Figures and figure supplements

Phosphorylation-mediated interactions with TOPBP1 couple 53BP1 and 9-1-1 to control the G1 DNA damage checkpoint

Nicolas Bigot et al
Figure 1. Identification and in vitro characterisation of TOPBP1 binding phosphorylation sites in 53BP1. (A) The TOPBP1/Rad4-binding motif matches only four potential sites out of over two hundred phosphorylation sites documented for 53BP1 (Hornbeck et al., 2004; Sharma et al., 2014). (B)
Fluorescence polarisation (FP) analysis shows no substantial interaction of fluorescently-labelled phospho-peptides derived from the putative phosphorylation sites centred on 53BP1-Thr344, Ser366 or Ser380 with the BRCT0,1,2 segment of TOPBP1. (C) A fluorescently-labelled phospho-peptide centred on 53BP1-Thr670 binds to the BRCT0,1,2 segment of TOPBP1 with high affinity in FP assays. Treatment with λ-phosphatase abolishes binding, confirming that the interaction is specific for the phosphorylated peptide. (D) Charge-reversal mutation of Lys155, which is implicated in phospho-binding in BRCT1 has little effect on binding of the pThr670 peptide to BRCT0,1,2, whereas mutation of the equivalent residue, Lys250 in BRCT2 substantially decreases the affinity. Mutation of both sites completely abolishes the interaction. (E) No binding of 53BP1-derived phospho-peptides centred on Thr670 or Thr334 was detected with the BRCT4,5 segment of TOPBP1. (F) Fluorescently-labelled phospho-peptides centred on 53BP1-Ser366 and Ser380 bind with modest affinity to the TOPBP1 BRCT4,5 segment. (G) Treatment of the 53BP1-Ser366 phosphopeptide with λ-phosphatase or charge-reversal mutation of Lys704, which is implicated in phospho-binding in BRCT5, abolishes interaction of the phosphopeptide to BRCT4,5.

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Figure 2. Crystal structures of TOPBP1 – 53BP1 phosphopeptide complexes. (A) Structure of TOPBP1 BRCT0,1,2 bound to a 53BP1-pT670 peptide. As predicted from the consensus motif and confirmed by the FP data, this peptide binds to BRCT2. TOPBP1 secondary structure is rainbow-coloured (N-terminus blue - > C terminus red).

(B) Interactions of 53BP1-pT670 peptide and TOPBP1-BRCT2. Dashed lines indicate hydrogen bonding interactions. See text for details.

(C) Structure of TOPBP1 BRCT4,5 bound to a 53BP1-pS366 peptide. Consistent with the FP data, the peptide binds to BRCT5.

(D) Interactions of 53BP1-pS366 peptide and TOPBP1-BRCT5. Dashed lines indicate hydrogen bonding interactions. See text for details.

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Figure 3. TOPBP1-binding sites on 53BP1 are phosphorylated in vivo. (A) Western blot of cell lysate from HeLa cells, showing induction of phosphorylation of 53BP1-Ser366 (top) and 53BP1-Thr670 (bottom) following irradiation. siRNA knockdown of 53BP1 eliminates the reactive bands in both cases, confirming the specificity of the antibody for 53BP1. (B) Imaging of irradiated eYFP-53BP1 WT U2OS cells with siRNA knockdown of endogenous 53BP1. 53BP1-pSer366 and 53BP1-pThr670 immunofluorescence signals co-localise in discrete foci with eYFP-53BP1 after IR (9Gy). Scale bar, 10 μm. (C) 53BP1-pSer366 and 53BP1-pThr670 immunofluorescent foci coincident with eYFP-53BP1 WT are lost in irradiated 53BP1 siRNA knocked-down stable eYFP-53BP1 U2OS cells expressing the S366A and T670A mutants, respectively. The α–53BP1-pThr670 antiserum has some additional low-affinity off-target reactivity unrelated to 53BP1 which is not evident when 53BP1 is present. The CDT1-RFP signal in nuclei indicates cells in G1. Scale bar, 5 μm.

