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Supplemental Information

ATR Signaling Uncouples the Role of RAD51 Paralogs in Homologous Recombination and Replication Stress Response

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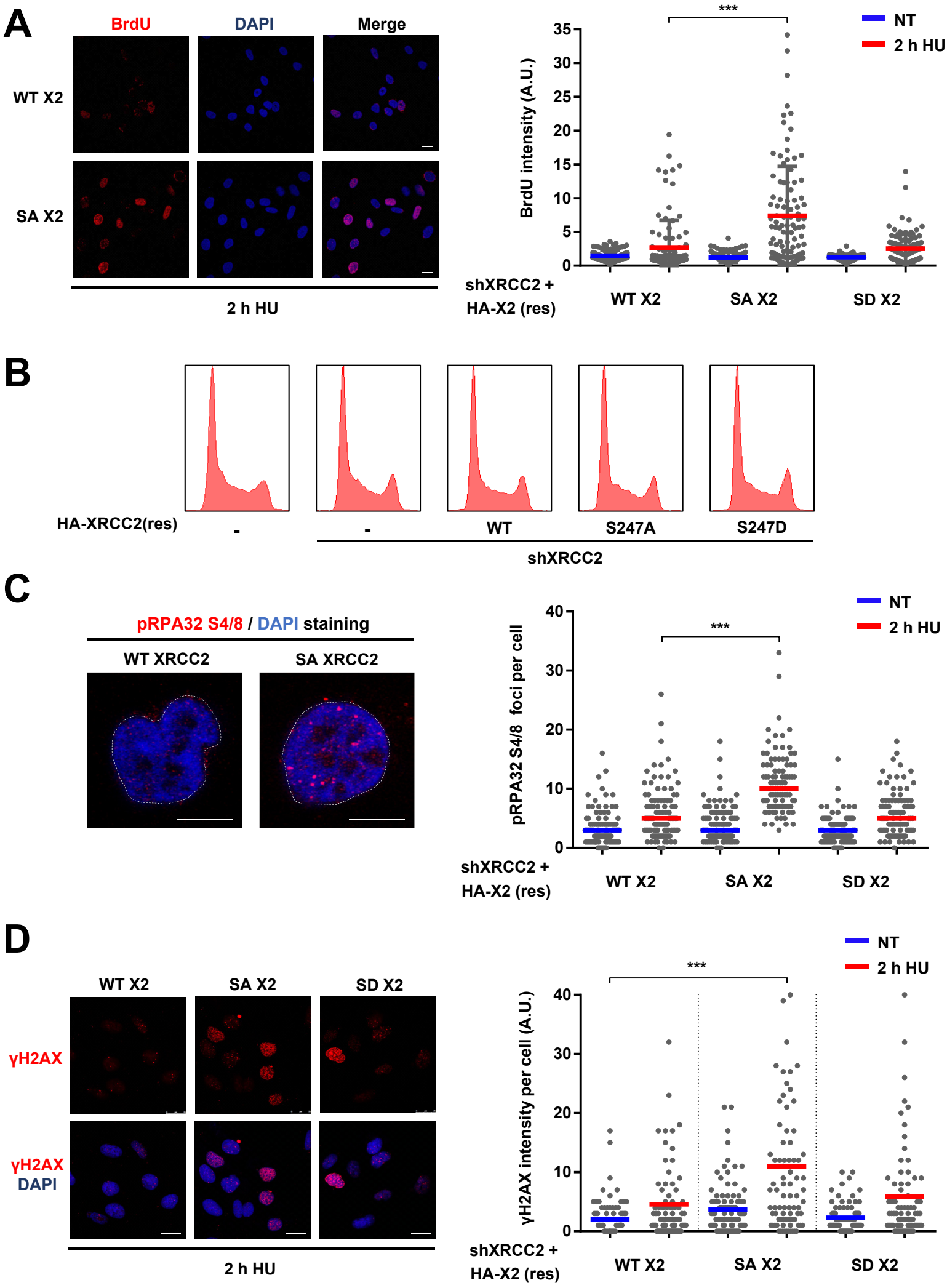
A
BrdU
DAPI
Merge
WT X2
SA X2
2 h HU
***
NT
2 h HU
BrdU intensity (A.U.)
35
30
25
20
15
10
5
0
shXRCC2 +
HA-X2 (res)
WT X2
SA X2
SD X2
B
HA-XRCC2(res)
-
-
WT
S247A
S247D
shXRCC2
C
pRPA32 S4/8 / DAPI staining
WT XRCC2
SA XRCC2
NT
2 h HU
***
pRPA32 S4/8 foci per cell
40
30
20
10
0
shXRCC2 +
HA-X2 (res)
WT X2
SA X2
SD X2
D
WT X2
SA X2
SD X2
γH2AX
γH2AX
DAPI
2 h HU
NT
2 h HU
***
γH2AX intensity per cell (A.U.)
40
30
20
10
0
shXRCC2 +
HA-X2 (res)
WT X2
SA X2
SD X2

