53BP1 promotes ATM activity through direct interactions with the MRN complex

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The Mre11/Rad50/Nbs1 (MRN) complex has a central function in facilitating activation of the ATM protein kinase at sites of DNA double-strand breaks (DSBs). However, several other factors are also required in human cells for efficient signalling through MRN and ATM, including the tumour suppressor proteins p53-binding protein 1 (53BP1) and BRCA1. In this study, we investigate the functions of these mediator proteins in ATM activation and find that the presence of 53BP1 and BRCA1 can amplify the effects of MRN when interactions between MRN and ATM are compromised. This effect is dependent on a direct interaction between MRN and the tandem breast cancer carboxy-terminal (BRCT) repeats in 53BP1, and is accompanied by hyper-phosphorylation of both Nbs1 and 53BP1. We also find that the BRCT domains of 53BP1 affect the overall structure of 53BP1 multimers and that this structure is important for promoting ATM phosphorylation of substrates as well as for the repair of DNA DSBs in mammalian cells.

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Introduction

After DNA double-strand breaks (DSBs) occur in eukaryotic cells, a rapid response initiates that leads to activation of cell cycle checkpoints and DNA repair. The ATM protein kinase has a central function in communicating this response to many downstream targets through phosphorylation (Lavin, 2008). The Mre11/Rad50/Nbs1 (MRN) complex has also been reported to be essential for this initial response, both in facilitating ATM signalling as well as in performing DNA repair (Stracker *et al*, 2004). The MRN complex mediates diverse functions in DNA DSB repair, including homologous recombination, non-homologous end joining (NHEJ), and telomere maintenance (Haber, 1998; D'Amours and Jackson, 2002; Symington, 2002; Deng *et al*, 2009; Dinkelmann *et al*, 2009; Xie *et al*, 2009). Hypomorphic

mutations in the Nbs1 and Mre11 genes are responsible for the human chromosome instability syndromes Nijmegen breakage syndrome (NBS) and ataxia-telangiectasia-like disorder, respectively (Carney *et al*, 1998; Varon *et al*, 1998; Stewart *et al*, 1999). Both syndromes show similar cellular and clinical phenotypes to that of Ataxia-Telangiectasia, which is caused by mutations in ATM gene, including radio-sensitivity, chromosome fragility, radioresistant DNA synthesis, and loss of damage-induced checkpoints (Lavin *et al*, 2005). Our earlier studies showed that the MRN complex is a sensor for DNA DSBs and facilitates activation of ATM by recruiting ATM to broken DNA molecules, facilitating the stable binding of substrates (Lee and Paull, 2004, 2005).

Although the MRN complex has been well characterized in the DNA damage response, many other proteins have been shown to be involved in DSB signalling, including several proteins that contain breast cancer carboxy-terminal (BRCT) domains. BRCT domains were first characterized in the C-terminus of BRCA1 (Koonin *et al*, 1996) and often function as phospho-specific-binding domains, although these domains have also been shown to interact with non-phosphorylated proteins (Derbyshire *et al*, 2002; Joo *et al*, 2002; Manke *et al*, 2003; Yu *et al*, 2003). BRCA1 and p53-binding protein 1 (53BP1) each contain two tandem BRCT repeats in their C-terminus and are considered to be in the ATM-dependent signalling pathway, as they co-localize with ATM and the MRN complex and are phosphorylated by ATM at DNA damage sites (Mochan *et al*, 2004; Gudmundsdottir and Ashworth, 2006).

BRCA1 is phosphorylated on serine 1387 and serine 1423 by ATM, and phosphorylation at these sites is important for the S-phase and the G2/M checkpoints in response to irradiation (IR), respectively (Xu *et al*, 2001, 2002). Moreover, Foray *et al* (2003) showed that cells lacking BRCA1 exhibit reduced levels of p53, c-Jun, Nbs1, Chk2, and CtIP phosphorylation after exposure to ionizing radiation. A study by Kitagawa *et al* (2004) suggested that BRCA1 may have a function in ATM-dependent phosphorylation with several other proteins, as both Nbs1 and BRCA1 are required for the phosphorylation of SMC1 by ATM in response to IR. Fabbro *et al* (2004) also showed an effect of BRCA1 on the phosphorylation of p53 on serine 15 by ATM, but did not observe effects on other substrates including Chk2 and c-Jun. Although it is not yet clear why these observations differ, the cumulative evidence suggests that BRCA1 has a function in mediating the ATM signal transduction pathway in mammalian cells.

53BP1 is also in this group of 'checkpoint mediators' and co-localizes with phosphorylated H2AX (γ -H2AX), Mdc1, the MRN complex, and BRCA1 after treatment with agents that cause DNA DSBs, such as IR and etoposide (Schultz *et al*, 2000; Anderson *et al*, 2001; Rappold *et al*, 2001; Xia *et al*, 2001). 53BP1 has been shown to interact with methylated lysine 20 in histone H4 as well as with methylated lysine 79 of histone H3, and this interaction was inhibited by the suppression of methylation, suggesting that 53BP1 binds directly to

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histones in response to changes in higher-order chromatin structure that expose methylated lysine residues (Huyen *et al*, 2004; Botuyan *et al*, 2006). Foci formation by both 53BP1 and BRCA1 is dependent on Mdc1 and the RNF8 ubiquitin ligase (Panier and Durocher, 2009), although the mechanism by which ubiquitylation affects interactions between the Tudor domain and methylated histones is not understood. 53BP1 has also been reported to have a direct function in DNA DSB repair through stimulation of NHEJ and by mediating long-range interactions between chromosomal ends (Iwabuchi *et al*, 2003; Xie *et al*, 2007; Difilippantonio *et al*, 2008; Dimitrova *et al*, 2008).

Figure 1 Stimulation of ATM phosphorylation of Chk2 and p53 by 53BP1 and BRCA1 with sub-optimal levels of the MRN complex. (A) Kinase assay with 0.2 nM dimeric ATM, 8 nM MRN, 200 nM GST-Chk2 substrate, 2.5 ng linear DNA, 1.8 nM BRCA1, and 30 nM 53BP1, probed with antibody directed against phospho-threonine 68 of Chk2. (B) Kinase assays in (A) except with 2 nM MRN. (C) Kinase assays in (A) except with 50 nM GST-p53 substrate, 10 ng of linear DNA, and varying MRN amount, probed with antibody to phosphoserine 15 of p53. Concentrations of MRN = 0.45, 0.9, 1.8, 3.6, 5.4, and 7.2 nM. (D) The results from (C) were quantitated and shown as a graphical representation, with the y axis showing levels of p53 phosphorylation (arbitrary units) and the x axis showing MRN concentration.

Although most of the recent studies of 53BP1 have focused on its functions in DNA repair, it is clear that 53BP1 also promotes ATM-mediated signalling. siRNA-mediated reduction of 53BP1 levels in human cells reduces the phosphorylation of p53, Chk2, BRCA1, and SMC1 by ATM, particularly after low doses of damage by ionizing radiation (DiTullio *et al*, 2002; Wang *et al*, 2002; Ward *et al*, 2003; Jowsey *et al*, 2004; Munoz *et al*, 2007). Mochan *et al* (2003, 2004) also showed that phosphorylation of various ATM targets was significantly reduced in NBS cells when 53BP1 function was suppressed using siRNA, but that this was not the case in cells expressing normal levels of wt MRN. Collectively, these results suggest that 53BP1 may act as a co-activator or mediator of ATM function and that its effects on ATM may be enhanced in situations in which the MRN complex is impaired or absent. 53BP1 is also phosphorylated by ATM after DNA damage and this phosphorylation is required for ATM-dependent signalling, although recruitment of 53BP1 to DNA damage sites is independent of its phosphorylation (Zgheib *et al*, 2005). When 15 conserved SQ/TQ sites in the N-terminus of 53BP1 were mutated, the repair functions of 53BP1 were abrogated, indicating the importance of these modifications (Ward *et al*, 2006). A recent study also showed that phosphorylation of 53BP1 on serine 25 was required for the binding of the hPTIP protein and for efficient phosphorylation of Chk2 on threonine 68 and BRCA1 on serine 1524 (Munoz *et al*, 2007).

