Dampening DNA damage checkpoint signalling via coordinated BRCT domain interactions

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Abstract

In response to DNA damage, checkpoint signalling protects genome integrity at the cost of repressing cell cycle progression and DNA replication. Mechanisms for checkpoint down-regulation are therefore necessary for proper cellular proliferation. We recently uncovered a phosphatase-independent mechanism for dampening checkpoint signalling, where the checkpoint adaptor Rad9 is counteracted by the repair scaffolds Slx4-Rtt107. Here, we establish the molecular requirements for this new mode of checkpoint regulation. We engineered a minimal multi-BRCT-domain (MBD) module that recapitulates the action of Slx4-Rtt107 in checkpoint down-regulation. MBD mimics the damage-induced Dpb11-Slx4-Rtt107 complex by synergistically interacting with lesion-specific phospho-sites in Ddc1 and H2A. We propose that efficient recruitment of Dpb11-Slx4-Rtt107 or MBD via a cooperative ‘two-site-docking’ mechanism displaces Rad9. MBD also interacts with the Mus81 nuclease following checkpoint dampening, suggesting a spatio-temporal coordination of checkpoint signalling and DNA repair via a combinatorial mode of BRCT-domains interactions.

Keywords BRCT domain; checkpoint; DNA damage; Dpb11; Slx4

Subject Categories DNA Replication, Repair & Recombination

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Introduction

The ability of eukaryotic cells to properly respond to genotoxic insults heavily relies on DNA damage checkpoint (DDC) signalling. Upon detection of a DNA lesion, triggering of a checkpoint response results in inhibitory effects on cell cycle progression (Weinert & Hartwell, 1988) and DNA synthesis (Santocanale & Diffley, 1998; Lopez-Mosqueda *et al*, 2010; Zegerman & Diffley, 2010), accompanied by transcriptional reprogramming (Allen *et al*, 1994; Huang *et al*, 1998; Bastos de Oliveira *et al*, 2012; Travesa *et al*, 2012) and striking elevations in dNTP levels (Zhou & Elledge, 1993; Zhao *et al*, 2001; Zhao & Rothstein, 2002; Lee *et al*, 2008; Davidson *et al*, 2012). More recently, DDC has been proposed to modulate the

action of nucleases such as Exo1 and Mus81 (Kai *et al*, 2005; Morin *et al*, 2008; Szakal & Branzei, 2013). Collectively, checkpoint functions help protect replication fork integrity and coordinate replication and DNA repair with other cell cycle events such as chromosome segregation (Branzei & Foiani, 2009). In budding yeast *Saccharomyces cerevisiae*, DDC is orchestrated mainly through the action of the upstream sensor kinase Mec1 (mammalian ATR), which then activates the downstream kinase Rad53 (functional analog of both mammalian CHK1/CHK2) for enforcement of a canonical checkpoint response. Transduction of signalling from Mec1 to Rad53 is mediated by protein adaptors, such as Rad9 and Mrc1, whose phosphorylation by Mec1 creates a docking site for the recruitment of Rad53 to DNA lesions (Sun *et al*, 1998; Durocher *et al*, 2000; Alcasabas *et al*, 2001; Schwartz *et al*, 2002; Pelliccioli & Foiani, 2005). Similarly, in mammals, signalling from ATR and the related kinase ATM to CHK1/CHK2 also requires the action of checkpoint adaptors such as MDC1, BRCA1 and Claspin (Cimprich & Cortez, 2008). Of importance, mutations in checkpoint kinases and adaptors have been associated with tumorigenesis and neurological disorders (Shiloh, 2003).

Recruitment of checkpoint adaptors to sites of lesions is a key step in mounting a checkpoint response. Differently than the Mec1/ATR kinase, which is recruited via interaction of its cofactor Ddc2/ATRIP with ssDNA-bound RPA, recruitment of checkpoint adaptors is a more complex and regulated process that involves multiple, and partially redundant, mechanisms (Ohouo & Smolka, 2012). For example, recruitment of the yeast Rad9 adaptor has been shown to be mediated by three distinct mechanisms: (i) binding of the tudor domain of Rad9 to methylated histone H3K79 (Wysocki *et al*, 2005), (ii) binding of BRCT (for BRCA1 C-Terminal homology) domains of Rad9 to histone H2A phosphorylated at serine 129 (phospho-H2A) (Hammet *et al*, 2007) and (iii) recognition of the phosphorylation sites in Rad9 (serine 462 and threonine 474) by BRCT domains 1/2 in Dpb11 (Pfander & Diffley, 2011). Notably, these three modes of adaptor recruitment are spatiotemporally distinct. H3K79 methylation is a constitutive mark spread throughout euchromatin (Nguyen & Zhang, 2011); phospho-H2A is induced by DNA damage and, in the case of double-strand break (DSB), has been shown to spread kilobases around the lesion (Shroff *et al*, 2004); and the Dpb11 adaptor appears to be more specifically located at the site of lesion, by binding to a damage-induced phosphorylation in the Ddc1 subunit of the 9-1-1 complex (Puddu *et al*, 2008). This arrangement

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of anchoring points likely allows recruitment of Rad9 to be highly regulated, such that activation of DDC can be fine-tuned to match the extent and type of DNA lesion.

