Molecular Cell, Volume *73*

Supplemental Information

Efficient Pre-mRNA Cleavage Prevents Replication-Stress-Associated Genome Instability

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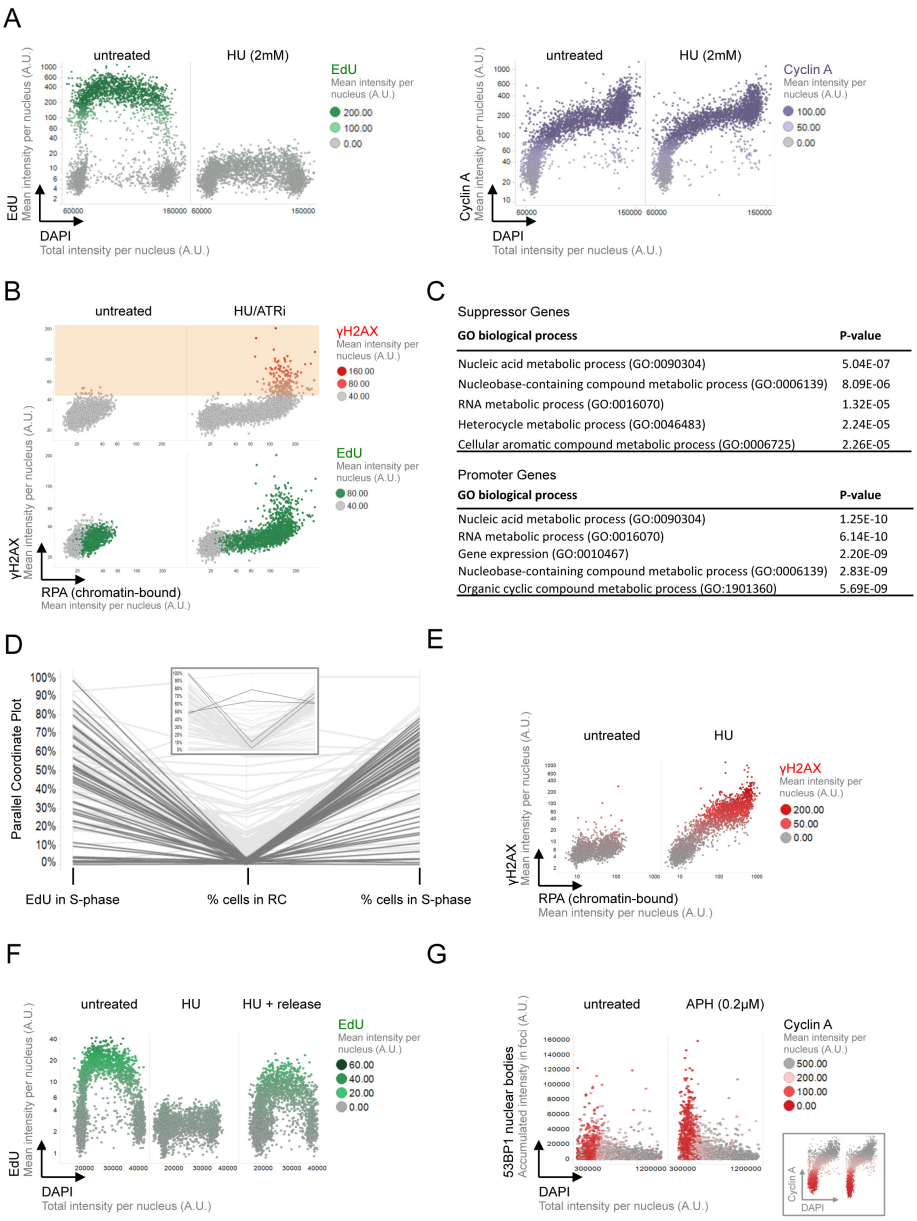

Figure S1: Convergent RNAi screens to identify high-confidence regulators of RS resilience. Related to Figure 1
(A) U-2 OS cells were treated with 2 mM HU for 1h as indicated and incorporation of EdU as well as Cyclin A levels were measured by QIBC.
(B) Cells were exposed to EdU for 20 min prior to a 1h HU/ATRi treatment and assessed for EdU, chromatin-bound RPA and γH2AX signaling by QIBC.
(C) GO analysis of identified suppressor genes (whose knockdown sensitizes to RC) and promoter genes (whose knockdown rescues from RC).
(D) Parallel coordinate plot of the screening results showing that neither the percentage of cells in S-phase nor EdU incorporation predicts RS sensitivity. The inlet highlights that cells with high S-phase percentage and high EdU incorporation can be resistant against RS, while intermediate levels of EdU incorporation can be associated with hypersensitivity to RS.
(E) Read-out for the second screen, which measured RS sensitivity upon HU treatment (2 mM, 4h). See Table S3 for results.
(F) Read-out for the third screen, which measured EdU recovery from a 2 h HU pulse. See Table S4 for results.
(G) Read-out for the fourth screen, which measured 53BP1 nuclear body formation in G1 cells as a consequence of mild RS (0.2 μM APH for 24h). Cells are color-coded by Cyclin A intensity to mark G1 cells. See Table S5 for results.

