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Removal of RTF2 from stalled replisomes promotes maintenance of genome integrity

Supplemental Information

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Supplemental Table 1: RNAi and primer sequences, related to STAR Methods Cell culture, transfection and viral transduction; and RNA preparation, reverse transcription, and real time PCR.

Figure S1

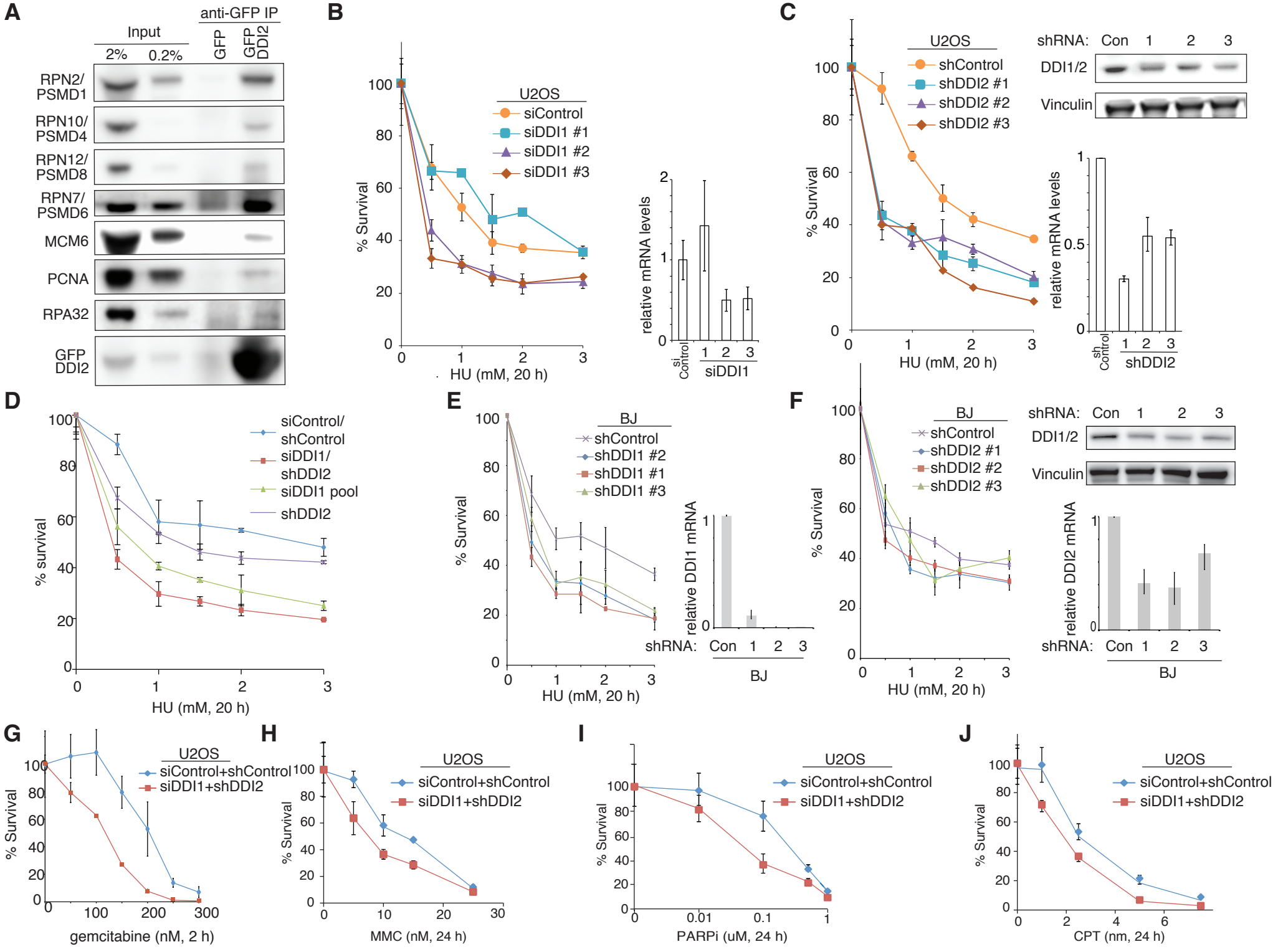

A
Input
anti-GFP IP
2%
0.2%
GFP
GFP DDI2
RPN2/ PSMD1
RPN10/ PSMD4
RPN12/ PSMD8
RPN7/ PSMD6
MCM6
PCNA
RPA32
GFP DDI2
B
U2OS
siControl
siDDI1 #1
siDDI1 #2
siDDI1 #3
% Survival
HU (mM, 20 h)
relative mRNA levels
si Control
siDDI1
C
U2OS
shControl
shDDI2 #1
shDDI2 #2
shDDI2 #3
shRNA: Con 1 2 3
DDI1/2
Vinculin
% Survival
HU (mM, 20 h)
relative mRNA levels
sh Control
shDDI2
D
siControl/ shControl
siDDI1/ shDDI2
siDDI1 pool
shDDI2
% survival
HU (mM, 20 h)
E
BJ
shControl
shDDI1 #2
shDDI1 #1
shDDI1 #3
% Survival
HU (mM, 20 h)
relative DDI1 mRNA
shRNA: Con 1 2 3
BJ
F
BJ
shControl
shDDI2 #1
shDDI2 #2
shDDI2 #3
shRNA: Con 1 2 3
DDI1/2
Vinculin
% Survival
HU (mM, 20 h)
relative DDI2 mRNA
shRNA: Con 1 2 3
BJ
G
U2OS
siControl+shControl
siDDI1+shDDI2
% Survival
gemcitabine (nM, 2 h)
H
U2OS
siControl+shControl
siDDI1+shDDI2
% Survival
MMC (nM, 24 h)
I
U2OS
siControl+shControl
siDDI1+shDDI2
% Survival
PARPi (uM, 24 h)
J
U2OS
siControl+shControl
siDDI1+shDDI2
% Survival
CPT (nm, 24 h)