We have recently proposed that regulation of Rad9 displacement from the site of lesion is also used as a regulatory mechanism, in this case to modulate down-regulation of DDC (Ohouo *et al*, 2013). We have shown that the Slx4 and Rtt107 repair scaffolds counteract Rad9-dependent activation of Rad53 and proposed that the Slx4-Rtt107 complex balances the engagement of Rad9 at sites of lesions by interacting with phospho-H2A and Dpb11 thus displacing Rad9. By competing with Rad9 that is engaged at a lesion site, Slx4 and Rtt107 dampen DDC signalling, providing a phosphatase-independent mechanism for down-regulating Rad53 activation that we named DAMP (dampens adaptor-mediated phospho-signalling). Interestingly, several of the protein–protein interactions important for establishing the DAMP mechanism appear to involve BRCT domains in at least three different proteins: Rad9, Dpb11 and Rtt107 (Fig 1A). BRCT domains often recognize phosphorylated motifs in targeted proteins and are commonly found in proteins involved in DNA damage signalling and repair (Reinhardt & Yaffe, 2013).

Failure to down-regulate the DDC strongly represses cell proliferation (Clerici *et al*, 2001). In yeast for example, Rad53 hyperactivation caused by lack of the Rad53 phosphatase Pph3 or by lack of Slx4 has been shown to increase sensitivity to genotoxins (O'Neill *et al*, 2007; Ohouo *et al*, 2010). Given the importance of proper checkpoint down-regulation, it is not surprising that multiple mechanisms for down-regulating checkpoint exist. While the role of phosphatases such as Pph3, Ptc2 and Ptc3 in down-regulating Rad53 signalling has been well documented [for review, see Heideker *et al* (2007)], much less is known about the DAMP mechanism. Here, we uncovered the molecular determinants of the DAMP mechanism and show that it is established via cooperative interactions mediated by BRCT domains in Rtt107 and Dpb11. We have engineered a minimal multi-BRCT-domain (MBD) module that fully recapitulates the action of the Dpb11-Slx4-Rtt107 complex in dampening checkpoint signalling. Efficient recruitment of Dpb11-Slx4-Rtt107 or MBD via a cooperative ‘two-site-docking’ mechanism explains how these BRCT-domain modules can displace the checkpoint adaptor Rad9 and down-regulate Rad53 signalling. We propose that this two-site-docking mechanism may be a general strategy for efficient and timely recruitment of multi-BRCT-domain protein complexes to DNA lesion sites.

Results

A working model for Slx4-Rtt107-dependent dampening of Rad53 signalling

We have previously shown that the Slx4-Rtt107 complex down-regulates Rad53 signalling by counteracting the Rad9 adaptor (Ohouo *et al*, 2013). We proposed that Slx4 and Rtt107 counteract Rad9 by binding to the Dpb11 scaffold and to phosphorylated histone H2A, both of which also interact with Rad9 to promote Rad53 activation at DNA lesions (Fig 1A). In our proposed model, BRCT domains of Dpb11 play a central role in mediating the competitive recruitment of Rad9 or Slx4-Rtt107 to the site of lesion where the 9-1-1 complex is loaded. While the pair of BRCT domains 3/4 of Dpb11 binds to a

phosphorylated residue in the 9-1-1 complex (threonine 602 in the Ddc1 subunit) (Puddu *et al*, 2008), the pair of BRCT domains 1/2 of Dpb11 was found to bind to phosphorylation sites in either Rad9 (Pfander & Diffley, 2011) or Slx4 (Ohouo *et al*, 2013). Here, we thoroughly tested the model depicted in Fig 1A and provide multiple lines of evidence supporting it. First, we predicted that efficient formation of the damage-induced Dpb11-Slx4-Rtt107 complex at lesion sites would be mediated, cooperatively, by Mec1 phosphorylation sites on H2A and Ddc1, which are expected to form anchoring points at the site of lesion. Consistent with this model, we found that mutation of either of these phosphorylation sites significantly reduced the Slx4-Dpb11 interaction (Fig 1B and C). Next, to test whether Dpb11 may indeed bridge Slx4 to Ddc1, as predicted in our model, we pulled down Slx4 and monitored the recovery of Ddc1. As shown in Fig 1D, we were able to specifically recover Ddc1 from Slx4 immunoprecipitates, consistent with our bridging model shown in Fig 1A. Noteworthy, the formation of the ternary complex (Ddc1-Dpb11-Slx4) is dependent on DNA damage (Fig 1D) and on phosphorylation of threonine 602 in Ddc1 (Fig 1E) previously shown to be recognized by BRCT 3/4 of Dpb11 (Wang & Elledge, 2002; Puddu *et al*, 2008). While these results support the model that Dpb11 bridges Slx4 to Ddc1 by recognizing these proteins via BRCT 1/2 and BRCT 3/4, respectively, a recent report showed that Dpb11 interaction with Slx4 can also be mediated by BRCT 3/4 (Gritenaite *et al*, 2014). Therefore, we performed experiments to check which BRCT domain plays a major role in mediating the Slx4-Dpb11 interaction in MMS-treated cells. As shown in Supplementary Fig S1, while we could indeed detect Slx4 in a pull-down using BRCT 3/4, we were able to detect significantly more Slx4 by pulling down BRCT 1/2. In addition, we constructed a series of Dpb11 mutants predicted to disrupt different BRCT domains [K55A in BRCT-1; R186A in BRCT-2 and K544A in BRCT-4; see Supplementary Fig S2 and Qu *et al* (2013) for details] and tested their binding to Slx4 when expressed in yeast cells following MMS treatment. Combined mutations in BRCT domains 1 and 2 (K55A/R186A) almost completely abolished the interaction with Slx4 (Fig 1F), further supporting that BRCT 1/2 plays a major role in the Slx4 interaction. Mutation in BRCT-4 moderately reduced interaction of Dpb11 with Slx4, which is also consistent with our model, as we showed in Fig 1B that recruitment of Dpb11 to Ddc1 helps promote robust Dpb11 interaction with Slx4. On the other hand, interaction of Dpb11 with Ddc1 was strongly affected by mutation in BRCT-4, as expected, and only mildly reduced by mutations in BRCT 1/2, which could also play a role in promoting efficient recruitment of Dpb11 to Ddc1 based on our model. Collectively, these results support the idea that Dpb11 functions to bridge Slx4 to the 9-1-1 complex, providing the basis for a competition-based model as illustrated in Fig 1A. We do not exclude the possibility that Dpb11 may also engage into distinct modes of BRCT-mediated interactions with Rad9 or Slx4, as recently proposed for the Crb2-Rad4 (orthologs of Rad9 and Dpb11, respectively) interaction in *Schizosaccharomyces pombe* (Qu *et al*, 2013).

