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Mechanism of 53BP1 activity regulation by RNA-binding TIRR and a designer protein

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Abstract

Dynamic protein interaction networks such as DNA double-strand break (DSB) signaling are modulated by post-translational modifications (PTMs). The DNA repair factor 53BP1 is a rare example of a protein whose PTM binding function can be switched on and off. 53BP1 is recruited to DSBs by recognizing histone lysine methylation in chromatin, an activity directly inhibited by 53BP1-binding protein TIRR. From X-ray structures of TIRR and a designer protein bound to 53BP1, we reveal a unique regulatory mechanism where an intricate binding area, centered on an essential TIRR arginine residue, blocks the methylated chromatin-binding surface of 53BP1 (the off switch). We find that abolishing TIRR-mediated regulation in cells via a separation-of-function 53BP1 mutation brings 53BP1 to a state of hyperactivation in response to DSBs, highlighting the key inhibitory function of TIRR. We show that this 53BP1 inhibition is relieved by TIRR-interacting RNA molecules, thus providing a proof-of-principle mechanism for RNA-triggered 53BP1 recruitment to DSBs (the on switch).

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Author Contributions

M.V.B., G.C., P.D., D.C., and G.M. conceived the study. M.V.B., G.C., P.D., C.D., A.D., S.C., M.E.B., N.P., J.R.T., B.B., D.Z., J.R.C., D.C., and G.M. performed the experiments and/or analyzed the data. G.M. wrote the manuscript with extensive input from M.V.B., G.C., P.D., and D.C. All authors edited the manuscript.

Competing interests

The authors declare no competing interests.

Introduction

Post-translational modifications (PTMs) control the functional assembly of numerous protein complexes. In the cascade of PTMs triggered by DNA double-strand breaks (DSBs) in mammals, the DNA damage response (DDR) p53-binding protein 1 (53BP1) is recruited to damaged chromatin by recognizing histone H2A ubiquitinated at Lys15 (H2AK15ub) and histone H4 dimethylated at Lys20 (H4K20me2) in the nucleosome core particle (NCP-ubme)^{1–5}. 53BP1 plays an important role in maintaining the balance between the non-homologous end joining (NHEJ) and homology-dependent DNA repair pathways^{6–8}. 53BP1 favors NHEJ over homology-dependent repair (HDR) by inactivating DNA end resection, the initiation step of HDR, and by blocking the recruitment of HDR factor BRCA1 to DSBs^{9,10}. Loss or inhibition of 53BP1 promotes HDR^{7,8,11}. As an activator of NHEJ, 53BP1 also promotes immunoglobulin class switch recombination (CSR)^{12,13}. 53BP1 represents a rare example of a protein whose PTM reader function can be inactivated. While 53BP1, via its tandem Tudor domain, recognizes the constitutive PTM H4K20me2 in damaged chromatin, in the absence of damage, the ability of 53BP1 to interact with H4K20me2 is inhibited by the protein TIRR¹⁴. The mechanism for the inhibitory function of TIRR is not known, and propositions for the mode of action of TIRR have been controversial^{14,15}. How 53BP1 can dissociate from TIRR in response to DNA damage is also unknown. Here we show that TIRR directly inhibits the interaction of 53BP1 with NCP-ubme. An X-ray structure of TIRR–53BP1 reveals an intricate binding area, centered on an arginine residue in TIRR, that blocks the histone binding surface of 53BP1 tandem Tudor domain. This unique binding mechanism is highly specific for 53BP1 as shown by mass spectrometry and mutagenesis. Based on the TIRR–53BP1 structure, we designed and validated a separation-of-function 53BP1 mutant inactive for binding TIRR, but fully functional for DSB recruitment. The “hyperactive” nature of this 53BP1 mutant demonstrates that a major function of TIRR is to keep 53BP1 in an inactive state in the absence of DNA damage. We also address the mechanism of 53BP1 dissociation from TIRR. TIRR being an RNA-binding protein^{16,17}, and non-coding RNAs having been implicated in the recruitment of 53BP1 to DSBs^{18–20}, we examined the possibility that RNA molecules produced in response to DNA damage could disassemble the TIRR–53BP1 complex. To first test this hypothesis with a well-controlled system, we engineered TIRR-related nucleotide- and RNA-binding and processing enzyme NUDT16 into a 53BP1-binding protein (NUDT16TI). Successful protein design was validated via X-ray structure determination of NUDT16TI–53BP1 and quantitative binding assays. Nucleotides dissociated 53BP1 from NUDT16TI, leading us to show that RNA molecules also disassembled the TIRR–53BP1 complex. RNA molecules could therefore serve as a trigger for 53BP1 chromatin recruitment in response to DNA damage.

Results

TIRR blocks the association of 53BP1 with the modified nucleosome core particle

The interaction of TIRR with the Tudor domains of 53BP1 (53BP1-Tudor) does not require lysine or arginine methylation and has an affinity that is higher than what is typical for PTM reader domains¹⁴. We recently showed that overexpression of TIRR in mammalian cells

abolished the formation of 53BP1 ionizing radiation-induced foci (IRIF)¹⁴. We therefore examined whether TIRR would inhibit the interaction of 53BP1 with its minimal chromatin substrate, NCP-ubme^{1,2}. While a GST-fused 53BP1 fragment encompassing the Tudor domains and ubiquitin-dependent recognition (UDR) motif readily interacted with NCP-ubme as previously reported^{2,3,21}, the same 53BP1 construct bound to TIRR had no affinity for NCP-ubme. Therefore, TIRR most likely directly blocks 53BP1 recruitment to chromatin (Fig. 1a).

The X-ray structure of TIRR–53BP1 reveals a binding interface centered on Arg107

To understand how TIRR inhibits 53BP1 binding to chromatin, we determined the X-ray structure of human TIRR in complex with 53BP1-Tudor, hereafter referred to as TIRR–53BP1, at 2.18 Å resolution (Table 1). In the crystal asymmetric unit there are two TIRR homodimers, each interacting with one 53BP1 molecule (Supplementary Fig. 1a). As dictated by symmetry, in solution each TIRR protomer should bind one 53BP1 molecule (Fig. 1b). Noticeably, the N-termini of the two 53BP1-Tudor molecules point away from the TIRR dimer and toward the previously identified upstream oligomerization region of 53BP1²² (Fig 1b). Oligomerization of 53BP1 is essential for its function, yet its precise oligomerization state had not been established when we started this study^{2,23}. We have now been able to reconstitute a stable 400-residue 53BP1 construct encompassing the oligomerization region and Tudor domains. As shown by sedimentation-velocity analytical ultracentrifugation (AUC), 53BP1 is a homodimer and is therefore expected to clamp a TIRR homodimer (Fig. 1c).

In the X-ray structure, TIRR masks the H4K20me2 binding surface of 53BP1, but its binding mode differs radically from that of H4K20me4 and involves a ~640-Å² intricate binding surface that includes the two Tudor domains (Fig. 1d). In the H4K20me2–53BP1 complex, the binding surface area is smaller (~257 Å²) with the interaction mainly mediated by the first Tudor domain and a few residues in the inter-Tudor region^{1,24} (Fig. 1e). A binding cage formed by four aromatic residues and Asp1521 in the first Tudor domain accommodates the dimethyl-lysine in H4K20me2 via cation- π interactions and an ion pair with the dimethylammonium group of K20me2 (Fig. 1e).