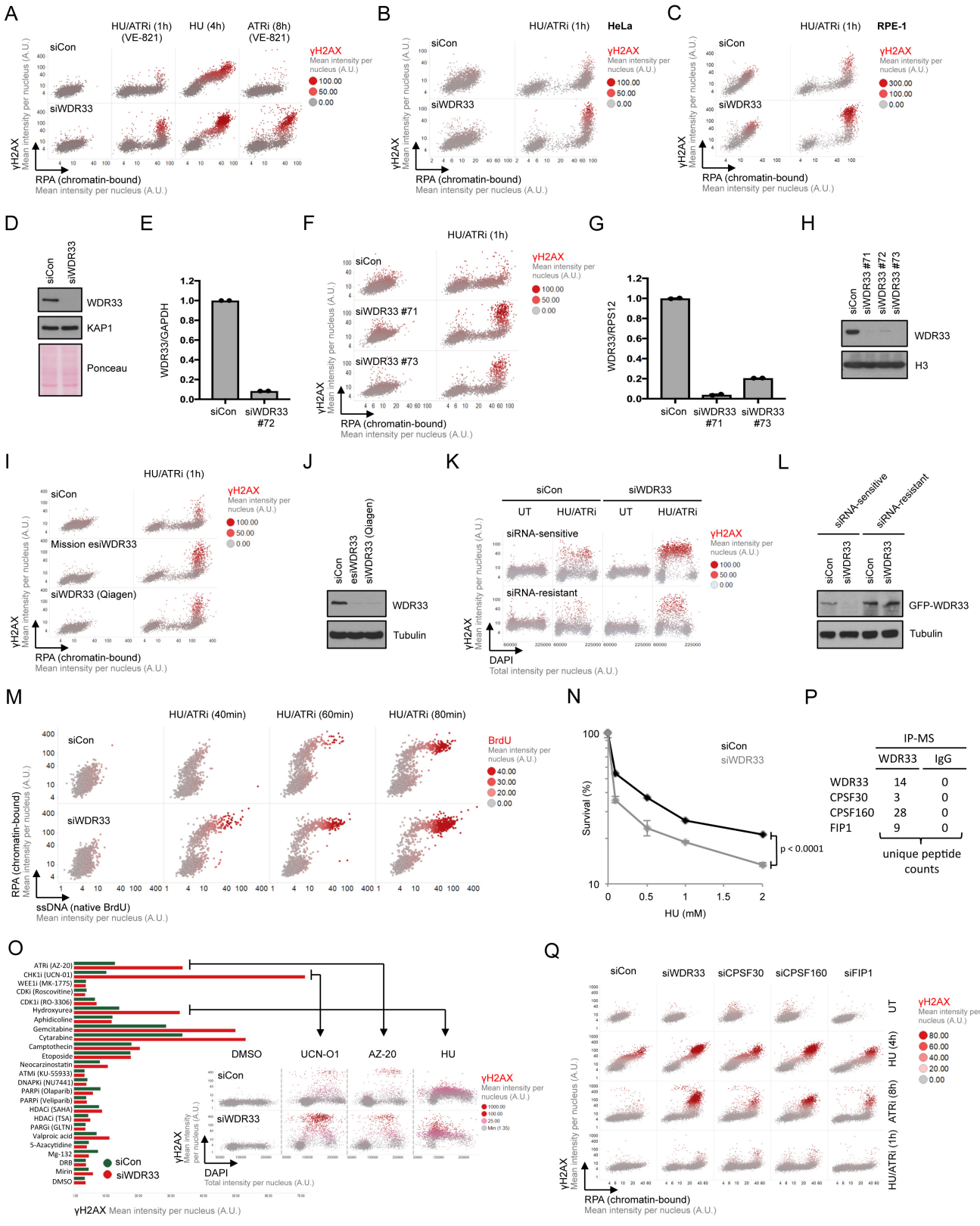
A
HU/ATRi (1h) (VE-821)
HU (4h)
ATRi (8h) (VE-821)
siCon
siWDR33
γH2AX
Mean intensity per nucleus (A.U.)
100.00
50.00
0.00
γH2AX Mean intensity per nucleus (A.U.)
RPA (chromatin-bound) Mean intensity per nucleus (A.U.)
B
HU/ATRi (1h)
HeLa
siCon
siWDR33
γH2AX
Mean intensity per nucleus (A.U.)
100.00
50.00
0.00
γH2AX Mean intensity per nucleus (A.U.)
RPA (chromatin-bound) Mean intensity per nucleus (A.U.)
C
HU/ATRi (1h)
RPE-1
siCon
siWDR33
γH2AX
Mean intensity per nucleus (A.U.)
300.00
100.00
0.00
γH2AX Mean intensity per nucleus (A.U.)
RPA (chromatin-bound) Mean intensity per nucleus (A.U.)
D
siCon
siWDR33
WDR33
KAP1
Ponceau
E
WDR33/GAPDH
siCon
siWDR33 #72
F
HU/ATRi (1h)
siCon
siWDR33 #71
siWDR33 #73
γH2AX
Mean intensity per nucleus (A.U.)
100.00
50.00
0.00
γH2AX Mean intensity per nucleus (A.U.)
RPA (chromatin-bound) Mean intensity per nucleus (A.U.)
G
WDR33/RPS12
siCon
siWDR33 #71
siWDR33 #73
H
siCon
siWDR33 #71
siWDR33 #72
siWDR33 #73
WDR33
H3
I
HU/ATRi (1h)
siCon
Mission esiWDR33
siWDR33 (Qiagen)
γH2AX
Mean intensity per nucleus (A.U.)
100.00
50.00
0.00
γH2AX Mean intensity per nucleus (A.U.)
RPA (chromatin-bound) Mean intensity per nucleus (A.U.)
J
siCon
esiWDR33
siWDR33 (Qiagen)
WDR33
Tubulin
K
siCon
siWDR33
UT
HU/ATRi
UT
HU/ATRi
siRNA-sensitive
siRNA-resistant
γH2AX
Mean intensity per nucleus (A.U.)
100.00
50.00
0.00
γH2AX Mean intensity per nucleus (A.U.)
DAPI Total intensity per nucleus (A.U.)
L
siRNA-sensitive
siRNA-resistant
siCon
siWDR33
siCon
siWDR33
GFP-WDR33
Tubulin
M
HU/ATRi (40min)
HU/ATRi (60min)
HU/ATRi (80min)
siCon
siWDR33
BrdU
Mean intensity per nucleus (A.U.)
40.00
30.00
20.00
0.00
RPA (chromatin-bound) Mean intensity per nucleus (A.U.)
ssDNA (native BrdU) Mean intensity per nucleus (A.U.)
N
siCon
siWDR33
Survival (%)
p < 0.0001
HU (mM)
P
IP-MS
WDR33
IgG
WDR33 14 0
CPSF30 3 0
CPSF160 28 0
FIP1 9 0
unique peptide counts
O
ATRi (AZ-20)
CHK1i (UCN-01)
WEE1i (MK-1775)
CDKi (Roscovitine)
CDK1i (RO-3306)
Hydroxyurea
Aphidicoline
Gemcitabine
Cytarabine
Camptothecin
Etoposide
Neocarzinostatin
ATMi (KU-55933)
DNAPKi (NU7441)
PARPi (Olaparib)
PARPi (Veliparib)
HDACi (SAHA)
HDACi (TSA)
PARGi (GLTN)
Valproic acid
5-Azacytidine
Mg-132
DRB
Mirin
DMSO
siCon
siWDR33
DMSO
UCN-O1
AZ-20
HU
γH2AX
Mean intensity per nucleus (A.U.)
DAPI Total intensity per nucleus (A.U.)
γH2AX Mean intensity per nucleus (A.U.)
Q
siCon
siWDR33
siCPSF30
siCPSF160
siFIP1
UT
HU (4h)
ATRi (8h)
HU/ATRi (1h)
γH2AX
Mean intensity per nucleus (A.U.)
80.00
60.00
40.00
20.00
0.00
γH2AX Mean intensity per nucleus (A.U.)
RPA (chromatin-bound) Mean intensity per nucleus (A.U.)
R
WDR33/GAPDH
siCon
siWDR33
CPSF30/GAPDH
siCon
siCPSF30
CPSF160/GAPDH
siCon
siCPSF160
FIP1/GAPDH
siCon
siFIP1
S
siCon
siWDR33
siCPSF30
siCPSF160
siFIP1
WDR33
H3