Functional rescue of *slx4Δ* cells via an Slx4-Dpb11 chimera

To functionally test our model and determine the minimal requirements for the ability of Slx4-Rtt107 to counteract Rad9 at lesion sites, we used a Dpb11-Slx4 chimera by fusing full-length Dpb11 with the C-terminal portion of Slx4 containing the S486A mutation

Figure 1. Dpb11 bridges Slx4 to the 9-1-1 complex.

A Working model for how the Slx4-Rtt107 complex modulates Rad53 signalling.

B Coimmunoprecipitation (CoIP) between Slx4-Myc and Dpb11-HA in MMS-treated cells lacking Ddc1 or expressing either wild-type or the T602A mutant form of Ddc1.

C CoIP between Slx4-Flag and Dpb11-HA in MMS-treated cells expressing either wild-type or the S129A mutant form of histone H2A.

D, E CoIP between Slx4-Flag and Ddc1-HA in MMS-treated cells. In (D), a plasmid was used for ectopic expression of Dpb11-Myc via an *ADH1* promoter. In (E), Dpb11 was expressed from the endogenous *DPB11* locus and driven by its own promoter.

F Dpb11 containing the mutations indicated in the schematic illustration (see also Supplementary Fig S2) was expressed from a plasmid in yeast cells containing Flag-tagged Slx4 or Ddc1 for CoIP experiments. AD: activation domain.

Data information: For (B, C), cells were treated with MMS 0.02% for 2 h. For (D, E, F), cells were incubated with MMS 0.033% for 2 h where indicated.

Source data are available online for this figure.

as well as mutation of 7 SQ/TQ sites (7MUT) (Fig 2A). We have previously shown that the S486A and the 7MUT mutations in Slx4 impair binding to Dpb11 and cause Rad53 hyperactivation and MMS sensitivity (Ohouo *et al*, 2010, 2013). If the major role of the

Dpb11-Slx4 interaction is indeed to bridge Slx4-Rtt107 to 9-1-1, the expectation is that the Dpb11-Slx4^{S486A/7MUT} chimera will rescue MMS sensitivity of *slx4Δ* cells back to wild-type levels. As predicted, this chimera was able to rescue the MMS sensitivity of an *slx4Δ*

Figure 2. A Dpb11-Slx4^{S486A/7MUT} chimera confers resistance to MMS treatment.

A Schematic illustration of the Dpb11-Slx4^{S486A/7MUT} chimera.
B Effect of the Dpb11-Slx4^{S486A/7MUT} chimera and the indicated mutants in the MMS sensitivity of an *slx4Δ* strain. Fourfold serial dilutions were spotted on SC-URA plates and grown for 2–3 days at 30°C.
C Immunoblots showing the phosphorylation status of Dpb11-HA (endogenously expressed copy) and Dpb11-Slx4^{S486A/7MUT} chimera-Flag after MMS treatment (0.02% for 2 h).
D, E Immunoblots showing the phosphorylation status of the Dpb11-Slx4^{S486A/7MUT} chimera-Flag in the indicated strains after MMS treatment (MMS 0.01% for 2 h).
 Source data are available online for this figure.