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Figure 3—figure supplement 1. Validation of 53BP1 siRNA western blots demonstrating depletion of 53BP1 protein in U2OS and RPE1 cells treated with siRNA targeted to 53BP1. DOI: https://doi.org/10.7554/eLife.44353.005
Figure 4. 53BP1 phosphorylation sites mediate interaction with TOPBP1 in vivo. (A) Four hours after 9Gy IR, TOPBP1 foci co-localise with eYFP-53BP1 WT in stably transfected U2OS cells depleted for endogenous 53BP1. Formation of co-localising TOPBP1 foci is greatly reduced in cells expressing eYFP-53BP1 S366A and T670A mutations, and the general distribution of TOPBP1 is more diffuse. The absence of substantial cyclin A immunofluorescence marks the nuclei of cells in G1. Scale bar, 10 μm. Comparable data for RPE1 cells is shown in Figure 4—figure supplement 1. (B) Statistical analysis of TOPBP1 and eYFP-53BP1 foci co-localisation per nucleus in irradiated G1 U2OS cells exemplified in A). Cells expressing S366A or T670A mutant eYFP-53BP1 show significantly lower levels of coincidence between TOPBP1 and eYFP-53BP1. More than 200 nuclei were counted per case. Median, mean (+), 10–90 percentiles and outliers are represented in boxplots. p values for the mutants relative to wild-type were calculated by a Kruskal-Wallis test corrected by Dunn’s multiple comparison test. (C) Figure 4 continued on next page
Effect of siRNA depletion of 53BP1 on S phase entry by incorporation of BrdU (green) following damage in U2OS cells. Cells that were already in S-phase prior to DNA damage incorporate EdU (yellow) and are not further analysed. Wild-type G1 cells (EdU-) show a robust G1/S checkpoint following irradiation, do not progress into S-phase and do not incorporate BrdU. G1 cells (EdU-) in which 53BP1 is knocked-down fail to checkpoint and progress into S-phase BrdU. EdU-/BrdU+ cells are indicated with arrowheads. Scale bars indicate 10 μm. Comparable data for RPE1 cells is shown in Figure 4—figure supplement 1. (D) 53BP1 siRNA knocked-down cells transfected with wild-type siRNA resistant HA-53BP1 show a G1/S checkpoint following irradiation, while those transfected with 53BP1 in which one or both TOPBP1-binding phosphorylation sites Ser 366 and Thr 670 are mutated, fail to checkpoint and progress into S-phase, incorporating BrdU. Cells that were in S-phase prior to irradiation incorporate EdU (yellow) and are not further analysed. Scale bars indicate 10 μm. Comparable data for RPE1 cells is shown in Figure 4—figure supplement 1. (E) Histogram of U2OS cells depleted of endogenous 53BP1 by siRNA, and transfected with either wild-type HA-53BP1 (WT) or HA-53BP1 with phosphorylation site mutants. The cell cycle phase distributions in the cells expressing mutant 53BP1 are significantly different (Chi-squared test) from that of the wild-type, with a shorter S-phase, and more cells in G2, consistent with a defective G1/S DNA damage checkpoint allowing progression into DNA replication in the presence of unrepaired damage.

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Figure 4—figure supplement 1. TOPBP1-53BP1 co-localization and G1/S Checkpoint defects in RPE1 cells. (A) Four hours after 9Gy IR, TOPBP1 foci co-localise with eYFP-53BP1 WT in stably transfected RPE1 cells depleted for endogenous 53BP1. Formation of co-localising TOPBP1 foci is greatly reduced in cells expressing eYFP-53BP1 S366A and T670A mutations, and the general distribution of TOPBP1 is more diffuse. The absence of substantial cyclin A immunofluorescence marks the nuclei of cells in G1. Scale bar, 10 μm. (B) G1/S checkpoint analysis. Incorporation of BrdU (green) in
EdU negative RPE1 cells (yellow) indicates their S phase entry. Control irradiated cells (2Gy) show a robust G1/S checkpoint arrest, with no progress into S-phase (no BrdU incorporation, see white arrows). Progress into S-phase is not inhibited in 53BP1 knocked-down cells following IR (red arrows). Images were acquired with an IX70 Core DeltaVision microscope. (C) Statistical analysis of data of observed as in A. Bars represent the means of percentage of BrdU+/EdU- cells from more than 2000 cells per case with error bars as standard error of the mean. (D) 53BP1 knocked-down cells transfected with eYFP-53BP1 show a G1/S checkpoint following irradiation, while those transfected with 53BP1 in which one or both TOPBP1-binding phosphorylation sites Ser 366 and/or Thr 670 are mutated, progress into S-phase and incorporate BrdU. (E) As B, bars represent means of percentage of BrdU+/EdU- cells from more than 500 eYFP-53BP1 transfected cells per case with error bars as standard errors of the mean. Kruskal-Wallis test corrected with a Dunn's multiple comparison was done. Adjusted p values are displayed when significant differences are observed.