Figure S2: WDR33 depletion sensitizes cells to replication drugs and checkpoint inhibitors. Related to Figure 2.
(A) U-2 OS cells were transfected with siRNA as indicated, treated with HU, ATRi VE-821, or both, and analyzed for their susceptibility to enter RC.
(B) HeLa cells were transfected with siRNA as indicated, treated with HU/ATRi and analyzed for their susceptibility to enter RC.
(C) RPE-1 cells were transfected with siRNA as indicated, treated with HU/ATRi (5 μM Az-20) and analyzed for their susceptibility to enter RC.
(D) Knockdown efficiency of siWDR33 transfection in U-2 OS cells as revealed by Western Blot.
(E) Knockdown efficiency as quantified by qPCR.
(F) U-2 OS cells were transfected with two additional siRNAs against WDR33 as indicated, treated with HU/ATRi and analyzed for their susceptibility to enter RC.
(G) Knockdown efficiency of WDR33 by the second set of siRNAs as quantified by qPCR.
(H) Knockdown efficiency of the three siRNAs against WDR33 (#71, #72, #73) as revealed by Western Blot.
(I) U-2 OS cells were transfected with a fourth siRNA against WDR33 from Qiagen or an esiRNA pool against WDR33 as indicated, treated with HU/ATRi and analyzed for their susceptibility to enter RC.
(J) Knockdown efficiencies as revealed by Western Blot.
(K) Monoclonal U-2 OS cell lines expressing either siRNA-sensitive or siRNA-resistant GFP-WDR33 were transfected with siRNA as indicated, treated with HU/ATRi for 1h and RC-associated γH2AX formation was quantified.
(L) Knockdown control of GFP-WDR33 expressing cells by Western Blot.
(M) U-2 OS cells were transfected with siRNA as indicated, incubated with BrdU for the last 24h, and RPA chromatin loading and exposure of ssDNA (visualized by native BrdU staining) were assessed upon HU/ATRi treatment.
(N) U-2 OS cells were transfected with siRNA as indicated, exposed to HU for 24h, and clonogenic survival was measured 10 days later. Normalized average colony numbers +/- SD are shown.
(O) Compound screen to assess drug sensitivities of WDR33-depleted cells. U-2 OS cells were transfected with siRNA as indicated and subjected to 24h drug treatments in 96 well imaging plates. Cell cycle resolved DNA damage signaling was assessed by γH2AX measurements. Cell population data for example conditions are shown on the right.
(P) Endogenous WDR33 was immunoprecipitated from U-2 OS cells and interacting cleavage and polyadenylation complex components were identified by mass spectrometry.
(Q) The polyadenylation specificity factor (PSF) components CPSF30, CPSF160 and FIP1 were depleted as indicated and RS sensitivity was assessed by quantification of entry into RC.
(R) Knockdown efficiencies of individual PSF components were assessed by qPCR.
(S) WDR33 protein levels upon depletion of different PSF components were analyzed by Western Blot.

