Figure S1

A

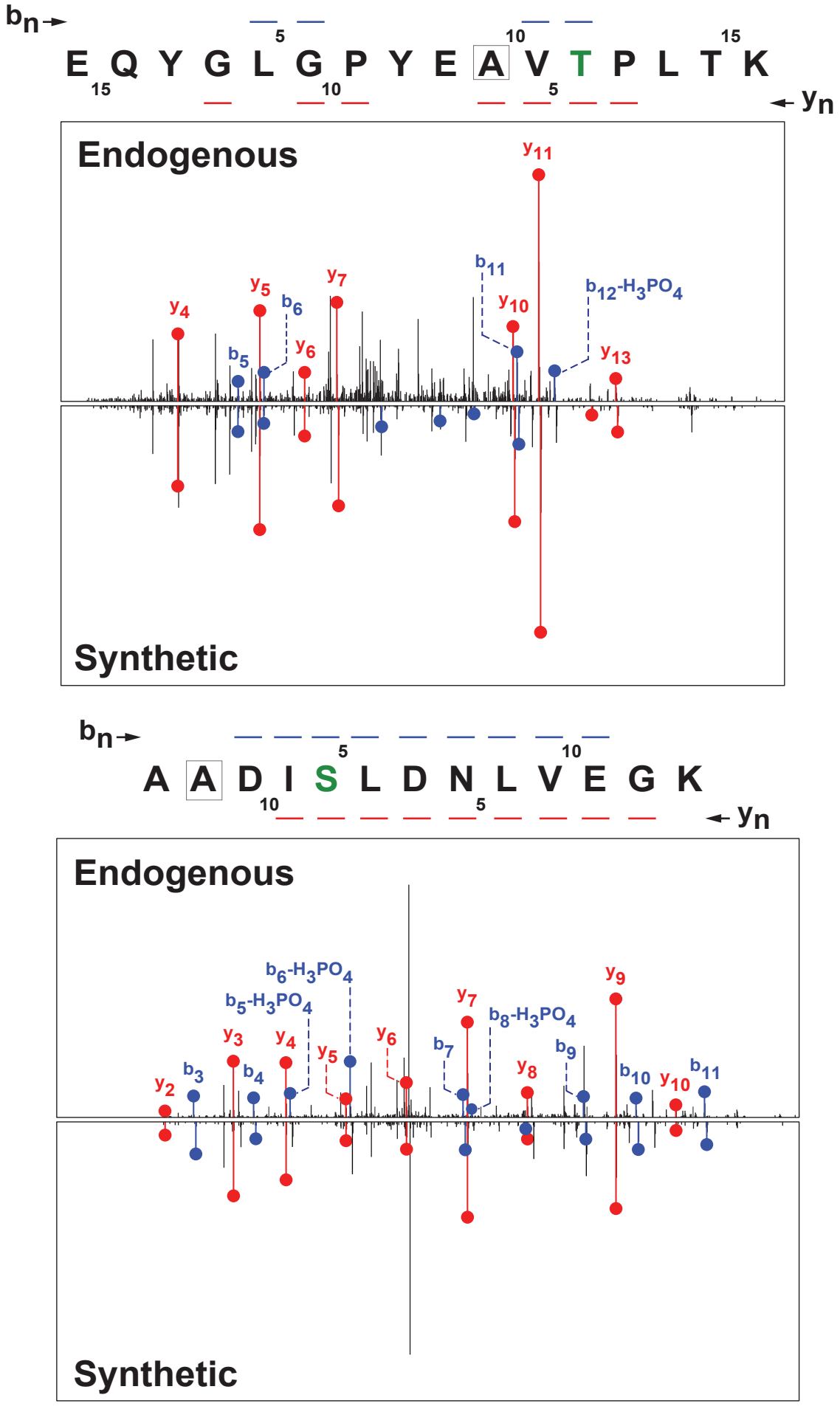
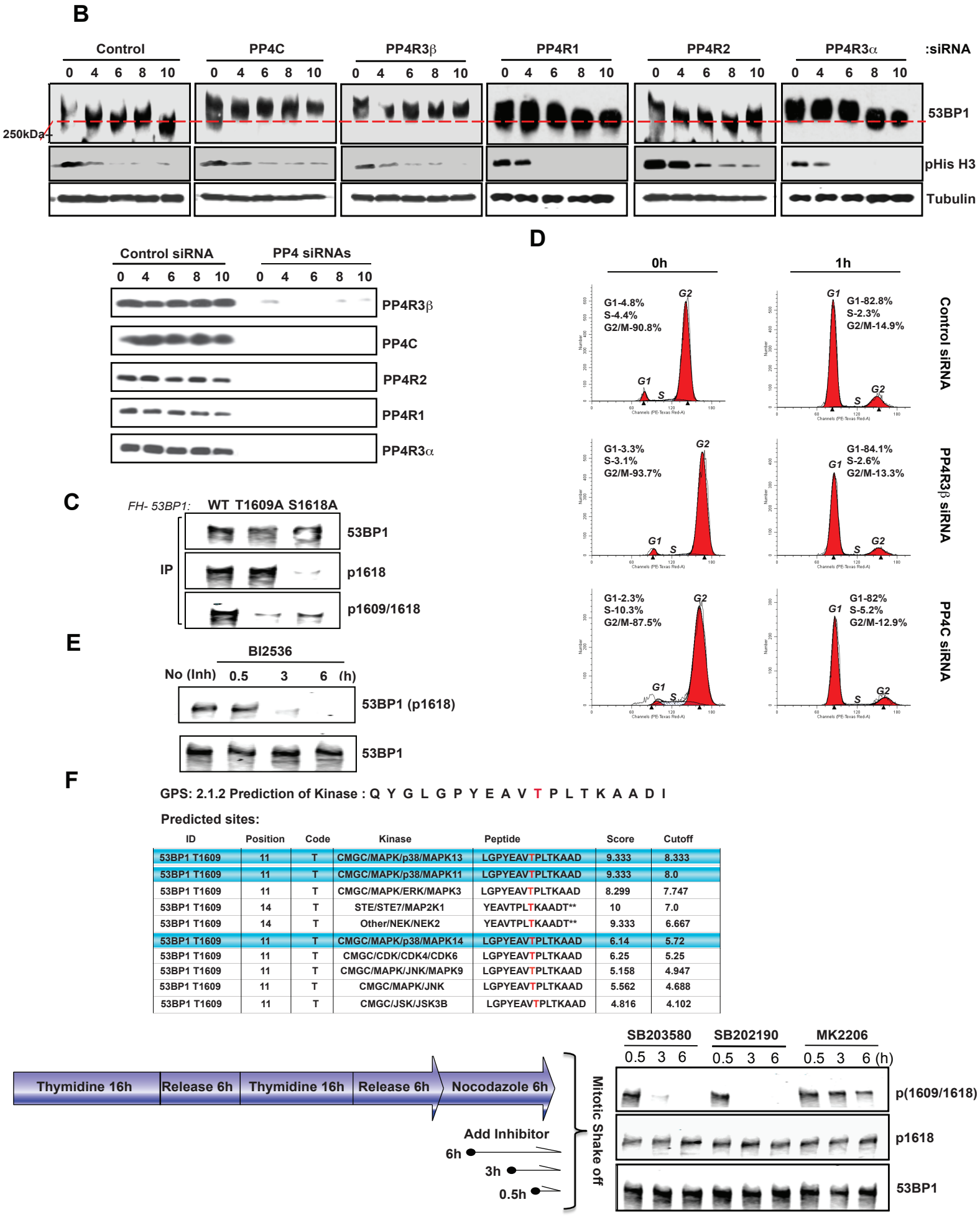