strain, bypassing the inability of Slx4^{S486A/7MUT} to bind to Dpb11 (Fig 2B) (see also Gritenaite *et al*, 2014). Interestingly, MMS sensitivity was rescued by a chimera carrying a mutation in BRCT-1 of Dpb11 (K55A) but not by a chimera with mutated BRCT-4 of Dpb11 (K544A). These results are consistent with the model that the Dpb11-Slx4^{S486A/7MUT} chimera rescues the MMS sensitivity of *slx4Δ* cells by bridging Rtt107 to 9-1-1 and displacing the Dpb11-Rad9 complex. Further supporting this model, we observed that both the Dpb11-Slx4^{S486A/7MUT} and Dpb11^{K55A}-Slx4^{S486A/7MUT} chimeras underwent extensive MMS-induced phosphorylation, as measured by reduced electrophoretic mobility, while the Dpb11^{K544A}-Slx4^{S486A/7MUT} chimera did not undergo reduced mobility shift (Fig 2C). We interpret this MMS-induced phosphorylation as an indication of recruitment of the chimera to sites of DNA lesions, as we previously showed that Mec1 and Rad53 extensively phosphorylate Dpb11 and Slx4 (Ohouo *et al*, 2010). Of importance, increased phosphorylation of the Dpb11-Slx4^{S486A/7MUT} chimera inversely correlated with phosphorylation of endogenous Dpb11 (Fig 2C), in agreement with the notion that recruitment of the chimera and the Dpb11-Rad9 complex is mutually exclusive. Finally, mutation of the phosphorylation site in H2A (S129) or in Ddc1 (T602), which are directly recognized by BRCT domains 5/6 of Rtt107 or BRCT domains 3/4 of Dpb11, respectively, significantly reduced the MMS-induced mobility shift of the chimera (Fig 2D and E). Taken together, these results are consistent with our proposed model for how Slx4-Rtt107 engage at sites of lesions.

A minimal multi-BRCT-domain module recapitulates the function of Slx4-Rtt107 in dampening Rad53 signalling

As shown in our working model (Fig 1A), the Dpb11-Rad9 and the Dpb11-Slx4-Rtt107 complexes are both anchored at a DNA lesion via two Mec1-targeted phospho-sites, Ddc1 T602 and H2A S129. To further test the notion that a key role of the Dpb11-Slx4-Rtt107 complex is to bridge 9-1-1 to H2A and compete out Rad9 from sites of lesions, we fused BRCT 5/6 of Rtt107 to BRCT 3/4 of Dpb11 (Fig 3A) with the prediction that this minimal multi-BRCT-domain module (herein referred as MBD) should compete out Rad9 and, consequently, rescue to some extent the MMS sensitivity of a strain lacking Slx4. Remarkably, expression of MBD fully rescued the MMS sensitivity of *slx4Δ* cells (Fig 3B), which correlated with decreased phosphorylation of Rad53, Dpb11 and Rad9, as well as reduced Dpb11-Rad9 interaction (Fig 3C and D). Of note, the mild sensitivity of *slx4Δ* cells to the UV-mimetic 4-NQO was also fully rescued by MBD (Supplementary Fig S3). Importantly, in these experiments, MBD expression was driven by the relatively weak *DPB11* promoter [Dpb11 protein is expressed at around 400 copies per cell (Mantiero *et al*, 2011)]. Furthermore, a point mutation in either pairs of BRCT domains in MBD was sufficient to prevent the ability of MBD to rescue the MMS sensitivity of *slx4Δ* cells (Fig 3E) and an alternative chimera containing the BRCT domain 1/2 of Dpb11 (instead of the BRCT domain 3/4) fused to BRCT 5/6 of Rtt107, herein referred as alt-MBD, could not rescue the MMS sensitivity of *slx4Δ* cells

Figure 3. A minimal multi-BRCT-domain module (MBD) recapitulates the DAMP mechanism.

A Schematic illustration of the MBD (Minimal multi-BRCT-domain module) chimera.

B Effect of MBD expression on the MMS sensitivity of *slx4Δ* cells.

C Immunoblot showing Rad53 phosphorylation status in *slx4Δ* cells expressing either MBD or Slx4 from a *pRS416* plasmid.

D CoIP between Dpb11 and Rad9 in wild-type cells after MMS treatment, in the presence or absence of a plasmid expressing MBD.

E Effect of expressing MBD or the indicated mutants on the MMS sensitivity of *slx4Δ* cells. Mutations in MBD indicated in parenthesis correspond to the equivalent residue in the full-length Dpb11 or Rtt107 proteins.

F CoIP of MBD-HA with Ddc1-Flag and Slx4-Flag upon MMS treatment.

G Counter-selection of indicated strains bearing an Slx4-expressing plasmid (pMBS213) containing the *URA3* gene in 5-FOA plates.

Data information: For (B, E), assay was performed as described in Fig 2B. For (C, D, F), cells were treated with MMS 0.02% for 2 h.

Source data are available online for this figure.