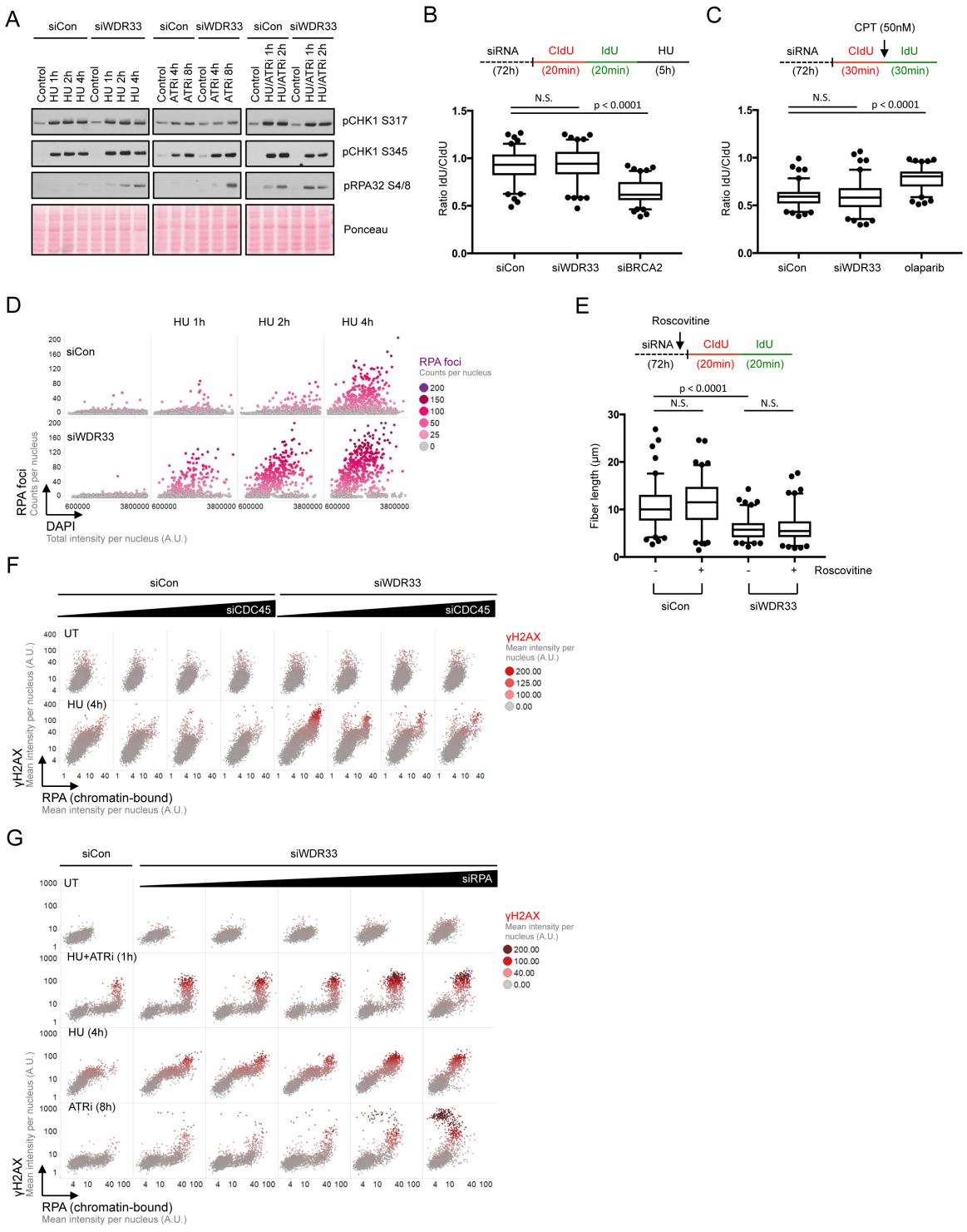
A
siCon
siWDR33
Control
HU 1h
HU 2h
HU 4h
ATRi 4h
ATRi 8h
HU/ATRi 1h
HU/ATRi 2h
pCHK1 S317
pCHK1 S345
pRPA32 S4/8
Ponceau
B
siRNA
(72h)
CldU
(20min)
IdU
(20min)
HU
(5h)
N.S.
p < 0.0001
Ratio IdU/CldU
siCon
siWDR33
siBRCA2
C
CPT (50nM)
(30min)
olaparib
D
HU 1h
HU 2h
HU 4h
RPA foci
Counts per nucleus
DAPI
Total intensity per nucleus (A.U.)
E
Roscovitine
Fiber length (µm)
F
siCDC45
UT
HU (4h)
γH2AX
Mean intensity per nucleus (A.U.)
RPA (chromatin-bound)
G
siRPA
HU+ATRi (1h)
ATRi (8h)

Figure S3: WDR33 depletion does not affect checkpoint activation, fork reversal and fork degradation. Related to Figure 3.
(A) Western Blot analysis of checkpoint activation (pCHK1) and entry into RC (pRPA32 S4/8) upon HU (left panels), ATRi (middle panels) and combined treatments (right panels).
(B) Nascent DNA degradation assay. Cells were treated with siRNA and CIdU/IdU as indicated followed by a 5h 4 mM HU challenge. Degradation of nascent DNA was measured by calculation of the IdU/CIdU ratio.
(C) Unrestrained fork progression assay. Cells were treated with siRNA and CIdU/IdU as indicated; fork progression in the presence of 50 nM CPT was assessed by calculation of the IdU/CIdU ratio. Olaparib (10 μM) was added 60 min prior to the CIdU/IdU pulse.
(D) RPA foci formation upon short-term HU-induced replication fork stalling in checkpoint-proficient control and WDR33-depleted cells.
(E) Replication fork speed with and without CDKi. Roscovitine was added 80 min prior to the CIdU/IdU labeling.
(F) Partial depletion of CDC45 rescues WDR33-deficient cells from RC. Cells were treated with siRNA as indicated (siCDC45 titration: 0 nM, 0.25 nM, 1.25 nM, 2.5 nM), exposed to HU, and analyzed by QIBC for entry into RC.
(G) Partial depletion of RPA exacerbates RC in WDR33-deficient cells. Cells were treated with siRNA as indicated (siRPA1 titration: 0 nM, 0.04 nM, 0.2 nM, 1 nM, 5 nM), exposed to HU, ATRi or HU/ATRi, and analyzed by QIBC for entry into RC.

