**53BP1: function and mechanisms of focal recruitment**

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**Abstract**

53BP1 (p53-binding protein 1) is classified as a mediator/adaptor of the DNA-damage response, and is recruited to nuclear structures termed foci following genotoxic insult. In the present paper, we review the functions of 53BP1 in DNA-damage checkpoint activation and DNA repair, and the mechanisms of its recruitment and activation following DNA damage. We focus in particular on the role of covalent histone modifications in this process.

**Introduction**

DSBs (double-strand breaks) are an extremely dangerous form of DNA damage for the cell, and can be caused by a wide range of agents ranging from γ-irradiation to the products of endogenous oxidative metabolism [1]. To prevent propagation of mutations that could lead to genome instability and cancer, it is vital that DNA damage is detected and repaired before DNA replication and cell division. This is facilitated by a signal transduction cascade known as the DNA-damage response (DDR) [2], which initiates processes such as apoptosis, cell-cycle arrests (termed checkpoints), DNA repair and senescence, as well as regulation of transcription and replication, processes designed to protect genomic integrity [2,3]. Indeed, the DDR is an integral player in tumour suppression, and many tumour cells display loss of functional DDR components [1,4–6].

The DDR is highly conserved from yeast to humans, and most of the essential players have been identified in both lower and higher eukaryotes [7,8]. These players have been loosely classified as DNA-damage sensors, mediators, transducers and effectors [2] (Figure 1). Sensors are defined as activities that detect DNA lesions. The nature of these sensors and the mechanism of DNA-damage detection are unclear; however, the earliest detectable DNA DSB-induced events involve the MRN [MRE11 (meiotic recombination 11)–Rad50–NBS1 (Nijmegen breakage syndrome 1)] complex [9] and the PIKKs (phosphoinositide 3-kinase-like kinases) ATM (ataxia telangiectasia mutated), ATR (ATM- and Rad3-related) and DNA-PK (DNA-dependent protein kinase) [10,11]. From these proteins, the DNA-damage signal is transmitted to transducer kinases such as CHK (checkpoint kinase) 1 and CHK2, which function in signal transduction cascades targeting downstream DDR components, as well as amplifying the DDR signal [12] (Figure 1). This signalling between sensors and transducers is thought to be facilitated by mediator or adaptor proteins, such as MDC1 (mediator of DNA-damage checkpoint 1), 53BP1 (p53-binding protein 1) and BRCA1 (breast cancer 1 early-onset), which are believed to enhance DDR signalling through as yet ill-defined mechanisms [13]. The activated sensor and transducer kinases then proceed to phosphorylate a number of downstream effector molecules, including p53 [8], resulting in appropriate biological responses (Figure 1).

In the present review, we focus on the mediator/adaptor protein 53BP1. We discuss the involvement of 53BP1 in checkpoint activation and DNA repair, the mechanisms of 53BP1 recruitment and activation following DNA damage, and the relationship between these processes and chromatin.

**53BP1 and its domain structure**

Mediator/adaptor proteins of the DDR include MDC1, 53BP1, BRCA1, TOPBP1 (topoisomerase II-binding protein 1), Claspin and PTIP (Pax transactivation domain-interacting protein). From these molecules displaying roles in both DDR signalling events and DNA repair (13 and see below). Apart from the E3 ubiquitin ligase activity of BRCA1 [19], these proteins have no characterized enzymatic activity, and instead may act as recruitment platforms for other DDR proteins [13,20].
Following genotoxic insult, DNA damage is detected by sensor proteins, which include the MRN complex and the PIKKs ATM, ATR and DNA-PK. The damage signal is then transmitted to the transducer kinases CHK1 and CHK2, and this signalling is facilitated by mediator/adaptor proteins such as MDC1, 53BP1 and BRCA1. Activated CHK1 and CHK2 kinases, as well as ATM and ATR, then phosphorylate downstream effector proteins, for example p53. This results in the cellular responses indicated which act to prevent DNA replication and cell division in the presence of DNA damage.

53BP1 function in checkpoint activation

Abrogation of 53BP1 results in modest G2/M cell cycle arrest defects in human and mouse cells. These defects are only detectable following low doses of IR (3 Gy) [31,32] and not following higher doses [31–34]. Partial intra-S-phase checkpoint defects have also been found in human cell lines following treatment with 53BP1 siRNA (small interfering RNA) and in mouse 53BP1−/− cells at IR doses between 5 and 20 Gy [26,32]. In contrast with these results, however, other groups have reported intact intra-S-phase and G2/M checkpoint arrest in the absence of 53BP1 [35–37]. Indeed one group found that 53BP1−/− MEFS (mouse embryonic fibroblasts) accumulated in G2-phase after IR, consistent with a defect in DNA repair [37]. No defects in the G1/S-phase checkpoint have been reported.