Figure S1: DDI1/2 depletion leads to decreased cellular survival after replication stress, related to Figure 1

(**A**) Validation of a subset of interactions identified by mass spectrometry by western blot following IP of GFP-tagged DDI2. (B) Graphs showing survival of U2OS cells transiently transfected with 3 independent siRNAs against DDI1 or luciferase control. Cells were treated with the indicated doses of HU for 20 h, washed, released, allowed to grow for 7 days, and counted. Relative DDI1 mRNA levels are shown. (**C**) Graphs showing survival of U2OS cells stably expressing 3 independent shRNAs against DDI2 or luciferase control. Cells were treated as above. Western blot and relative DDI2 mRNA levels are shown. (**D**) Graph showing relative survival of U2OS cells treated with shDDI2, siDDI1, or a combination of the two, following treatment with indicated doses of HU for 20 h. **(E)** Graphs showing survival of BJ cells transduced with 3 independent shRNAs against DDI1 or luciferase control. Cells were treated with the indicated doses of HU for 20 h, washed, released, allowed to grow for 7 days, and counted. (**F**) Graphs showing survival of BJ cells stably expressing 3 independent shRNAs against DDI2 or luciferase control. Cells were treated as above. Western blot and relative DDI2 mRNA levels are shown. (**G-J**) Graphs showing survival of U2OS cells transiently transfected with a pool of siRNAs against DDI1 or control and stably expressing shDDI2 #1 or control following 2 h treatment with indicated doses of gemcitabine **(G)** or 24 hrs of MMC **(H),** PARP inhibitor olaparib **(I)**, or camptothecin **(J)**. Cells were counted 7 days after treatment**.**

