Supplementary Videos

Supplementary Videos (1-3) Kinetic quantitation of 53BP1 recruitment to DNA lesions. To quantify the kinetics of 53BP1 recruitment to DSBs, a multi-photon laser (MPL) system was used. U2OS cells, stably expressing full-length wild-type (WT) or phospho-mutant 53BP1 (ED or AA) were synchronized in mitosis and released into G1. Representative videos of single cells show the time and scale. Video S1 is WT 53BP1, S2 is 53BP1-ED and S3 is 53BP1-AA.

Supplementary Videos (4-8) Monitoring 53BP1recruitment to chromatin mitotic cells. U2OS cells expressing RFP-H2B were transfected with wild-type or mutant GFP-53BP1 constructs. For aphidicolin experiments (S4 and S5), transfected cells were treated with aphidicolin and released to regular medium prior to imaging. For irradiation experiments (S6, S7 and S8), transfected cells were synchronized in mitosis with Nocodazole, irradiated and released to regular medium prior to imaging. Time from anaphase onset to the first appearance of discrete 53BP1 foci was quantified. Representative videos of single cells are shown. Video S4 is WT 53BP1 and S5 is 53BP1-AA in aphidicolin treated cells. Video S6 is WT 53BP1 and S7 and S8 are 53BP1-AA in irradiated cells.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Cell Culture, Antibodies, and Reagents

U2OS, HeLa, and RPE1-Fucci, 53BP1-/- MEFs cells were grown in DMEM supplemented with 10% (v/v) FBS. U2OS cells stably expressing GFP-RNF168was grown in McCoy 5A supplemented with 10% (v/v) FBS. BRCA1-mutant ovarian line, UWB1.289 (ATCC) and grown in RPMI-1640 supplemented with 50% MEGM (Mammary Epithelial Growth Medium) from Clonetics/Lonza and 3% (v/v) FBS. Antibodies used were against PP4R1 (Bethyl), PP4R2 (Bethyl), PP4R3α (Bethyl), PP4R3β (Bethyl), PP4C (Bethyl), Phospho-histone H3 (Cell Signaling), 53BP1 (Cell Signaling), pS1618-53BP1 (Cell Signaling), γ-H2AX (Cell Signaling), γ-H2AX (Mouse, Millipore), KU70 (Santa Cruz), Histone H3 (Cell Signaling), Dimethyl-Histone H4 (Lys20) (Abcam), Histidine (Clontech), GST (Santa Cruz), Histone H4 (Santa Cruz), Ubiquitin (Cell Signaling), RPA2 (Cell Signaling), FLAG (Sigma) and Tubulin (Sigma). Rabbit polyclonal anti-pT1609/S1618-53BP1 antibody was produced by Antagene (Sunnyvale, CA) with a peptide, Cys - NRLREQYGLGPYEAV(p)TPLTKAADI(p)SLDN. SB203580 and SB202190 (p38 MAPK inhibitors), MK-2206 (Akt inhibitor), BI2536 (Plk1 inhibitor) and NU7441 (DNA-PK inhibitor) were obtained from Selleckchem. Nocodazole, Thymidine and Aphidicolin were obtained from Sigma-Aldrich.

siRNAs and Plasmids Transfection

The PP4 siRNAs were described previously (Lee et al., 2010a). To replace endogenous 53BP1 with WT or phospho-mutants, HeLa and UWB1.289 cells were reverse transfected with 53BP1 siRNA by RNAiMAX and after 30 h, transfected with siRNA-resistant 53BP1 plasmids using Lipofectamine 2000 (Invitrogen). We constructed phospho-mutants of 53BP1 (T1609A, S1618A, T1609A/S1618A, T1609E, S1618D, T1609E/S1618D) by QuickChange II XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions.