In yeast, the 53BP1 homologues Rad9 and Crb2 have indispensable roles in DNA-damage signalling and cell-cycle arrest [39,40]. Rad9 in particular is necessary for the G1, intra-S-phase and G2/M cell cycle delay induced by DNA damage, and plays an important role in activating the yeast homologues of both CHK1 and CHK2 [40–42]. However, from the above, it appears that, unlike Rad9, 53BP1 is not absolutely required for these processes in higher cells. This raises the possibility that 53BP1 may act redundantly with other proteins, perhaps the other Rad9/Crb2 homologues MDC1 and BRCA1. Several studies have shown this to be the case [17,18,43], with recent reports demonstrating that these proteins have overlapping as well as unique functions in DNA-damage signalling [17]. For example, of the three proteins, only 53BP1 mediates ATM autophosphorylation, whereas all three mediate CHK2 phosphorylation following IR [17]. Thus it is likely that, in higher cells, MDC1, 53BP1 and BRCA1 are required to fulfil functions which are performed by just one protein (Rad9 or Crb2) in yeast.
Figure 2  (A) Domain structure of the mediator/adaptor proteins MDC1, 53BP1 and BRCA1 of higher cells, as well as S. cerevisiae Rad9. The main structural features of the proteins are indicated as coloured boxes. Numbers indicate amino acids. Post-translational modifications are omitted for ease of presentation. Each schematic diagram is drawn to scale. (B) Detailed schematic diagram of 53BP1, showing domains and functionally significant regions. FHA, forkhead-homology-associated; GAR, glycine/arginine-rich region; OD, oligomerization domain. The minimal region required for efficient 53BP1 foci formation is amino acids 1220–1711, and includes the oligomerization domain, the MDC1-interacting region and the Tudor domains. Circles and squares indicate residues shown to be phosphorylated by MS or Western blot analysis (see text for details): circles denote phosphorylation sites which correspond to S/TQ PIKK consensus sequences, with red circles indicating phosphorylation induced by IR and white circles indicating sites whose phosphorylation levels remains unchanged following IR; white squares denote phosphorylation sites that correspond to S/TP potential CDK consensus sequences. The schematic diagram is drawn to scale.

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Role of 53BP1 in DNA repair

Knockout or knockdown of 53BP1 results in genome instability, as typified by increased levels of chromatin gaps, breaks and exchanges, as well as aneuploidy and tetraploidy in the absence of exogenous DNA damage [37]. Also, mouse and DT40 53BP1−/− cell lines exhibit sensitivity to exogenous DNA damage, indicating that 53BP1 is involved in DNA repair [26,35]. Indeed, the 53BP1 Tudor domains are reported to stimulate the ligase activity of the DNA ligase VI/XRCC4 (X-ray repair complementing defective repair in Chinese-hamster cells 4) complex in vitro [44], although it is thought that 53BP1 is dispensable for core NHEJ (non-homologous end-joining). Instead, it is required, along with H2AX (histone 2AX), NBS1 and MRE11 [45], for ATM-dependent NHEJ of DSBs that are repaired with late kinetics following DNA damage and are thought to be present in heterochromatin [46]. It is interesting to note that Rad9 is also necessary for efficient repair of DSB by NHEJ [47].

Recently, a fascinating report by Dimitrova et al. [48] has identified another role for 53BP1 in NHEJ. The authors demonstrated that 53BP1 influences chromatin mobility at the environment surrounding a DNA damage focus [48]. Specifically, the rate of fusion of decapped telomeres by NHEJ was examined through use of a conditional knockout of the mouse telomere-protecting protein, TRF2 (telomeric-repeat-binding factor 2). This showed that the presence of 53BP1 at deprotected telomeres increased chromatin mobility, thereby increasing the likelihood that two telomere ends, normally separated in G1-phase when the NHEJ pathway of DSB repair is most active, would come within sufficient proximity to allow an NHEJ reaction to occur [48]. 53BP1 also increases the rate of class switch recombination [49,50] and long-range V(D)J recombination [51], both end-joining process involving DNA ends that are not in close proximity. Difilippantonio et al. [51] suggest that this is again through modulation of chromatin mobility, and may be facilitated by the interaction of 53BP1 with dynein motor proteins [52]. It is also likely that 53BP1 acts in NHEJ repair at heterochromatin [46] through a similar mechanism, to increase chromatin mobility in these regions. The change in chromatin mobility required 53BP1 recruitment to the dysfunctional telomeres via its Tudor domains [48]. However a Tudor domain-independent interaction, possibly involving MDC1, also plays a role in this process [48].