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Figure 4—figure supplement 2. TOPBP1-53BP1 co-localization and G1/S Checkpoint defects in U2OS cells. (A) Data underlying Figure 4C, but shown as separate channels. (B) Data underlying Figure 4D, but shown as separate channels.
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Figure 5. Damage checkpoint signalling through ATR is perturbed in 53BP1 phosphorylation site mutants. (A) ATR activated by phosphorylation on Thr1989 (pATR) forms immunofluorescent foci that co-localise with transfected eYFP-53BP1 in irradiated G1 U2OS cells with siRNA knockdown of 53BP1 mutants.

Figure 5 continued on next page.
endogenous 53BP1. However, co-localisation of ATR foci with eYFP-53BP1 foci is lost in cells expressing eYFP-53BP1 constructs with S366A and T670A mutations. The absence of substantial cyclin A immunofluorescence marks the nuclei of cells in G1. Scale bar, 5 μm. (B) Statistical analysis of pATR and eYFP-53BP1 foci co-localisation per nucleus in irradiated G1 U2OS cells exemplified in (A) More than 100 nuclei were counted per case. Median, mean (+), 10–90 percentiles and outliers are represented in boxplots. p values for the mutants relative to wild-type were calculated by a Kruskal-Wallis test corrected by Dunn’s multiple comparison test. (C) CHK1 forms distinct immunofluorescent foci in irradiated G1 RPE1 cells transfected with a control scrambled siRNA (SCR). On siRNA knockdown of 53BP1, CHK1 no longer forms discrete foci, but takes on a diffuse pan nuclear distribution. The absence of substantial cyclin A immunofluorescence marks the nuclei of cells in G1. Scale bar, 5 μm. (D) CHK1 focus formation in irradiated G1 RPE1 cells with siRNA knockdown of endogenous 53BP1, is rescued by expression of siRNA-resistant wild-type eYFP-53BP1 but not by eYFP-53BP1 constructs with S366A and T670A mutations. Scale bar, 5 μm. (E) Phosphorylation of the ATR/CHK1 target site, TP53-Ser15, is evident in the nuclei of irradiated U2OS cells stably expressing the wild-type eYFP-53BP1 and depleted for endogenous 53BP1. This signal is significantly diminished in cells expressing eYFP-53BP1 constructs with S366A or T670A mutations. Scale bar, 10 μm. (F) Statistical analysis of mean α-TP53-Ser15 immunofluorescence per nucleus in irradiated G1 U2OS cells exemplified in (E) More than 100 nuclei were counted per case. Median, mean (+), 10–90 percentiles and outliers are represented in boxplots. p values for the mutants relative to wild-type were calculated by a Kruskal-Wallis test corrected by Dunn’s multiple comparison test. (G) p21/CDKN1A and TP53 nuclear signals are decreased after irradiation in U2OS cells expressing eYFP-53BP1 S366A and T670A mutants and depleted of endogenous 53BP1 compared to a wild-type eYFP-53BP1 control. Neither the TP53-pSer15 (E) nor total TP53 immunofluorescence signals show any pattern of co-localisation with 53BP1, confirming that direct interaction of the two proteins is not significant in the context of DNA damage signalling (Cuella-Martin et al., 2016). Scale bar, 10 μm. (H) Statistical analyses of mean α-p21/CDKN1A (left) and α-TP53 (right) immunofluorescence per nucleus in irradiated G1 U2OS cells exemplified in (G) More than 100 nuclei were counted per case. Median, mean (+), 10–90 percentiles and outliers are represented in boxplots. p values for the mutants relative to wild-type were calculated by a Kruskal-Wallis test corrected by Dunn’s multiple comparison test.
Figure 5—figure supplement 1. pATM focus colocalisation with 53BP1 is unaffected by mutations in Ser366 or Thr670. (A) Representative pictures of pATM foci formation 2 hr post IR (9Gy) in RPE1 cells after a 72 hr knockdown period with a 3'UTR 53BP1 siRNA. Wide field pictures are taken with an IX70 Core DeltaVision microscope (scale bar, 40 μm) and magnifications display absence of foci in knocked-down cells (scale bar, 5 μm). Boxplots (right)
represent the pATM foci counts per nucleus following 53BP1 depletion in RPE1 cells 2 hr post IR (9Gy). The median, mean (+), 10–90 percentiles and outliers are presented. The pATM foci formation decrease after knockdown is considered significant (p<0.0001) by a Mann-Whitney test. More than 700 nuclei were counted for each case. (B) pATM foci co-localise with 53BP1 in irradiated U2OS cells with siRNA knockdown of endogenous 53BP1 and transfected with siRNA-resistant eYFP-53BP1. co-localisation is not substantially disrupted by the presence of S366A or T670A mutations in 53BP1. Scale bar, 10 μm. Boxplots (right) statistical analysis of images exemplified in B. More than 200 nuclei were counted per case. Median, mean (+), 10–90 percentiles and outliers are represented in boxplots. p values for the mutants relative to wild-type were calculated by a Kruskal-Wallis test corrected by Dunn’s multiple comparison test. (C) as B. but for RPE1 cells. More than 300 cells per case were counted. Scale bar, 10 μm.