Figure S1

Figure S1. XRCC2 phosphorylation limits the accumulation of DNA damage during mild replication stress. Related to Figures 1 and 2. (A) Representative images and quantification of XRCC2 depleted U2OS cells expressing indicated shRNA resistant XRCC2 variants stained for BrdU under non-denaturing conditions. Data are presented as mean intensity ± SD. Scale bars: 20 μm. (B) Cell cycle analysis using propidium iodide (PI) staining in U2OS cells depleted of XRCC2 and complemented with indicated shRNA resistant XRCC2 variants. (C) Representative images and quantification of XRCC2 depleted U2OS cells expressing indicated shRNA resistant XRCC2 variants stained for pRPA32 S4/8. Cells were treated with 2 mM HU for indicated times prior to pre-extraction, fixation and immunofluorescence staining. Scale bars: 10 μm. (D) Representative images and quantification of XRCC2 depleted U2OS cells expressing indicated shRNA resistant XRCC2 variants stained for γH2AX. Cells were treated with 2 mM HU for indicated times prior to pre-extraction, fixation and immunofluorescence staining. A.U., arbitrary units. Scale bars: 25 μm. HA-X2 (res), shRNA resistant HA-tagged XRCC2. Student's t-test, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. n.s., non-significant.

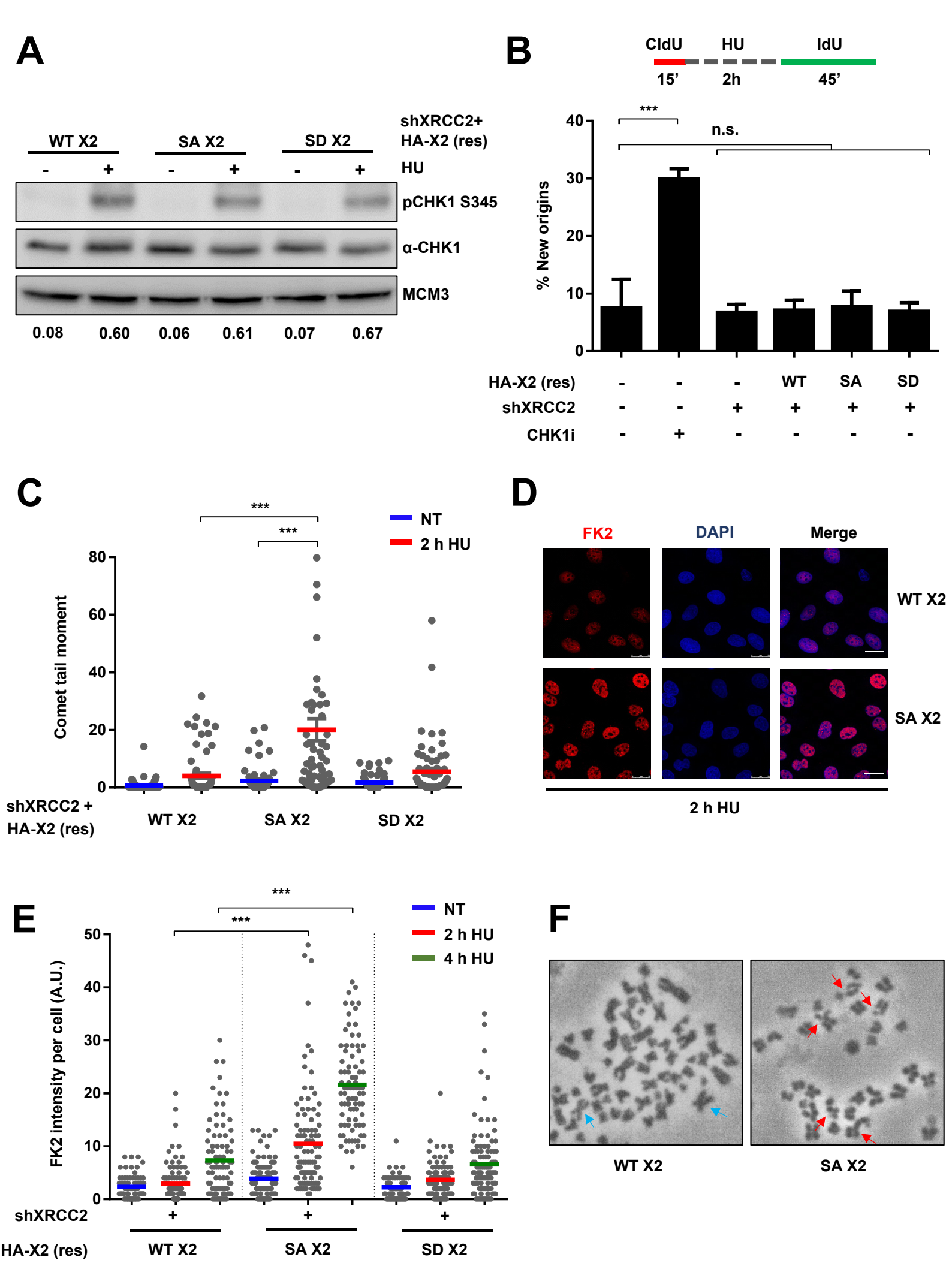
A
WT X2
SA X2
SD X2
shXRCC2+
HA-X2 (res)
- + - + - +
HU
pCHK1 S345
α-CHK1
MCM3
0.08 0.60 0.06 0.61 0.07 0.67
B
CldU
HU
IdU
15'
2h
45'
***
n.s.
% New origins
0
10
20
30
40
HA-X2 (res) - - - WT SA SD
shXRCC2 - - + + + +
CHK1i - + - - - -
C
***
***
NT
2 h HU
Comet tail moment
0
20
40
60
80
shXRCC2 +
HA-X2 (res)
WT X2
SA X2
SD X2
D
FK2
DAPI
Merge
WT X2
SA X2
2 h HU
E
***
***
NT