bn→
E Q Y G L G P Y E A V T P L T K
←yn
Endogenous
y11
b11
y5
y7
b12-H3PO4
y4
b6
y10
b5
y6
y13
Synthetic
bn→
A A D I S L D N L V E G K
←yn
Endogenous
b6-H3PO4
b5-H3PO4
y7
y9
b8-H3PO4
y3
y4
y6
b7
b9
y5
b3
b4
y8
b10
y10
b11
y2
Synthetic

Figure S1



ID	Position	Code	Kinase	Peptide	Score	Cutoff
53BP1 T1609	11	T	CMGC/MAPK/p38/MAPK13	LGPYEAVTPLTKAAD	9.333	8.333
53BP1 T1609	11	T	CMGC/MAPK/p38/MAPK11	LGPYEAVTPLTKAAD	9.333	8.0
53BP1 T1609	11	T	CMGC/MAPK/ERK/MAPK3	LGPYEAVTPLTKAAD	8.299	7.747
53BP1 T1609	14	T	STE/STE7/MAP2K1	YEAVTPLTKAADT**	10	7.0
53BP1 T1609	14	T	Other/NEK/NEK2	YEAVTPLTKAADT**	9.333	6.667
53BP1 T1609	11	T	CMGC/MAPK/p38/MAPK14	LGPYEAVTPLTKAAD	6.14	5.72
53BP1 T1609	11	T	CMGC/CDK/CDK4/CDK6	LGPYEAVTPLTKAAD	6.25	5.25
53BP1 T1609	11	T	CMGC/MAPK/JNK/MAPK9	LGPYEAVTPLTKAAD	5.158	4.947
53BP1 T1609	11	T	CMGC/MAPK/JNK	LGPYEAVTPLTKAAD	5.562	4.688
53BP1 T1609	11	T	CMGC/JSK/JSK3B	LGPYEAVTPLTKAAD	4.816	4.102

S1, Related to Figure 1

S1A. We used isotope-encoded synthetic phosphopeptide analogs to verify sequence assignment and localization of the phosphorylated residues for pT1609 *(top panel)* and pS1618 *(bottom panel)*. The MS/MS spectra of tryptic peptide derived from immunoprecipitated endogenous 53BP1 (upper spectra) and of isotope-encoded synthetic analog (lower spectra) illustrate the alignment of the major b-type (blue) and y-type (red) fragment ions containing the original peptide N- and C- termini, respectively, for both phosphorylated peptides. Peptide sequences above each spectra are annotated to show phosphorylated residues (green), isotope-encoded amino-acid (boxed) and sequence-specific fragments detected in each MS/MS spectrum (with blue and red underlines).