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Figure 5—figure supplement 2. Validation of pATR antibody in U2OS cells. (A) Western blot demonstrating the specificity of the pATR-pT1989 phospho-specific antibody used for immunofluorescence. The strong band observed in irradiated U2OS cells treated with a control siRNA is not present in irradiated cells treated with a specific siRNA to ATR. (B) Immunofluorescent foci of pATR are evident in irradiated U2OS cells treated with a scrambled control siRNA and to a lesser extent in similarly treated unirradiated cells. Transfection with an siRNA targeting ATR significantly diminishes the observation of pATR foci in both irradiated and non-irradiated cells. Scale bar, 2 μm. (C) Statistical analysis of images exemplified in B. Boxplots (right) represent the pATR foci counts per nucleus. The median, mean (+), 10–90 percentiles and outliers are presented. The pATR foci formation decrease after knockdown is considered significant (p<0.0001) by a Mann-Whitney test. More than 100 nuclei were counted for each case.

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Figure 6. TOPBP1 physically couples 9-1-1 and 53BP1 complexes. (A) Schematic of domain architecture of TOPBP1 and interactions. The selective phosphorylation-dependent interactions of 9-1-1 and 53BP1 with different BRCT domains allow for the possibility of their simultaneous interaction with a single TOPBP1 molecule and their collaborative participation in ATR interaction. (B) 53BP1 and RAD9 immunofluorescence foci partially co-localise in irradiated RPE1 cells. Scale bar, 5 μm. (C) Proximity ligation assay (PLA) events (red) for RAD9 and 53BP1 demonstrating the occurrence of RAD9 and 53BP1 molecules within 30–40 nm of each other within the nuclei of irradiated RPE1 cells. Scale bar representing 50 μm and 5 μm are indicated. (D) Scatter plot of PLA events per nucleus for RAD9 – 53BP1 proximity as a function of nuclear Hoechst signal for irradiated (top) and non-irradiated (bottom) RPE1 cells. The PLA signal is predominantly seen in G1 cells (lower Hoechst staining) and is significantly increased by irradiation of the cells. (E) Statistical analysis of PLA events per nucleus in irradiated RPE1 cells shown in D, showing a significant increase in PLA signals on irradiation. More than 500 nuclei were counted per case.
Figure 6 continued