Figure S2

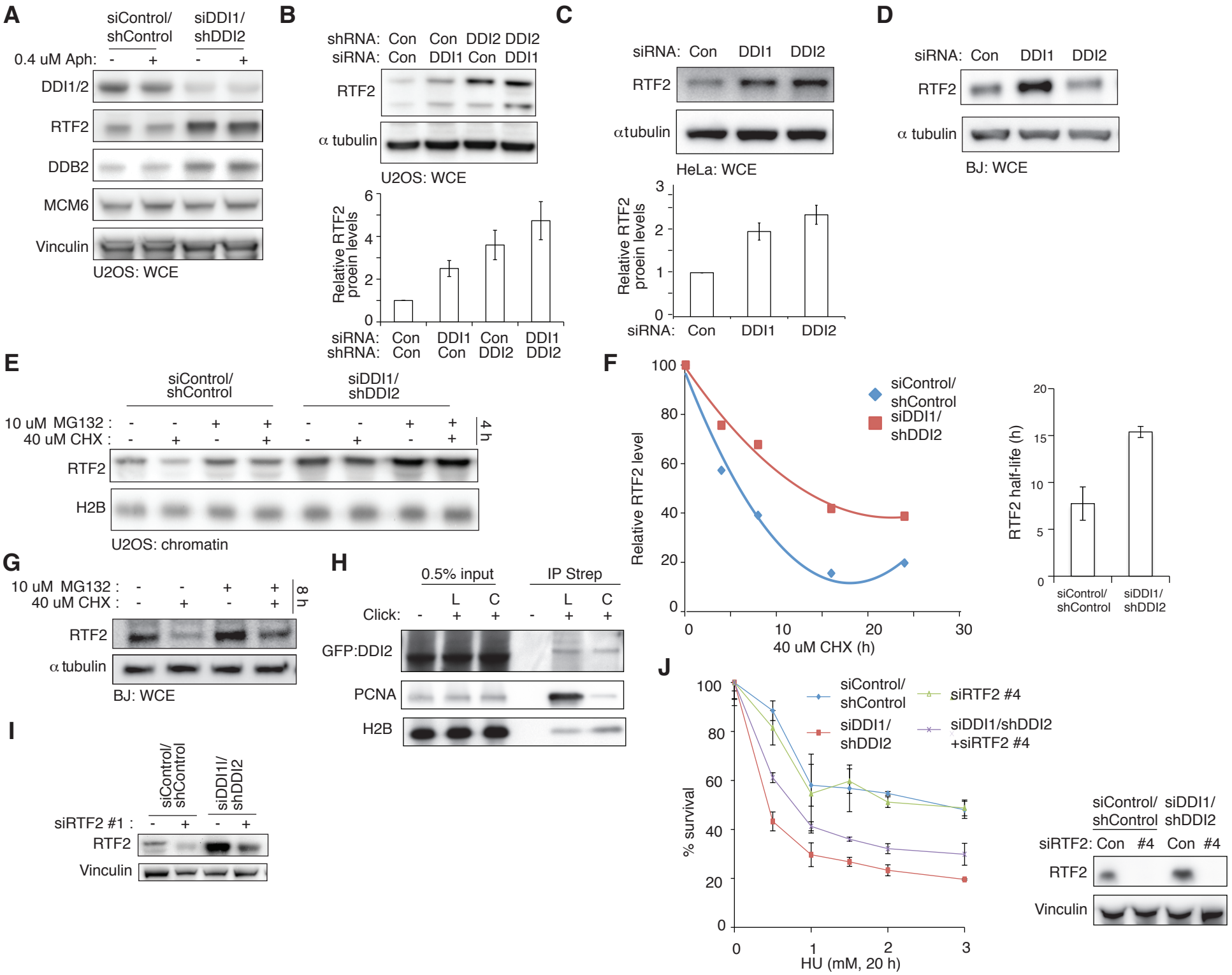

A
siControl/ shControl
siDDI1/ shDDI2
0.4 uM Aph: - + - +
DDI1/2
RTF2
DDB2
MCM6
Vinculin
U2OS: WCE
B
shRNA: Con Con DDI2 DDI2
siRNA: Con DDI1 Con DDI1
RTF2
α tubulin
U2OS: WCE
Relative RTF2 proein levels
siRNA: Con DDI1 Con DDI1
shRNA: Con Con DDI2 DDI2
C
siRNA: Con DDI1 DDI2
RTF2
αtubulin
HeLa: WCE
Relative RTF2 proein levels
siRNA: Con DDI1 DDI2
D
siRNA: Con DDI1 DDI2
RTF2
α tubulin
BJ: WCE
E
siControl/ shControl
siDDI1/ shDDI2
10 uM MG132 : - - + + - - + +
40 uM CHX : - + - + - + - +
4 h
RTF2
H2B
U2OS: chromatin
F
siControl/ shControl
siDDI1/ shDDI2
Relative RTF2 level
40 uM CHX (h)
RTF2 half-life (h)
siControl/ shControl
siDDI1/ shDDI2
G
10 uM MG132 : - - + +
40 uM CHX : - + - +
8 h
RTF2
α tubulin
BJ: WCE
H
0.5% input
IP Strep
L C L C
Click: - + + - + +
GFP:DDI2
PCNA
H2B
I
siControl/ shControl
siDDI1/ shDDI2
siRTF2 #1 : - + - +
RTF2
Vinculin
J
siControl/ shControl
siDDI1/ shDDI2
siRTF2 #4
siDDI1/shDDI2 +siRTF2 #4
% survival
HU (mM, 20 h)
siControl/ shControl
siDDI1/ shDDI2
siRTF2: Con #4 Con #4
RTF2
Vinculin

Figure S2: DDI1/2 depletion results in RTF2 stabilization; related to Figure 2

(A) Western blot showing stabilization of RTF2 and DDB2 in whole cell lysates from U2OS cells following depletion of DDI1/2. (**B**) Western blot and quantification showing stabilization of RTF2 in U2OS cells by depletion of DDI1 alone, DDI2 alone, or both. Error bars represent SEM n=3. (**C**) Western blot and quantification showing RTF2 levels in HeLa cells following depletion of DDI1 or DDI2. Error bars represent SEM n=3. (**D**) Western blot showing RTF2 levels in BJ fibroblasts following depletion of DDI1 or DDI2. (**E**) Western blot showing stabilization of RTF2 on chromatin from U2OS cells following knockdown of DDI1+2 and/or treatment with proteasome inhibitor. Cells were exposed to 4 h of cycloheximide, MG-132, or a combination of the two drugs. (**F**) Graphs showing half-life of RTF2 in the presence of cycloheximide in control cells compared to DDI1/2 depletion. Error bars represent SEM n=at least 3. (**G**) Western blot showing stabilization of RTF2 in whole cell lysates from BJ cells following treatment by proteasome inhibitor. Cells were exposed to 8 h of cycloheximide, MG-132, or a combination of the two drugs. (**H**) Western blot showing iPOND analysis of H2B, PCNA, and GFP:DDI2 presence on chromatin following 10' EdU label (L) or 10' EdU label followed by 50' thymidine chase (C). **(I)** Western blot showing RTF2 levels in cells used in sensitivity assay shown in Figure 2H. (**J**) Graph showing relative survival of U2OS cells treated with shDDI2 and siDDI1 or control plus or minus siRNA knockdown of RTF2 with si #4. Cells were exposed to indicated doses of HU for 20 h. Western blot showing RTF2 level in these cells.