(Supplementary Fig S4). As predicted by our model, MBD was capable of robustly interacting with Ddc1, but not Slx4, in a CoIP experiment (Fig 3F). Finally, as Slx4 also plays established roles in DNA repair in conjunction with the structure-specific nuclease Slx1 (Fricke & Brill, 2003), we addressed whether MBD specifically

rescues the checkpoint regulatory function of Slx4 but not the Slx4 function in Slx1-mediated DNA repair. For that, we examined whether MBD is able to rescue the lethality caused by deletion of both *SLX4* and *SGS1*, a helicase also known to display synthetic lethality with Slx1 (Mullen *et al*, 2001). Our prediction was that

MBD would not rescue this synthetic lethality, for the following reasons: (i) while deletion of *SLX1* also results in synthetic lethality with *sgs1Δ*, Slx1 has no role in the DAMP mechanism (Ohouo *et al*, 2013); (ii) lack of Rtt107, a protein that is required for DAMP (Fig 1A), does not result in lethality in *sgs1Δ* cells (Zappulla *et al*, 2005). Indeed, we found that expression of MBD does not rescue the lethality of cells lacking *SLX4* and *SGS1* (Fig 3G). Taken together, these results support our model that Slx4 has an important role in checkpoint down-regulation that is independent from its role in Slx1-mediated DNA repair and show that the MBD-mediated rescue is dependent on the cooperative action of both BRCT 3/4 of Dpb11 and BRCT 5/6 of Rtt107, mimicking what occurs by the phospho-mediated assembly of the Dpb11-Slx4-Rtt107 complex. We therefore propose that a 'two-site-docking' mechanism established by cooperative interactions of two pairs of BRCT domains with two lesion-specific anchoring points (Ddc1-T602 and H2A-S129) promotes efficient recruitment of the Dpb11-Slx4-Rtt107 complex to sites of lesions to displace Rad9. The results are consistent with the idea that the cooperative nature of multi-BRCT domain interactions, as shown for MBD, is important for these modules to have a robust and highly specific effect at sites of lesions.

Modulation of Rad53 signalling via MBD is dosage dependent, localized and specific for the Rad9 branch of DDC signalling

To better understand additional features of MBD-mediated modulation of DDC signalling, we asked whether MBD action is dosage dependent. We expressed MBD with the *CPY* and *ADH1* promoters (Fig 4A), which are stronger than the *DPB11* promoter, and are referred here as C-MBD and A-MBD, respectively. As shown in Fig 4B, while C-MBD could still efficiently rescue MMS sensitivity of *slx4Δ* cells, A-MBD could only provide partial rescue. The high level of MBD expression through the *ADH1* promoter was correlated with stronger reduction in Rad53 and Rad9 phosphorylation (Fig 4C), and expression of A-MBD conferred enhanced MMS sensitivity in wild-type cells (Fig 4D). Also, we observed reduced levels of checkpoint-dependent Slx4 phosphorylation in wild-type cells expressing A-MBD (Supplementary Fig S5). Taken together, these results show that modulation of DDC signalling via MBD is dosage dependent and that increasing levels of MBD prevent a proper level of Rad53 activation to be reached.

Next, to address how specific the action of MBD is at DNA lesions, we monitored the effect of MBD expression on localization of Slx4, Dpb11 and Rad9 to sub-nuclear foci. Interestingly, MBD expression resulted in a decrease in the number of Dpb11 foci but did not affect the number of Rad9 or Slx4 foci (Fig 4E). This result is consistent with the notion that MBD is specifically impairing the recruitment of Dpb11, which is dependent on the 9-1-1 complex that is loaded at the 5' end of a double/single-stranded DNA junction. On the other hand, MBD expression does not impact Slx4-Rtt107 and Rad9 as they can also be recruited via phospho-H2A (Hammet *et al*, 2007; Li *et al*, 2012), which likely forms a long platform spreading kilobases around the site of lesion (Downs *et al*, 1999; Shroff *et al*, 2004). In addition, Rad9 can also be recruited via methylated H3K79 that is believed to be constitutively present throughout chromatin (Grenon *et al*, 2007). These findings reveal the specificity of MBD action and

support the model that the Dpb11-mediated engagement of Rad9 or Slx4 to the 9-1-1 complex is the key point of regulation during checkpoint dampening. Consistent with this model, we could detect a robust interaction between MBD and Ddc1 in a CoIP experiment (Fig 3F). Finally, to confirm the prediction that MBD is specifically and efficiently affecting the Rad9 branch of the pathway for Rad53 activation, we expressed MBD in *mrc1Δ* cells and noticed that it rendered cells extremely sensitive to replication stress (Fig 4F). It is well established that in *mrc1Δ* cells, the activation of the checkpoint is dependent on Rad9 (Alcasabas *et al*, 2001). Remarkably, overexpression of MBD with the *ADH1* promoter resulted in a strong growth defect in *mrc1Δ* cells even in the absence of genotoxins (Fig 4G), suggesting that the essential function of Rad53 is being affected. Collectively, these data further support the initial model depicted in Fig 1A and reveal key features of checkpoint dampening via a multi-BRCT-module that mimics the action of the Dpb11-Slx4-Rtt107 complex.