53BP1 foci formation following DNA damage

53BP1 undergoes nuclear relocalization to focal structures of unknown architecture following IR, presumably to facilitate the checkpoint and repair functions detailed above [53–57]. The recruitment of 53BP1 into focal structures after IR is a highly complex process involving many regulatory steps and multiple post-translational modifications of various proteins. Efficient 53BP1 focal recruitment depends on a number of upstream factors, including phosphorylation of H2AX at Ser159 [58], recruitment of both MDC1 [59–61] and the E3 ubiquitin ligase RNF8 [62–64], methylation of histones H3 and H4 [23,65] and Tip60 HAT (histone acetyltransferase) activity [66].

The model for the recruitment of 53BP1 to focal structures is as follows: in response to DNA DSB induction, H2AX is phosphorylated by the PIKKs to form γH2AX [67]. MDC1 then binds to this modified histone residue via its BRCT domains [68]. MDC1, once phosphorylated in an ATM-dependent manner, then recruits RNF8 to the DSB site [62–64], where it catalyses one or more ubiquitylation events, including Lys119 of H2AX [69]. This γH2AX-dependent MDC1-regulated recruitment of RNF8 is required for subsequent focal recruitment of 53BP1 [63,70], a process which also requires binding of 53BP1 to methylated histone residues (see below). However, it is important to note that γH2AX is not required for the initial recruitment of 53BP1 immediately following DNA damage [58]. Instead, this histone mark is required for the sustained retention of these foci [26,33,71]. The mechanism of this transient γH2AX-independent 53BP1 recruitment is unknown, but it is interesting to speculate that it could occur via methylated histone residues.

Recently, a direct interaction between the MDC1 BRCT domains and amino acids 1288–1409 of 53BP1 (Figure 2) has also been shown to be required for efficient 53BP1 focus formation [72]. In addition, 53BP1 focal recruitment requires Tip60 HAT activity, which can acetylate histone residues and therefore may facilitate 53BP1 recruitment through formation of a more open chromatin structure [66].

The minimal region of 53BP1 required for focal recruitment has been mapped to amino acids 1220–1711, encompassing the tandem Tudor domain (amino acids 1486–1602) [23,44] and a region required for 53BP1 oligomerization [73] (Figure 2). Similarly to Rad9 and Crb2 [74,75], oligomerization of 53BP1 is required for efficient 53BP1 focus formation, although the exact function of oligomerization in 53BP1 focal recruitment remains unclear. The BRCT domains and the N-terminus of 53BP1 are not required for focal recruitment [34].

In yeast model systems, it has been shown that S. cerevisiae Rad9 is recruited to DNA DSBs via binding of its Tudor domains to H3K79me [methylated H3K79 (Lys79 of histone H3)] [76,77], whereas S. pombe Crb2 is recruited via H4K20me [methylated H4K20 (Lys20 of histone H4)] [78]. Strikingly, both of these histone modifications have been implicated in the recruitment of 53BP1 [23,65], and this is discussed in detail in the next section.

The role of histone methylation in the recruitment of 53BP1

A function for H3K79me in the recruitment of 53BP1 was first reported by Huyen et al. [23], who showed that the Tudor domain of 53BP1 binds to H3K79me both in vitro and in vivo. Point mutations in the Tudor domain abolished this interaction, and furthermore these mutated proteins failed to relocalize to DNA damage-induced foci. Importantly, foci
formation in U2OS cells was reduced by siRNA knockdown of hDot1L (human Dot1-like, also known as KMT4 [79]), the HMTase (histone methyltransferase) that catalyses the methylation of H3K79. Indeed loss of Dot1 in S. cerevisiae results in loss of Rad9 hyperphosphorylation in G1-phase cells and concomitant loss of the G1 checkpoint [76,77,80]. Together, these results strongly suggest that 53BP1 requires methylation of H3K79 by hDot1L/KMT4 for recruitment via its Tudor domain.

Botuyan et al. [65] also showed that an intact Tudor domain is required for 53BP1 recruitment to foci. However, this study found that the domain displays affinity for H4K20me, whereas the affinity between 53BP1 and H3K79me was negligible. In agreement with these in vitro data, siRNA knockdown of hDot1L/KMT4 in HeLa cells had no effect on 53BP1 foci formation. 53BP1 foci were also unaffected in hypomorphic Dot1L/KMT4 mutant MEFs [65], in which only monomethylation of H3K79, and not di- and tri-methylation (H3K79me2/3), is present [81]. Instead, reduction of H4K20 methylation levels through knockdown of Set8/KMT5A, an H4K20 HMTase, abrogated 53BP1 recruitment [65]. In addition, other groups have shown that knockdown of the H4K20 HMTases Suv420h1/KMT5B and Suv420h2/KMT5C in HeLa cells [82], or knock-out of these enzymes in mouse, also decreases 53BP1 foci formation [83]. Although the percentage of cells exhibiting 53BP1 foci was similar to wild-type cells at later time points following IR, the percentage of cells with foci was reduced at 1–5 min after IR exposure [83]. These results bear similarity to those found in S. pombe, in which H4K20 methylation is required for Crb2 focus formation and efficient checkpoint activation [78].