To characterize the functions of 53BP1 and BRCA1 in ATM-dependent phosphorylation events, we tested the effects of recombinant human 53BP1 and BRCA1 in ATM kinase assays using purified components *in vitro*. Our results show that both proteins are required for ATM-dependent phosphorylation of p53 on serine 15 and Chk2 on Threonine 68 in the presence of sub-optimal levels of the MRN complex and that the mediators can also complement an MRN complex that is impaired in ATM association. These effects are dependent on a direct interaction between 53BP1 and the MRN complex through the BRCT domains of 53BP1. These interactions also promote optimal phosphorylation of Nbs1 and 53BP1 and are required for efficient DNA DSB repair.

Results

53BP1 and BRCA1 amplify the effects of MRN on ATM kinase activity

53BP1 and BRCA1 have been shown to affect a subset of ATM phosphorylation events in human cells expressing wild-type MRN (DiTullio *et al*, 2002; Wang *et al*, 2002; Foray *et al*, 2003; Fabbro *et al*, 2004). To determine the effects of 53BP1 and BRCA1 on ATM kinase activity with wild-type MRN, we added 53BP1 and BRCA1 to our standard kinase assay with purified, recombinant ATM as shown in Figure 1A. Under these conditions, the wild-type MRN complex stimulates ATM kinase activity by 80–100-fold in a reaction that requires linear DNA, as we have earlier shown in this system (Lee and Paull, 2005). The addition of 53BP1 and BRCA1 to the MRN complex and ATM stimulated Chk2 phosphorylation by ATM by approximately three-fold.

Mochan *et al* (2003) reported a reduction in ATM phosphorylation events in NBS cells when 53BP1 expression was suppressed, but relatively little effect of 53BP1 reduction in wild-type cells. As NBS cells contain very low levels of MRN

containing an N-terminal-truncated Nbs1 (Maser *et al*, 2001), this suggests that the effects of 53BP1 and BRCA1 on ATM may only be evident under conditions in which the amounts of MRN are limiting. To investigate this hypothesis, we tested the effects of 53BP1 and BRCA1 in ATM kinase assays containing four-fold lower levels of MRN complex compared with the optimized reaction (Figure 1B). With this amount of MRN (2 nM), ATM activation is minimal. Under these conditions, the addition of 53BP1 and BRCA1 stimulated ATM by ~14-fold with Chk2 as a substrate, making the reaction nearly as efficient as standard reactions with higher levels of MRN. Similar to the standard reaction, the 53BP1/BRCA1-stimulated reaction still requires the MRN complex and linear DNA. These results suggest that 53BP1 and BRCA1 complement low levels of MRN in stimulating ATM-dependent phosphorylation.

Our results with low levels of MRN suggest that there must be a direct relationship between the concentration of MRN and the effects of 53BP1 and BRCA1 on ATM. To examine this issue, we tested ATM kinase activity on p53 in the presence of both 53BP1 and BRCA1 with varying concentrations of MRN. We found that ATM kinase activity increased cooperatively with MRN concentration and that 53BP1 and BRCA1 facilitated ATM substrate phosphorylation most significantly with MRN in the 1–4 nM range (Figure 1C; quantification in Figure 1D). 53BP1 and BRCA1 thus amplify the effects of low levels of MRN on ATM substrate phosphorylation.

MRN lacking the C-terminus of Nbs1 can be complemented by the addition of 53BP1 and BRCA1

Earlier studies have shown that the MRN complex interacts with ATM through the C-terminus of Nbs1 and that a short motif in this region is required for the recruitment of ATM to the sites of DNA DSBs (Nakada *et al*, 2003; Falck *et al*, 2005; You *et al*, 2005). To confirm this in our purified system *in vitro*, we expressed and purified MRN complexes containing a mutant of Nbs1 that lacks the 20 C-terminal amino acids MRN(Δ C). We found that the MRN(Δ C) complex failed to stimulate ATM kinase activity on p53, using phosphorylation of serine 15 as a readout for ATM activity (Figure 2A).

Expression of an Nbs1 allele lacking the C-terminal 20 amino acids in cells derived from NBS patients was shown earlier to reduce, but not eliminate ATM-mediated phosphorylation of its substrates (Falck *et al*, 2005). Mice expressing the same human Nbs1(Δ C) allele also show relatively normal levels of ATM-mediated phosphorylation events, although they do show a specific deficiency in apoptosis (Difilippantonio *et al*, 2007; Stracker *et al*, 2007). In considering why we observe a much stronger effect of deleting the Nbs1 C-terminus in our kinase assays *in vitro* compared with human cells and mouse cells, we hypothesized that other cellular factors may be compensating for loss of the C-terminal residues *in vivo*. As 53BP1 and BRCA1 are thought to be ‘mediators’ in ATM-dependent signalling pathways, we tested the effects of purified recombinant 53BP1 and BRCA1 in the ATM kinase assay with the MRN(Δ C) complex. As shown in Figure 2B and C, 53BP1 and BRCA1 stimulated ATM kinase activity up to 70-fold on p53 and Chk2 in an MRN(Δ C)-dependent manner. This suggests that 53BP1 and BRCA1 can compensate for loss of the ATM interaction motif in the MRN complex. The MRN(Δ C) complex was still

Figure 2 Complementation of the MRN complex lacking the C-terminus of Nbs1 (Nbs1 Δ C) by 53BP1 and BRCA1. (A) Kinase assays as in Figure 1C except with 1.35, 2.7, and 5.4 nM wild-type MRN or MRN(Δ C). (B) Kinase assays as in Figure 1C, except with 5.4 nM MRN(Δ C). (C) Kinase assays as in Figure 1A, except with 8 nM MRN(Δ C). (D) Kinase assays as in Figure 2A, except with 5.4 nM wt MRN or MRN(S1202R).

required for this reaction, as 53BP1 and BRCA1 have no effect on ATM activity in the absence of MRN. We also tested an earlier studied MRN complex that contains a catalytic mutation in Rad50, MR(S1202R)N. This mutant MRN complex shows defects in all ATP-dependent activities (Moncalian *et al*, 2004), fails to unwind DNA ends, and does not stimulate ATM kinase activity *in vitro* (Lee and Paull, 2005). When we added 53BP1 and BRCA1 to ATM kinase assays in the presence of the MR(S1202R)N complex, there was no stimulation of p53 phosphorylation by ATM (Figure 2D), suggesting that 53BP1 and BRCA1 cannot generally compensate for all deficiencies in the MRN complex, but that the effect is specific to the MRN(Δ C) mutant. We focus on 53BP1 in the rest of the work, as it seems to have a more dominant effect on ATM activation.

Figure 3 Interaction of 53BP1 with the MRN complex. (A) Wild-type MRN was incubated with wild-type 53BP1 either separately (top) or together (bottom) for 1 h before gel filtration (Superose 6), SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and western blotting with antibodies directed against 53BP1, Rad50, and Nbs1 as indicated. (B) Biotinylated 53BP1 was incubated with MRN or ATM for 1 h before adding streptavidin-coated magnetic beads. Associated MRN and ATM were detected by western blotting. The input lanes have 1/20 of the amounts of ATM, MRN, and 53BP1 used in the reactions. (C) HA-tagged 53BP1 was expressed in human 293 cells by transient transfection, and lysates were incubated with an antibody directed against the HA epitope. Antibody-bound proteins were immunoprecipitated with streptavidin-conjugated magnetic beads, which were incubated with biotinylated anti-rabbit secondary antibody. Associated proteins were detected by western blotting.

53BP1 interacts with the MRN complex and ATM

To determine the mechanistic basis of ATM stimulation by 53BP1, we investigated whether physical associations occur between 53BP1 and the MRN complex using gel filtration. Earlier analysis of the MRN complex by gel filtration indicates that it separates as an extremely large complex of ~1.2 MDa (Lee *et al*, 2003). In comparison, 53BP1 seems to separate as an even larger species of >1.2 MDa, although this could be strongly affected by the shape of this protein if it is elongated. Incubation of 53BP1 with the MRN complex caused a subset of the MRN complex to elute earlier, coincident with 53BP1 (Figure 3A). This result suggests that 53BP1 directly interacts with the MRN complex in the absence of DNA. To confirm this interaction, we performed *in vitro* binding assays with purified recombinant 53BP1, the MRN complex, and ATM. Biotinylated full-length 53BP1 was incubated with the MRN complex or ATM and then isolated with streptavidin-coated magnetic beads. We found that 53BP1 associated with the MRN complex and also independently with ATM (Figure 3B). To further confirm this interaction in cells, we transiently

over-expressed HA-tagged 53BP1 in human 293 cells, followed by immunoprecipitation with an anti-HA antibody. The MRN complex was immunoprecipitated with 53BP1 in the presence and absence of DNA damage, showing that this is a constitutive interaction (Figure 3C). We also confirmed the presence of ATM in 53BP1-immunoprecipitated material (Supplementary Figure S4), consistent with the presence of 53BP1 in large complexes with ATM and MRN in cells.