Figure S3

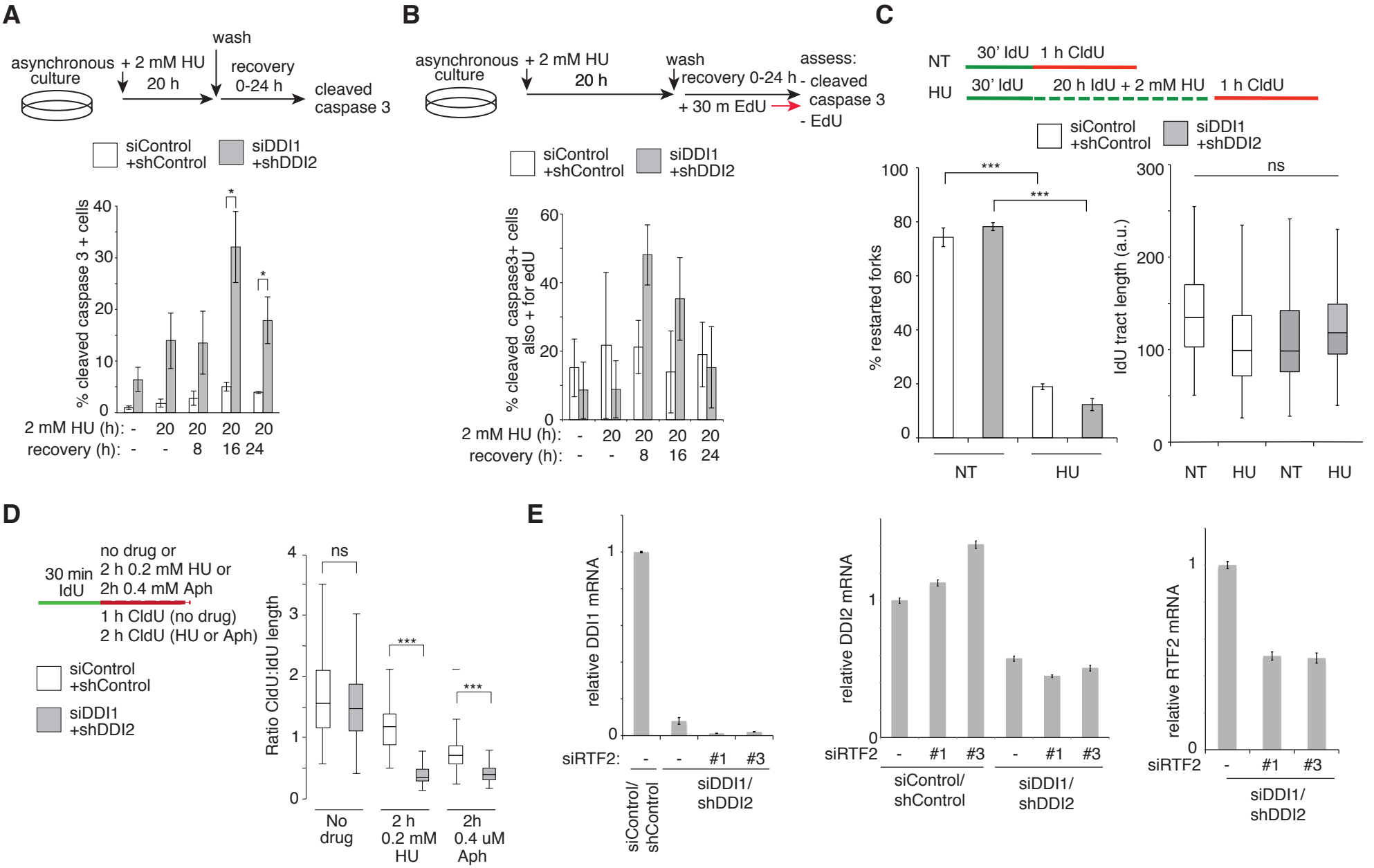
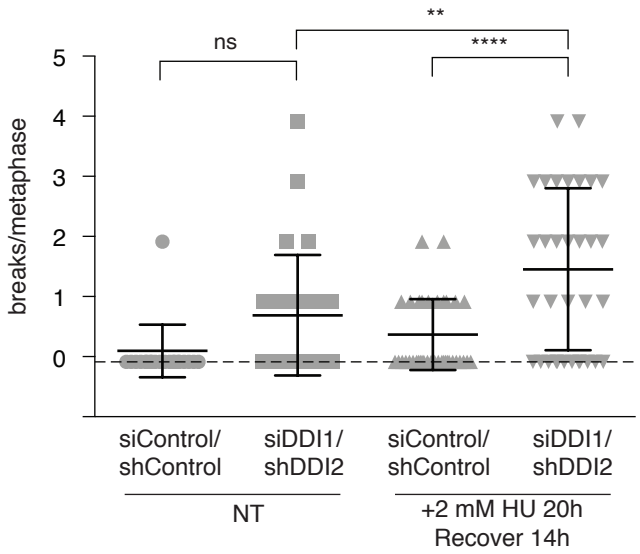