In TIRR, a loop comprised of residues 101 to 107 connecting two antiparallel β -strands is the main interface with 53BP1 (Figs 1d, 2a). Central to this binding site is TIRR Arg107, which occupies a unique intermolecular cavity assembled from Trp24 of TIRR and Tyr1502, Asp1521 and Met1584 of 53BP1 (Fig. 2a,c). The guanidinium group of Arg107 establishes hydrogen bonds with the carboxyl group of Asp1521 and carbonyl group of Met1584. TIRR Gly21 amide group and Trp24 side chain HE1 atom are hydrogen bonded to the carbonyl and carboxyl groups of Asp1521, respectively. TIRR Pro105 contributes van der Waals intermolecular contacts and CH- π interactions with its side chain sandwiched between the aromatic rings of 53BP1 Tyr1500 and Phe1553 (Fig. 2a,d). The carbonyl group of Pro105 forms a hydrogen bond with the hydroxyl group of Tyr1502. TIRR His106 HE2 atom may be transiently hydrogen bonded to the backbone carbonyl group of 53BP1 Glu1551. TIRR residues 102 to 104 do not interact with 53BP1, but Leu101 contacts the side chain of 53BP1 Trp1495 (Fig. 2a). Distant from the aforementioned binding loop, TIRR Leu20

contacts Tyr1523 while TIRR Lys10 participates in van der Waals interaction with the aromatic ring of Trp1495 (Fig. 2b,e). Additionally, the ammonium group of Lys10 is hydrogen bonded to the carbonyl group of Trp1495 and to the hydroxyl group of Tyr1523 (Fig. 2b,e). In the other protomer, the ammonium group of TIRR Lys151 is positioned favorably to form salt bridges with the carboxyl groups of evolutionarily conserved 53BP1 residues Glu1549 and Glu1551, an observation consistent with nuclear magnetic resonance (NMR) chemical shift perturbations indicating that Lys151 is in the vicinity of the binding interface¹⁴ (Fig. 1d). However, these salt bridges are not essential for complex formation since the K151E mutation does not disrupt the interaction of 53BP1 with TIRR in cells¹⁴.

53BP1 and TIRR undergo conformational changes upon complex formation

There are substantial conformational changes in 53BP1 resulting from its interaction with TIRR. The 53BP1 loop (residues 1495–1499) harboring Trp1495, a residue essential for methyl-lysine recognition, is flipped by about 180°, and Trp1495 aromatic ring is reoriented to interact with TIRR Lys10 and Leu101 (Figs 1d–f, 2b,e). Tyr1523 in the methyl-lysine binding cage of 53BP1 also undergoes a drastic reorientation in the complex with TIRR, so does Phe1553 as well as the loop (residues 1547–1553) harboring this residue (Figs 1d–f, 2b,e). Noticeably, the side chains of Trp1495 and Tyr1523 in apo 53BP1 are highly flexible, an indication of the structural malleability of 53BP1 (refs^{1,25}). There are also changes in TIRR upon interaction with 53BP1. In the X-ray structures of apo TIRR and TIRR protomers not interacting with 53BP1 in TIRR–53BP1 crystals, Lys10 and residues 103–107 in the 53BP1-binding loop are highly flexible as can be deduced from the poor electron density for these regions of the protein. The same residues are more rigid in the TIRR–53BP1 complex as evidenced by the well-defined electron density. The selective broadening of NMR spectroscopy signals we previously observed for ¹³C-methyl-labeled Lys10 upon TIRR–53BP1 complex formation further highlights the reduced flexibility of this key interface residue¹⁴. There is good agreement between the binding interface mapped using solution NMR spectroscopy¹⁴ and the inter-molecular contacts in the high-resolution X-ray structure of TIRR–53BP1, indicating that these contacts are not artifacts of crystallization.

The functional effects of mutations in TIRR and 53BP1 validate the 3D structure of TIRR–53BP1

It is noteworthy that although not an enzyme, TIRR is evolutionarily related to Nudix hydrolase NUDT16 (47.9% amino acid sequence identity and 60.5% homology)^{16,26,27}. NUDT16 is an RNA nucleotide diphosphatase that processes the 5′ m⁷GpppG cap from small nucleolar RNAs and cytoplasmic messenger RNAs²⁸. In addition, NUDT16 hydrolyzes inosine diphosphate (IDP) into inosine monophosphate (IMP) and inorganic phosphate, thereby limiting inosine incorporation in RNA^{27,29,30}. NUDT16 also processes ADP-ribosylation of proteins^{31,32}. Intriguingly, although NUDT16 does not bind 53BP1 (see below), the TIRR loop residues mediating interaction with 53BP1 are conserved in NUDT16 (Fig. 3a). A major difference between TIRR and NUDT16, however, is the lack of a histidine (His106 in TIRR) between Pro104 and Arg105 in NUDT16, corresponding to Pro105 and Arg107 in TIRR (Fig. 3a). As a result, the loop in NUDT16 is shorter and rigid, and locked in a conformation incompatible for binding 53BP1 (Supplementary Fig. 2).

which contrasts with the flexible nature of the 53BP1-binding loop in TIRR as discussed above.

Guided by the TIRR–53BP1 structure and structural resemblance between TIRR and NUDT16, we incorporated mutations in 53BP1 and TIRR, and evaluated their effects on TIRR–53BP1 complex formation *in vitro* using proteins expressed in *E. coli* and in mammalian cells (Fig. 3b). Mutating several interfacial residues in 53BP1 greatly diminished its interaction with TIRR (Fig. 3b). Replacement of TIRR residues 101 to 107 in the 53BP1-binding loop by the corresponding residues in NUDT16 abolished formation of the TIRR–53BP1 complex (Fig. 3b). Single point TIRR mutations K10E and R107S likewise abolished binding to 53BP1 while H106G, H106E and deletion of H106 (H106^Δ) greatly diminished the interaction (Fig. 3b). Furthermore, mass spectrometric analysis of proteins associated with wild type TIRR and TIRR modified to harbor the loop residues of NUDT16 (see above) demonstrated that the loop motif in TIRR is highly specific for 53BP1 interaction (Supplementary Fig. 3 and Supplementary Table 1). As discussed above, the K151E mutation, expected to disrupt salt bridges between TIRR and 53BP1 (Fig. 1d), did not have any obvious effect on the interaction. From a functional standpoint, we previously observed that overexpression of TIRR in mammalian cells blocked 53BP1 recruitment to DSBs thereby impairing 53BP1 function in the DDR¹⁴. Unlike wild type TIRR, expression of the K10E and R107S mutants in RPE1 cells did not prevent 53BP1 IRIF formation (Fig. 3c).

A separation-of-function mutation creates a hyperactive form of 53BP1

Residues Trp1495 and Asp1521 in the first Tudor domain of 53BP1 participate in critical intermolecular contacts in the TIRR–53BP1 and H4K20me2–53BP1 structures (Fig. 2a,b,e). Consequently, the W1495A and D1521A mutations markedly diminished 53BP1 binding to both TIRR (Fig. 3b) and H4K20me2^{1,14}. These mutations also abolished 53BP1 IRIF formation^{1,14}. To create a version of 53BP1 that would not bind TIRR but would still be recruited to DNA damage sites, we opted to mutate Phe1553 in the second Tudor domain. This residue is rigid and buried at the intermolecular interface in the TIRR–53BP1 structure (Fig. 4a). In contrast, Phe1553 is flexible in the 53BP1–H4K20me2 structure as inferred from the two conformations modeled in the electron density (Fig. 1e,f). In one of the conformations, the phenyl ring points away from H4K20me2, suggesting that Phe1553 is not essential for the 53BP1–H4K20me2 interaction. As we expected, mutating Phe1553 to an arginine prevented the interaction of 53BP1 Tudor domains with TIRR (Fig. 4b). The formation of intense IRIF by the minimal focus-forming region (FFR, residues 1220–1711) of 53BP1 F1553R, indicative of interaction with H4K20me2, and the markedly diminished interaction of F1553R with TIRR in immunoprecipitation assays validated this separation-of-function mutant in mammalian cells (Fig. 4b). For negative controls, we also mutated two solvent accessible residues, Glu1551 and Tyr1552, adjacent to Phe1553. As predicted from the TIRR–53BP1 structure, the E1551R and Y1552R mutations had virtually no effect on the interaction of 53BP1 with TIRR in cells (Fig. 4b).