2 h HU
4 h HU
FK2 intensity per cell (A.U.)
0
10
20
30
40
50
shXRCC2
+ + +
HA-X2 (res)
WT X2
SA X2
SD X2
F
WT X2
SA X2

Figure S2

Figure S2. XRCC2 phosphorylation fosters genome integrity during replication stress. Related to Figure 2. (A) Western blot for pCHK1 S345 activation in indicated U2OS cells upon treatment with 2 mM HU for 4 h. Ratio of phosphorylated to total CHK1 is indicated below the respective lanes. (B) Frequency of new origin firing in indicated U2OS cells. U2OS cells were sequentially labelled with CldU and IdU with a 2 h HU pulse (2 mM) in between. Green-only tracts were counted as newly fired origins. CHK1 inhibition was included as a positive control for increased origin firing. New origins are shown as percentage of all labelled tracks. DNA fiber labeling protocol is shown. Data are presented as mean ± SD. (C) Cells were treated with 2 mM HU for indicated times and collected to perform neutral comet assay. Data are presented as mean tail moment ± SEM. (D) Representative images of XRCC2 depleted U2OS cells expressing indicated shRNA resistant XRCC2 variants stained for FK2. Cells were treated with 2 mM HU for indicated times prior to pre-extraction, fixation and immunofluorescence staining. Scale bars: 25 µm. (E) Quantification of FK2 intensity in cells as in (D). A.U., arbitrary units. HA-X2 (res), shRNA resistant HA-tagged XRCC2. (F) Representative images for metaphase spreads in XRCC2 depleted U2OS cells expressing indicated shRNA resistant XRCC2 variants. Blue and red arrows indicate normal chromosomes and chromosomal aberrations respectively. Student's t-test, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. n.s., non-significant.