A
asynchronous culture
+ 2 mM HU 20 h
wash
recovery 0-24 h
cleaved caspase 3
siControl +shControl
siDDI1 +shDDI2
% cleaved caspase 3 + cells
2 mM HU (h): - 20 20 20 20
recovery (h): - - 8 16 24
B
asynchronous culture
+ 2 mM HU 20 h
wash
recovery 0-24 h
+ 30 m EdU
assess:
- cleaved caspase 3
- EdU
siControl +shControl
siDDI1 +shDDI2
% cleaved caspase3+ cells also + for edU
2 mM HU (h): - 20 20 20 20
recovery (h): - - 8 16 24
C
NT 30' IdU 1 h CldU
HU 30' IdU 20 h IdU + 2 mM HU 1 h CldU
siControl +shControl
siDDI1 +shDDI2
% restarted forks
***
***
NT HU
IdU tract length (a.u.)
ns
NT HU NT HU
D
30 min IdU
no drug or
2 h 0.2 mM HU or
2h 0.4 mM Aph
1 h CldU (no drug)
2 h CldU (HU or Aph)
siControl +shControl
siDDI1 +shDDI2
Ratio CldU:IdU length
ns
***
***
No drug
2 h 0.2 mM HU
2h 0.4 uM Aph
E
relative DDI1 mRNA
siRTF2: - - #1 #3
siControl/ shControl
siDDI1/ shDDI2
relative DDI2 mRNA
siRTF2 - #1 #3 - #1 #3
siControl/ shControl
siDDI1/ shDDI2
relative RTF2 mRNA
siRTF2 - #1 #3
siDDI1/ shDDI2

Figure S3: Response to replication stress is altered in cells depleted of DDI1/2; related to Figure 3

(A) Analysis of the percentage of apoptotic cells as determined by cleaved caspase-3 staining in control or DDI1/2 depleted cells following synchronization by 20h 2mM HU treatment and release. (**B**) Analysis of the percentage of cleaved caspase 3-positive cells that are also positive for EdU incorporation. Error bars represent SEM n=3. (**C**) Schematic of experimental setup in which cells were exposed to consecutive pulses of IdU and CldU -/+ 20 h of 2 mM HU coincubated with IdU. Graph showing the percent of restarted forks (IdU:CldU jointly labeled/[IdU:CldU jointly labeled + only IdU labeled]) under these conditions. Graph showing total IdU tract length after 30' or 30' + 20h HU + IdU. (**D**) Analysis of fork processivity defined by the ratio of CldU to IdU label length of continuing forks measured using fibers from control or DDI1/2 depleted cells subjected to the indicated drug treatment during CldU labeling. (**E**) Relative DDI1 and DDI2 mRNA levels with and without concurrent knockdown of RTF2.

Figure S4


**
ns
****
5
4
3
2
1
0
breaks/metaphase
siControl/
shControl
siDDI1/
shDDI2
siControl/
shControl
siDDI1/
shDDI2
NT
+2 mM HU 20h
Recover 14h

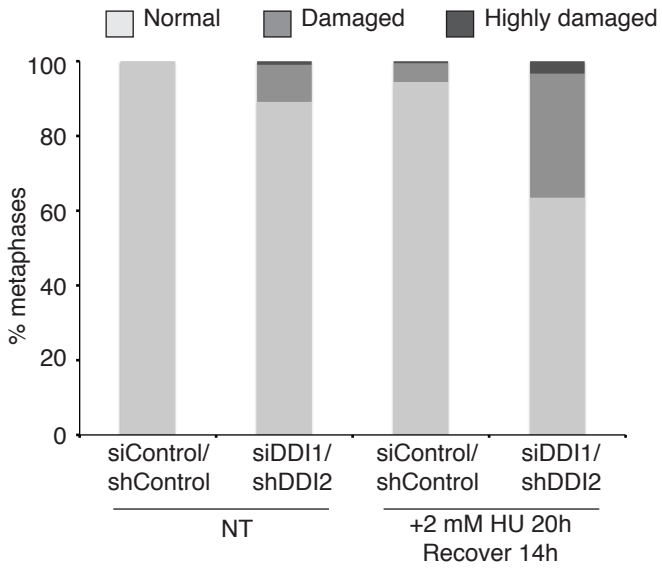

Normal
Damaged
Highly damaged
100
80
60
40
20
0
% metaphases
siControl/
shControl
siDDI1/
shDDI2
siControl/
shControl
siDDI1/
shDDI2
NT
+2 mM HU 20h
Recover 14h

Figure S4: DDI1/2 depletion leads to increased chromosomal damage after acute replication stress, related to Figure 4

Quantification of the number of breaks per countable metaphase, and the proportion of metaphases classified as normal, damaged, or highly damaged, in control or DDI1/2 depleted cells that were exposed to 20 h 2 mM HU and released for 14 h in the presence of the apoptosis inhibitor zVAD-FMK.