S1B. Impact of PP4C and PP4R3β silencing on 53BP1 phosphorylation. HeLa cells were transfected with siRNAs against PP4C or the indicated PP4 regulatory subunits and synchronized in mitosis with 100 ng/ml Nocodazole for 8 hrs. Cells were released from mitosis and harvested at the indicated timepoints in G1 phase. 53BP1 mobility shift was assessed by gel electrophoresis. Mitotic exit was monitored using phospho-histone H3 (pHistoneH3) antibody. Tubulin was used as a loading control. The 250 kDa marker is shown as a reference. Cells harvested to assess 53BP1 mobility shift were also analyzed in parallel by immunoblot for knockdown of PP4C and the indicated PP4 regulatory subunits.

S1C. Phospho-53BP1 antibody specificity. The phospho-antibody against pT1609/pS1618-53BP1 is specific for the combination of pT1609/pS1618 and does not recognize individual phospho-residues. HeLa cells expressing FH-53BP1 WT or indicated phosphomutants were synchronized in mitosis and harvested by mitotic shake-off. Anti-FLAG agarose beads were used to immunopreciptate WT 53BP1 or indicated phosphomutants. The immunopreciptate was analyzed by immunoblotting using antibodies against 53BP1, 53BP1-p1618, and 53BP1 p(1609/1618).

S1D. FACS plots of propidium iodide (PI) staining of HeLa cells transfected with the indicated siRNAs. At 60h after siRNA transfection, cells were incubated in Nocodazole for 8h. Resulting mitotic cells were released into media without drug and harvested at 0 and 1h. Peaks for G1, S, and G2/M phases are indicated in addition to percent cells in each phase. By 1h after release 80-85% of cells exit mitosis and enter G1.

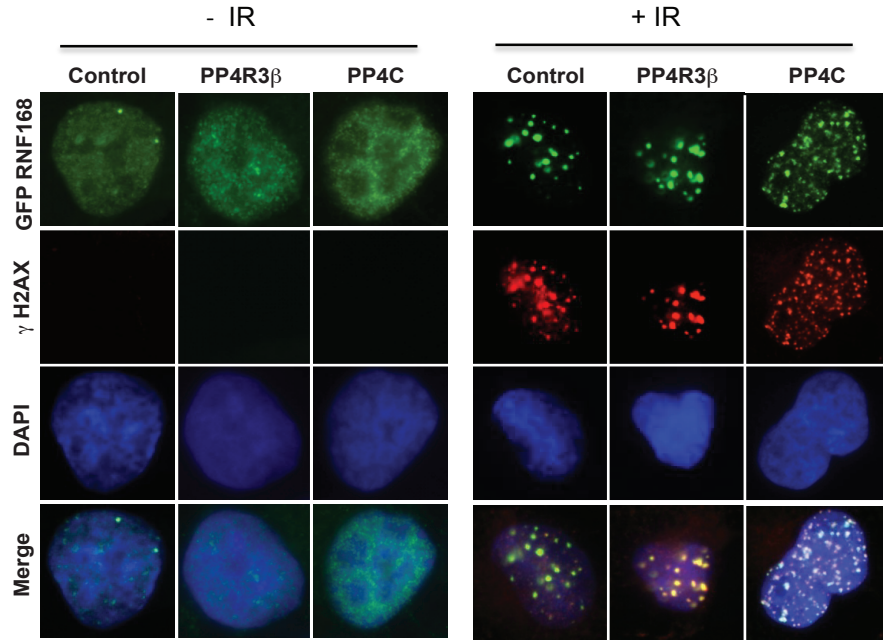
S1E. S1618 of 53BP1 is phosphorylated by PLK-1. HeLa cells were treated with PLK-1 inhibitor (BI-2536, 20nM) at various timepoints during 6h incubation with Nocodazole. Mitotic cells were harvested and lysates were used for immunoblotting with antibodies against p1618-53BP1 and 53BP1.

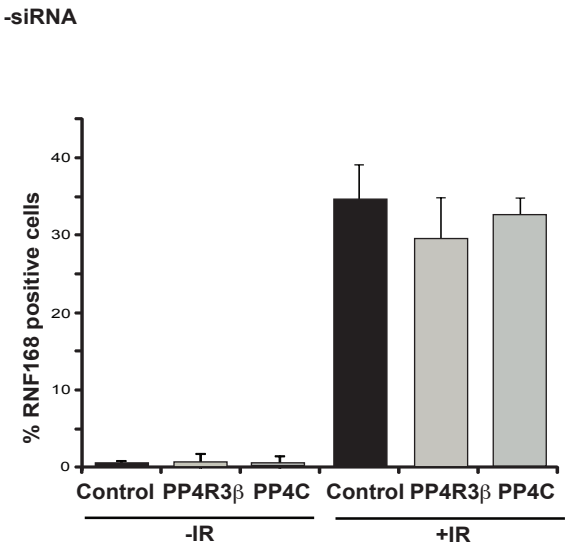
S1F. T1609 is phosphorylated by p38-MAPK. T1609 of 53BP1 is phosphorylated by p38 MAPK during mitosis. *Upper panel*: Group-based Prediction System (GPS 2.1.2) was

employed to predict kinase(s) that phosphorylate T1609 of 53BP1. *Lower left panel*: Experimental scheme for inhibiting p38 MAPK in mitotic cells. HeLa cells were synchronized in mitosis by a double-thymidine block followed by Nocodazole treatment for 6hr. During the 6hr Nocodazole treatment to arrest cells in mitosis, SB203580/SB202190 (0.5 μM, p38 kinase inhibitors) or MK-2206 (0.5 μM, Akt inhibitor) were added at three different time points. . MK-2206 was used as a negative control. *Lower right panel*: Immunoblot of cells treated with the indicated kinase inhibitors during Nocodazole arrest. Antibodies against 53BP1 (p1618) and 53BP1 p were used for immunoblotting.