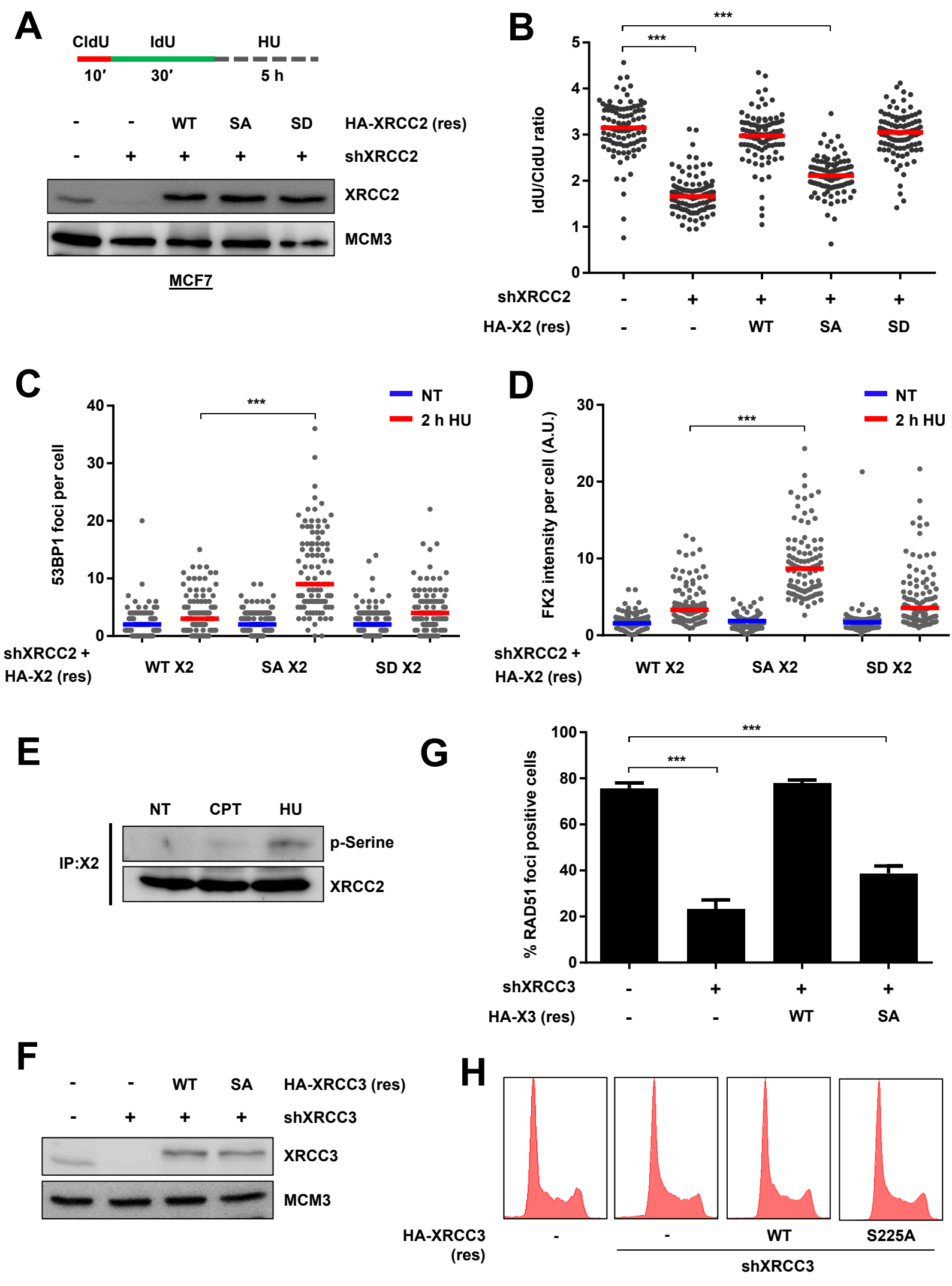
A
CldU
IdU
HU
10′
30′
5 h
- - WT SA SD HA-XRCC2 (res)
- + + + + shXRCC2
XRCC2
MCM3
MCF7
B
***
***
IdU/CldU ratio
shXRCC2 - + + + +
HA-X2 (res) - - WT SA SD
C
***
NT
2 h HU
53BP1 foci per cell
shXRCC2 +
HA-X2 (res)
WT X2
SA X2
SD X2
D
***
NT
2 h HU
FK2 intensity per cell (A.U.)
shXRCC2 +
HA-X2 (res)
WT X2
SA X2
SD X2
E
NT CPT HU
IP:X2
p-Serine
XRCC2
G
***
***
% RAD51 foci positive cells
shXRCC3 - + + +
HA-X3 (res) - - WT SA
F
- - WT SA HA-XRCC3 (res)
- + + + shXRCC3
XRCC3
MCM3
H
HA-XRCC3 (res) - - WT S225A
shXRCC3