Figure S5

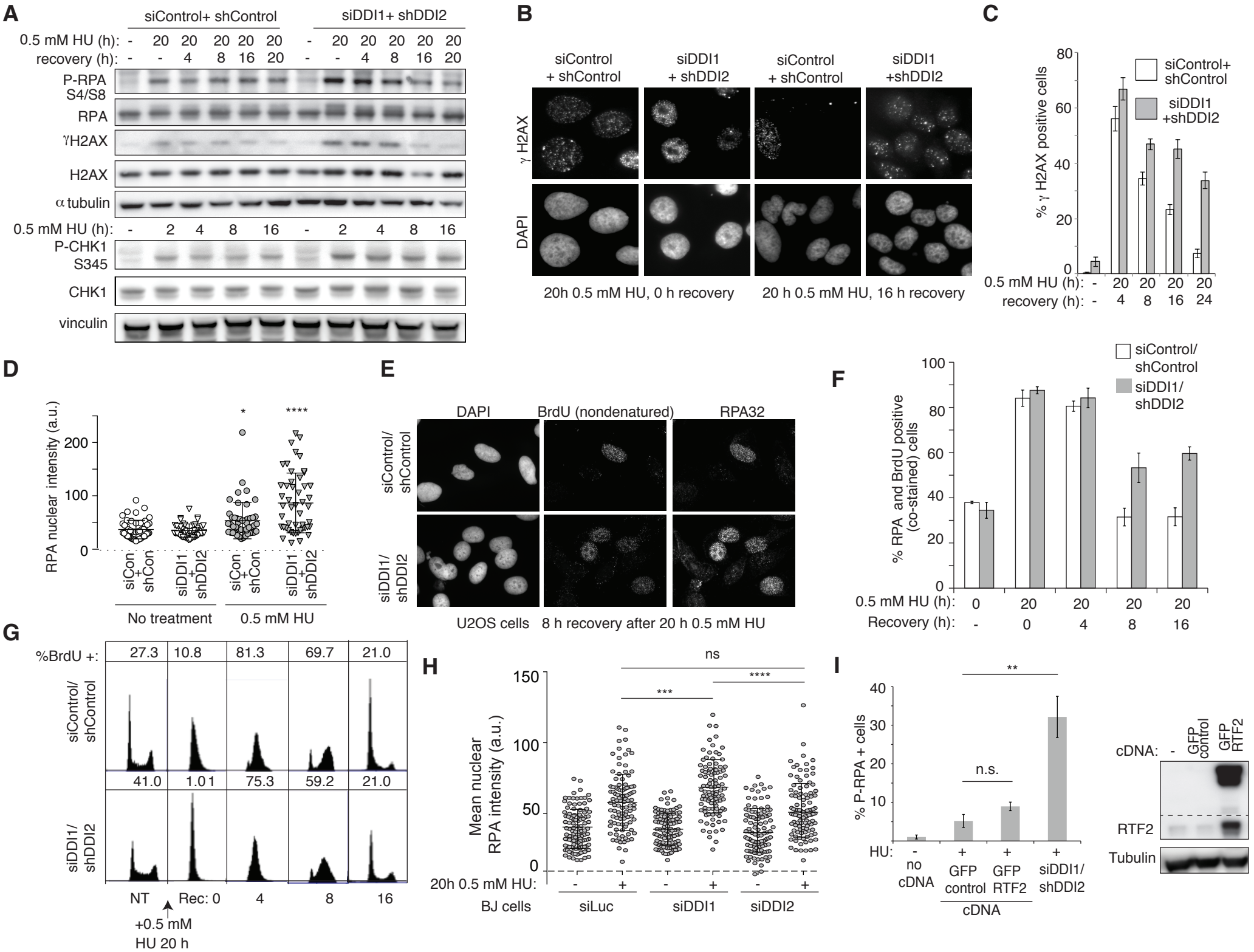

A
siControl+ shControl
siDDI1+ shDDI2
0.5 mM HU (h): - 20 20 20 20 20 - 20 20 20 20 20
recovery (h): - - 4 8 16 20 - - 4 8 16 20
P-RPA S4/S8
RPA
γH2AX
H2AX
α tubulin
0.5 mM HU (h): - 2 4 8 16 - 2 4 8 16
P-CHK1 S345
CHK1
vinculin
B
siControl + shControl
siDDI1 + shDDI2
siControl + shControl
siDDI1 +shDDI2
γ H2AX
DAPI
20h 0.5 mM HU, 0 h recovery
20 h 0.5 mM HU, 16 h recovery
C
% γ H2AX positive cells
siControl+ shControl
siDDI1 +shDDI2
0.5 mM HU (h): - 20 20 20 20
recovery (h): - 4 8 16 24
D
RPA nuclear intensity (a.u.)
siCon + shCon
siDDI1 + shDDI2
No treatment
0.5 mM HU
E
DAPI
BrdU (nondenatured)
RPA32
siControl/ shControl
siDDI1/ shDDI2
U2OS cells 8 h recovery after 20 h 0.5 mM HU
F
% RPA and BrdU positive (co-stained) cells
siControl/ shControl
siDDI1/ shDDI2
0.5 mM HU (h): 0 20 20 20 20
Recovery (h): - 0 4 8 16
G
%BrdU +: 27.3 10.8 81.3 69.7 21.0
siControl/ shControl
41.0 1.01 75.3 59.2 21.0
siDDI1/ shDDI2
NT Rec: 0 4 8 16
+0.5 mM HU 20 h
H
Mean nuclear RPA intensity (a.u.)
ns
****
***
20h 0.5 mM HU:
BJ cells siLuc siDDI1 siDDI2
I
% P-RPA + cells
**
n.s.
HU: - + + +
no cDNA
GFP control
GFP RTF2
siDDI1/ shDDI2
cDNA
cDNA: - GFP control GFP RTF2
RTF2
Tubulin

Figure S5: Heightened DNA damage signaling and ssDNA during replication stress under conditions of DDI1/2 depletion, related to Figure 5