It is difficult to resolve these conflicting histone methylation results in higher cells, especially as substantial evidence for a function of each of these methylated sites has been reported. However, it should be noted that different transformed cell lines (U2OS [23] and HeLa [65,82]) were used in each RNAi (RNA interference) study. It is possible that the genome instability generated during transformation has altered the relative importance of H3K79 and H4K20 methylation to 53BP1 recruitment in these cells. This raises the intriguing possibility that H3K79me and H4K20me may function redundantly in higher cells. Note that both γH2A and H3K79me chromatin marks are required for the localization and checkpoint functions of Rad9 in S. cerevisiae, a model system in which H4K20me does not occur [84]. Conversely, H3K79me does not occur in S. pombe [85], and instead Crb2 functions require H4K20 methylation as well as γH2A [85,86]. In human cells, γH2AX, H3K79me and H4K20me have all been implicated in 53BP1 localization to IRIF (IR-induced foci) [23,58,65]. It is therefore very probable that higher cells have evolved to utilize H2AX phosphorylation plus two histone methylation marks in the function of 53BP1, with H3K79me and H4K20me perhaps having overlapping or redundant roles, whereas, in lower eukaryotes, just H2A phosphorylation and one methylation mark are required for the chromatin localization and function of 53BP1 orthologues.

These methylation marks are present on chromatin independently of DNA damage. So how do methylated H3K79 and H4K20 specifically recruit proteins to sites of DSBs? It was initially proposed that H3K79 methylation (in human cells) and/or H4K20 methylation (in S. pombe) function in sensing of DNA damage [23,78]. As illustrated in Figure 3, these residues are present on the surface of the nucleosome, and are thought to be buried in higher-order chromatin structures [23,78,87]. It was postulated that DNA damage induces passive relaxation of chromatin structure surrounding the break site, allowing 53BP1 (and its yeast homologues) to bind H3K79me/H4K20me and so sense DNA damage [23,78]. This model is attractive, because the rapid exposure of these marks in response to DNA damage, as opposed to their generation, would allow rapid DNA damage signalling. However, it appears that this may not be the case. Activation of PIKKs and formation of γH2AX occur as normal in the absence of H3K79me in S. cerevisiae, or H4K20me in S. pombe and higher cells [65,80,85], indicating that this methylation mark alone is not required for activation of the DDR. It is possible that γH2AX and MDC1 foci formation, together with RNF8 and Tip60 activity result in a more open chromatin structure in which H3K79/H4K20me are accessible to 53BP1, allowing stable focal retention of the protein at the break site, and also facilitating specific recruitment of 53BP1 to DSB sites [64].

The requirement of H3K79 and H4K20 methylation for the cell-cycle arrest and DNA-repair functions of 53BP1 are not well characterized, although recently roles for the Tudor domains [48,51,88,89] and Suv420h1/2 enzymes in DNA repair [83] have been found. However, these marks are required for efficient checkpoint activation, DNA repair and localization of Rad9/Crb2 to DNA DSBs in both S. cerevisiae [76,77] and S. pombe [78,85]. Interestingly,
methylated histone residues are not required for all aspects of Rad9/Crb2 function. A histone modification-independent pathway, involving cell-cycle-regulated phosphorylation of Rad9/Crb2, and the TOPBP1 homologues Cut5 (S. pombe) and Dpb11 (S. cerevisiae) is also important [85,90].

Together, these data point to great complexity in the recruitment of 53BP1 to DSBs. Multiple protein–protein interactions, many post-translational modifications (of both chromatin and non-chromatin proteins) and distinct enzymatic activities all play important roles in the choreography of 53BP1 recruitment. Furthermore, the regulation of this recruitment varies at different stages during the DDR.

Conclusions
Much progress has been made recently in understanding the role of 53BP1 in the DDR and the maintenance of genomic integrity. However, further work remains to dissect the precise function of this protein in the DDR. The exact mechanism of 53BP1 focal recruitment must be resolved, as this event is clearly required for the repair function of 53BP1. The relative roles of H3K79 methylation and H4K20 methylation, and the influence of the methylation state (mono-, di- or tri-methylation) of these residues must also be addressed, as well as the functional relationship between 53BP1 and other DDR components, especially the mediators MDC1 and BRCA1, in cell-cycle arrest and DNA repair.

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