Figure S3

Figure S3. ATR signaling uncouples the roles of RAD51 paralog complexes in replication stress response and DSB repair. Related to Figures 2 and 3. (A) Western blot shows depletion of endogenous XRCC2 and expression of shRNA-resistant HA-tagged XRCC2 variants in MCF7 cells. HA-X2 (res), shRNA resistant HA-tagged XRCC2; WT, wild-type; SA, S247A; SD, S247D. Top, Schematic to study fork degradation in MCF7 cells. (B) IdU to CldU tract length ratios in indicated MCF7 cells to study fork degradation following HU treatment (4 mM). (C and D) Quantification of 53BP1 foci (C) and FK2 intensity (D) in XRCC2 depleted MCF7 cells expressing indicated shRNA resistant XRCC2 variants. Cells were treated with 2 mM HU for indicated times prior to pre-extraction, fixation and immunofluorescence staining. A.U., arbitrary units. (E) HeLa cells were used to analyze phosphorylation of XRCC2 and where indicated, DNA damage was induced with HU (2 mM) or CPT (5 μM) for 1 h. Cell extracts were prepared and immunoprecipitated (IP) using XRCC2 antibody. Following IP, the proteins were immunoblotted with indicated antibodies. (F) Western blot shows depletion of endogenous XRCC3 and expression of shRNA-resistant HA-tagged XRCC3 variants in U2OS cells. HA-XRCC3 (res), shRNA resistant HA-tagged XRCC3; WT, wild-type; SA, S225A. (G) Quantification of RAD51 foci positive cells in XRCC3 depleted U2OS cells expressing shRNA resistant HA-tagged WT/S225A XRCC3, exposed to 5 Gy IR, and 1 h of recovery. Cells with more than 10 RAD51 foci were considered positive. Data are presented as mean ± SD. (H) Cell cycle analysis using propidium iodide (PI) staining in XRCC3 depleted U2OS cells expressing shRNA resistant HA-tagged WT/S225A XRCC3. Student's t-test, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. n.s., non-significant.