Figure S2

A

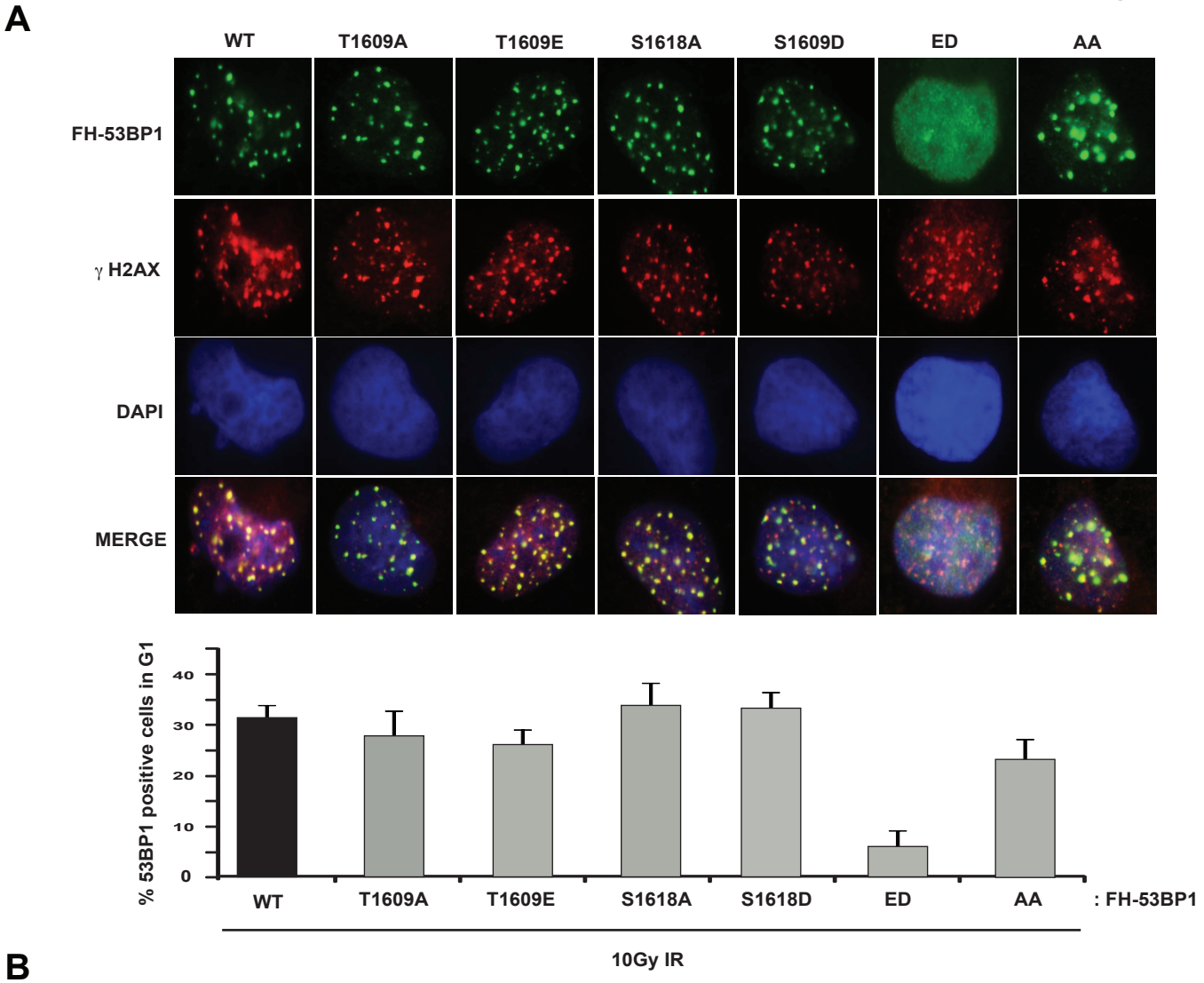

- IR
+ IR
Control
PP4R3β
PP4C
Control
PP4R3β
PP4C
GFP RNF168
γ H2AX
DAPI
Merge

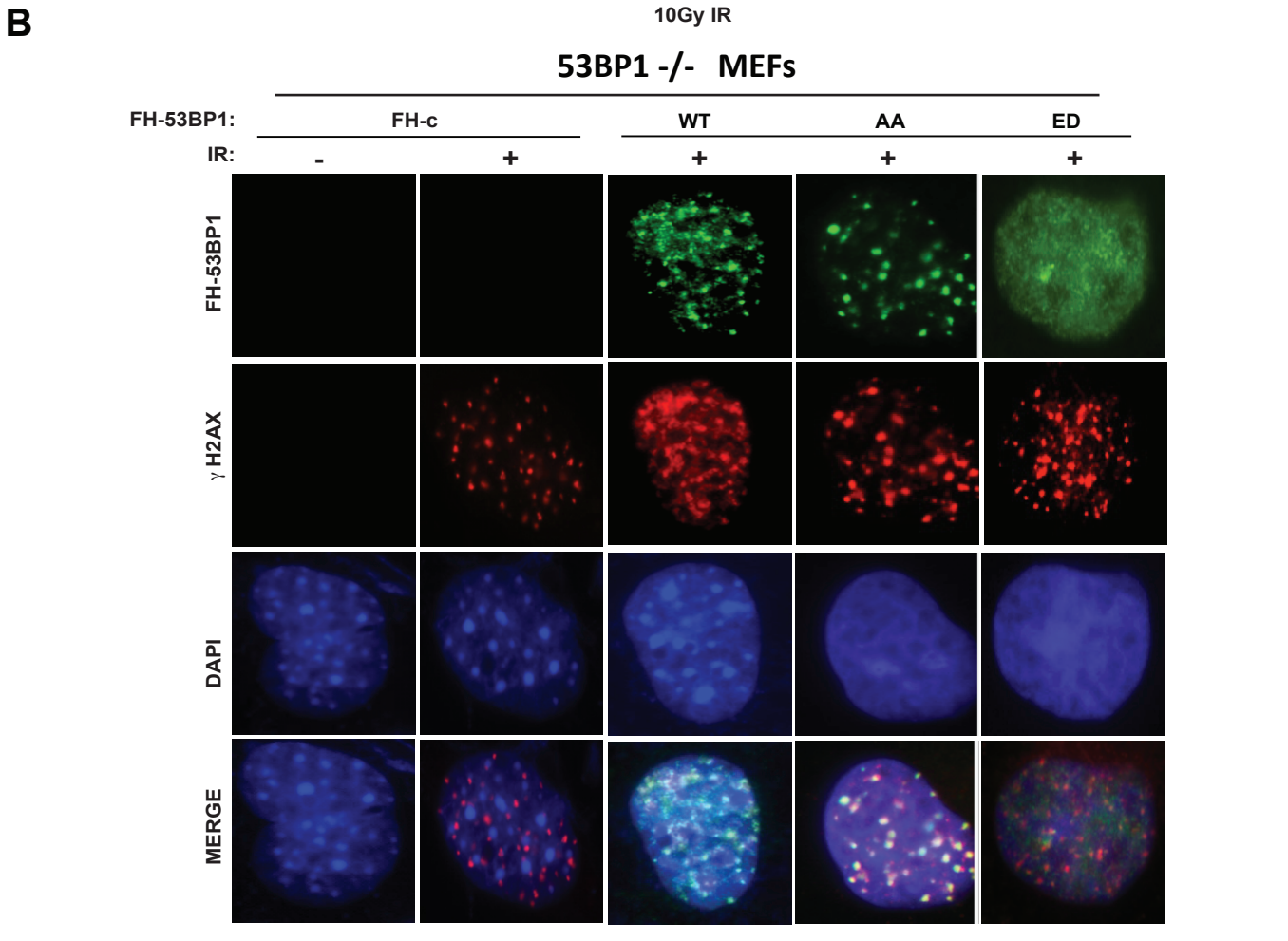

-siRNA
% RNF168 positive cells
40
30
20
10
0
Control
PP4R3β
PP4C
Control
PP4R3β
PP4C
-IR
+IR

S2, related to Figure 2

RNF168 foci formation is not affected by silencing PP4C and PP4R3β. *Left panel:* U2OS cells expressing GFP-RNF168 were transfected with siRNAs against PP4C or PP4R3β. Cells were synchronized in mitosis, exposed to 10 Gy IR immediately following release, and fixed for immunofluorescence. Representative images are shown. *Right panel:* Quantification of RNF168 foci positive cells (>100 cells were quantified per condition.) Data are expressed as mean ±S.D. γ-H2AX staining was used to visualize DSBs.

Figure S3


A
WT
T1609A
T1609E
S1618A
S1609D
ED
AA
FH-53BP1
γ H2AX
DAPI
MERGE
% 53BP1 positive cells in G1
40
30
20
10
0
WT
T1609A
T1609E
S1618A
S1618D
ED
AA
: FH-53BP1
10Gy IR