(**A**) Immunoblot analysis of whole cell extract to assess phosphorylated RPA32 and γH2AX levels. Control or DDI1/2 depleted cells were treated with 0.5 mM HU for 20 h, released, and allowed to recover for indicated duration. (**B**) Representative immunofluorescence images of γH2AX foci in control or DDI1/2 depleted cells following release from 20 h treatment with 0.5 mM HU. (**C**) Quantification of γH2AX-positive cells at the indicated timepoints following release from 20h treatment with 0.5 mM HU. (**D**) Quantification of mean nuclear intensity of chromatin-bound RPA staining in control or DDI1/2 depleted cells treated with 0.5 mM HU for 20 h and extracted with 0.25% Triton-X. (**E**) Representative image of ssDNA in U20S cells 8 hours after release from 20h treatment with 0.5 mM HU as assessed by immunofluorescence imaging of RPA or staining of BrdU signal performed under non-denaturating conditions. (**F**) Quantification of ssDNA accumulation and persistence in U2OS following 20 h treatment with 0.5 mM HU and release for the indicated duration. Cells with at least 5 RPA and BrdU positive foci were counted as positive. Error bars represent SEM n=3. (**G**) Analysis of cell cycle progression in U2OS cells exposed to 20 h 0.5 mM HU and released for the indicated length of time. (**H**) Quantification of mean nuclear RPA intensity of Triton extracted BJ fibroblasts following 20 h treatment with 0.5 mM HU and no release. (**I**) Graph showing % of phospho-RPA cells by immunofluorescence in cells expressing GFP control, overexpressed GFP:RTF2, or with DDI1/2 knockdown. Only cells visibly expressing GFP:RTF2 were counted. Western blot showing level of overexpressed RTF2.