BRCT domains of 53BP1 interact with the MRN complex through Rad50

53BP1 is a large protein with several distinct domains (Figure 4A). The Tudor domain is known to be required for interactions with methylated histone H4 lysine 20 (Botuyan *et al*, 2006) and was also earlier reported to bind to methylated histone H3 lysine 79 (Huyen *et al*, 2004). Ward *et al* (2006) identified a dimerization domain in 53BP1 and showed that it is required for oligomerization of 53BP1 and the efficient repair of DNA DSBs. The BRCT domains of 53BP1 were earlier shown to bind to p53 (Iwabuchi *et al*, 1994), but it is not known whether they have other binding partners. To determine whether these domains are required for the interaction with the MRN complex, we performed *in vitro* binding assays with deletion mutants of 53BP1. Wild-type full-length 53BP1 and 53BP1 lacking the dimerization domain (Δ Dimer) associated with the MRN complex (Figure 4B). However, when the BRCT domains were deleted, this interaction was dramatically reduced, suggesting that the BRCT motif is required for the interaction. To confirm this, we purified the BRCT domains separately as a GST-fusion protein (GST-BRCT) and performed binding assays with the MRN complex (Figure 4C). The GST-BRCT fragment strongly associated with the MRN complex, but GST expressed alone did not. We then investigated which component of the MRN complex interacts with 53BP1 using Mre11 and Mre11/Rad50 (MR) complexes in binding assays with the GST-BRCT fragment. The BRCT domains bound to the immobilized MR complex, but not to Mre11 or Nbs1 alone (Figure 4D and data not shown), suggesting that the interaction between 53BP1 and the MRN complex occurs through the Rad50 component.

Tandem BRCT domains promote 53BP1 self-interaction

Earlier studies showed that 53BP1 is oligomerized through its dimerization domain, amino-acids 1231 to 1270 (Adams *et al*, 2005; Ward *et al*, 2006). However, Usui *et al* (2009) suggested that Rad9, the orthologue of 53BP1 in budding yeast, is oligomerized through an interaction between the C-terminal tandem BRCT domains and the N-terminal SQ/TQ cluster domain (SCD). To investigate the function of dimerization domain and the BRCT domains in oligomerization of 53BP1, we analysed the elution profiles of 53BP1 deletion mutants using gel filtration. As shown above, full-length 53BP1 separates as an extremely large complex by gel filtration. As expected, the peak of the 53BP1(Δ Dimer) mutant was shifted by several fractions later in the elution, indicating a significantly smaller molecular mass (Figure 5A). The 53BP1(Δ BRCT) mutant exhibited an intermediate profile, with ~50% of the protein separating similar to the wild-type protein and the rest distributed similar to the 53BP1(Δ Dimer) protein. 53BP1 lacking both the BRCT domains and the dimerization region exhibited only the

Figure 4 The BRCT domains of 53BP1 are required for interaction with Rad50. (A) A schematic diagram of the 53BP1 protein. 53BP1 is a 220-kDa protein, consisting of 1972 amino acids. 15 ATM/ATR-dependent phosphorylation sites are located in the N-terminus of 53BP1, '15SQ/TQ' (Ward *et al*, 2006), and the identified domains within 53BP1 consist of the dimerization domain (Ward *et al*, 2006), a glycine/arginine-rich (GAR) motif (Boisvert *et al*, 2005), the Tudor domain (Iwabuchi *et al*, 2003), a nuclear localization signal (NLS) (Adams *et al*, 2005), and the Tandem BRCT domains (Bork *et al*, 1997). (B) Binding assays with 53BP1 and MRN were performed as in Figure 3B except with various 53BP1 mutants as indicated. (C) Binding assays with MRN and the BRCT domains of 53BP1. Biotinylated MRN was incubated with a GST-BRCT fragment or GST alone for 1 h before adding streptavidin-coated magnetic beads. After incubation and washing of the beads, the GST-BRCT fragment was detected by western blotting using an antibody directed against GST. (D) Binding assays between GST-BRCT and Mre11/Rad50 (MR) or the Mre11 protein alone. GST-BRCT was incubated with MR or the Mre11 protein ('+' = 10 nM; '++' = 20 nM) for 1 h and immunoprecipitated with streptavidin-coated magnetic beads, on which anti-Flag antibody was immobilized. Associated proteins on the beads were detected by western blotting as indicated.

smaller peak (Figure 5A). This suggests that the BRCT domains of 53BP1 contribute to its tertiary or quaternary structure, although the dimerization domain is clearly having a major function also. In contrast, mutations of 15 SQ/TQ sites in the N-terminus of 53BP1 had no effect on its size as measured by gel filtration.

Self-association of 53BP1 dependent on the BRCT domain suggests that either the BRCT domains homodimerize, as was

suggested for yeast Rad9 (Soulier and Lowndes, 1999), or that the BRCT domains associate with a different region of 53BP1, as was also shown for Rad9 (Usui *et al*, 2009). We tagged the tandem BRCT domain fragment with two different epitope tags and performed binding assays, but found that the BRCT fragment does not directly interact with another BRCT fragment (data not shown). We then examined whether GST-tagged BRCT domains interact with full-length 53BP1

Figure 5 Requirement of BRCT domains of 53BP1 for its self-interaction. **(A)** Elution profiles of various 53BP1 truncation mutants by gel filtration. Fractions from the Superose 6 separations were analysed by SDS-PAGE and western blotting using an antibody directed against 53BP1. **(B)** Binding assays as in Figure 4C, except with biotinylated full-length 53BP1 and a biotinylated fragment of the C-terminus of 53BP1 (a.a. 868 to 1972) and GST-BRCT from 53BP1, measuring binding of the BRCT fragment using an antibody directed against GST. **(C)** Binding assays as in **(B)** except with a GST-BRCT fragment containing the S1853A mutation in the linker between the BRCT domains. **(D)** Kinase assays as in Figure 1C, except with 1.1 nM wt MRN and various mutant 53BP1 complexes as indicated.

using a binding assay (Figure 5B). Immobilized 53BP1 interacts with the GST-BRCT fragment, but not with the GST tag alone, suggesting that the BRCT domains mediate interactions of 53BP1 with itself. The N-terminus of 53BP1 is dispensable for this interaction, as the GST-BRCT protein bound equivalently to a C-terminal fragment containing a.a. 868 to 1972, as it did to full-length 53BP1 (Figure 5B). We also found that this interaction was not mediated by the dimerization domain, as the 53BP1(ΔDimer) mutant also interacts with the GST-BRCT fragment similar to full-length 53BP1 (data not shown).

Usui *et al* (2009) showed that disrupting the BRCT linker between the two BRCT repeats in Rad9 by mutating Ser-1129 to Ala disrupts interaction of the BRCT domains with a phosphorylated Rad9 SCD. To check whether 53BP1 also shows a similar effect, we purified a GST-tagged BRCT fragment containing the analogous mutation (S1853A) and

performed binding assays with full-length 53BP1. However, BRCT(S1853A) also interacts with 53BP1 similar to wt BRCT (Figure 5C). Consistent with this result, we did not observe any significant deficiencies in BRCT-MRN or BRCT-53BP1 interactions after treatment of the MRN complex or 53BP1 with a phosphatase (Supplementary Figure S2). We also made several other mutations in the BRCT domains of 53BP1 based on the crystal structure of 53BP1 bound to p53 and comparisons with the BRCT domains of BRCA1 bound to phosphopeptides (Joo *et al*, 2002; Shiozaki *et al*, 2004), and found that none of these mutations affect binding of the BRCT domains to the MRN complex or to full-length 53BP1 (Supplementary Figure S3). These results suggest that the tandem BRCT domains of 53BP1 not only interact with the MRN complex, but also have the capacity for self-interaction and that both of these interactions are independent of phosphorylation.