B
53BP1 -/- MEFs
FH-53BP1:
FH-c
WT
AA
ED
IR:
-
+
+
+
+
FH-53BP1
γ H2AX
DAPI
MERGE

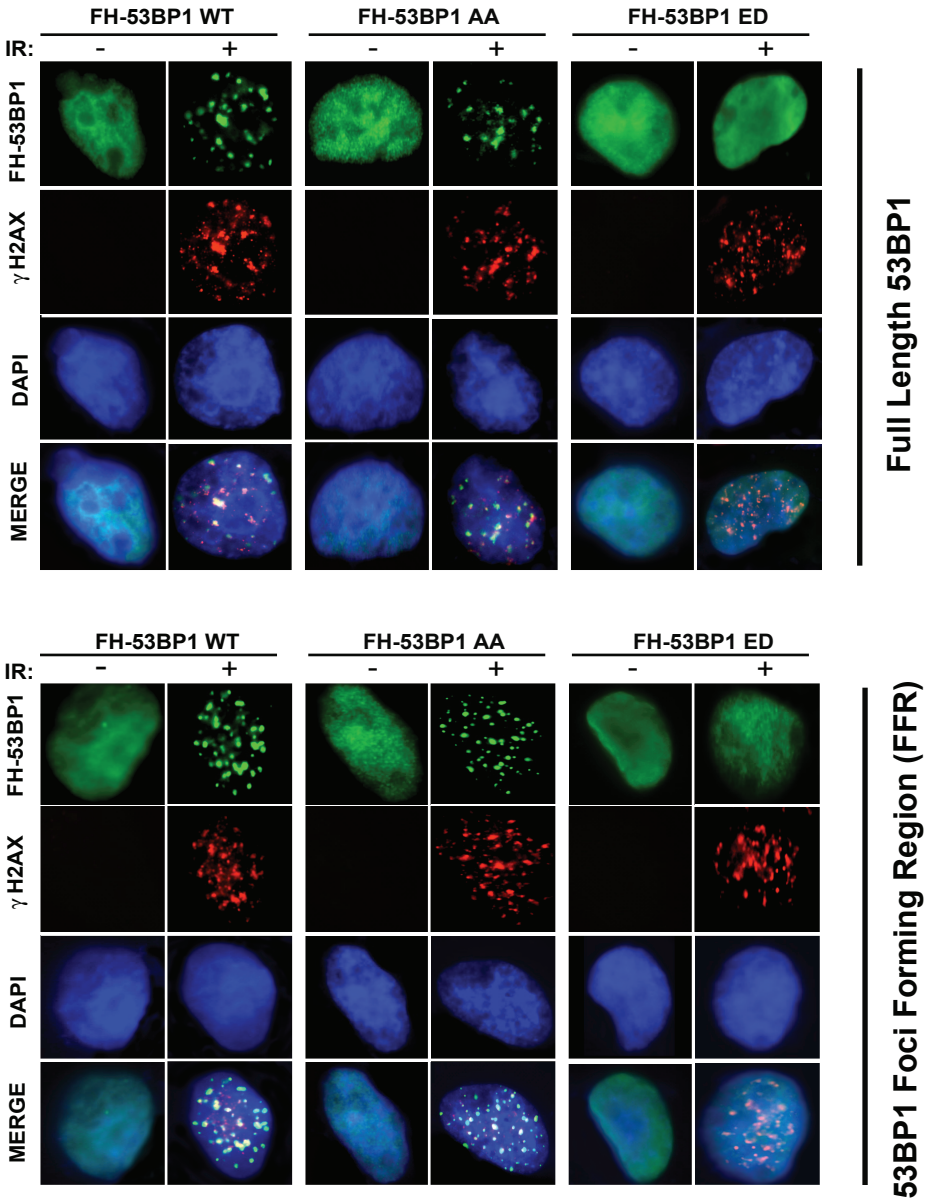
S3, related to Figure 3

3A. Individually mutating T1609 and S1618 has no impact on 53BP1 foci formation. *Upper panel:* HeLa cells expressing FH-53BP1 WT or the indicated single or double phospho-mutants were treated with Nocodazole , exposed to 10Gy IR and processed for immunofluorescence as in Suppl. Fig 2A. 53BP1-ED does not form foci as expected. *Lower panel:* Quantitation of 53BP1 foci positive cells (>100 cells were quantified per condition.) γ-H2AX staining was used to visualize DSBs. Cells with >10 foci were counted as positive.

3B. 53BP1-WT and AA but not 53BP1-ED forms foci in 53BP1 -/- MEFs. FH-53BP1 WT and the indicated phosphomutants were expressed in 53BP1-deficient mouse embryonic fibroblasts (MEFs) and exposed to 10 Gy IR. FH-c (Empty vector) was transfected as a control. Cells were fixed and stained with anti-FLAG antibody. γ-H2AX was used to visualize DSBs.

Figure S4

A


FH-53BP1 WT
FH-53BP1 AA
FH-53BP1 ED
IR:
-
+
FH-53BP1
γH2AX
DAPI
MERGE
Full Length 53BP1
53BP1 Foci Forming Region (FFR)