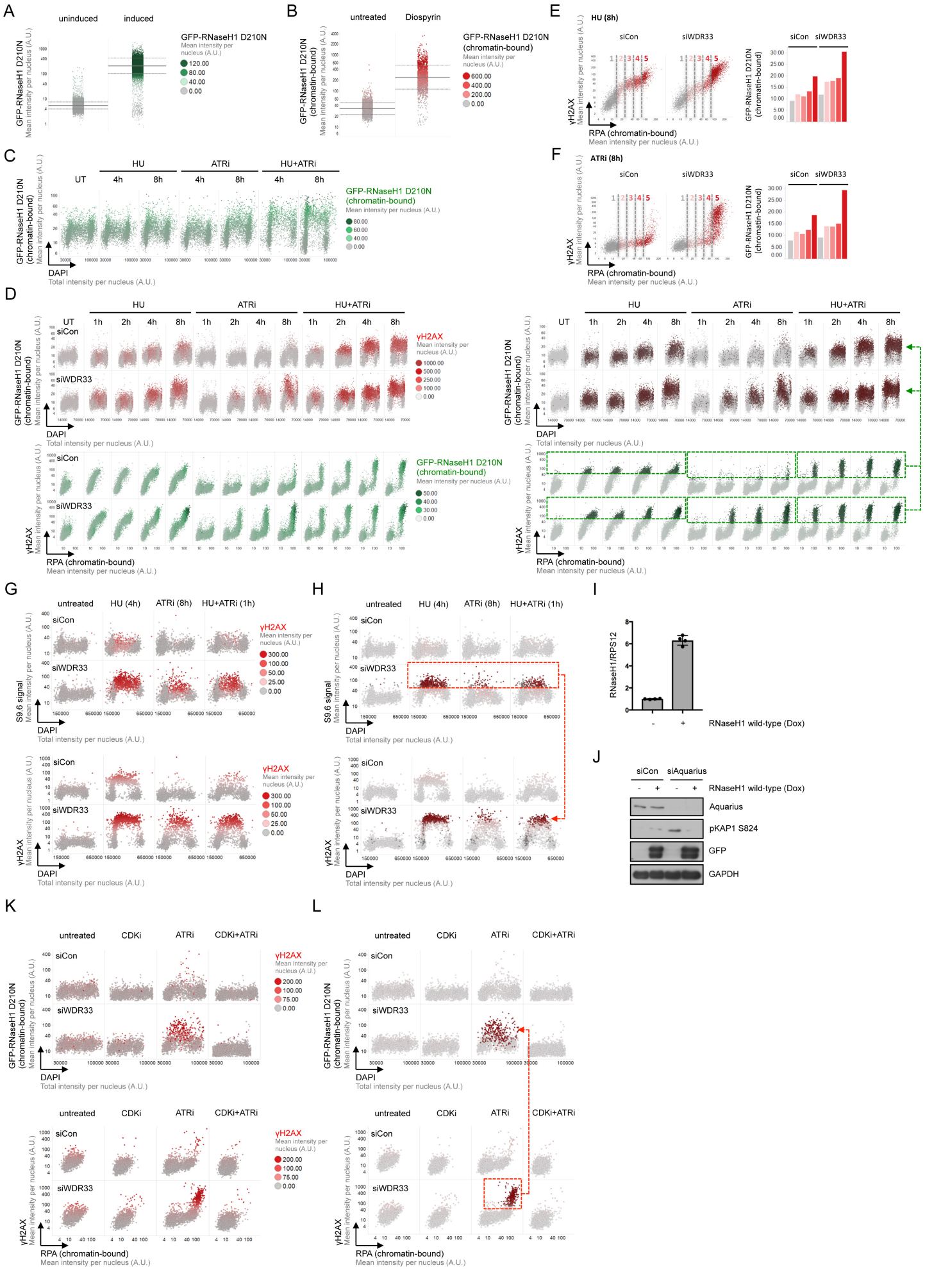
A
uninduced
induced
GFP-RNaseH1 D210N
Mean intensity per nucleus (A.U.)
120.00
80.00
40.00
0.00
B
untreated
Diospyrin
GFP-RNaseH1 D210N (chromatin-bound)
600.00
400.00
200.00
0.00
E
HU (8h)
siCon
siWDR33
γH2AX
RPA (chromatin-bound)
F
ATRi (8h)
C
UT
HU
ATRi
HU+ATRi
4h
8h
DAPI
Total intensity per nucleus (A.U.)
D
1h
2h
siCon
siWDR33
G
untreated
HU (4h)
ATRi (8h)
HU+ATRi (1h)
S9.6 signal
H
I
RNaseH1/RPS12
RNaseH1 wild-type (Dox)
J
siAquarius
Aquarius
pKAP1 S824
GFP
GAPDH
K
CDKi
CDKi+ATRi
L