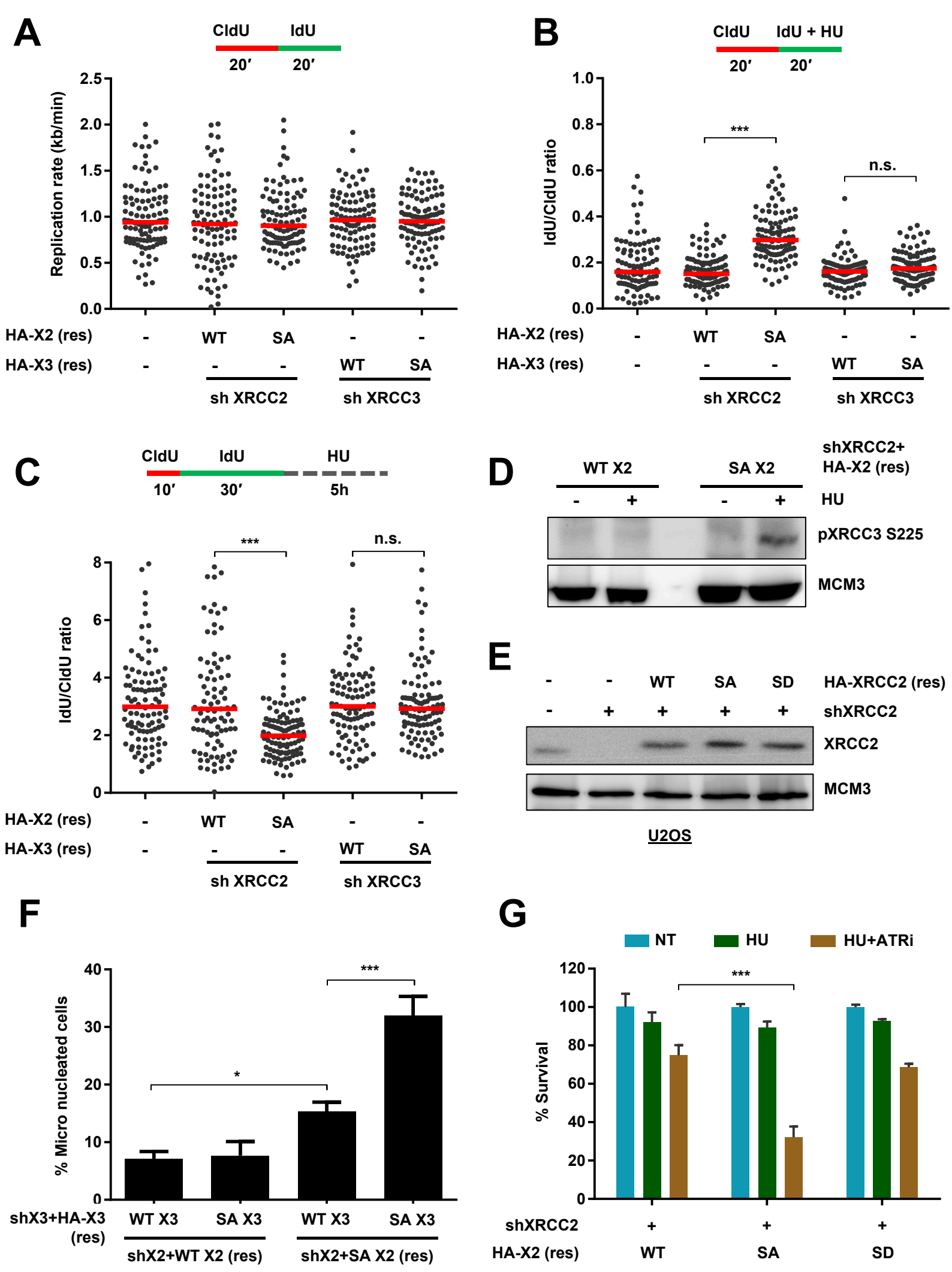
A
CldU
IdU
20′
20′
Replication rate (kb/min)
2.5
2.0
1.5
1.0
0.5
0.0
HA-X2 (res)
HA-X3 (res)
WT
SA
sh XRCC2
sh XRCC3
B
CldU
IdU + HU
20′
20′
IdU/CldU ratio
1.0
0.8
0.6
0.4
0.2
0.0
***
n.s.
C
CldU
IdU
HU
10′
30′
5h
8
6
4
2
0
D
shXRCC2+
HA-X2 (res)
WT X2
SA X2
HU
pXRCC3 S225
MCM3
E
HA-XRCC2 (res)
shXRCC2
XRCC2
MCM3
U2OS
F
% Micro nucleated cells
40
30
20
10
0
*
shX3+HA-X3
(res)
WT X3
SA X3
shX2+WT X2 (res)
shX2+SA X2 (res)
G
NT
HU
HU+ATRi
% Survival
120
100
80
60
40
20
0
shXRCC2
HA-X2 (res)
SD