MBD modulates Rad53 signalling independently of Mus81-Mms4

Activation of Rad53 has been proposed to antagonize the function of the Mus81 nuclease (Szakal & Branzei, 2013). In this manner, Rad53 hyper-activation in *slx4Δ* cells would lead to persistent repression of Mus81 function. Consistent with this notion, we found that expression of MBD in *slx4Δ* cells rescues chromosomal defects seen by electrophoretic analysis of chromosome plugs after transient exposure to MMS (Fig 5A). This defect is characteristic of *mus81Δ* cells (Saugar *et al*, 2013), and in cells lacking *SLX4*, such defect has been attributed to inefficient resolution of joint molecules (JMs) formed between sister chromatids, with the idea that Slx4 helps facilitate Mus81 function (Gritenaite *et al*, 2014). Consistent with this view, we found that expression of MBD could not provide any rescue of MMS sensitivity in cells lacking both Slx4 and Mus81 (Fig 5B). Our findings therefore support that MBD is promoting Mus81 function by dampening Rad53 activation. It has been proposed that inhibition of Mus81 by Rad53 signalling is established indirectly, via regulation of the CDK and Cdc5 kinases (Szakal & Branzei, 2013). However, a recent report showed that Dpb11 can physically interact with the Mms4 subunit of the Mus81-Mms4 complex specifically during G2/M (Gritenaite *et al*, 2014). The authors proposed that formation of an Slx4-Rtt107-Dpb11-Mms4-Mus81 complex has a role in directly regulating the action of Mus81-Mms4 for the resolution of DNA repair intermediates formed in response to MMS treatment. We found that MBD can interact with Mms4 in G2/M cells (Fig 5C). Of note, a point mutation in MBD corresponding to K544A in Dpb11 (BRCT-4 of Dpb11) significantly reduced interaction of MBD with Mms4, indicating that the pair of BRCT 3/4 of Dpb11 possibly recognizes phosphorylated Mms4. Strengthening this notion, we could not detect Mms4 when pulling down alt-MBD (which has Dpb11 BRCT 1/2 instead of 3/4) (Supplementary Fig S6). These results, in combination with our data showing that Dpb11 BRCT 1/2 plays a prominent role in mediating the interaction with Slx4, help explain how an Slx4-Rtt107-Dpb11-Mms4-Mus81 complex is formed in G2/M. Furthermore, these results reveal the highly coordinated nature of the cell cycle-specific interactions that are mediated by the BRCT domains of Dpb11. Importantly, we decided to rule out the possibility that regulation of

Figure 4. Modulation of Rad53 signalling via MBD is dosage dependent, localized and specific for the Rad9 branch of DDC signalling.

A Immunoblot showing MBD-HA expression under the control of different promoters.

B Effect of increased MBD expression on the MMS sensitivity of *slx4* Δ cells.

C Immunoblots showing the effect of increased MBD expression on Rad53 and Rad9 phosphorylation status after MMS treatment.

D Effect of increased MBD expression on the MMS sensitivity of wild-type cells.

E Effect of MBD expression levels on the number of Dpb11, Rad9 or Slx4 foci. Foci were scored manually, and for the nuclear foci formation experiments, the means and standard deviations from three independent experiments are shown.

F Effect of C-MBD expression on the sensitivity of *mrc1* Δ cells to replication stress.

G Same number of WT or *mrc1* Δ cells were transformed with equimolar concentrations of vector control or vector for the expression of A-MBD and directly grown on SC-URA plates.

Source data are available online for this figure.

Rad53 activation via MBD expression could somehow be mediated by the interaction of MBD with Mms4. We monitored Rad53 activation in cells lacking Mus81 and/or Slx4 and found that MBD could still dampen checkpoint signalling (Fig 5D). These results show that

lack of Mus81 does not lead to Rad53 hyperactivation and that dampening of checkpoint signalling via MBD is independent of Mus81. Altogether, these findings support our working model of how Dpb11-Slx4-Rtt107 complex and MBD promote the DAMP

Figure 5. MBD dampens checkpoint signalling independently of Mus81 action.

A Asynchronous (ASY) cells were treated with 0.033% MMS for 2 h and then released in MMS-free media. Recovery of fully replicated chromosomes was analysed by pulse-field gel electrophoresis (PFGE).
B Effect of MBD expression on MMS sensitivity of indicated cells.
C CoIP experiment showing the binding of Mms4 to MBD or MBD containing a mutation (corresponding to K544A in Dpb11) shown to destabilize BRCT-4. Cells were grown until log phase and then arrested in metaphase with nocodazole.
D Immunoblot showing Rad53 phosphorylation status after MMS treatment (0.01% for 2 h) in the indicated strains expressing or not MBD.
 Source data are available online for this figure.

mechanism. It remains to be determined whether the ability of MBD to interact with Mms4 has any role in the resolution of MMS-induced JMs independently of Rad53 regulation.

Temporal dynamics of DAMP

While both MBD and the 'physiological' Dpb11-Slx4-Rtt107 complex are able to promote the DAMP mechanism, a key difference between them is that formation of the later module is regulated. The Slx4-Dpb11 interaction is dependent on CDK and checkpoint-mediated phosphorylation events (Ohouo *et al*, 2010, 2013; Gritenaite *et al*, 2014), which are expected to impact establishment of the cooperative BRCT-mediated interactions and recruitment to sites of lesions. For example, stronger binding of Slx4 to Dpb11 should result in more cooperative binding to phosphorylated Ddc1-T602 and H2A-S129 at lesions and more efficient recruitment. On the other hand, MBD does not require phosphorylation to assemble a module capable of docking at both phosphorylated Ddc1-T602 and H2A-S129. As Slx4 requires CDK phosphorylation that builds up during S-phase to interact with Dpb11, we predicted that 'physiological' DAMP (via phospho-dependent Slx4-Dpb11 interaction) occurs later than a

'synthetic' DAMP (via MBD). Temporal analysis of the dynamics of Rad53 activation during S-phase progression in the presence of MMS showed that this is indeed the case, as Rad53 activation was delayed in MBD expressing cells (Fig 6A). Notably, overexpression of MBD led to a stronger impairment of Rad53 activation.