To investigate the functional consequences of BRCT domain and dimerization domain deletions in ATM-dependent signalling, we compared the mutants with full-length 53BP1 in ATM kinase assays (Figure 5D). Full-length 53BP1 and 53BP1(15AQ) stimulate ATM kinase activity towards p53 in the presence of low levels of MRN, BRCA1, and DNA (Figure 5D). However, the 53BP1(ΔDimer) and 53BP1(ΔBRCT) mutants show significantly reduced ability to stimulate ATM. As both the 53BP1(ΔBRCT) mutant and the 53BP1(ΔDimer) mutant show severe defects in the stimulation of ATM, these results together suggest that 53BP1 oligomerization and interactions with the MRN complex are both required for optimal activation of ATM.

53BP1-MRN interactions promote hyper-phosphorylation of Nbs1 and 53BP1

Nbs1 is phosphorylated by ATM after DNA damage and this phosphorylation is required for efficient ATM-dependent signalling and S-phase checkpoint control (Lim *et al*, 2000; Buscemi *et al*, 2001; Nakanishi *et al*, 2002; Yazdi *et al*, 2002). 53BP1 is heavily phosphorylated by ATM after DNA damage (Rappold *et al*, 2001; Morales *et al*, 2003; Matsuoka *et al*, 2007) and phosphorylation of 53BP1 promotes efficient repair of DNA DSBs (Ward *et al*, 2006). Considering the interactions between 53BP1, the MRN complex, and ATM that we have identified, we also tested the effects of 53BP1 on ATM-mediated phosphorylation of the MRN complex itself and of 53BP1. We found that Nbs1 is hyper-phosphorylated in the presence of wt 53BP1 (Figure 6A). We do observe Nbs1 phosphorylation *in vitro* with ATM and MRN, but 53BP1 dramatically increases the efficiency of this phosphorylation, similar to the hyper-phosphorylation that occurs in human cells after DNA damage (Gatei *et al*, 2000; Lim *et al*, 2000). The 53BP1 mutant lacking the BRCT domain is less effective at inducing this hyper-phosphorylation (Figure 6B). Full-length 53BP1 itself is also strongly phosphorylated in the presence of the MRN complex and DNA, but the 53BP1(ΔBRCT) is impaired in this phosphorylation. These results suggest that intact BRCT domain is required for efficient Nbs1 and 53BP1 phosphorylation.

A recent study also showed that 53BP1 is phosphorylated on Ser-25, which promotes phospho-specific interactions with the PTIP protein (Munoz *et al*, 2007). Disruption of this interaction caused defects in ATM-dependent signalling and hyper-sensitivity to DNA damage in human cells (Jowsey

Figure 6 The BRCT domains of 53BP1 promote hyper-phosphorylation of 53BP1 and Nbs1. (A) Kinase assays with 8 nM MRN, 100 nM 53BP1, 10 ng of linear DNA, and 0.2 nM dimeric ATM, probed with antibody directed against phospho-SQ/TQ. (B) Kinase assays as in (A), except with 53BP1(ΔBRCT). (C) Kinase assays as in (B), except with antibody directed against phosphoserine 25 of 53BP1.

et al, 2004; Munoz *et al*, 2007). On the basis of this, we examined whether phosphorylation on Ser-25 of 53BP1 is also stimulated by the MRN complex. As expected, we observed 53BP1 phosphorylation on Ser-25 by ATM in the presence of MRN and DNA, but the 53BP1(ΔBRCT) mutant was much less efficiently phosphorylated (Figure 6C). All of the ATM phosphorylation of 53BP1 we have observed is in the N-terminus, as the signal is completely eliminated by the 15AQ mutations (Ward *et al*, 2006) (data not shown).

53BP1(ΔBRCT) defects in DNA repair

Here we show that the tandem BRCT domains of 53BP1 are important for MRN association, for the promotion of ATM phosphorylation events, and for the overall structure of 53BP1. It is possible that these domains may affect the repair of DNA DSBs, either indirectly through ATM signalling, or more directly through MRN association or unknown mechanisms. Therefore, we further investigated the function of the BRCT domains on 53BP1 in cells. 53BP1 or ATM deletion results in failure to effect the slow component of DSB repair (Riballo *et al*, 2004). Thus, a defect in the repair of ~15% of induced lesions, evident at later time points after IR, is observed. To observe this subtle DSB repair defect, 53BP1-deficient mouse embryonic fibroblasts (MEFs) were transfected with human full-length 53BP1 cDNA or 53BP1 containing a deletion of the BRCT domains (ΔBRCT). As a measure of DNA repair, we analysed average numbers of γ-H2AX foci per cell after ionizing radiation exposure, a method earlier shown to be both accurate and highly sensitive in non-dividing cells (Riballo *et al*, 2004; Goodarzi *et al*, 2009). To avoid potential effects of over-expression, only cells with 53BP1 levels quantitatively comparable with the wild-type control were scored. Consistent with earlier findings, we observed a DSB repair defect in 53BP1 knockout MEFs, and as expected, expression of human full-length 53BP1 compensates for the loss of

Figure 7 53BP1(ΔBRCT) defects in DNA repair. Wild type (MDC + / +) or 53BP1 knockout MEFs were transfected with full-length human 53BP1 (h53BP1^{FL}) tagged with HA or human 53BP1 missing the two C-terminal BRCT domains (h53BP1^{ΔBRCT}). A total of 16 h post-transfection, cells were irradiated with 3 Gy IR and harvested 0.5, 6, 16, or 24 h later. Cells were fixed and stained with anti-γ-H2AX (green) and anti-HA (red) and DAPI (blue). H2AX foci numbers were quantified for each condition by blind counting and scoring only HA-positive cells for samples transfected with 53BP1 constructs. All results represent the mean and SD of three experiments. To compare the expression of the two transfected constructs with each other and to endogenous 53BP1 levels, either anti-HA signal or anti-53BP1 signal was quantified by ImageJ software analysis (see graph insert). (B) Cells from (A) were irradiated with 4 Gy and, 8 h post-IR, were treated with 50 ng/μl Calyculin-A for 0.5 h to induce PCC in G₂-phase cells. Cells were then harvested, fixed, and processed for chromosome break analysis. Chromosome breaks were scored per 100 chromosomes counted. All results represent the mean and s.d. of three experiments.

53BP1 (Figure 7A). Significantly, under conditions in which mutant expression levels were matched to normal, the 53BP1(ΔBRCT) mutant was unable to complement the DSB repair defect conferred by 53BP1 loss. These results provide strong evidence that the BRCT domains of 53BP1 are indeed important for ATM-dependent DNA DSB repair. We also

confirmed this repair deficiency using a premature chromosome condensation (PCC) assay that directly measures DNA DSBs (Deckbar *et al*, 2007) and found that the 53BP1 (Δ BRCT) allele exhibits a marked increase in DNA breaks similar to the vector control (Figure 7B).

Discussion

We show in this study that the 'mediator' proteins 53BP1 and BRCA1 can directly promote ATM kinase activity on multiple substrates. The magnitude of this effect is proportional to the level of MRN complex in the reaction, consistent with the idea that 53BP1 facilitates productive interactions between MRN and ATM and that these interactions are limiting when MRN concentrations fall below a critical threshold. This seems to be the case in cells from NBS patients, in which the 657del5 mutation in the NBS1 gene generates a C-terminal fragment of Nbs1 and also significantly lowers the overall levels of the MRN complex (Maser *et al*, 2001). An earlier study showed that siRNA knockdown of 53BP1 in NBS cells resulted in a marked reduction in Chk2 and Smc1 phosphorylation after ionizing radiation (Mochan *et al*, 2003), in agreement with this interpretation.

The rescue of ATM phosphorylation events can also be seen with normal levels of MRN containing a C-terminal truncation of Nbs1 (MRN Δ C). The 20 amino-acid fragment deleted in this mutant was identified as a critical ATM-binding region of Nbs1 and was also shown to be important for ATM phosphorylation of substrates and autophosphorylation in human cells as well as in *Xenopus* extracts (Falck *et al*, 2005; You *et al*, 2005). The MRN Δ C mutant is unable to promote ATM phosphorylation *in vitro* with purified components, consistent with its proposed function in ATM binding. The simplest model to explain these observations is that 53BP1 (and also possibly BRCA1) binds to both MRN and ATM and compensates for either low levels of MRN or for deficiencies in ATM association that occur with the deletion of the C-terminus of Nbs1. We propose that, in this way, cells can maintain very efficient ATM signalling that is not drastically affected by small changes in the levels of MRN. Our findings with 53BP1 and Brca1 suggest that these and perhaps other mediators may act to bridge the MRN Δ C complexes with ATM *in vivo*, which may explain the relatively mild phenotype of the Nbs1 Δ C mutants in tissue culture and in mouse models (Difilippantonio *et al*, 2007; Stracker *et al*, 2007).