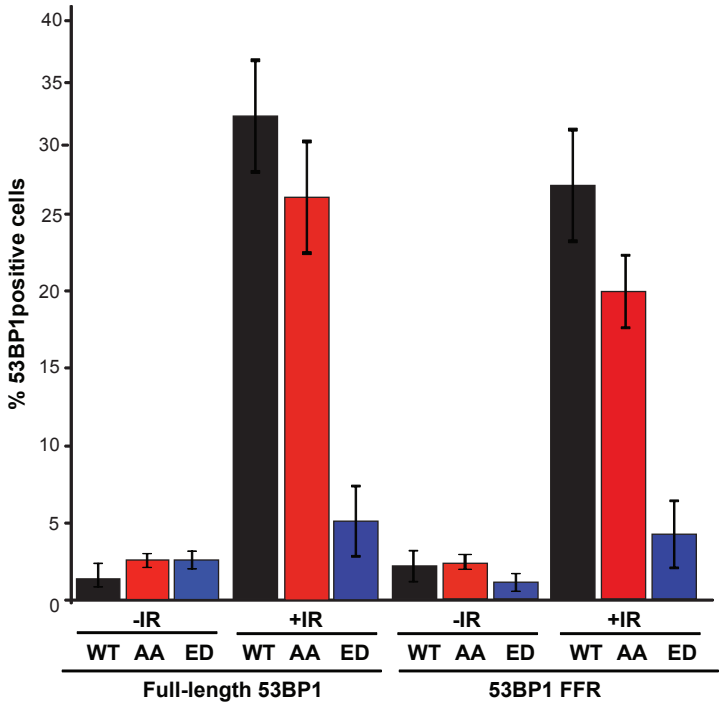
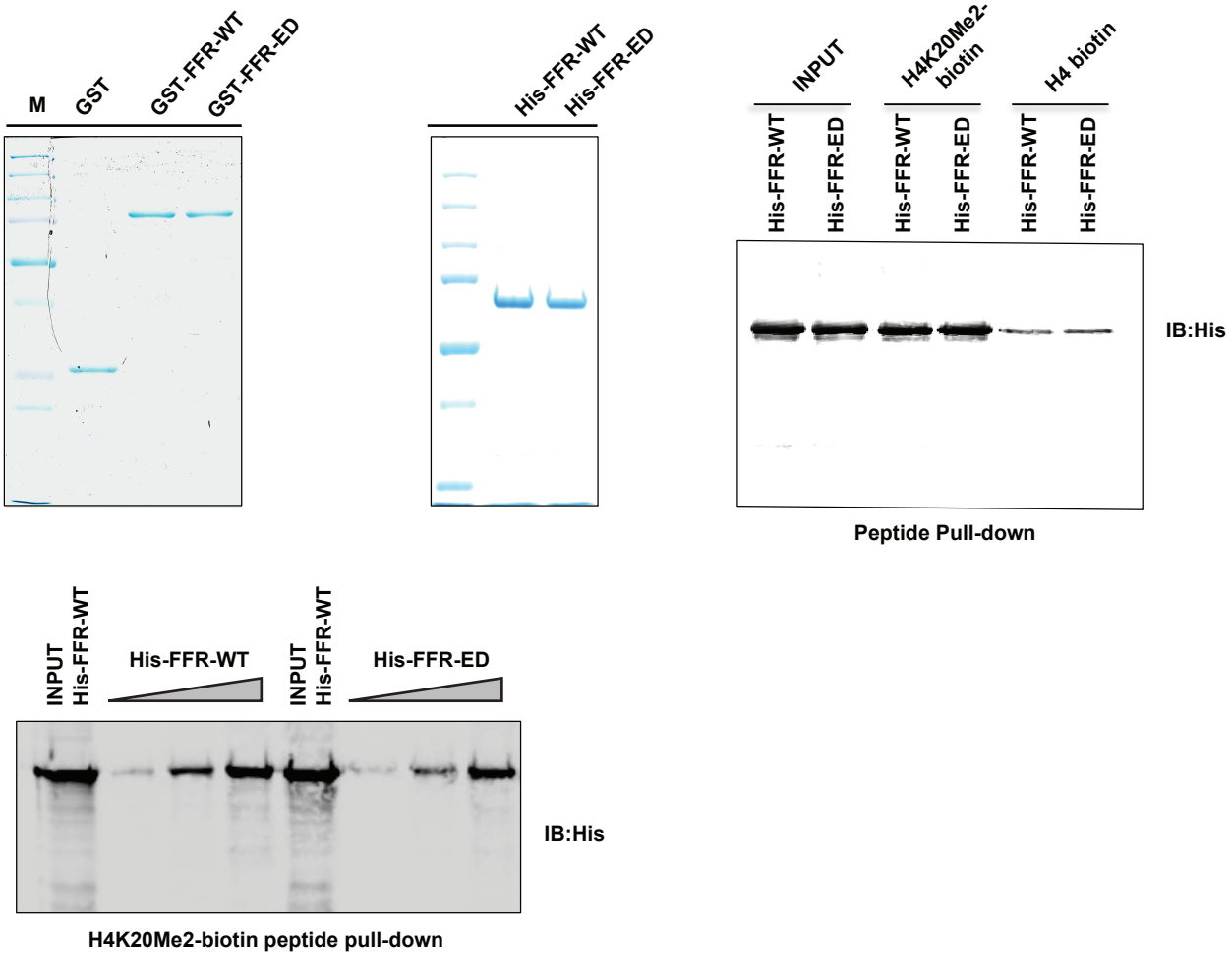

% 53BP1positive cells
40
35
30
25
20
15
10
5
0
-IR
+IR
WT AA ED
Full-length 53BP1
53BP1 FFR

