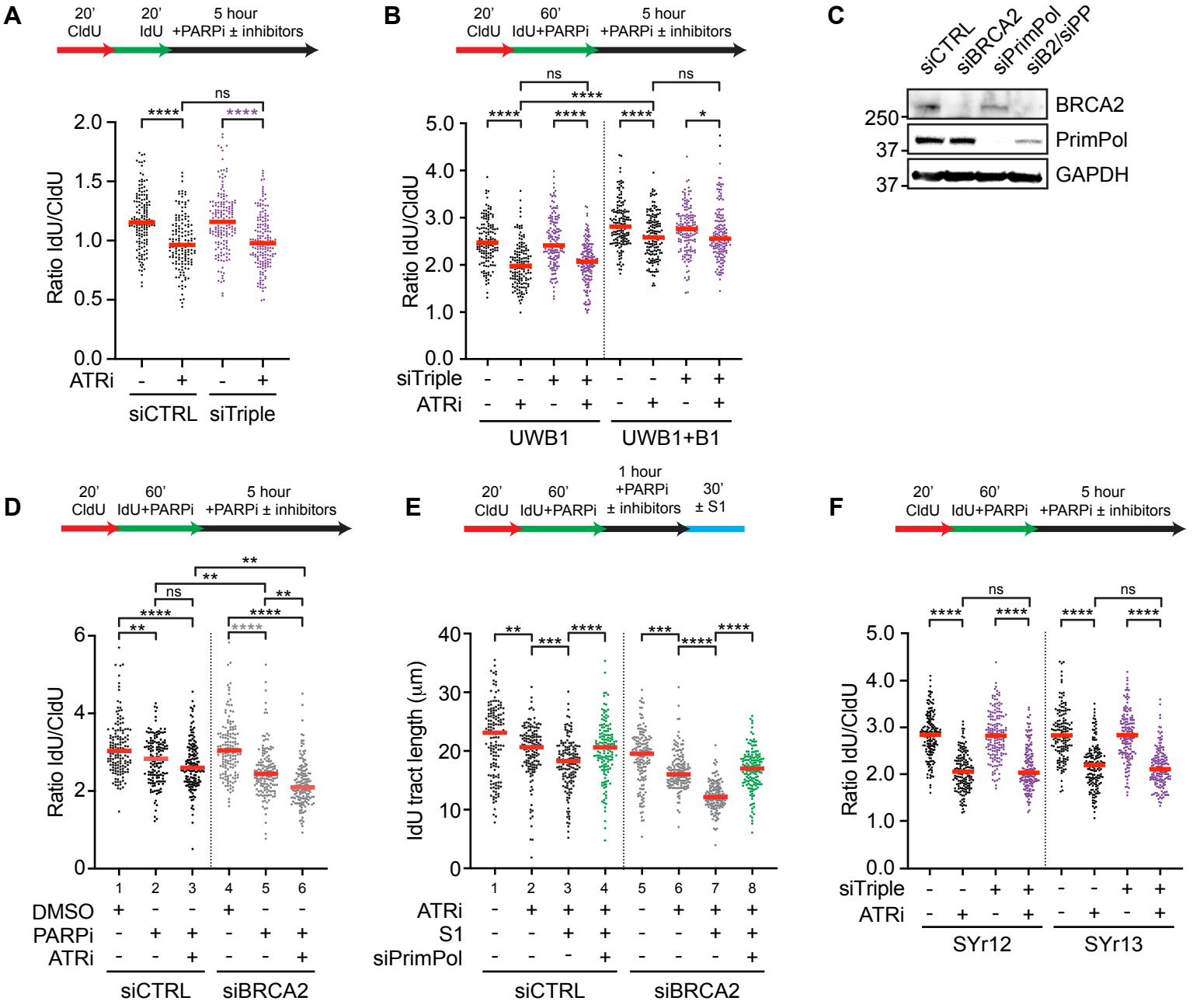
A
20' CldU
20' IdU
5 hour +PARPi ± inhibitors
Ratio IdU/CldU
ATRi
siCTRL
siTriple
B
20' CldU
60' IdU+PARPi
5 hour +PARPi ± inhibitors
Ratio IdU/CldU
siTriple
ATRi
UWB1
UWB1+B1
C
siCTRL
siBRCA2
siPrimPol
siB2/siPP
BRCA2
PrimPol
GAPDH
250
37
37
D
20' CldU
60' IdU+PARPi
5 hour +PARPi ± inhibitors
Ratio IdU/CldU
DMSO
PARPi
ATRi
siCTRL
siBRCA2
E
20' CldU
60' IdU+PARPi
1 hour +PARPi ± inhibitors
30' ± S1
IdU tract length (µm)
ATRi
S1
siPrimPol
siCTRL
siBRCA2
F
20' CldU
60' IdU+PARPi
5 hour +PARPi ± inhibitors
Ratio IdU/CldU
siTriple
ATRi
SYr12
SYr13