We focus on 53BP1 in this study and find that the tandem BRCT domains at the C-terminus mediate a direct interaction with the MRN complex through Rad50. The BRCT motif of 53BP1 was initially shown to interact with p53 in a two-hybrid assay (Iwabuchi *et al*, 1994) and the structure of the motif was subsequently solved in complex with the DNA-binding domain of p53 (Derbyshire *et al*, 2002). It could be that this domain has multiple binding partners as we find that the same motif is capable of interacting with 53BP1 as well (see below). However, the BRCT domains are not just sticky motifs that bind non-specifically to every protein, as we do not observe interactions with Mre11 or GST and it does not associate with itself. The BRCT domains are essential for stimulating ATM kinase activity under low MRN conditions, which we postulate is due to the MRN-BRCT interaction.

In an earlier study, 53BP1 knockout MEFs were complemented with human 53BP1 or various deletion mutants and analysed for cell cycle checkpoints and DNA repair after exposure to ionizing radiation (Ward *et al*, 2006). A deletion mutant lacking the BRCT domains was tested and found to be similar in both respects to the wild-type allele. In our study, we also investigated the effects of BRCT deletion and observed a significant DSB repair defect, based on the levels of PCC breaks at 8 h post-IR and the levels of γ -H2AX foci remaining after 16 and 24 h post-IR. This subset of unrepaired breaks is similar in magnitude and timing to that remaining in cells lacking ATM, Mre11, Nbs1, H2AX, or 53BP1 (Riballo *et al*, 2004). This ATM-dependent DSB repair pathway was subsequently shown to resolve DSBs located within heterochromatin, requiring the ATM-dependent phosphorylation of the KAP1 nuclear co-repressor (Goodarzi *et al*, 2008). The basis underlying the difference between our results and those of Ward *et al* is unclear, but it is possible that the assay is sensitive to levels of the MRN complex, the heterochromatic content, or the level of 53BP1 and that over-expression of 53BP1 mutant alleles could mask the subtle DSB repair defect.

It is noteworthy that there is an absolute requirement of 53BP1 in ATM-dependent DSB repair, which is distinct to its stimulatory function in ATM activation. This raises the possibility that these BRCT domains of 53BP1 could mediate an effect on DNA repair directly through their interaction with the MRN complex, a primary responder at sites of DSBs, in a manner that is greater than the stimulation of ATM kinase activity that we described here. Further, 53BP1 was recently shown to have unusual effects on the dynamics of chromatin at sites of uncapped telomeres and to be required for distal end-joining events during V(D)J recombination (Difilippantonio *et al*, 2008; Dimitrova *et al*, 2008), in addition to its earlier characterized function in class switch recombination (Manis *et al*, 2004; Ward *et al*, 2004). Deficiency in ATM or Nbs1 also results in the appearance of aberrant V(D)J-joining intermediates and a deficiency in class switching during lymphocyte development (Bredemeyer *et al*, 2006; Dinkelmann *et al*, 2009; Helmink *et al*, 2009).

In addition to increasing phosphorylation of substrates in the ATM kinase assay, we show here that the BRCT domains of 53BP1 induce a dramatic increase in the phosphorylation of both Nbs1 and 53BP1 itself. Nbs1 phosphorylation has been shown to be important for S-phase cell cycle control (Gatei *et al*, 2000; Lim *et al*, 2000; Wu *et al*, 2000; Zhao *et al*, 2000), and 53BP1 phosphorylation in the N-terminus is essential for the effects of 53BP1 on DNA repair (Ward *et al*, 2006). Phosphorylation of serine 25 of 53BP1 in particular was recently shown to regulate the binding of the hTIP protein to 53BP1 that promotes ATM signalling and DNA repair (Munoz *et al*, 2007). However, it should be noted that mutation of 15 SQ/TQ phosphorylation sites in the N-terminus of 53BP1 had a much stronger effect on levels of γ -H2AX foci after IR treatment compared with a BRCT deletion (Ward *et al*, 2006). Thus, an effect of the BRCT domains on phosphorylation could only be partial in comparison with the complete removal of these sites.

The dimerization region between amino-acids 1231 and 1270 was earlier shown to be essential for coimmunoprecipitation of 53BP1 with itself (Adams *et al*, 2005; Ward *et al*, 2006), and our results are certainly consistent with this

region having a major function in mediating inter-molecular interactions between molecules of 53BP1. However, during the course of our investigation of 53BP1 and MRN, we observed that removal of the tandem BRCT motif at the C-terminus of 53BP1 also partially disrupts its structure. This is visible in gel filtration by the appearance of an additional smaller peak of protein (Figure 5A). On the basis of these results, we hypothesized that the BRCT motif may mediate an intra-molecular interaction within 53BP1, which we confirmed in binding assays that show association between the BRCT domains and the C-terminus of 53BP1.

This model of 53BP1 associations is distinct from that proposed for the Rad9 protein in *Saccharomyces cerevisiae*, which is an orthologue of 53BP1. The BRCT domains in Rad9 were shown to bind to a phosphorylated region in the N-terminus after DNA damage and in this way mediate oligomerization of Rad9 that is necessary for Rad53 activation (Usui *et al*, 2009). In contrast, the accumulation of 53BP1 that is manifested as cytologically visible foci depends on the Tudor domain, but not on the BRCT domains (Iwabuchi *et al*, 2003; Morales *et al*, 2003), and the association that we show here between the BRCT motif and the C-terminus of 53BP1 is not phosphorylation dependent.

As deletion of the BRCT domains from 53BP1 dramatically alters its structure, we conclude that the domains are constitutively bound to their binding site within 53BP1. In this bound form (whether it is intra- or inter-molecular), the BRCT domains are able to interact with MRN and stimulate ATM activity on substrates. In contrast, the BRCT domains expressed alone as a GST-BRCT fusion bind to MRN, but do not stimulate ATM activity on substrates other than Nbs1 (Supplementary Figure S5). This is likely because the BRCT fragment alone is missing the binding interface for ATM, and/or the BRCT domains must be in their 53BP1-bound form to stimulate ATM through the MRN complex.

53BP1 is a multifunctional protein with several important domains. It is not clear how binding of 53BP1 in the vicinity of DSBs through the Tudor domain promotes long-range NHEJ events or why the dimerization domain is required for 53BP1 to be active in cells. However, in this study, we have identified a function for the BRCT domains in promoting ATM-dependent phosphorylation events through the MRN complex, a function that has been widely reported in mouse and human cells, but whose mechanism has been elusive. Our results also highlight the redundancy that has evolved in the DNA damage response, in that the function of the BRCT domains may not be fully apparent in a normal cell expressing high levels of the MRN complex. Although the MRN complex is an essential component of ATM activation by DSBs, it is clear that other factors such as 53BP1 and BRCA1 have important regulatory and compensatory functions in this process *in vivo*.

Materials and methods

Expression constructs

See Supplementary data

Protein purification

Wild type and mutant MRN and subcomplexes were purified as described (Bhaskara *et al*, 2007). Dimeric ATM was made by transient transfection of expression constructs into 293T cells using

calcium phosphate and purified as described earlier (Lee and Paull, 2006).

53BP1 and BRCA1 were expressed separately in Sf21 cells as earlier described for BRCA1 with some modifications (Paull and Gellert, 2000). To prepare the lysate, the cells were thawed and resuspended in 40 ml of lysis buffer: 100 mM NaCl, 25 mM Tris-HCl pH 8.0, 0.5% Tween 20, 10% glycerol, 1 mM dithiothreitol (DTT), and 2 mM phenylmethylsulfonylfluoride (PMSF). The mixture was resuspended briefly with a Dounce homogenizer, and then sonicated (three times 20 s). After a 1-h centrifugation at 100 000g, the supernatant was loaded onto a 1-ml column of anti-Flag M2 agarose (Sigma). The column was run in buffer A (lysis buffer without the PMSF) and washed with 5 ml buffer A containing 0.5 M lithium chloride. The column was washed into buffer A again and Flag-tagged 53BP1 or BRCA1 were eluted with 5 ml buffer A containing 0.1 mg/ml Flag peptide (Sigma). Fractions containing 53BP1 or BRCA1 were dialysed against buffer A before storage in small aliquots at -80°C .