Figure S6

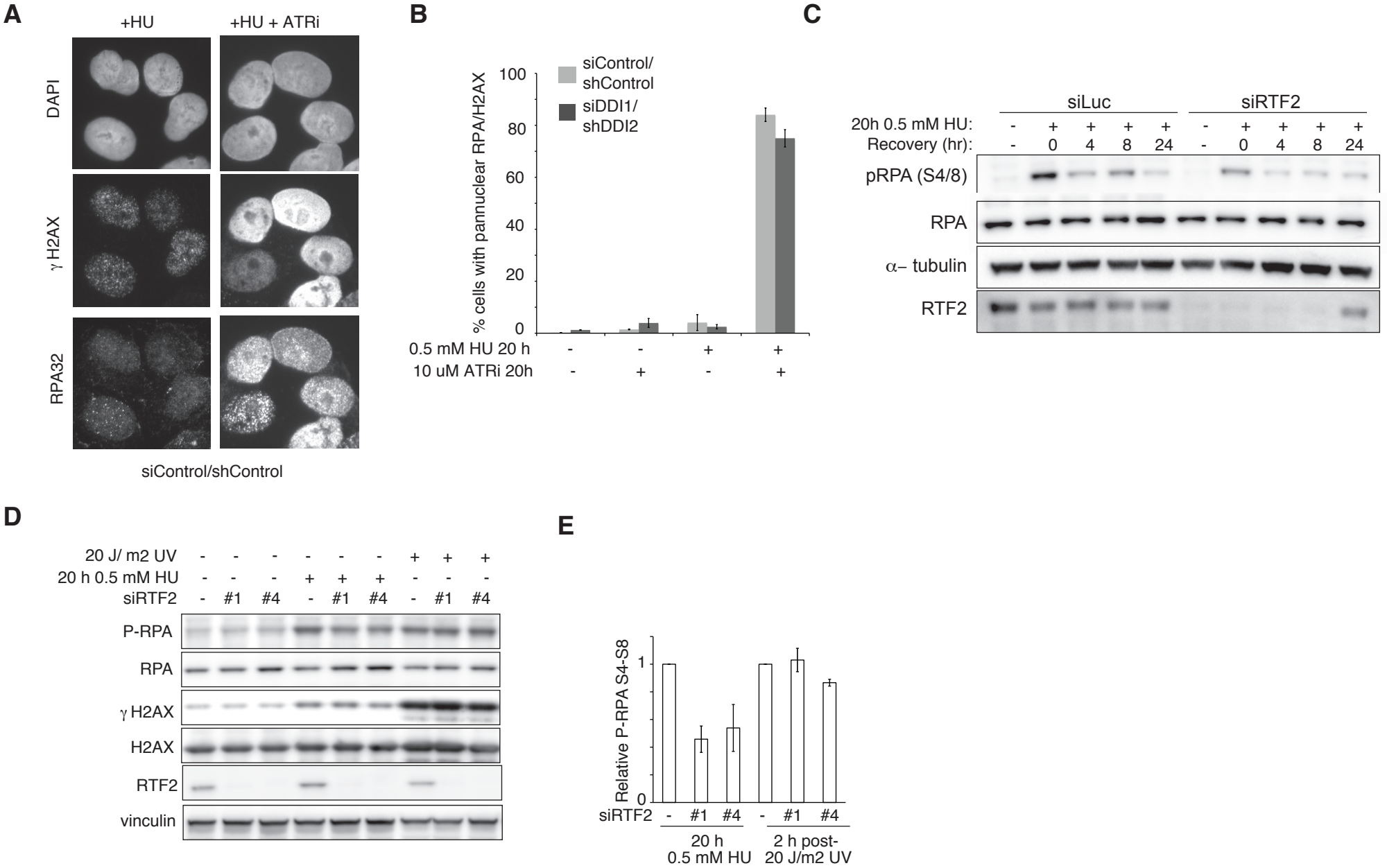

A
+HU
+HU + ATRi
DAPI
γ H2AX
RPA32
siControl/shControl
B
siControl/
shControl
siDDI1/
shDDI2
% cells with pannuclear RPA/H2AX
100
80
60
40
20
0
0.5 mM HU 20 h - - + +
10 uM ATRi 20h - + - +
C
siLuc
siRTF2
20h 0.5 mM HU: - + + + + - + + + +
Recovery (hr): - 0 4 8 24 - 0 4 8 24
pRPA (S4/8)
RPA
α– tubulin
RTF2
D
20 J/ m2 UV - - - - - - + + +
20 h 0.5 mM HU - - - + + + - - -
siRTF2 - #1 #4 - #1 #4 - #1 #4
P-RPA
RPA
γ H2AX
H2AX
RTF2
vinculin
E
Relative P-RPA S4-S8
1
0
siRTF2 - #1 #4 - #1 #4
20 h
0.5 mM HU
2 h post-
20 J/m2 UV

Figure S6: RTF2 is required for robust RPA phosphorylation in response to replication stress induced by HU; related to Figure 5

(**A**) Representative immunofluorescence images of focal and pancellular γH2AX staining in U2OS cells following treatment with 20h 0.5 mM HU +/- the ATR inhibitor VE-821. (**B**) Quantification of U2OS cells with pancellular γH2AX and RPA staining following treatment with 20h 0.5 mM HU +/- the ATR inhibitor VE-821. Error bars represent SEM n=3. (**C**) Analysis of DNA damage signaling and ssDNA by western blotting whole cell extracts from BJ fibroblasts with antibodies against phosphorylated RPA (S4/S8). Cells treated with siControl and siRTF2 #1 were treated with 20 h 0.5 mM HU and allowed to recover for the indicated times. **(D)** Analysis of DNA damage signaling and ssDNA by western blotting with antibodies against phospho-RPA. Cells treated with siControl, siRTF2 #1, and siRTF2 #4 were treated with 20 h 0.5 mM HU or 20 J/m2 UV (2 h post-irradiation). (**E**) Quantification of phosphorylated-RPA levels relative to total RPA. Error bars represent SEM n=3. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001 by ANOVA.

Supplemental Table 1: RNAi and primer sequences, related to STAR Methods Cell culture, transfection and viral transduction; and RNA preparation, reverse transcription, and real time PCR.

RNAi materials		
DDI1 siRNA 1 ccggagacaucaauguuccaucgat	Invitrogen	HSS181016
DDI1 siRNA 2 ggaaauuacacauucagucauggat	Invitrogen	HSS140552
DDI1 siRNA 3 ccuacggccucaaagauggcgauat	Invitrogen	HSS140553
DDI2 siRNA 1 gaatagauuucaguaguautt	Ambion	s38861
DDI2 siRNA 2 gcaaagauagaagaaagauatt	Ambion	s38862
DDI2 siRNA 3 gcagaaagguguaacauaatt	Ambion	s38863
DDI2 shRNA 1 uggaaaucgauacagcuca	Open Biosystems	V3LHS_328065
DDI2 shRNA 2 aggcagaaugccaauguca	Open Biosystems	V3LHS_32806
DDI2 shRNA 3 aguaugagguugcacaaca	Open Biosystems	V3LHS_328069
MRE11 siRNA 1 gauagacauuaguccgguutt	Ambion	s8959
MRE11 siRNA 2 cccgaaaugucacuacuaatt	Ambion	s8960
MRE11 siRNA 3 cgacugcgaguggacuauatt	Ambion	s8961
BRCA2 siRNA 1 uacguuuuuaggugaagcctg	Ambion	s224695
BRCA2 siRNA 2 uuccguuuaauuucaacugta	Ambion	s2083
BRCA2 siRNA 3 uugcgaaauauguauaaucca	Ambion	s2084
RTF2 siRNA 1 gaagaaggucgagaagguugacaaa	Invitrogen	MSS227432
RTF2 siRNA 2 ucaaagccggguaccacacaagatt	Invitrogen	MSS227433
RTF2 siRNA 3 gcccaauggaacuacuguacccuaa	Invitrogen	MSS227434
RTF2 siRNA 4 caaagaugccgucauugaatt	Invitrogen	s226737

qPCR primer sequences		
Gene	Forward Sequence	Reverse Sequence
Beta-Actin	gctacgagctgcctgacg	ggctggaagagtgcctca
DDI1	tggaacacaacgtgctacct	atctgtctgggggctgtct
DDI2	tcgatgtagtgtgtgtgtactgc	ccagtgaggtagattctttaccactt
RTF2	tgctgaagacaaggatggag	tgaaacagactctgctgcct