Figure S6 (related to Figure 6). Fork reversal is not required for ATRi-induced nascent DNA degradation from PARPi-induced ssDNA gaps

(**A-B**) ATRi-induced nascent DNA degradation in PARPi-treated cells is independent of fork reversal in U2OS (A), BRCA1-deficient UWB1 and UWB1 complemented with wild-type BRCA1 (UWB1+B1) cells (B). Cells were sequentially labeled in CldU (50 μM) followed by IdU (100 μM) in the presence of PARPi (Olaparib, 10 μM). Cells were then incubated in media containing PARPi (Olaparib, 10 μM) with or without ATRi (VE-821, 10 μM) for 5 h following 48 h knockdown of HLTF, SMARCAL1, and ZRANB3 (siTriple) and processed for fiber assay analysis. (**C**) Western blot confirmation of siRNA-mediated knockdown of BRCA2 and PrimPol. (**D**) Inhibition of ATR enhances nascent DNA degradation in BRCA2-deficient cells. U2OS cells were sequentially labeled in CldU (50 μM) followed by IdU (100 μM) in the presence of PARPi (Olaparib, 10 μM). Cells were then incubated in media containing DMSO or PARPi (Olaparib, 10 μM) with or without ATRi (VE821, 10 μM) for 5 h and processed for fiber assay analysis following 48 h knockdown of BRCA2. (**E**) Inhibition of ATR increases ssDNA gaps at forks generated by PrimPol in BRCA2-deficient cells. U2OS cells were sequentially labeled in CldU (50 μM) followed by IdU (100 μM) in the presence of PARPi (Olaparib, 10 μM). Cells were then incubated in media containing PARPi (Olaparib, 10 μM) with or without ATRi (VE-821, 10 μM) for 1 h followed by S1 nuclease digestion and processed for fiber assay analysis following 48 h knockdown of BRCA2 and PrimPol. Number (n) of fibers quantified >250 across two biological replicates. Significance was calculated using Mann-Whitney Ranked Sum Test with **<.01, ***<.001, ****<.0001 in D-E. (**F**) ATRi-induced nascent DNA degradation in PARPi-treated cells is independent of fork reversal in BRCA1-deficient, PARPi-resistant SYr12 and SYr13 cells. Cells were sequentially labeled in CldU (50 μM) followed by IdU (100 μM) in the presence of PARPi (Olaparib, 10 μM). Cells were then incubated in media containing PARPi (Olaparib, 10 μM) with or without ATRi (VE-821, 10 μM) for 5 h following 48 h knockdown of HLTF, SMARCAL1, and ZRANB3 (siTriple) and processed for fiber assay analysis. Number (n) of fibers quantified >300 across two biological replicates. Significance was calculated using Mann-Whitney Ranked Sum Test with *<.05, ****<.0001 in A-B and F.

Table S1. A list of the siRNAs used in this study	
BRCA2	GGAUUAUACAUAUUUCGCATT
Control siRNA	Silencer Negative Control No.1 siRNA, 4404021
CtIP	GCUAAAACAGGAACGAAUCTT
DNA2	GAGUCACAAUCGAAGGAUATT
EXO1	GCCUGAGAAUAAUAUGUCUTT
HLTF	GGAAUAUAAUGUUAACGAUTT
MRE11	CCAAAAGACAUUAUCCAUUTT
MUS81	CAGCCCUGGUGGAUCGAUA
PrimPol-1	GAAGAGCCCAUAAGAGUAATT
PrimPol-2	GAGACUAUUUCAUCGACAATT
SMARCAL1	AGAGAGAAGUUUUUAGUAUTT
SLX4	AAACGUGAAUGAAGCAGAA
TIMELESS	GCAGCAUGAUGAGACCUAUTT
TIPIN	GCAAAGCUGCUGAGUAAUATT
ZRANB3	GACUCGUUACAUUAGGAUATT