To purify biotinylated proteins, cells were co-infected with baculovirus expressing BirA. Purification procedures for biotinylated proteins were the same as for the non-biotinylated proteins.

The GST-fusion proteins were purified identically to the GST-Brcal fragments as described earlier (Paull *et al*, 2001). The GST-p53, GST-Chk2, GST-BRCT(53BP1), and GST-BRCT(53BP1, S1853A) proteins were further purified by separation on a Superdex 200 gel filtration column (GE) in buffer A. Protein concentrations were determined by Bradford assay (Pierce) and by quantitation of protein preparations with standards on colloidal Coomassie-stained SDS-PAGE gels using the Odyssey system (LiCor).

Kinase assay

All ATM kinase assays were performed in kinase buffer: 50 mM HEPES, pH 7.5, 50 mM potassium chloride, 5 mM magnesium chloride, 10% glycerol, 1 mM ATP, and 1 mM DTT for 90 min at 30°C in a volume of 40 μl as described earlier (Lee and Paull, 2006). A total of 10 ng DNA was used in kinase assays with GST-p53; 2.5 ng were used with GST-Chk2. MRN concentrations were varied in some experiments as described in the Figure legends. Phosphorylated Chk2(thr68) and p53(ser15) were detected as described earlier (Lee and Paull, 2006) using phospho-specific antibodies from Cell signaling (2661S) and Calbiochem (PC461), and phospho-specific SQ/TQ and phospho-Ser25 (53BP1) antibodies were obtained from Cell signaling (2851S) and R&D Systems (AF3405), respectively.

In vitro binding assays

Biotinylated proteins (100 nM 53BP1, including wild type and mutants, or 20 nM MRN) were incubated with putative interacting partners (20 nM MRN, 5 nM ATM, or 20 nM GST-BRCT, including wild type and S1853A) in buffer A for 1 h at 30°C in a final volume of 100 μl and then incubated with streptavidin-coated magnetic beads (Dyna) and 0.2% CHAPS (Sigma), whereas rotating at 4°C for 15 min. Beads with associated proteins were washed three times with buffer A containing 0.2% CHAPS and bound proteins were eluted by boiling the beads in SDS loading buffer, and analysed by SDS-PAGE and western blotting using antibodies directed against the Flag epitope (Sigma, F3165), 53BP1 (Cell Signaling, 4937), Rad50 (Genetex, GTX70228), Nbs1 (Genetex, MSNBS10PX1), and the GST epitope (Santa Cruz, SC-459).

Immunoprecipitation from cell lysates

HA-tagged 53BP1 was expressed by transient transfection of human 293 cells (ATCC, CRL-1573) in one dish ($245 \times 245 \times 20$) using calcium phosphate as described earlier (Lee and Paull, 2006). Cells were harvested and resuspended in 1 ml of lysis buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP40, 5 mM EDTA, 10% glycerol, and 2 mM PMSF. The mixture was resuspended briefly with a Dounce homogenizer, and then sonicated (three times 20 s). Polyclonal anti-HA antibodies directed against 53BP1 (Bethyl, A190-108A) were added and incubated at 4°C overnight. Although incubating lysates with the antibody, biotin-conjugated rabbit IgG secondary antibody (Abcam, Ab6021) was also incubated with streptavidin-coated magnetic beads (Dyna) at 4°C overnight and washed with buffer A three times. IgG-immobilized beads were then added to lysates and incubated for 4 h at 4°C . Beads with associated proteins were washed three times with buffer A containing 0.2% CHAPS, and bound proteins were eluted by boiling the beads in SDS loading buffer, and analysed by SDS-PAGE and western blotting.

Gel filtration

A total of 200 nM wt 53BP1 or mutant 53BP1 were incubated with 30 nM MRN or buffer A at 30°C for 1 h in final volume of 60 µl and then the samples were directly loaded on a Superose 6 PC 3.2/30 column (GE) equilibrated in buffer A. Samples from the 50-µl fractions were run on 6% SDS-PAGE gels, transferred to PVDF membranes (Immobilon-FL, Millipore), and probed with antibodies directed against 53BP1 (Cell Signaling, 4937), Rad50 (Genetex, GTX70228), and Nbs1 (Genetex, MSNBS10PX1), followed by detection with IRDye 800 anti-mouse (Rockland, 610-132) or Alexa Fluor 680 anti-rabbit (Invitrogen, A21076) secondary antibodies. Western blots were analysed and quantitated using a Licor Odyssey system.

DNA repair analysis

Wild-type and 53BP1^{−/−} MEFs were grown as described (Riballo *et al*, 2004). 53BP1 constructs were pCMH6K-53BP1-FL (full length, 1–1972) or pCMH6K-53BP1-deltaBRCT (1–1710), originally cloned and reported by Iwabuchi *et al* (1994). Expression was achieved using 1 µg of plasmid per 2×10^5 of logarithmically growing cells by Metafectene-Pro (Biontex, Germany)-mediated transfection (according to the manufacturer's instructions). Cells were then plated onto glass slides in 2 ml media; 24 h later, cells were

irradiated, harvested, and processed for immunofluorescence as described earlier (Goodarzi *et al*, 2008), using anti-H2AX (ab18311) and anti-HA (ab9110) from Abcam, UK. PCC assays were performed as described earlier (Deckbar *et al*, 2007).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