Figure S4: WDR33-depleted cells accumulate RNA:DNA-hybrids when entering RC. Related to Figure 4.
(A) GFP-RNaseH1 D210N cells were induced for 24h as indicated and GFP levels were measured by QIBC.
(B) GFP-RNaseH1 D210N cells were induced for 24h and chromatin retention of mutant RNaseH1 upon RNA:DNA-hybrid formation by Diospyrin was assessed.
(C) Cell cycle resolved RNA:DNA-hybrid formation upon different RS treatments was measured by quantification of chromatin-bound GFP-RNaseH1 D210N.
(D) Extended time course of RNA:DNA-hybrid formation upon different RS treatments in siCon and siWDR33 cells. Chromatin-bound GFP-RNaseH1 D210N, γH2AX and chromatin-bound RPA were measured simultaneously in the same cell populations (left panels). Cells in RC were marked based on their γH2AX and chromatin-bound RPA levels, and their RNA:DNA-hybrid levels were highlighted (right panel). For HU-treated samples, cells with highest levels of ATR-independent γH2AX formation are marked. Note that compared to non-highlighted cells, cells in RC show consistently higher levels of RNA:DNA-hybrids.
(E) The 8h HU samples from (D) were sub-divided further in five sub-populations from low to high RPA loading, and their average levels of RNA:DNA-hybrids are depicted. Note the sharp increase in RNA:DNA-hybrids in the fifth sub-population with RPA exhaustion.
(F) The 8h ATRi samples from (D) were sub-divided further in five sub-populations from low to high RPA loading, and their average levels of RNA:DNA-hybrids are depicted. Note the sharp increase in RNA:DNA-hybrids in the fifth sub-population with RPA exhaustion.
(G) Validation of RNA:DNA-hybrid measurements in control and siWDR33 cells using the S9.6 antibody.
(H) RNA:DNA-hybrid-positive S-phase cells have maximum levels of γH2AX. Cells with detectable levels of S9.6-marked RNA:DNA-hybrids from (G) were selected (top panels) and their γH2AX levels analyzed (lower panels). Note again the tight association between RNA:DNA-hybrids and high γH2AX levels.
(I) Induction of wild-type GFP-RNaseH1 measured by qPCR.
(J) Confirmation of the functionality of wild-type GFP-RNaseH1 over-expression by monitoring pKAP1 as marker of R-loop-induced DNA damage in siAquarius cells (Sollier et al., 2014).
(K) CDKi rescues cells from RC and reduces RNA:DNA-hybrid formation. RNA:DNA-hybrids were assessed by quantification of chromatin-retained, catalytically inactive GFP-RNaseH1 D210N in siCon and siWDR33 cells with and without CDKi AZD5438 (top panels). In the same cells, RC was also analyzed (bottom panels). All treatments were done for 8h.
(L) Cells in RC from (K), marked by maximum chromatin-loading of RPA and highest γH2AX levels, were selected (lower panels) and their RNA:DNA-hybrid levels are shown (top panels). All treatments were done for 8h. Note that cells in RC are the ones with highest RNA:DNA-hybrid levels.

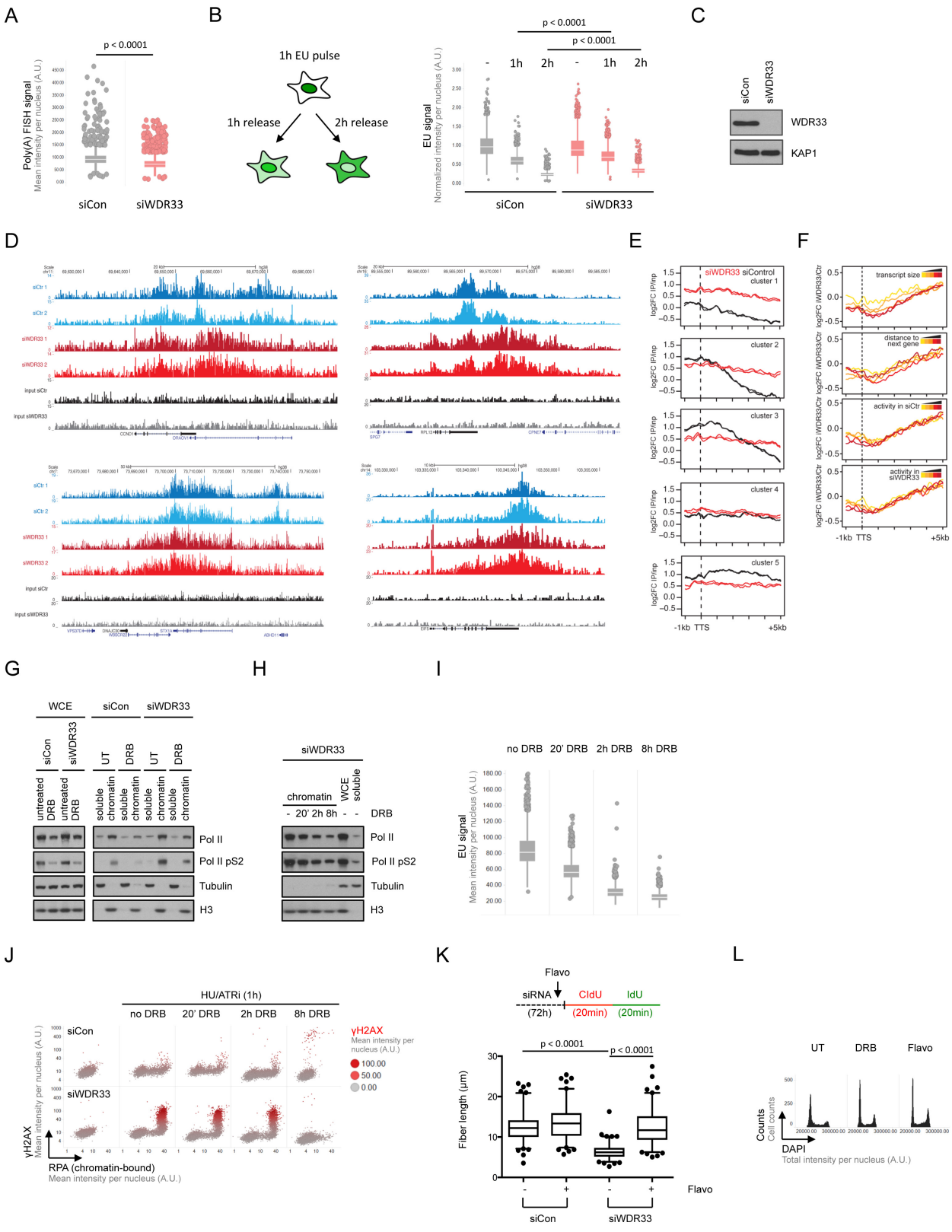
A
p < 0.0001
Poly(A) FISH signal
Mean intensity per nucleus (A.U.)
siCon
siWDR33
B
1h EU pulse
1h release
2h release
p < 0.0001
p < 0.0001
EU signal
Normalized intensity per nucleus (A.U.)
-
1h
2h
-
1h
2h
siCon
siWDR33
C
siCon
siWDR33
WDR33
KAP1
D
siCtr 1
siCtr 2
siWDR33 1
siWDR33 2
input siCtr
input siWDR33
E
siWDR33 siControl
cluster 1
cluster 2
cluster 3
cluster 4
cluster 5
log2FC IP/inp
-1kb TTS +5kb
F
transcript size
distance to next gene
activity in siCtr
activity in siWDR33
log2FC iWDR33/Ctr
-1kb TTS +5kb
G
WCE
siCon
siWDR33
siCon
siWDR33
UT
DRB
UT
DRB
untreated
DRB
untreated
DRB
soluble
chromatin
soluble
chromatin
soluble
chromatin
soluble
chromatin
Pol II
Pol II pS2
Tubulin
H3
H
siWDR33
chromatin
WCE soluble
- 20' 2h 8h - -
DRB
Pol II
Pol II pS2
Tubulin
H3
I
no DRB
20' DRB
2h DRB
8h DRB
EU signal
Mean intensity per nucleus (A.U.)
J
HU/ATRi (1h)
no DRB
20' DRB
2h DRB
8h DRB
siCon
siWDR33
γH2AX
Mean intensity per nucleus (A.U.)
100.00
50.00
0.00
γH2AX
Mean intensity per nucleus (A.U.)
RPA (chromatin-bound)
Mean intensity per nucleus (A.U.)
K
Flavo
siRNA
(72h)
CldU
(20min)
IdU
(20min)
p < 0.0001
p < 0.0001
Fiber length (μm)
Flavo
siCon
siWDR33
L
UT
DRB
Flavo
Counts
Cell counts
DAPI
Total intensity per nucleus (A.U.)