We previously observed that loss of TIRR markedly reduced the nuclear soluble levels of 53BP1 and resulted in enhanced phosphorylation and association of 53BP1 with its effector

proteins after induction of DNA damage¹⁴. Remarkably, 53BP1 F1553R mimics the loss of TIRR, giving rise to a “hyperactive” form of 53BP1 as supported from the following results. First, salt fractionation assays with F1553R revealed a very distinct decrease in nuclear soluble 53BP1 (Fig. 4c). Second, in response to ionizing radiation, 53BP1 F1553R became efficiently phosphorylated at Ser25 and Ser29, and associated more strongly than wild type 53BP1 with known 53BP1 effector proteins such as PAX transactivation domain-interacting protein (PTIP) and topoisomerase II β binding protein 1 (TopBP1) (Fig. 4d). Noticeably, this “hyperactivity” of 53BP1 F1553R correlated with a significant increase in mobility in cells compared to wild type 53BP1 as shown using fluorescence recovery after photobleaching (FRAP) assays (Fig. 4e). The value of halftime recovery ($t_{1/2}$) was 55.6 sec for cells expressing wild type 53BP1, which is more than 3 times slower than for the cells expressing 53BP1 F1553R (15.5 sec). This increased mobility of 53BP1 F1553R mutant may be the foundation for its “hyperactive” state.

Next, we checked if, akin to loss of TIRR or ectopic expression of histone H2A Lys15-specific E3 ubiquitin ligase RNF168 (refs^{14,33}), 53BP1 F1553R would impact DNA repair. To test this possibility, we probed the influence of F1553R on the cell killing effect of a poly-(ADP-ribose) polymerase inhibitor (PARPi) on DNA repair-deficient cells. We recently showed that loss of 53BP1 function caused by TIRR overexpression conferred resistance to the PARPi olaparib via reactivation of the HDR pathway in mouse embryonic fibroblasts (MEFs) carrying hypomorphic *BRCA1*-11 alleles¹⁴. In contrast, TIRR-depletion, or RNF168 overexpression, increased the sensitivity of *BRCA1*-mutant MEFs to olaparib^{14,33}. We therefore examined whether the F1553R 53BP1 mutation, which activates 53BP1, would sensitize cells to olaparib. We reconstituted *53BP1*^{-/-} MEFs with wild type (WT) or F1553R human 53BP1 (Fig. 5a). As anticipated, BRCA1 depletion in the WT-expressing MEFs made the cells more sensitive to olaparib (Fig. 5b). Expression of F1553R in the context of BRCA1 depletion further sensitized these cells to olaparib, thereby phenocopying the TIRR-deficient phenotype (Fig. 5c). Consistent with increased inactivation of HDR by F1553R, the number of HDR protein RAD51 foci per cell was smaller in 53BP1 RPE1 cells reconstituted with F1553R relative to wild type 53BP1 (Fig. 5d,e). Like the TIRR-deficient cells or cells expressing TIRR K10E¹⁴, a mutant that does not bind 53BP1, F1553R-expressing 53BP1^{-/-} MEFs were defective in DNA damage repair. There was increased persistence of DSBs as gauged from the kinetics of histone γ H2AX IRIF formation (Fig. 5f).

The binding of 53BP1 to damaged chromosome ends is necessary for fusion of uncapped telomeres³⁴. Hence, 53BP1 activity at telomeres can be quantified by telomeric fluorescence *in situ* hybridization (FISH) assays. Errors in telomeric replication³⁵ or choice of DSB repair pathways (classical versus alternative NHEJ) may lead to telomere loss in fused chromosomes³⁶. Therefore, we scored chromosome fusions by monitoring the FISH signal as a marker of telomere loss or processing in cells expressing the dominant-negative TRF2 B M allele (Fig. 5g). TRF2 B M promotes NHEJ at chromosome ends³⁷. We transiently expressed TRF2 B M in 53BP1 RPE1 cells reconstituted with wild type 53BP1 or F1553R mutant. Metaphases were prepared from these cells and scored for chromosome end-to-end fusions. 53BP1 deficiency resulted in diminished fusion events at telomeres induced by transient TRF2 B M expression, and the total numbers of

chromosome fusions were comparable in wild type and F1553R 53BP1 (Fig. 5g). Intriguingly, the F1553R mutant led to a significant increase in telomere-free fusions. This observation suggests that the TIRR–53BP1 interaction could prevent the loss of uncapped telomeres prior to NHEJ-mediated fusion. The underlying mechanism for this phenotype remains unclear but could involve telomeric replication, which would be disrupted by the F1553R 53BP1 mutant.

To examine the function of TIRR–53BP1 interactions in regulating NHEJ, we monitored the effect of the 53BP1 F1553R on CSR in cultures of primary splenic B cells harvested from *53BP1*^{−/−} mice. Class switching to IgG1 was analyzed in *53BP1*^{−/−} B cells stimulated with anti-CD40 antibody and IL-4 upon reconstitution with retroviruses that express wild type 53BP1, the F1553R mutant, or as a negative control, the H4K20me2-binding defective W1495A mutant (Fig. 5h). Note that to avoid the inefficient packaging of large 53BP1 inserts in retroviral particles, all rescue constructs expressed a truncated 53BP1 protein (53BP1-BRCT, residues 1–1711) that supports wild type CSR frequencies³⁸. As expected, wild type 53BP1 expression efficiently rescued class switching in *53BP1*^{−/−} B cells, while the W1495A mutant could not (Fig. 5h and Supplementary Fig. 4), consistent with its inability to interact with DSB sites¹. In contrast, the fact that 53BP1 F1553R-reconstitution rescued CSR to levels comparable to wild type (Fig. 5h) indicates that TIRR binding is not involved in CSR regulation. This observation is in line with previous findings that increased expression of 53BP1 or RNF168 enhances PARP inhibitor sensitivity, but not CSR³³, reinforcing the idea that 53BP1 stimulates productive CSR and inactivates mutagenic DNA repair via distinct pathways³⁹. Together these results with the F1553R mutant show that the impact of TIRR on 53BP1 activity is complex and context dependent, but overall the TIRR–53BP1 interaction significantly influences genomic stability.

A nucleotide- and RNA-binding and processing enzyme can be engineered to bind 53BP1

While TIRR forms a tight complex with 53BP1 and regulates 53BP1 function in cells, it is not known how TIRR is removed from 53BP1 in response to DNA damage. Because TIRR is an RNA-binding protein and because it has been shown that small non-coding RNAs (ncRNAs) play a role in the recruitment of 53BP1 to DNA damage sites^{18–20,40}, it is conceivable that RNA molecules could help displace 53BP1 from TIRR. Since the RNA targets of TIRR have not been identified and since the nucleic acid binding and RNA processing activities of NUDT16 are well understood, we attempted to engineer NUDT16 into a 53BP1-binding protein as a proxy to probe the possibility that nucleic acids would interfere with 53BP1 binding. The TIRR 53BP1-binding loop was introduced in NUDT16 and NUDT16 Arg5 (corresponding to Lys10 in TIRR) was replaced by a lysine. Astoundingly, while NUDT16 has no affinity for 53BP1, the engineered protein (NUDT16 Tudor-interacting or NUDT16TI) binds 53BP1 with a K_d of 1.2 μ M at 22 °C in the presence of 150 mM NaCl, which is similar to the affinity of TIRR for 53BP1 (Fig. 6). We used isothermal titration calorimetry (ITC) for the affinity measurements.