Median, mean (+), 10–90 percentiles and outliers are represented in boxplots. p values for the irradiated versus non-irradiated cells were calculated by a Mann-Whitney test. (F) Statistical analysis of PLA events per nucleus in irradiated RPE1 cells transfected either with a control scrambled siRNA (SCR) or an siRNA directed against TOPBP1 (Figure 6—figure supplement 1C). A very significant decrease in PLA signal between 53BP1 and RAD9 when TOPBP1 is knocked down. More than 200 nuclei were counted per case. Median, mean (+), 10–90 percentiles and outliers are represented in boxplots. p values for the irradiated versus non-irradiated cells were calculated by a Mann-Whitney test. Comparable data for U2OS cells is presented in Figure 6—figure supplement 1B. (G) Statistical analysis of PLA events per nucleus between RAD9 and eYFP, in irradiated U2OS cells with siRNA knockdown of endogenous 53BP1, transfected with either wild-type eYFP-53BP1 or eYFP-53BP1 with S366A and/or T670A mutations. More than 200 nuclei were counted per case. Median, mean (+), 10–90 percentiles and outliers are represented in boxplots. p values for the mutant versus wild-type eYFP-53BP1 constructs were calculated by a Mann-Whitney test.

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Figure 6—figure supplement 1. RAD9 - 53BP1 Proximity Ligation Assay in U2OS Cells. (A) Scatter plot of PLA events per nucleus for RAD9 – 53BP1 proximity as a function of nuclear Hoechst signal for irradiated U2OS cells in the presence of either a control scrambled siRNA (top) or an siRNA

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Figure 6—figure supplement 1 continued

directed against TOPBP1 (bottom). The PLA signal in the control is predominantly seen in G1 cells (lower Hoechst staining). (B) Statistical analysis of PLA events in A., showing a very significant decrease in PLA signal between 53BP1 and RAD9 when TOPBP1 is knocked down compared to the control siRNA treated cells. More than 150 nuclei were counted per case. Median, mean (+), 10–90 percentiles and outliers are represented in boxplots. p values for the irradiated versus non-irradiated cells were calculated by a Mann-Whitney test. (C) Western blots demonstrating depletion of TOPBP1 protein in U2OS and RPE1 cells treated with siRNA targeted to TOPBP1. (D) Analysis of 53BP1 (left) and RAD9 (centre) foci counts per nucleus in the presence of siRNA knockdown of TOPBP1, and a non-targeting scrambled siRNA control. No significant difference in 53BP1 focus formation is seen between the targeted and non-targeted siRNA treatments. RAD9 foci numbers are decreased in the presence of siTOPBP1, but still present. Consistent with the PLA data, co-localisation of those RAD9 foci with 53BP1 is diminished. More than 200 nuclei were counted per case. Median, mean (+), 10–90 percentiles and outliers are represented in boxplots. p values for the targeting versus scrambled siRNA were calculated by a Mann-Whitney test.

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Figure 7. A model for ATR activation through phospho-dependent interaction of 53BP1 and TOPBP1. (A) Following irradiation, the Mre11-Rad50-Nbs1 (MRN) complex is recruited to broken ends of a DNA double-strand break, and facilitates recruitment and activation of ATM, which phosphorylates H2AX-Ser139 to generate the γ-H2AX signal. (B) Limited resection of the broken ends by MRN and CtIP (not shown), provides binding sites for the ssDNA-binding RPA complex, and for loading of the RAD9-RAD1-HUS1 checkpoint clamp (9-1-1) at the dsDNA.
Figure 7 continued

ssDNA junction by the RAD-RFC clamp loader. The γ-H2AX signal leads to recruitment of MDC1 and RNF168 (not shown) resulting in H2A ubiquitylation and consequent recruitment of 53BP1, which interacts with multiple post-translational modifications on nucleosomes in the vicinity of the break. (C) Phosphorylation of 53BP1-Ser366 and Thr670 by an as yet unidentified kinase, facilitates 53BP1 interaction with TOPBP1, which can simultaneously bind 9-1-1 via the phosphorylated C-terminus of RAD9, leading to recruitment and activation of ATR and CHK1. Whether the 53BP1 and ATR-ATRIP complexes bridged by TOPBP1 are on the same side of a break, or on opposite sides of a break as shown here, remains to be determined.

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