References

- Adams MM, Wang B, Xia Z, Morales JC, Lu X, Donehower LA, Bochar DA, Elledge SJ, Carpenter PB (2005) 53BP1 oligomerization is independent of its methylation by PRMT1. *Cell Cycle* **4**: 1854–1861
- Anderson L, Henderson C, Adachi Y (2001) Phosphorylation and rapid relocalization of 53BP1 to nuclear foci upon DNA damage. *Mol Cell Biol* **21**: 1719–1729
- Bhaskara V, Dupre A, Lengsfeld B, Hopkins BB, Chan A, Lee JH, Zhang X, Gautier J, Zakian VA, Paull TT (2007) Rad50 adenylate kinase activity regulates DNA tethering by Mre11/Rad50 complexes. *Mol Cell* **25**: 647–661
- Boisvert FM, Rhie A, Richard S, Doherty AJ (2005) The GAR motif of 53BP1 is arginine methylated by PRMT1 and is necessary for 53BP1 DNA binding activity. *Cell Cycle* **4**: 1834–1841
- Bork P, Hofmann K, Bucher P, Neuwald AF, Altschul SF, Koonin EV (1997) A superfamily of conserved domains in DNA damage-responsive cell cycle checkpoint proteins. *FASEB J* **11**: 68–76
- Botuyan MV, Lee J, Ward IM, Kim JE, Thompson JR, Chen J, Mer G (2006) Structural basis for the methylation state-specific recognition of histone H4-K20 by 53BP1 and Crb2 in DNA repair. *Cell* **127**: 1361–1373
- Bredemeyer AL, Sharma GG, Huang CY, Helmink BA, Walker LM, Khor KC, Nuskey B, Sullivan KE, Pandita TK, Bassing CH, Sleekman BP (2006) ATM stabilizes DNA double-strand-break complexes during V(D)J recombination. *Nature* **442**: 466–470
- Buscemi G, Savio C, Zannini L, Micciche F, Masnada D, Nakanishi M, Tauchi H, Komatsu K, Mizutani S, Khanna K, Chen P, Concannon P, Chessa L, Delia D (2001) Chk2 activation dependence on Nbs1 after DNA damage. *Mol Cell Biol* **21**: 5214–5222
- Carney JP, Maser RS, Olivares H, Davis EM, Le Beau M, Yates III JR, Hays L, Morgan WF, Petrini JH (1998) The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response. *Cell* **93**: 477–486
- D'Amours D, Jackson SP (2002) The Mre11 complex: at the crossroads of dna repair and checkpoint signalling. *Nat Rev Mol Cell Biol* **3**: 317–327
- Deckbar D, Birraux J, Krempler A, Tchouandong L, Beucher A, Walker S, Stiff T, Jeggo P, Lobrich M (2007) Chromosome breakage after G2 checkpoint release. *J Cell Biol* **176**: 749–755
- Deng Y, Guo X, Ferguson DO, Chang S (2009) Multiple roles for MRE11 at uncapped telomeres. *Nature* **460**: 914–918
- Derbyshire DJ, Basu BP, Serpell LC, Joo WS, Date T, Iwabuchi K, Doherty AJ (2002) Crystal structure of human 53BP1 BRCT domains bound to p53 tumour suppressor. *EMBO J* **21**: 3863–3872
- Difilippantonio S, Celeste A, Kruhlak MJ, Lee Y, Difilippantonio MJ, Feigenbaum L, Jackson SP, McKinnon PJ, Nussenzweig A (2007) Distinct domains in Nbs1 regulate irradiation-induced checkpoints and apoptosis. *J Exp Med* **204**: 1003–1011
- Difilippantonio S, Gapud E, Wong N, Huang CY, Mahowald G, Chen HT, Kruhlak MJ, Callen E, Livak F, Nussenzweig MC, Sleekman BP, Nussenzweig A (2008) 53BP1 facilitates long-range DNA end-joining during V(D)J recombination. *Nature* **456**: 529–533
- Dimitrova N, Chen YC, Spector DL, de Lange T (2008) 53BP1 promotes non-homologous end joining of telomeres by increasing chromatin mobility. *Nature* **456**: 524–528
- Dinkelmann M, Spehalski E, Stoneham T, Buis J, Wu Y, Sekiguchi JM, Ferguson DO (2009) Multiple functions of MRN in end-joining pathways during isotype class switching. *Nat Struct Mol Biol* **16**: 808–813
- DiTullio Jr RA, Mochan TA, Venere M, Bartkova J, Sehested M, Bartek J, Halazonetis TD (2002) 53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer. *Nat Cell Biol* **4**: 998–1002
- Fabbro M, Savage K, Hobson K, Deans AJ, Powell SN, McArthur GA, Khanna KK (2004) BRCA1-BARD1 complexes are required for p53Ser-15 phosphorylation and a G1/S arrest following ionizing radiation-induced DNA damage. *J Biol Chem* **279**: 31251–31258
- Falck J, Coates J, Jackson SP (2005) Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. *Nature* **434**: 605–611
- Foray N, Marot D, Gabriel A, Randrianarison V, Carr AM, Perricaudet M, Ashworth A, Jeggo P (2003) A subset of ATM- and ATR-dependent phosphorylation events requires the BRCA1 protein. *EMBO J* **22**: 2860–2871
- Gatei M, Young D, Cersaletti KM, Desai-Mehta A, Spring K, Kozlov S, Lavin MF, Gatti RA, Concannon P, Khanna K (2000) ATM-dependent phosphorylation of nibrin in response to radiation exposure. *Nat Genet* **25**: 115–119
- Goodarzi AA, Noon AT, Deckbar D, Ziv Y, Shiloh Y, Lobrich M, Jeggo PA (2008) ATM signaling facilitates repair of DNA double-strand breaks associated with heterochromatin. *Mol Cell* **31**: 167–177
- Goodarzi AA, Noon AT, Jeggo PA (2009) The impact of heterochromatin on DSB repair. *Biochem Soc Trans* **37**(Pt 3): 569–576
- Gudmundsdottir K, Ashworth A (2006) The roles of BRCA1 and BRCA2 and associated proteins in the maintenance of genomic stability. *Oncogene* **25**: 5864–5874
- Haber JE (1998) The many interfaces of Mre11. *Cell* **95**: 583–586
- Helmink BA, Bredemeyer AL, Lee BS, Huang CY, Sharma GG, Walker LM, Bednarski JJ, Lee WL, Pandita TK, Bassing CH, Sleekman BP (2009) MRN complex function in the repair of chromosomal Rag-mediated DNA double-strand breaks. *J Exp Med* **206**: 669–679