Designer protein NUDT16TI recapitulates the 53BP1 binding mode of TIRR

The X-ray structure of NUDT16TI–53BP1, which we determined to a resolution of 2.49 Å (Fig. 6a, Supplementary Fig. 1b and Table 1), demonstrates that we recreated a binding

interface comparable to that in TIRR–53BP1 even if NUDT16 is significantly smaller than TIRR (Supplementary Fig. 5a,b and Fig. 6a). NUDT16TI is a homodimer and in the crystal each protomer is bound to one 53BP1-Tudor molecule. Akin to the TIRR–53BP1 complex (Fig. 1b), the N-termini of the two 53BP1-Tudor molecules point in the same direction toward the upstream oligomerization region of 53BP1. In solution, a 400-residue 53BP1 homodimeric construct interacts with one NUDT16TI homodimer as shown by sedimentation-velocity AUC (Fig. 6b). An added benefit of studying NUDT16TI is the high stability of the purified protein, allowing the quantitative characterization of several mutants using ITC, which was not possible with TIRR because of its propensity to precipitate over time (Fig. 6c). In agreement with the NUDT16TI–53BP1 structure, the R106S (R107 in TIRR) mutation in NUDT16TI abolished binding while the K5S (K10 in TIRR) mutation or deletion of His105 (His106 in TIRR) decreased the affinity by ~14- or ~38-fold, respectively (Fig. 6c). Selected mutations in 53BP1 – W1495A, D1521A, F1553R – also abolished 53BP1 interaction with NUDT16TI, further validating the binding interface identified in the crystal structure (data not shown).

Nucleotides and RNA molecules displace 53BP1 from designer protein NUDT16TI and TIRR

We showed that NUDT16TI retains wild type affinity for its nucleic acid substrate IMP²⁷ ($K_d \sim 15 \mu\text{M}$) (Fig. 7a). It is noteworthy that the nucleotide-binding site in NUDT16 and NUDT16TI maps to a small area of a large positively charged surface of TIRR that forms a plausible RNA-binding channel (Fig. 7a,b). Remarkably, an unbiased high-resolution mapping of RNA-binding regions in the nuclear proteome identified this channel and the adjacent 53BP1-binding loop (present study) as the RNA binding site of TIRR¹⁷, suggesting that 53BP1 and RNA could compete for the same binding area in TIRR (Fig. 7c).

Next, we asked whether IMP would interfere with the interaction of NUDT16TI with 53BP1. In the presence of excess IMP to saturate the complex, the K_d of NUDT16TI for 53BP1 increased by ~3 fold (Fig. 7a). This decreased affinity is readily explained by a steric effect as the hairpin motif formed by residues 1549–1552 of 53BP1 points toward the IMP (and RNA) binding site in NUDT16TI (notice Glu1551 in Fig. 7a). That a single nucleotide can significantly decrease the affinity of NUDT16TI for 53BP1 suggested that a longer RNA molecule could prevent binding of 53BP1 to TIRR. Indeed, this is what we found. RNA molecules from cell extracts blocked the interaction of 53BP1 with TIRR (Fig. 7d). In our current assay, RNAs extracted from irradiated or non-irradiated cells had indistinguishable effect. In the future, more elaborate investigations will need to be devised to possibly isolate ionizing radiation-specific RNAs that dissociate the TIRR–53BP1 complex. Our studies of TIRR and NUDT16TI provide a proof of principle for the involvement of RNAs in the recruitment of 53BP1 to DNA damage sites.

Discussion

Our studies reveal in near atomic detail a hitherto unobserved regulation mechanism where the PTM reader function of a protein, 53BP1, is inhibited by another protein, TIRR. We successfully engineered a nucleotide- and RNA-binding and processing enzyme, NUDT16,

to make it interact with 53BP1 (NUDT16TI) while preserving its nucleic acid binding properties. With this designer system, we unambiguously validated the TIRR–53BP1 binding mechanism by recreating this mechanism from “first principles.” The structures were also validated by mutagenesis and led us to design a separation of function 53BP1 mutant (F1553R) that cannot bind TIRR but retains affinity for chromatin. The hyperactive nature of 53BP1 F1553R highlights the important role of TIRR in maintaining 53BP1 in an inactive state in the absence of DNA damage.

The regulation of 53BP1 recruitment to DNA damage sites is complex as it involves constitutive (H4K20me2) as well as DNA damage-induced (H2AK15ub) PTMs, factors like JMJD2A, L3MBTL1, RAD18 and RNF169 that compete with 53BP1 for reading these PTMs^{21,41–46}, acetylation of H2AK15 that blocks 53BP1 recruitment⁴⁷, and the direct inhibition of 53BP1 PTM reader function by TIRR¹⁴. 53BP1 forms a tight complex with TIRR, and therefore probing how 53BP1 dissociates from TIRR is of utmost importance to understand how 53BP1 is recruited to DNA damage sites. There is evidence that RAP1-interacting factor 1 (RIF1), which binds phosphorylated 53BP1 in response to DNA damage, is involved in the dissociation process¹⁴, but how this happens is not known. RIF1 has not been shown to directly bind TIRR. It was recently reported that ncRNAs control the DDR and contribute to 53BP1 recruitment^{18–20}. 53BP1-Tudor has affinity for RNA molecules^{20,40}. It is however unlikely that such RNAs would be involved in dissociating 53BP1 from TIRR as the RNAs would be expected to mask the histone binding surface of 53BP1 required for its recruitment to damaged chromatin. TIRR being both an inhibitor of 53BP1 and an RNA-binding protein, relief of 53BP1 state of inhibition by a TIRR-binding RNA molecule would be a simple and plausible mechanism. In addition, we note that RIF1 also binds RNAs¹⁷. In response to DNA damage, RIF1 interacts with phosphorylated 53BP1 in the soluble nuclear fraction and could possibly shuttle RNAs to TIRR leading to its dissociation from 53BP1. It is noteworthy that recombinant TIRR co-purifies with nucleic acids and requires nucleic acid removal by treatment with benzonase and purification at high salt concentration to be able to bind 53BP1 (data not shown). The TIRR–53BP1 and NUDT16TI–53BP1 structures that we determined show overlap between the nucleic acid- and 53BP1-binding surfaces, further supporting the relief of inhibition mechanism. We showed that a single nucleotide diminishes the interaction of NUDT16TI with 53BP1 and that RNA molecules extracted from cells displace 53BP1 from TIRR. The RNA sequence(s) recognized by TIRR are not known and its RNA-binding mechanism remains to be established. Interestingly, human cleavage factor Im (CFI_m), a component of the pre-mRNA 3′ processing complex, contains a Nudix-like subunit (CFI_m25) that, like TIRR, binds but does not hydrolyze RNA⁴⁸. Nevertheless, the mechanism for TIRR RNA-binding cannot be inferred from the X-ray structure of the CFI_m25–RNA complex⁴⁸ as the two proteins do not share the same RNA-binding surfaces. Since the nuclear environment is rich in RNA, we speculate that TIRR recognizes a specific RNA motif with high affinity, which allows dissociation of the tight TIRR–53BP1 complex in response to DNA damage.

In totality, our work with TIRR and a designer protein provides an experimental proof of principle that in response to DNA damage an RNA molecule could trigger the dissociation of TIRR from 53BP1, and thereby promote 53BP1 recruitment to DSBs. Work is in progress to further test this mechanism.