To gain a better understanding of the temporal coordination of the events during 'physiological' DAMP, we monitored the Dpb11-Rad9 and Dpb11-Slx4 interactions in synchronized cells during replication stress response. We were able to detect the Rad9-Dpb11 interaction very early in S-phase, as early as 20 min following release of cells from an alpha-factor arrest (Fig 6B). On the other hand, we could only detect the Dpb11-Slx4 interaction 25 min after release. Importantly, at later time points, we could observe a decrease in the Dpb11-Rad9 interaction, whereas the Dpb11-Slx4 interaction was sustained. This result suggests that there is a temporal coordination of Dpb11 interactions as replication forks encounter MMS-induced lesions. A conceivable model is that Dpb11 first binds to Rad9 to activate Rad53 at fork-proximal regions, and as forks bypass and progress far from the lesion site, Slx4 and Rtt107 are then recruited to displace Rad9 that is engaged onto the 9-1-1 complex (see working model in Supplementary Fig S7). Consistent

Figure 6. Temporal dynamics of DAMP.

- A Dynamics of Rad53 activation during S-phase in the presence of MBD. *slx4Δ* cells carrying plasmids expressing Slx4, MBD or A-MBD were arrested with alpha-factor and then released in media containing 0.015% MMS. Cells were collected at different time points following release.
- B Temporal dynamics of the Dpb11-Rad9 and Dpb11-Slx4 interactions during early S-phase. Cells were arrested with alpha-factor and then released in media containing 0.02% MMS. Cells were collected starting 20 min following release, which coincides with the start of DNA synthesis.
- C CoIP between Dpb11 and Rad9 or Slx4 from cells treated with 0.04% MMS for 1 h.
- D Effect of the S462A and T474A mutations in Rad9 (known to disrupt Rad9 binding to Dpb11) on the MMS sensitivity of cells expressing the *slx4-S486A* mutant.
- E Alpha-factor-arrested cells were released in MMS 0.02% for 45 min and then transferred to MMS-free media containing nocodazole for the indicated times. MBD was expressed from a plasmid (*pMB5798*) and monitored for its interaction with Ddc1 and Mms4 using CoIP.

Source data are available online for this figure.

with this model and the idea that Slx4 displaces Rad9, we could detect that the amount of Dpb11 co-immunoprecipitated with Slx4 is at least 10 times higher than the amount of Dpb11 co-immunoprecipitated with Rad9 after MMS treatment for 60 min (Fig 6C). The overall abundance of Rad9 in the cell is higher than that of Slx4 (see input in Supplementary Fig S1A), strengthening the notion that 60 min in the replication stress response, Slx4 more avidly interacts

with Dpb11 than Rad9 does. The importance of Slx4 in counteracting the action of the Dpb11-Rad9 complex is further supported by the fact that mutation of the two phospho-sites in Rad9 (S462 and T474) that mediate interaction with Dpb11 leads to the rescue of the MMS sensitivity of cells carrying the S486 mutation in Slx4 (Fig 6D). Following checkpoint dampening, additional coordination of Dpb11 interactions is apparently established, as the Dpb11-Slx4-Rtt107

Figure 7. Dpb11 as a central multi-BRCT-domain module in the coordination of replication initiation, checkpoint signalling and DNA repair. See Discussion for details.

complex was reported to interact with Mus81-Mms4 in G2/M (Gritenaite *et al*, 2014). As we have shown that both Ddc1 and Mms4 are recognized by BRCT 3/4 of Dpb11 (for Ddc1 see Supplementary Fig S1B and Fig 1F, for Mms4 see Fig 5C), we predicted that as the Dpb11-Mms4 interaction increases in G2/M, the Dpb11-Ddc1 interaction should decrease. To test this, we used MBD (which only has BRCT 3/4, but lacks BRCT 1/2 of Dpb11) as bait in experiments with cells arrested in MMS and released from MMS treatment in media containing nocodazole. While we could detect an increase in the MBD-Mms4 interaction following release of cells from MMS treatment into media containing nocodazole, the MBD-Ddc1 interaction was reduced (Fig 6E). This is consistent with the model that these Dpb11 interactions are mutually exclusive. Overall, the findings reported here support the working model depicted in Fig 1A for how the DAMP mechanism is established and help elucidate how Dpb11 may coordinate DDC signalling and DNA repair (Fig 7).

Discussion

Despite the importance of DDC for cell survival and genome maintenance, the inability to properly down-regulate a checkpoint response can severely compromise cell growth. Given the importance of DDC down-regulation, multiple mechanisms exist to modulate and fine-tune the activation state of checkpoint kinases. Our recent finding of the DAMP mechanism provides an example of a phosphatase-independent mechanism for counteracting checkpoint signalling (Ohouo *et al*, 2013). Here, we have examined the molecular requirements for this new mechanism of checkpoint regulation. We demonstrate that we can engineer a minimal BRCT-domain module that recapitulates the action of the Dpb11-Slx4-Rtt107 complex in down-regulating Rad53 signalling. The availability of this minimal module allowed us to rigorously test our working model for how DAMP works. Our findings support the existence of a competition-based mechanism through which Slx4 and Rtt107 counteract the checkpoint adaptor Rad9 to dampen checkpoint signalling.