Figure S4

B

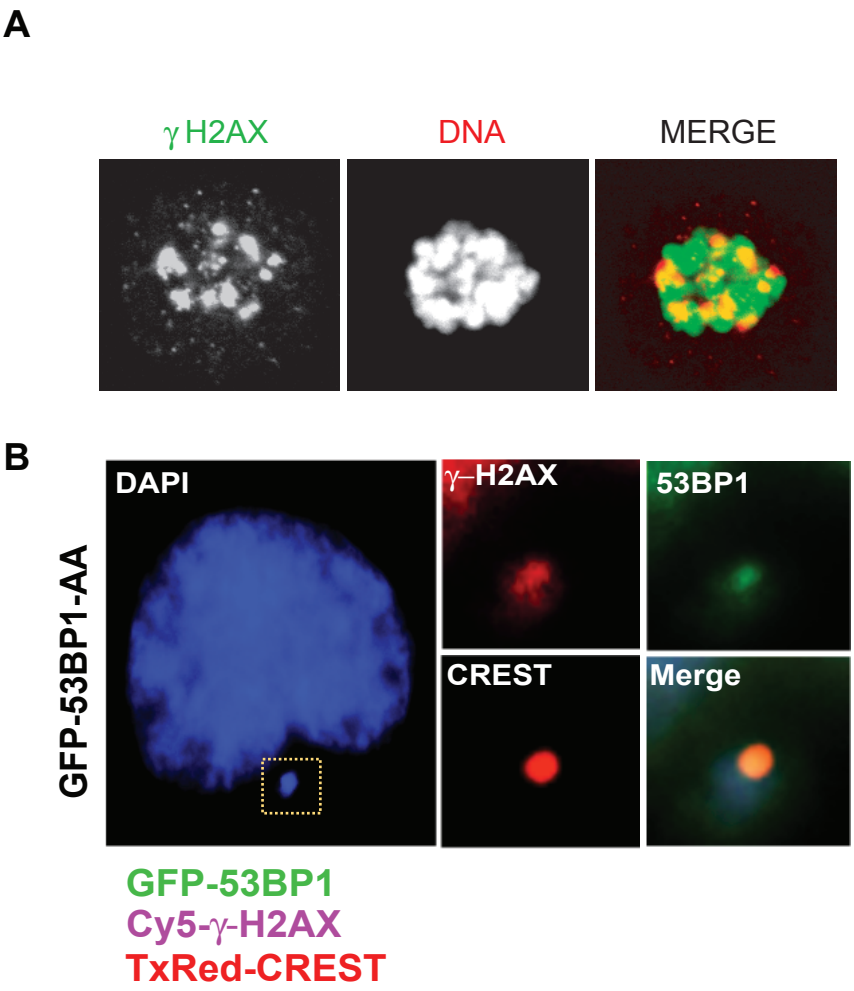

M
GST
GST-FFR-WT
GST-FFR-ED
His-FFR-WT
His-FFR-ED
INPUT
H4K20Me2-biotin
H4 biotin
His-FFR-WT
His-FFR-ED
His-FFR-WT
His-FFR-ED
His-FFR-WT
His-FFR-ED
IB:His
Peptide Pull-down
INPUT
His-FFR-WT
His-FFR-WT
INPUT
His-FFR-WT
His-FFR-ED
IB:His
H4K20Me2-biotin peptide pull-down

S4, related to Figure 5

4A. Full-length 53BP1 and focus forming region (FFR)-53BP1 show no difference in foci-forming ability. *Upper and middle panel:* Full-length or FFR-53BP1 constructs (WT, AA, or ED) were transfected in HeLa cells followed by treatment of cells with Nocodazole and exposure to 10 Gy IR. Cells were allowed to enter G1 and fixed and stained for immunofluorescence after 6h. *Lower panel:* Quantification of 53BP1 foci in HeLa cells expressing either full-length or FFR-53BP1 with or without exposure to 10 Gy IR. More than 100 cells were quantified. Data are shown as mean ± S.D.

4B. 53BP1-ED mutation does not impair binding of 53BP1 to a biotinylated H4K20me2 peptide. *Upper left panel:* Purified GST-fusion proteins used in Figure 4C and 4D resolved on 12% SDS-PAGE. *Upper middle panel:* Purification of His-fusion proteins resolved on 8% SDS-PAGE. *Upper right panel:* Streptavidin pull-downs of the indicated His-fusion protein with either biotinylated H4K20me2 or H4 peptide. Input represents 33% of the His-fusion protein amount used in the pull-down assays. *Lower panel:* Pull-downs of increased amounts of the indicated His-fusion protein with biotinylated H4K20me2 peptide. Input represents 33% of the His-fusion protein amount used in the pull-down assays.

Figure S5


A
γ H2AX
DNA
MERGE
B
GFP-53BP1-AA
DAPI
γ–H2AX
53BP1
CREST
Merge
GFP-53BP1
Cy5-γ-H2AX
TxRed-CREST

S5, related to Figure 6

5A. DSB foci formation in mitotic cells exposed to 0.5 Gy IR. U2OS cells expressing RFP-H2B were arrested in Nocodazole for 6 h and stained for γ-H2AX (green).

5B. Representative micrographs of GFP-53BP1-AA-expressing U2OS cells showing micronucleus stained with CREST and γ-H2AX at 6 h after release from Nocodazole-induced mitotic arrest. Cells were exposed to 0.5 Gy IR during mitosis.