Methods

Plasmids for protein expression in *E. coli*

Human 53BP1 encompassing the tandem Tudor domain and the UDR sequence (residues 1484–1635) was cloned in a GST-based vector as reported²¹. A shorter 53BP1 construct containing only the Tudor domains (residues 1484–1603) was cloned in a modified pET vector encoding an N-terminal His₆-tag cleavable by the tobacco etch virus (TEV) protease as reported¹. Human TIRR (residues 6–211), codon optimized for *E. coli* expression, was cloned in a pBB75 vector with a non-cleavable C-terminal His₆-tag and without a tag. Another version of TIRR not codon-optimized for *E. coli* expression was cloned in a pET-based vector that adds a non-cleavable C-terminal His₆-tag to the protein. NUDT16 and NUDT16TI were cloned in pET-based vectors encoding an N-terminal His₆-tag cleavable by TEV protease or PreScission protease or a non-cleavable C-terminal His₆-tag. NUDT16TI was also cloned in pBB75 without a tag. All mutations were introduced by standard site-directed mutagenesis.

Protein preparation

The proteins were expressed or co-expressed in BL21(DE3) *E. coli* cells grown at 37 °C in LB media to an OD₆₀₀ of ~0.6 and induced with 0.5 mM isopropyl-β-D-thiogalactoside at 15 °C for ~16 h. Harvested cells were lysed using a microfluidizer (Avestin Emulsiflex C5), incubated with 250 U of benzonase (EMD Millipore) per 1 L of cell culture in the presence of 1.5 mM MgCl₂ for 1 h at 4 °C, and then centrifuged. Supernates were loaded into appropriate purification columns.

For the binding assays, His₆-tagged 53BP1 was first purified by Ni²⁺-nitrilotriacetic acid (NTA) agarose chelation chromatography (Qiagen) using a standard solution of 50 mM sodium phosphate (NaPi), pH 7.5 and 300 mM NaCl with different imidazole concentrations to bind (5 mM), wash (20 mM) and elute (250 mM) the protein. The various version of His₆-tagged TIRR, NUDT16 and NUDT16TI were purified similarly but at high salt, with 1 M and 500 mM NaCl during wash and elution, respectively. N-terminal His₆-tags were next cleaved from the proteins by 4 °C overnight incubation with TEV protease or PreScission protease and 10 mM DTT. Samples were then concentrated and purified by size exclusion chromatography using HiLoad 16/60 Superdex 75 column (GE Healthcare) and running buffer of 50 mM NaPi, pH 7.5, 500 mM NaCl. Samples were concentrated and buffer exchanged in 20 mM Tris-HCl, pH 7.5 with 300 or 500 mM NaCl.

To prepare the TIRR–53BP1 and NUDT16TI–53BP1 complexes, His₆-tagged 53BP1 and untagged TIRR or NUDT16TI were co-expressed and co-purified by Ni²⁺-NTA (Qiagen) at high salt like above. After the tag on 53BP1 was cleaved as above, the samples were purified by size exclusion chromatography using HiLoad 16/60 Superdex 75 column (GE Healthcare) and running buffer of 50 mM NaPi, pH 7.5, 500 mM NaCl. Samples were next incubated with ~100 μL Ni²⁺-NTA (Qiagen) for 30 min at 4 °C in a nutator, re-injected into HiLoad 16/60 Superdex 75 column (GE Healthcare) with a running buffer of 25 mM HEPES, pH 7.5, 500 mM NaCl, and then concentrated in the presence of 2 mM TCEP.

For GST pull down assays, GST and GST-tagged 53BP1 were first purified by passing through a GSTPrep FF 16/10 column (GE Healthcare), washing with PBS, pH 7.3 and eluting with PBS, pH 8.0, 20 mM glutathione. On the other hand, GST-tagged 53BP1 co-expressed with C-terminally His₆-tagged TIRR was first Ni²⁺-NTA (Qiagen) purified at high salt as above. Subsequently, all proteins were further purified by size exclusion chromatography using HiLoad 16/60 Superdex 75 or 200 columns (GE Healthcare) and running buffer of 50 mM NaPi, pH 7.5, 500 mM NaCl.

The nucleosome core particle ubiquitylated at histone H2A Lys15 and bearing a dimethylation mimic at histone H4 Lys20 (H4K₂₀me₂) (NCP-ubme) was prepared as previously reported²¹.

To probe the displacement of TIRR from 53BP1 by RNA molecules, 1 µg of purified recombinant Flag-tagged 53BP1 (residues 1220–1631) was bound to Anti-Flag agarose beads (Sigma) and incubated with recombinant His₆-tagged TIRR (1 µg) in TGEN150 buffer (20 mM Tris-HCl, pH 7.65, 150 mM NaCl, 3 mM MgCl₂, 10% glycerol and 0.01% NP-40) for 1 h at room temperature on a roller. Identical aliquots of the washed slurry were next incubated in TGEN150 buffer with 200 ng and 1 µg of nuclear RNAs purified from untreated and from 5 Gy-irradiated HeLa cells, and without RNA as a control, for 1 h at room temperature on a roller. The final volume of the incubation mixtures was 200 µL. The beads were washed five times with TGEN150 buffer and proteins retained on the beads were resolved and identified using 12% SDS/PAGE and Western blot.

GST pull-down assay

GST pull-down assays were performed by first mixing 40 µL of 50% GSH slurry (Clontech) in buffer 1 (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% NP-40, 0.1% BSA) and bait (3 µg GST or equimolar amounts of mutant T1609E/S1618E GST-53BP1, and wild-type GST-53BP1, free or in complex with TIRR with C-terminal His₆-tag) for 1 h on a nutator at 4 °C. Beads were then washed 3 times with buffer 1 (1 mL, 5 min), centrifuging (21,000 g, 2 min) between washes. Input NCP-ubme (36 µg) was added to the GST beads with immobilized baits and mixed for 2 h on a nutator at 4 °C. Beads were washed 5× with buffer 1, removing NP-40 and BSA in buffer 1 in the last wash, and resuspended with 40 µL of 2× Laemli dye. Beads were boiled for 2 min and 10 µL of the supernate was loaded onto a 4–20% TGX gel (Biorad). Protein bands were transferred onto a nitrocellulose membrane (Trans-Blot Turbo System, Biorad) and processed for Western blot analysis. The membrane was blocked (5% non fat milk in TBS, 1 h, room temperature), incubated with primary antibody (1:1000 dilution in 1% BSA in TBS, overnight 4 °C), washed 5 × 5 min with TBST, incubated with HRP-conjugated secondary antibody (1:10,000 dilution in 1% non fat milk in TBS, 1 h, room temperature), washed 5 × 5 min with TBST, and developed with an ECL reagent for imaging using a ChemiDoc MP system (Biorad).

The sources and dilutions for the antibodies used for Western blot analysis of the GST pull-down assays are as follow: anti-ubiquitin (Cell Signaling P4D1, 1:1000); anti-H2A (Millipore 07146, 1:1000 dilution); anti-K15 ubiquitylated H2A (a gift from Dr. Zhiguo Zhang, 1:500); anti-GST (Santa Cruz sc-138, 1:1000), anti-mouse HRP-conjugated (Cell

Signaling 7076, 1:10,000 dilution) and anti-rabbit HRP-conjugated (BioRad 172–1019, 1:10,000 dilution).

X-ray crystallography

Crystals were grown by the hanging drop method, mixing 1.5 μ L of the protein sample and 1.5 μ L of the reservoir solution for the drop, and suspending 2–4 of these drops over 0.5 mL of reservoir solution. Crystals of apo TIRR (residues 6–211) and TIRR bound to 53BP1 tandem Tudor domain (residues 1484–1603) were obtained using a sample of 10–20 mg/mL of co-expressed TIRR–53BP1 tandem Tudor domain and reservoir solution 1 (0.06 M citric acid, 0.04 M Bis-Tris-Propane, pH 4.1, 16% PEG 3,350) and solution 2 (0.1 M MES, pH 5.5, 0.2 M calcium acetate, 7% isopropanol), respectively. Crystals of NUDT16TI–53BP1 tandem Tudor complex were obtained using 15–20 mg/mL of co-expressed proteins and reservoir solution 3 (0.1 M sodium citrate tribasic dehydrate, pH 5.6, 0.2 M ammonium acetate, 10% PEG 4,000). All crystals were obtained at 22 °C.