Figure S5: Rescue of RS sensitivity by inhibition of transcription. Related to Figure 5.
(A) Cells were transfected with siRNA as indicated and mRNA polyadenylation was assessed by poly(A) FISH.
(B) Cells were transfected with siRNA as indicated, exposed to EU for 1h and released into fresh medium for 1h or 2h. Nuclear EU intensities were measured.
(C) The supernatants from the RNA Pol II pS2 ChIP-seq samples were analyzed by Western Blot to control the WDR33 knockdown.
(D) ChIP-seq-derived RNA Pol II pS2 binding profiles in duplicates for four additional genomic regions. Input reads are shown at the bottom.
(E) Average RNA Pol II pS2 binding for the five gene clusters from -1kb to +5kb of the TTS.
(F) RNA Pol II pS2 occupancy sub-divided by transcript size, distance to next gene, gene activity in siCon, and gene activity in siWDR33.
(G) Chromatin fractionation to globally assess RNA Pol II loading upon WDR33 depletion. Where indicated, cells were treated with DRB for the last 8h.
(H) Time-course of DRB-induced RNA Pol II removal from chromatin.
(I) Time-course of DRB-induced transcriptional inhibition measured by EU incorporation.
(J) Rescue of RC in WDR33-depleted cells by DRB treatment.
(K) Rescue of replication fork speed in WDR33-depleted cells by flavopiridol. Where indicated, cells were treated with flavopiridol for the last 8h.
(L) QIBC-derived DAPI-based cell cycle profiles of cells treated with DRB or flavopiridol for 8h.

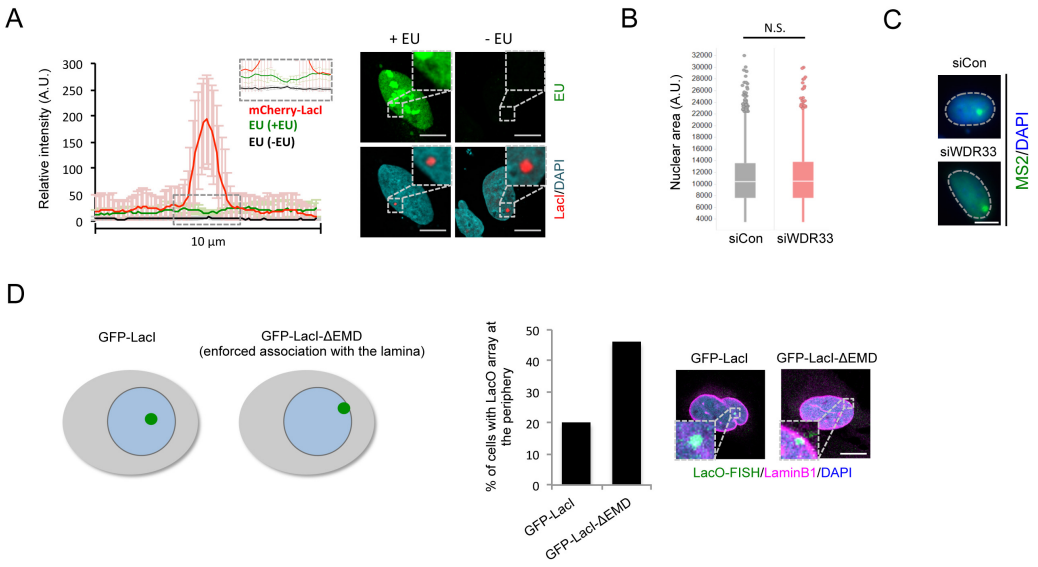

Figure S6: WDR33 deficiency is linked to gene re-localization and chromosome fragility at the nuclear periphery. Related to Figure 6.

(A) Analysis of transcriptional activity at the LacI-marked locus of the LacO/LacI system using EU Click-iT in EU-labeled (+EU) and -unlabeled (-EU) cells.