Figure S4

Figure S4. Cells defective in XRCC2 phosphorylation undergo early XRCC3 S225 activation to promote cell survival. Related to Figures 3 and 4. (A) Replication rate in indicated U2OS cells. Each dot represents one fiber. A minimum of 100 DNA fibers were analyzed for each condition. HA-X2 (res), shRNA resistant HA-tagged XRCC2. (B) Quantification of IdU to CldU tract length ratios in indicated U2OS cells to study fork slowdown in the presence of HU (500 μM). (C) Quantification of IdU to CldU tract length ratios in indicated U2OS cells to study fork degradation following HU treatment (4 mM). (D) Analysis of XRCC3 S225 phosphorylation in indicated U2OS cells treated with 2 mM HU for 2 h. (E) Western blot showing expression of HA-tagged, shRNA resistant WT, S247A and S247D XRCC2 variants in XRCC2 depleted U2OS cells at levels comparable to endogenous levels. (F) Quantification of micronuclei in indicated U2OS cells after treatment with HU (150 μM) for 24 h. Data are presented as mean ± SD. (G) Survival of indicated U2OS cells upon exposure to 2 mM HU for 4 h, with or without continuous treatment of 1 μM ATRi (VE-821). Data are representative of 3 independent experiments. Data are presented as mean ± SD. DNA fiber labeling protocol is shown for individual panels. A minimum of 100 DNA fibers were analyzed for each condition. Student's t-test, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. n.s., non-significant.

Table S1. Sequence of primers for generation of XRCC2 and XRCC3 constructs. Related to STAR Methods

PRIMER NAME	SEQUENCE (5′ - 3′)
XRCC2 Forward	ATAGAGGATCCATGTGTAGTGCCTTCCAT
XRCC2 Reverse (HA-his tag)	ATAGAGAATTCTCAGTGATGGTGGTGATGGTGTGCATAGTCGGGGACGTC ATAGGGGTAACAAAATTCAACCCCACT
XRCC2 (shX2#1 resistant) Forward	GACTATCGCCTGGTTCTTTTCGCGACCACGCAGACCATCATGCAGAAAGCC TCGAGCTCA
XRCC2 (shX2#1 resistant) Reverse	TGAGCTCGAGGCTTTCTGCATGATGGTCTGCGTGGTCGCGAAAAGAACCAG GCGATAGTC
XRCC2 S247A Forward	AAACAAGATGATGCACAAAGCAGCAAC
XRCC2 S247A Reverse	GTTGCTGCTTTGTGCATCATCTTGTTT
XRCC2 S247D Forward	AAACAAGATGATGACCAAAGCAGCAAC
XRCC2 S247D Reverse	GTTGCTGCTTTGGTCATCATCTTGTTT
XRCC3 (shX3#1 resistant) Forward	ATAGAGGATCCATGGATTTGGATCTACTGGACCTGAATCCCAGGATCATCG CCGCGATCAAAAAAGCCAAACTGAAATCG
XRCC3 Reverse (HA-his tag)	ATAGAGAATTCTCAGTGATGGTGGTGATGGTGTGCATAGTCGGGGACGTC ATAGGGGTAGTGGGACTGGGTCCCAGG
XRCC3 S225A Forward	TGTGAATTTGACGCCCAGGCCTCCGCC
XRCC3 S225A Reverse	GGCGGAGGCCTGGGCGTCAAATTCACA