Crystals of apo TIRR, TIRR–53BP1 and NUDT16TI–53BP1 were cryoprotected in 25% PEG 3,350 in H₂O, 30% glycerol in H₂O, and 25% xylitol in solution 3, respectively, and were quick-frozen in cryoloops (Hampton Research) with liquid nitrogen. The space group of the apo TIRR crystals is P1 with 2 molecules per asymmetric unit. The space group of the TIRR–53BP1 crystals is P1 with 2 molecules of 53BP1 and 4 molecules of TIRR in the asymmetric unit. The space group of the NUDT16TI–53BP1 crystals is P4₃ 2₁ 2, with 2 molecules of 53BP1 and 2 molecules of NUDT16TI in the asymmetric unit.

X-ray diffraction data for apo TIRR were obtained with a Rigaku Micromax-007/R-Axis IV⁺⁺ X-ray diffractometer. For TIRR–53BP1 and NUDT16TI–53BP1, data were collected at the 19-ID beamline of the Advanced Photon Source at Argonne National Laboratory, IL. All diffraction patterns were indexed, integrated, and scaled with HKL2000 (ref⁵¹). To compensate for radiation damage sensitivity, data from four different crystals of TIRR–53BP1 were scaled and merged. Initial phases for apo TIRR, TIRR–53BP1 and NUDT16TI–53BP1 were obtained by molecular replacement using the atomic coordinates of NUDT16L1 (PDB 3KVH), NUDT16 (PDB 2XSQ) and 53BP1 (PDB ID 2G3R) as search models in PHENIX⁵². The initial models were completed with manual building in COOT⁵³ and refined in PHENIX. Statistics of the final structures are shown in Table 1. Molecular representations were generated with PyMol⁵⁴.

Analytical ultracentrifugation (AUC)

Sedimentation-velocity measurements were performed at 30,000 or 40,000 r.p.m. using a Beckman Coulter Optima AUC instrument and an An-50 Ti rotor. Data were obtained after 15 h of centrifugation at 20 °C by monitoring the absorbance between sample and blank at 230, 280 or 290 nm. Protein samples were in 50 mM sodium phosphate buffer, pH 7.5, with 300 mM NaCl (Fig. 1c) or 100 mM NaCl (Fig. 6b). Data were analyzed using Sedfit⁵⁵.

Isothermal titration calorimetry (ITC)

ITC experiments were carried out at 22 °C using an iTC200 calorimeter (MicroCal, Malvern). Samples were buffer-exchanged extensively with 20 mM Tris-HCl, pH 7.5, 20

mM MgCl₂, 150 mM NaCl. 53BP1 in the calorimeter injection syringe at a concentration of 0.4 mM was delivered as a series of 2 µl injections every 3 min (iTC200) to the reaction cell containing NUDT16 or NUDT16TI, wild type and mutants, at a concentration of 20 µM. To probe, the NUDT16TI–53BP1 interaction in the presence of inosine 5'-monophosphate (IMP, Sigma), IMP was added in both the cell and injection syringe at 16 mM final concentration. For the titration of NUDT16 and NUDT16TI with IMP, IMP was at a concentration of 1.5 mM and NUDT16 or NUDT16TI was at a concentration of 50 µM. The measurements were paired with control titrations for heat of dilution. Data were analyzed using a one-site model with Levenberg-Marquardt nonlinear regression programmed in Origin 7.0 software (OriginLab Corporation).

Cell culture and antibodies

Cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum. Parental cells were confirmed to be free of any mycoplasma contamination. Mouse antibodies employed were against Flag M2 (Sigma F1804), β-Tubulin ((Sigma T8328)), cyclin A (Santa Cruz sc-271682) and γH2AX (Millipore 05-636). Rabbit antibodies were against TopBP1 (Bethyl Laboratories A300-111A), PTIP (Bethyl Laboratories A300-370A), 53BP1 (Cell Signaling 4937S), 53BP1 (Santa Cruz sc-22760), TIRR (Sigma HPA-044186), phospho53BP1 (S25/29) (Cell Signaling 2674S).

Immunofluorescence

Cells were grown on glass coverslips, fixed in 4% formaldehyde in PBS for 15 min at room temperature and blocked and permeabilized for 1 h in PBS containing 0.3% Triton-X100, 1% BSA, 10% fetal bovine (or 3% goat serum). Incubation with primary and secondary antibodies (Alexa Fluor, Molecular Probes) were made in PBS containing 1% BSA and 0.1% Triton-X100 for 1 h at room temperature. Coverslips were mounted using DAPI Fluoromount-G (SouthernBiotech).

FRAP Assay

FRAP analysis was performed on stably expressing GFP-53BP1-BRCT WT and GFP-BRCT F1553R HeLa cell lines. Cells were plated on 35 mm round glass bottom dishes 24 h prior to FRAP. Dishes were placed in an environmentally controlled closed chamber system (Tokai Hit) which was maintained at 37 °C and 5% CO₂ during imaging. The chamber system was mounted on an inverted confocal microscope (FluoView 1000, Olympus). A defined circular area in a single nucleus, termed as the region of interest (ROI) was photobleached using a 488 laser set at 80% power. Time-lapse images were acquired at an interval of 5 sec at 10% laser power for 400 sec. Average fluorescent intensities in the bleached region were normalized against intensities in the unbleached area of the same nucleus. The adjusted fluorescence intensity at each time point is represented as the fraction of the pre-bleach intensity at the ROI. The fluorescence intensity curve was plotted using the mean of 5 ROIs, each in a different nucleus in different fields.

Double immunoaffinity purification

TIRR harboring C-terminal Flag- and HA-epitope tags (TIRR-FH) was stably expressed in cells by retroviral transduction. Viral vector transduction, cell fractionation and purification of the protein complexes were carried out as reported previously⁵⁶. The identification of proteins using mass spectrometry was done at Harvard Medical School Taplin Biological Mass Spectrometry Facility.

Immunoprecipitation

Cells were lysed for 30 min in 20 mM Tris-HCl, pH 7.65, 250 mM NaCl, 0.5% NP-40, 5 mM EDTA, 5% glycerol and protease and phosphatase inhibitor cocktails (Roche). Protein concentrations from cleared supernatant were estimated using the Bradford dye-binding method (Biorad). Five hundred µg of whole-cell extracts were incubated on a roller for 16 h at 4°C with an anti-Flag antibody (Sigma). Resins were washed five times with TGEN150 buffer before elution in 0.1 M glycine, pH 2.9. Eluted proteins were analyzed by immunoblotting.

Cell viability assay

To measure the sensitivity of cells expressing 53BP1 F1553R to PARP inhibition by olaparib, *53BP1*^{-/-} MEFs (kindly provided by Dr. Penny Jeggo, University of Sussex) were transduced with the retroviral pOZ vector, empty or containing the cDNA of 53BP1, with or without the F1553R mutation, and lacking the BRCT domains. Selected clones were transfected twice in 24-h intervals using Lipofectamine (Invitrogen) with a control siRNA (AAGCCGGUAUGCCGGUUAAGU) or an siRNA directed against *BRCA1* (CAGCAGUUUAUUGCUCAUUGA). The cells were seeded into 96-well plates 24 h after the second transfection. The day after plating, olaparib (ChemieTek) was serially diluted in media that was added to the wells. Five days later, olaparib was removed and the cells were incubated in drug-free media for 72 h. The number of viable cells in culture was then determined from the ATP-based CellTiter-Glo luminescent cell viability assay (Promega) using a luminescence microplate reader (CLARIOstar, BMG Labtech). For each olaparib concentration, data were plotted as a percentage of cell survival in drug-free media.