(B) Nuclear area of siCon and siWDR33 cells used to quantify the closest distance of the center of the LacO array to the nuclear periphery.

(C) Example images of the MS2 reporter to mark nascent transcripts in siCon and siWDR33 cells.

(D) Control for the re-localization of the LacO array to the nuclear periphery upon binding by GFP-LacI-ΔEMD. The percentage of cells with the LacO array visualized by DNA-FISH at the nuclear periphery based on LaminB1 co-staining was quantified. Scale bar, 10 µm.

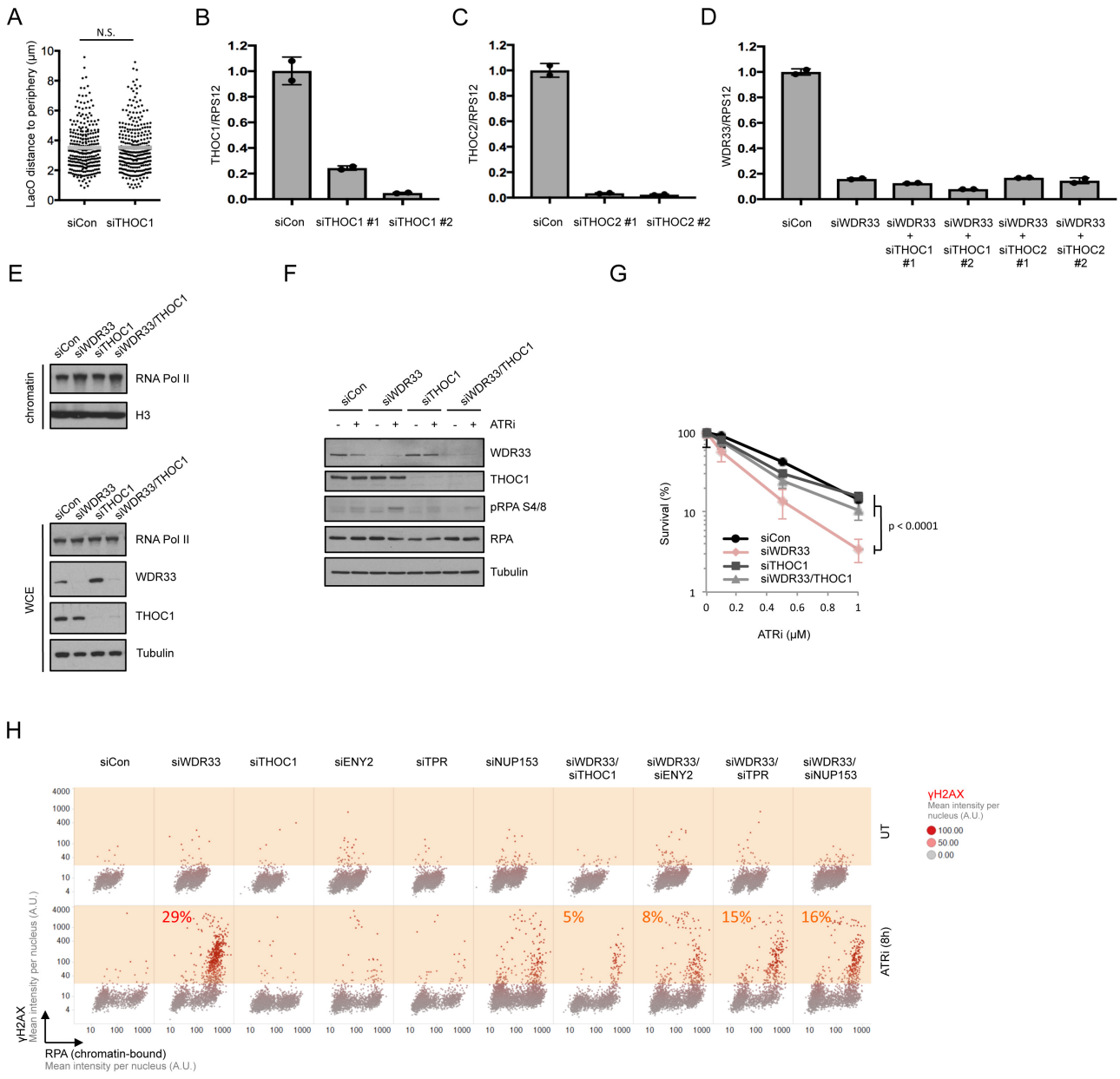

Figure S7: Rescue of RS sensitivity by co-depletion of the THO complex. Related to Figure 7.

(A) Quantification of the closest distance of the center of the LacO array to the nuclear periphery upon depletion of THOC1.

(B) Knockdown efficiencies of two different siRNAs targeting THOC1 were assessed by qPCR.

(C) Knockdown efficiencies of two different siRNAs targeting THOC2 were assessed by qPCR.

(D) Knockdown efficiency of WDR33 upon co-depletion with THOC1/2 was assessed by qPCR.

(E) Chromatin and whole cell extracts to assess RNA Pol II chromatin loading upon co-depletion of WDR33 and THOC1.

(F) Western Blot analysis of pRPA S4/8 upon single and combined WDR33 and THOC1 depletion. Cells were transfected and treated with ATRi for 8h as indicated.

(G) U-2 OS cells were transfected with siRNA as indicated, exposed to ATRi for 24h, and clonogenic survival was measured 10 days later. Average colony numbers ± SD are shown.

(H) Co-depletion of THOC1, ENY2, TPR or NUP153 rescues RS sensitivity in WDR33-deficient cells. U-2 OS cells were transfected as indicated and exposed to ATRi for 8h. While depletion of THOC1, ENY2, TPR or NUP153 did not alleviate RPA loading and DNA damage signaling in WDR33-proficient cells, it partially rescued WDR33-deficient cells from RC. Percentages of cells in RC are provided.