- Huyen Y, Zgheib O, Ditullio Jr RA, Gorgoulis VG, Zacharatos P, Petty TJ, Sheston EA, Mellert HS, Stavridi ES, Halazonetis TD (2004) Methylated lysine 79 of histone H3 targets 53BP1 to DNA double-strand breaks. *Nature* **432**: 406–411
- Iwabuchi K, Bartel PL, Li B, Marraccino R, Fields S (1994) Two cellular proteins that bind to wild-type but not mutant p53. *Proc Natl Acad Sci USA* **91**: 6098–6102
- Iwabuchi K, Basu BP, Kysela B, Kurihara T, Shibata M, Guan D, Cao Y, Hamada T, Imamura K, Jeggo PA, Date T, Doherty AJ (2003) Potential role for 53BP1 in DNA end-joining repair through direct interaction with DNA. *J Biol Chem* **278**: 36487–36495
- Joo WS, Jeffrey PD, Cantor SB, Finnin MS, Livingston DM, Pavlentic NP (2002) Structure of the 53BP1 BRCT region bound to p53 and its comparison to the Brca1 BRCT structure. *Genes Dev* **16**: 583–593
- Jowsey PA, Doherty AJ, Rouse J (2004) Human PTIP facilitates ATM-mediated activation of p53 and promotes cellular resistance to ionizing radiation. *J Biol Chem* **279**: 55562–55569
- Kitagawa R, Bakkenist CJ, McKinnon PJ, Kastan MB (2004) Phosphorylation of SMC1 is a critical downstream event in the ATM-NBS1-BRCA1 pathway. *Genes Dev* **18**: 1423–1438
- Koonin EV, Altschul SF, Bork P (1996) BRCA1 protein products. Functional motifs. *Nat Genet* **13**: 266–268
- Lavin MF (2008) Ataxia-telangiectasia: from a rare disorder to a paradigm for cell signalling and cancer. *Nat Rev Mol Cell Biol* **9**: 759–769
- Lavin MF, Birrell G, Chen P, Kozlov S, Scott S, Gueven N (2005) ATM signaling and genomic stability in response to DNA damage. *Mutat Res* **569**: 123–132
- Lee J-H, Ghirlando R, Bhaskara V, Hoffmeyer MR, Gu J, Paull TT (2003) Regulation of Mre11/Rad50 by Nbs1: effects on nucleotide-dependent DNA binding and association with ATLD mutant complexes. *J Biol Chem* **278**: 45171–45181
- Lee JH, Paull TT (2004) Direct activation of the ATM protein kinase by the Mre11/Rad50/Nbs1 complex. *Science* **304**: 93–96
- Lee JH, Paull TT (2005) ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. *Science* **308**: 551–554
- Lee JH, Paull TT (2006) Purification and biochemical characterization of ataxia-telangiectasia mutated and Mre11/Rad50/Nbs1. *Methods Enzymol* **408**: 529–539
- Lim DS, Kim ST, Xu B, Maser RS, Lin J, Petrini JH, Kastan MB (2000) ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway. *Nature* **404**: 613–617
- Manis JP, Morales JC, Xia Z, Kutok JL, Alt FW, Carpenter PB (2004) 53BP1 links DNA damage-response pathways to immunoglobulin heavy chain class-switch recombination. *Nat Immunol* **5**: 481–487
- Manke IA, Lowery DM, Nguyen A, Yaffe MB (2003) BRCT repeats as phosphopeptide-binding modules involved in protein targeting. *Science* **302**: 636–639
- Maser RS, Zinkel R, Petrini JH (2001) An alternative mode of translation permits production of a variant NBS1 protein from the common Nijmegen breakage syndrome allele. *Nat Genet* **27**: 417–421
- Matsuoka S, Ballif BA, Smogorzewska A, McDonald III ER, Hurov KE, Luo J, Bakalarski CE, Zhao Z, Solimini N, Lerenthal Y, Shiloh Y, Gygi SP, Elledge SJ (2007) ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* **316**: 1160–1166
- Mochan TA, Venere M, DiTullio Jr RA, Halazonetis TD (2003) 53BP1 and NFB1/MDC1-Nbs1 function in parallel interacting pathways activating ataxia-telangiectasia mutated (ATM) in response to DNA damage. *Cancer Res* **63**: 8586–8591
- Mochan TA, Venere M, DiTullio Jr RA, Halazonetis TD (2004) 53BP1, an activator of ATM in response to DNA damage. *DNA Repair (Amst)* **3**: 945–952
- Moncalian G, Lengsfeld B, Bhaskara V, Hopfner KP, Karcher A, Alden E, Tainer JA, Paull TT (2004) The rad50 signature motif: essential to ATP binding and biological function. *J Mol Biol* **335**: 937–951
- Morales JC, Xia Z, Lu T, Aldrich MB, Wang B, Rosales C, Kellems RE, Hittelman WN, Elledge SJ, Carpenter PB (2003) Role for the BRCA1 C-terminal repeats (BRCT) protein 53BP1 in maintaining genomic stability. *J Biol Chem* **278**: 14971–14977
- Munoz IM, Jowsey PA, Toth R, Rouse J (2007) Phospho-epitope binding by the BRCT domains of hPTIP controls multiple aspects of the cellular response to DNA damage. *Nucleic Acids Res* **35**: 5312–5322
- Nakada D, Matsumoto K, Sugimoto K (2003) ATM-related Tel1 associates with double-strand breaks through an Xrs2-dependent mechanism. *Genes Dev* **17**: 1957–1962
- Nakanishi K, Taniguchi T, Ranganathan V, New HV, Moreau LA, Stotsky M, Mathew CG, Kastan MB, Weaver DT, D'Andrea AD (2002) Interaction of FANCD2 and NBS1 in the DNA damage response. *Nat Cell Biol* **4**: 913–920
- Panier S, Durocher D (2009) Regulatory ubiquitylation in response to DNA double-strand breaks. *DNA Repair (Amst)* **8**: 436–443
- Paull TT, Cortez D, Bowers B, Elledge SJ, Gellert M (2001) Direct DNA binding by Brca1. *Proc Natl Acad Sci USA* **98**: 6086–6091
- Paull TT, Gellert M (2000) A mechanistic basis for Mre11-directed DNA joining at microhomologies. *Proc Natl Acad Sci USA* **97**: 6409–6414
- Rappold I, Iwabuchi K, Date T, Chen J (2001) Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage-signaling pathways. *J Cell Biol* **153**: 613–620
- Riballo E, Kuhne M, Rief N, Doherty A, Smith GC, Recio MJ, Reis C, Dahm K, Fricke A, Krempler A, Parker AR, Jackson SP, Gennery A, Jeggo PA, Lobrich M (2004) A pathway of double-strand break rejoining dependent upon ATM, Artemis, and proteins locating to gamma-H2AX foci. *Mol Cell* **16**: 715–724
- Schultz LB, Chehab NH, Malikzay A, Halazonetis TD (2000) p53 binding protein 1 (53BP1) is an early participant in the cellular response to DNA double-strand breaks. *J Cell Biol* **151**: 1381–1390
- Shiozaki EN, Gu L, Yan N, Shi Y (2004) Structure of the BRCT repeats of BRCA1 bound to a BACH1 phosphopeptide: implications for signaling. *Mol Cell* **14**: 405–412
- Soulier J, Lowndes NF (1999) The BRCT domain of the *S. cerevisiae* checkpoint protein Rad9 mediates a Rad9-Rad9 interaction after DNA damage. *Curr Biol* **9**: 551–554
- Stewart GS, Maser RS, Stankovic T, Bressan DA, Kaplan MI, Jaspers NG, Raams A, Byrd PJ, Petrini JH, Taylor AM (1999) The DNA double-strand break repair gene *hMre11* is mutated in individuals with an ataxia-telangiectasia-like disorder. *Cell* **99**: 577–587
- Stracker TH, Morales M, Couto SS, Hussein H, Petrini JH (2007) The carboxy terminus of NBS1 is required for induction of apoptosis by the MRE11 complex. *Nature* **447**: 218–221
- Stracker TH, Theunissen JW, Morales M, Petrini JH (2004) The Mre11 complex and the metabolism of chromosome breaks: the importance of communicating and holding things together. *DNA Repair (Amst)* **3**: 845–854
- Symington LS (2002) Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair. *Microbiol Mol Biol Rev* **66**: 630–670
- Usui T, Foster SS, Petrini JH (2009) Maintenance of the DNA-damage checkpoint requires DNA-damage-induced mediator protein oligomerization. *Mol Cell* **33**: 147–159
- Varon R, Vissinga C, Platzer M, Cerosaletti KM, Chrzanowska KH, Saar K, Beckmann G, Seemanova E, Cooper PR, Nowak NJ, Stumm M, Weemaes CM, Gatti RA, Wilson RK, Digweed M, Rosenthal A, Sperling K, Concannon P, Reis A (1998) Nibrin, a novel DNA double-strand break repair protein, is mutated in Nijmegen breakage syndrome. *Cell* **93**: 467–476
- Wang B, Matsuoka S, Carpenter PB, Elledge SJ (2002) 53BP1, a mediator of the DNA damage checkpoint. *Science* **298**: 1435–1438
- Ward I, Kim JE, Minn K, Chini CC, Mer G, Chen J (2006) The tandem BRCT domain of 53BP1 is not required for its repair function. *J Biol Chem* **281**: 38472–38477
- Ward IM, Minn K, van Deursen J, Chen J (2003) p53 binding protein 53BP1 is required for DNA damage responses and tumor suppression in mice. *Mol Cell Biol* **23**: 2556–2563
- Ward IM, Reina-San-Martin B, Olaru A, Minn K, Tamada K, Lau JS, Cascalho M, Chen L, Nussenzweig A, Livak F, Nussenzweig MC, Chen J (2004) 53BP1 is required for class switch recombination. *J Cell Biol* **165**: 459–464
- Wu X, Ranganathan V, Weisman DS, Heine WF, Ciccone DN, O'Neill TB, Crick KE, Pierce KA, Lane WS, Rathbun G, Livingston DM, Weaver DT (2000) ATM phosphorylation of Nijmegen breakage syndrome protein is required in a DNA damage response. *Nature* **405**: 477–482

- Xia Z, Morales JC, Dunphy WG, Carpenter PB (2001) Negative cell cycle regulation and DNA damage-inducible phosphorylation of the BRCT protein 53BP1. *J Biol Chem* **276**: 2708–2718
- Xie A, Hartlerode A, Stucki M, Odate S, Puget N, Kwok A, Nagaraju G, Yan C, Alt FW, Chen J, Jackson SP, Scully R (2007) Distinct roles of chromatin-associated proteins MDC1 and 53BP1 in mammalian double-strand break repair. *Mol Cell* **28**: 1045–1057
- Xie A, Kwok A, Scully R (2009) Role of mammalian Mre11 in classical and alternative nonhomologous end joining. *Nat Struct Mol Biol* **16**: 814–818
- Xu B, Kim S, Kastan MB (2001) Involvement of Brca1 in S-phase and G(2)-phase checkpoints after ionizing irradiation. *Mol Cell Biol* **21**: 3445–3450
- Xu B, O'Donnell AH, Kim ST, Kastan MB (2002) Phosphorylation of serine 1387 in Brca1 is specifically required for the Atm-mediated S-phase checkpoint after ionizing irradiation. *Cancer Res* **62**: 4588–4591
- Yazdi PT, Wang Y, Zhao S, Patel N, Lee EY, Qin J (2002) SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint. *Genes Dev* **16**: 571–582
- You Z, Chahwan C, Bailis J, Hunter T, Russell P (2005) ATM activation and its recruitment to damaged DNA require binding to the C terminus of Nbs1. *Mol Cell Biol* **25**: 5363–5379
- Yu X, Chini CC, He M, Mer G, Chen J (2003) The BRCT domain is a phospho-protein binding domain. *Science* **302**: 639–642
- Zgheib O, Huyen Y, DiTullio Jr RA, Snyder A, Venere M, Stavridi ES, Halazonetis TD (2005) ATM signaling and 53BP1. *Radiother Oncol* **76**: 119–122
- Zhao S, Weng YC, Yuan SS, Lin YT, Hsu HC, Lin SC, Gerbino E, Song MH, Zdzienicka MZ, Gatti RA, Shay JW, Ziv Y, Shiloh Y, Lee EY (2000) Functional link between ataxia-telangiectasia and Nijmegen breakage syndrome gene products. *Nature* **405**: 473–477