Telomere FISH

RPE1 (53BP1^{-/-}) cells stably expressing 53BP1 (wild type or F1553R mutant) were transduced with pWZL-TRF2 B M retrovirus (Addgene #18013)⁵⁷. Seven days later, cells at 80% confluency were treated with 0.1 µg/mL colcemid for 2 h before trypsinization and neutralization in media. Cell pellets were incubated in 0.075 M fresh KCl for 10 min then fixed in 3:1 methanol:acetic acid solution followed by one methanol:acetic acid wash. Metaphases were dropped on slides on a humidified heat block at 42 °C and air-dried. They were fixed in 4% formaldehyde, treated with 1 mg/mL pepsin for 10 min at 37 °C, fixed again in 4% formaldehyde and then dehydrated with sequential rinses in 70, 90 and 100% ethanol for 5 min each. Telomere PNA-FISH was performed with 20 nM Cy3-TelC probe (PNA Bio Inc.) in hybridization solution (0.5% blocking reagent (Roche), 70% formamide in 10 mM Tris-HCl, pH 7.2) overnight at 4°C following 5 min denaturation at 80 °C. Following washes, slides were dehydrated in sequential ethanol wash and mounted in

ProLong Gold. Images were captured on a Zeiss Imager Z1 and the percentage of chromosome fusions per chromosome was measured.

Mice

All experiments involved 8–10 week old and sex-matched littermate control animals on an inbred C57BL/6 background. *53BP1*^{-/-} mice (MGI:2654201) were generated and described elsewhere⁵⁸. All experiments were approved by the University of Oxford Ethical Review Committee and performed under a UK Home Office Licence.

Primary B cell isolation, culture and reconstitution

B cells were purified from red blood cell-lysed single-cell suspensions of four mouse spleens by magnetic negative selection using a B Cell Isolation Kit (Miltenyi Biotec). B cells (7.5×10^5 cells per well in a 6-well plate) were cultured in RPMI supplemented with 10% FCS, 100 U/mL penicillin, 100 ng/mL streptomycin, 2 mM L-glutamine, 1× MEM nonessential amino acids, 1 mM sodium pyruvate and 50 μM β-mercaptoethanol. B cells were stimulated with 10 ng/mL mouse recombinant IL-4 (Peprotech), and agonist anti-CD40 antibody (0.5 μg/mL; Miltenyi Biotec; FGK45.5). Cultures were grown at 37 °C with 5% CO₂ under ambient oxygen conditions. Filtered retroviral supernatants harvested 48 h following co-transfection of BOSC23 cells with 7 μg pCL-Eco and 7 μg pMX-DEST-GFP-derived plasmids were used to infect IL-4/anti-CD40 antibody-stimulated B cell cultures in the presence of polybrene (2.5 μg/mL) and HEPES (20 mM) by spinoculation ($850 \times g$ for 90 min at 30 °C). After a rest period of 4 to 6 h, viral supernatants were removed, and replaced with IL-4/anti-CD40 antibody-supplemented culture medium. Three days later, transduced B cells were analyzed using a FACSCanto (BD Biosciences); analysis was performed using FlowJo Software v10 (TreeStar). Cells were resuspended in PBS with 2% BSA and 0.025% sodium azide, blocked with Mouse BD Fc Block (1:500, BD Pharmingen 553141), and immunostained with biotinylated anti-mouse IgG1 (1:100, BD Pharmingen 553441; clone A85-1) and Streptavidin APC (1:500, Thermo Fisher 17-4317-82). Live/dead cells were discriminated after staining with Zombie Aqua viability dye (1:200; BioLegend 423102). Surface IgG1 expression was determined in gated cell populations positive for the expression of an eGFP retroviral reporter.

Statistical analysis

The statistical analyses were performed with GraphPad Prism 5.0 using a two-tailed Student's t-test (Mann-Whitney test).

Data availability

Atomic coordinates and structure factors were deposited in the Protein Data Bank with accession codes 6D0L, 6CO1 and 6CO2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. TIRR blocks 53BP1 binding to NCP-ubme by masking the histone-binding surface of 53BP1

a, GST pull-down assays of NCP-ubme by GST-53BP1(Tudor-UDR) in the absence and presence of TIRR. GST and GST-53BP1 T1609E/S1618E (TS/EE) mutant^{49,50} were used as negative controls. IB, immunoblot. H2AK15ub-H2B represents fused histones H2B and H2A ubiquitylated at H2A Lys15.

b, Surface and cartoon representation of a TIRR dimer interacting with two 53BP1-Tudor molecules. The model was generated by symmetry from the X-ray structure of TIRR–53BP1.

c, Sedimentation-velocity AUC analysis of 53BP1 (residues 1204–1603) at 10 μ M without and with addition of 0.1% sodium dodecyl sulfate (SDS).

d, Cartoon and stick representation of the TIRR–53BP1 binding interface highlighting the binding loop in TIRR (black dashed circle). 53BP1 residues for which there is a major change in conformation compared to the H4K20me2–53BP1 structure (panel e) are marked with a red star.

e, Cartoon and stick representation of the H4K20me2–53BP1 binding interface highlighting the aromatic binding cage in 53BP1 (black dashed circle).

f, Overlay of selected 53BP1 side chains in the H4K20me2–53BP1 (gray) and TIRR–53BP1 (yellow) structures highlighting the conformational changes in 53BP1. The side chains of TIRR Arg107 and H4 dimethylated Lys20 are also shown.

Figure 2. TIRR and 53BP1 anchor sites at the TIRR–53BP1 interface

a, Cartoon and stick representation of the TIRR–53BP1 interface centered on TIRR Arg107 in the binding loop.

b, Cartoon and stick representation of the TIRR–53BP1 interface centered on TIRR Lys10.

c–e, Highlights of the intermolecular interactions of TIRR Arg107, Pro105 and Lys10 with 53BP1. In **e**, the H4K20me2 binding cage of 53BP1 is shown for comparison.

Figure 3. Structure-based mutations in TIRR and 53BP1 affect complex formation and regulation of 53BP1 DSB recruitment

a, Amino acid sequence alignment of TIRR 53BP1-binding loop (red) with the corresponding region of NUDT16. The red stars indicate 53BP1-binding TIRR residues.

b. Top: Co-purification of *E. coli*-produced TIRR–53BP1 complexes prepared with indicated 53BP1 mutations. 53BP1 was His₆-tagged and TIRR untagged. Middle: Co-purification of *E. coli*-produced TIRR–53BP1 complexes prepared with indicated TIRR mutations. 53BP1 was His₆-tagged and TIRR untagged. Bottom: U2OS cells stably expressing Flag- and HA-tagged (FH) wild type TIRR, indicated TIRR mutants and NUDT16, were lysed and analyzed by immunoprecipitation and immunoblotting using Flag and 53BP1 antibodies.

c. Quantification of 53BP1 IRIF 90 min after 1 Gy irradiation of RPE1 cells transiently transfected with empty vector, wild type TIRR and indicated TIRR mutants (error bars represent mean $\pm$ s.d., $n = 2$ independent transfections. Representative images are shown. Scale bar = 10 μ m. NS (not significant) indicates a P value > 0.05 as determined by 2-tailed Mann-Whitney test.