53BP1siRNAs were as follows: 53BP1, siRNA #1 sense 5´-AGAACGAGGAGACGGUAAUAGUGGG-3´, antisense 5´-CCCACUAUUACCGUCUCCUCGUUCU-3´. The siRNA-resistant 53BP1 expression vector has been previously described (Noon et al., 2010) and obtained from Penelope A. Jeggo in University of Sussex. We used the following primers: 53BP1 T1609A-F:
5´-CTTGGCCCCTATGAAGCAGTAGCACCTCTTACAAA-3´
53BP1 T1609A-R:
5´-TTTGTAAGAGGTGCTACTGCTTCATAGGGGCCAAG-3´
53BP1 T1609E-F:
5´-CTTGGCCCCTATGAAGCAGTAGAGCCTCTTACAAAGGCAGCAG-3´
53BP1 T1609E-R,
5´-CTGCTGCCTTTGTAAGAGGCTCTACTGCTTCATAGGGGCCAAG-3´
53BP1 S1618A-F:
5´-CTTACAAAGGCAGCAGATATCGCCTTAGACAATTTGGTGGAAGG-3´
53BP1 S1618A-R:
5´-CCTTCCACCAAATTGTCTAAGGCGATATCTGCTGCCTTTGTAAG-3´

53BP1 S1618D-F:
5´-CTTACAAAGGCAGCAGATATCGAC TTAGACAAT TTGGTGGAAGG-3’
53BP1 S1618D-R:
5´-CCTTCCACCAAATTGTC TAAGTCGATATCTGCTGCCTTTGTAAG-3’
53BP1 I1617S-F:
5´- CTCTTACAAAGGCAGCAGATAGCAGCTTAGACAATTTGGTG -3´
53BP1 I1617S –R:
5´- CACCAAATTGTCTAAGCTGCTATCTGCTGCCTTTGTAAGAG -3´

Purification of recombinant 53BP1 fragments
The cDNAs encoding wild-type or mutant 53BP1-FFR were PCR-amplified and subcloned into pGEX5X-1 (GE Healthcare) or pET-15b (Novagen) vectors. GST and His fusion proteins were produced by incubating cells at 25°C for 16h in presence of 0.1mM IPTG. Cells were lysed by sonication in lysis buffer [50mM Tris-HCl pH 7.5, 300mM NaCl, 1mM DTT, 1X protease inhibitor cocktail (Roche)]. Cleared extracts were incubated for 1h at 4°C with glutathione sepharose 4B (GE Healthcare) or Ni-NTA agarose resin (Life Technologies) to purify GST- or His-fusion proteins, respectively. Proteins were eluted in lysis buffer containing 20mM glutathione (GST proteins) or 250mM Immidazole (His proteins) and dialyzed in 50mM Tris-HCl pH 7.5, 150mM NaCl and 10% glycerol.

Pull-down Assays
Nucleosome pull-downs were done by incubating 15µl of ubiquitination reaction with 3µg of GST proteins immobilized on glutathione sepharose 4B (GE Healthcare)
in TNB buffer (50mM Tris–HCl pH 8.0, 150mM NaCl, 0.05% NP-40, 0.1% BSA). After 2h at 4°C, beads were washed four times with TNB buffer and once with 50mM Tris–HCl pH 8.0, 150mM NaCl, before elution of retained proteins with Laemmli sample buffer and analysis by immunoblotting.

For some assays, GST proteins were first phosphorylated in vitro using a mitotic extract prepared from Nocodazole-treated HeLa cells homogenized in 3 pellet volumes of extraction buffer [50mM Tris pH 7.4, 250mM NaCl, 1mM EDTA, 1mM DTT, 0.1% Triton X-100, 1X protease and phosphatase inhibitor cocktail (Roche)]. The lysate was solubilized with a 25G needle and spun for 30 min at 16,000 g. GST fusion proteins were phosphorylated for 60 min at 30°C in a final assay volume of 25µl containing 50mM Tris–HCl pH 7.5, 10mM MgCl2, 0.1mM EDTA, 2mM DTT, 0.01% Brij35, 1mM ATP, 5µl of mitotic extract, 1X phosphatase cocktail inhibitor (Roche) and 0.6 µg of GST proteins. In some case, a combination of 100nM BI2536 and 10□M SB202190 kinase inhibitors was also added in the phosphorylation reaction.

Peptide pull downs were performed by incubating the indicating amount of his-FFR-53BP1 proteins with 2µg of biotinylated histone H4-derived peptide in TNB buffer. After 2h at 4°C, 10 µl of streptavidin-Dynabeads (Dynal) were added and reaction was further incubated 30 min at 4°C. Beads were next treated as described above for nucleosome pull-downs.