Figure 4. A separation-of-function 53BP1 mutation leads to a “hyperactive” form of 53BP1

a, Cartoon and stick representation of the TIRR–53BP1 interface centered on 53BP1 Phe1553. Residues mutated in panel **b** are highlighted with red rectangles.

b, Top: Co-purification of *E. coli*-produced TIRR–53BP1 complexes with wild type (WT) and F1553R 53BP1 mutant. Flag immunoprecipitation using RPE1 cells expressing 53BP1 FFR (WT and mutants). Schematic shows the location of the mutations. Bottom: IRIF of 53BP1 (WT and mutants) were visualized 1 h after irradiation at 5 Gy. Scale bar = 10 μ m.

c, Immunoblot of WT and mutant 53BP1 in nuclear proteins salt-extracted from HeLa cells.

d, Immunoblot of FH-tagged 53BP1 (Flag-53BP1) partner proteins pulled-down from indicated cells. HeLa cells deprived of endogenous TIRR (HeLa TIRR Δ) were stably transduced with FH-53BP1, and in parallel, for the control, HeLa cells were stably transduced with FH-53BP1 and the F1553R 53BP1 mutant. FH-53BP1 partners were purified from total nuclear extracts (nuclear soluble and chromatin extracts) 90 min after a 10 Gy irradiation and analyzed by immunoblotting with indicated antibodies. The star marks a non-specific band. 53BP1 phosphorylated on serine residues 25 and 29 is labeled phos53BP1(S25/29).

e, Top: FRAP assays of cells expressing GFP-tagged 53BP1-BRCT Δ and 53BP1-BRCT F1553R mutant. Relative fluorescence recovery curves were plotted using the mean of five regions of interest, each in a different nucleus from five different cells derived from three

different experiments. (Error bars = s.d.). Bottom: Representative images of the recovery kinetics of GFP-53BP1-BRCT cells. Shown are images before bleaching, immediately after the photobleach event, and later in the time course. The photobleached regions are indicated by white dashed circles.

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Figure 5. Assessing the 'hyperactivity' of 53BP1 F1553R mutant in multiple contexts

a, Immunoblot analysis of selected *53BP1*^{-/-} MEF clones transduced with empty vector (EV), 53BP1-BRCT (WT) or 53BP1-BRCT F1553R.

b, Survival assay of siRNA-transfected *53BP1*^{-/-} MEFs reconstituted with wild type 53BP1-BRCT and treated with olaparib (mean $\pm$ s.d., $n = 2$ independent experiments.)

c, Survival assay of *BRCA1* siRNA-transfected *53BP1*^{-/-} MEFs reconstituted with the indicated constructs and treated with olaparib (mean $\pm$ s.d., $n = 2$ independent experiments.) The heat map represents the statistical analysis ($n = 2$ independent experiments, 2-tailed Mann-Whitney test) of the survival of indicated cells with respect to cells expressing 53BP1-BRCT (WT).

d, Immunoblot analysis of selected 53BP1-null RPE1 clones transduced with empty vector (EV), 53BP1 (WT) or 53BP1 F1553R.

e, Quantification of the number of RAD51 foci in 53BP1- and 53BP1 F1553R-transduced RPE1 cells 6 h after a 2 Gy irradiation (mean $\pm$ s.d., $n = 2$ independent experiments).

f, Kinetics of γ H2AX foci formation in indicated MEF cell lines after a 2 Gy irradiation (mean $\pm$ s.d., $n = 2$ independent experiments).

g, Quantification of chromosome fusions harboring or not a telomeric signal after TRF2 B M expression in the indicated RPE1-derived clones. $n = 10$ metaphases scored per clone over two experiments. NS, not significant. Long and short arrows in representative pictures indicate fusions with or without telomeric signal, respectively. Scale bar = 15 μ m.

h, Class switch recombination (CSR) in stimulated B cells harvested from *53BP1*^{-/-} mice and infected with GST- (control) or indicated 53BP1 mutant-expressing retrovirus. Data indicate IgG1-positive events represented as a percentage of IgG1-positive cells in infected B cells (eGFP reporter-positive). *n* = 2 independent experiments, each performed with 2 mice, error bars represent mean ± s.d. The CSR experiments were done in the context of 53BP1-BRCT .

Figure 6. An engineered RNA- and nucleotide-processing enzyme, NUDT16TI, binds tightly to 53BP1

a, X-ray structure of NUDT16TI–53BP1 complex.

b, Sedimentation-velocity AUC analysis of NUDT16TI (Top), and 53BP1 (residues 1204–1603), free and bound to NUDT16TI (Bottom). All proteins were at a concentration of 5 μ M.

c, ITC of the interactions of NUDT16 and NUTD16TI, wild type and mutants, with 53BP1-Tudor. Corresponding amino acids in TIRR are indicated in blue. The parameter n is the stoichiometry of binding. K_d s are reported with s.d. determined by nonlinear least-squares analysis.

Figure 7. Nucleotides and RNA molecules dissociate the NUDT16TI–53BP1 and TIRR–53BP1 complexes

a, Left: Surface representation of NUDT16TI in the NUDT16TI–53BP1 structure. Two bound IMP molecules are positioned based on the X-ray structure of NUDT16–IMP²⁷. The side chain of 53BP1 Glu1551 pointing toward IMP is shown in stick representation. Right: ITC of the interaction of NUDT16 and NUDT16TI with IMP, and with 53BP1 in the presence of IMP. The parameter n is the stoichiometry of binding. K_d s are reported with s.d. determined by nonlinear least-squares analysis.

b, X-ray structure of TIRR–53BP1 highlighting the electrostatic potential surface of TIRR, from -5 kT/e (red) to 5 kT/e (blue).

c, X-ray structure of TIRR–53BP1 highlighting the RNA-binding region in TIRR (yellow) identified by protein-RNA photocrosslinking and quantitative mass spectrometry¹⁷.

d, Displacement of TIRR from 53BP1 by RNA molecules. Recombinant Flag-tagged 53BP1 (residues 1220–1631) bound to Anti-Flag agarose beads was first incubated with recombinant TIRR and, after washing the beads, with 200 ng (lanes 3 and 5) or 1 μg (lanes 4 and 6) of nuclear RNAs purified from non-treated (lanes 3 and 4) or 5 Gy-irradiated HeLa cells (lanes 5 and 6). Lane 2 is a control incubation with no RNA. Each incubation mixture had a final volume of 200 μL .

Table 1

Data collection and refinement statistics

	TIRR PDB 6D0L	TIRR-53BP1 PDB 6CO1	NUDT16TI-53BP1 PDB 6CO2
Data collection			
Space group	P1	P1	P43 21 2
Cell dimensions			
<i>a</i> , <i>b</i> , <i>c</i> (Å)	41.89, 46.36, 48.80	61.84, 77.03, 77.24	73.69, 73.69, 276.45
α , β , γ (°)	92.30, 108.07, 105.75	85.24, 86.07, 86.09	90.00, 90.00, 90.00
Resolution (Å)	38.00-1.97 (2.04-1.97)*	38.31-2.18 (2.26-2.18)	41.61-2.49 (2.58-2.49)
<i>R</i> _{merge}	0.084 (0.338)	0.133 (0.862)	0.127 (1.110)
<i>I</i> / σ <i>I</i>	15.1 (5.1)	17.9 (4.1)	20.5 (2.2)
Completeness (%)	93.7 (84.1)	99.6 (97.7)	99.9 (99.6)
Redundancy	3.7 (3.7)	14.4 (12.7)	42.9 (30.4)
Refinement			
Resolution (Å)	38-1.97	38.31-2.18	41.61-2.49
No. reflections	22070	73587	27761
<i>R</i> _{work} / <i>R</i> _{free}	0.17/0.21	0.16/0.20	0.22/0.27
No. atoms	3523	9484	4957
Protein	3172	8464	4644
Ligand/ion			
Water	351	1020	313
<i>B</i> -factors			
Protein	21.68	25.65	38.32
Ligand/ion			
Water	27.77	31.46	32.75
R.m.s. deviations			
Bond lengths (Å)	0.006	0.003	0.002
Bond angles (°)	1.05	0.90	0.43

* Values in parentheses are for highest-resolution shell. One crystal was used for determining the TIRR structure, four crystals for the TIRR-53BP1 structure and one crystal for the NUDT16TI-53BP1 structure.