DAMP is established via the cooperative action of BRCT domains

The fact that the minimal MBD module, which lacks any *SLX4* sequence, can fully rescue the MMS sensitivity of *slx4Δ* cells (Fig 3B and E) strongly supports that BRCT domain-mediated interactions are the minimal requirement for establishing a DAMP mechanism. Our results are consistent with the notion that the well-established interactions of Rtt107's BRCT 5/6 with phospho-H2A (Williams *et al*, 2010; Li *et al*, 2012) and Dpb11's BRCT 3/4 with phospho-Ddc1 (Wang & Elledge, 2002; Puddu *et al*, 2008; Pfander & Diffley, 2011) are directly enforcing DAMP. Importantly, point mutations that disrupt only one of these BRCT interactions severely compromise the ability of the Dpb11-Slx4^{S486A/7MUT} chimera to be robustly phosphorylated and to prevent phosphorylation of endogenous Dpb11 in response to DNA damage (Fig 2C–E), likely reflecting the inability of the mutated chimera to be efficiently recruited to the site of lesion. Furthermore, disruption of one of the BRCT domains of the MBD is enough to abolish its ability to down-regulate Rad53 signalling (Fig 3E). We therefore concluded that the combined action of these BRCT domain interactions confer the ability of MBD to efficiently compete with Rad9 and down-regulate DDC. In line with these findings, physiological DAMP would be established by the formation of a Dpb11-Slx4-Rtt107 complex capable of interacting with the phospho-H2A and phospho-Ddc1 anchoring points in a cooperative manner, similar to MBD. It is tempting to speculate that the coordination of these interactions provides advantages for precise spatio-temporal control of Slx4-Rtt107 recruitment. Because phospho-H2A may spread through kilobases around the site of lesion, while 9-1-1 is specifically loaded at the 5' end of primer-template junction at the lesion site, we speculate that a long tract of phospho-H2A at chromatin serves as a platform for initial recruitment and to guide Dpb11-Slx4-Rtt107 (or MBD) to the lesion site. Interestingly, Rad9 is recruited through a similar mechanism, but because the Dpb11-Rad9 interaction is presumably more transient than the Dpb11-Slx4 interaction, the latter will be able to be more efficiently recruited.

A spatio-temporal model for DAMP

It is important to mention that the work presented here is focused on the response to MMS treatment, which generates replication blocks that can be bypassed by a moving replication fork, leaving the lesion and an ssDNA gap behind the fork (Branzei & Foiani, 2010). As exemplified in our detailed working model (Supplementary Fig S7), we propose that early in the response, as forks encounter a lesion, the Rad9 adaptor is rapidly recruited to the site of lesion to mediate Rad53 activation. At this initial stage, key events include the recruitment of Mec1 to the lesion and subsequent phosphorylation of histone H2A and Ddc1 by Mec1 to create the anchoring sites for the recruitment of Dpb11 and Rad9. We speculate that the ability of Rad9 to be constitutively bound to chromatin via the H3K79 methylation mark enables Rad9 to be rapidly recruited to the phospho-H2A platform and, subsequently, to the site of lesion. In contrast, Slx4 and Rtt107 do not recognize methylated H3K79, which may explain why these proteins are recruited later on (Fig 6B). Following checkpoint activation, we propose that Slx4 and Rtt107 become more efficiently recruited and able to compete out Rad9 due to a presumably stronger interaction with Dpb11 (Fig 6C). The rapid formation of the Rad53-activating complex (Ddc1-Dpb11-Rad9) at the fork is likely important for rapidly inhibiting nucleases, such as Exo1, from processing fork structures (Morin *et al*, 2008), in addition to arresting the cell cycle and inhibiting late origin firing. Once the replication fork bypasses the lesion and recombination-dependent intermediates accumulate (such as intermediates derived from template-switching events), checkpoint down-regulation would be important to allow cell cycle progression and proper execution of mitotic events such as the resolution of JMs via the Mus81 nuclease. Therefore, we propose that DAMP occurs post-replicatively to disengage Rad9 from the site of lesion and disrupt the Ddc1-Dpb11-Rad9 complex. Consistent with this idea, recent ChIP-seq data show that Slx4 and Rtt107 accumulate on chromatin behind replication forks as they progress through the genome of MMS-treated cells (A Balint, T Kim, D Gallo, JR Cussiol, FB Oliveira, A Yimit, J Ou, R Nakato, A Gurevich, K Shirahige, MB Smolka, Z Zhang, G Brown, manuscript submitted).

The Slx4-Dpb11 interaction facilitates Mus81-Mms4 action

Cells that lack DAMP (such as *slx4Δ* cells) are hypersensitive to MMS and accumulate JMs (Mullen *et al*, 2001; Gritenaite *et al*, 2014). This is consistent with the idea that a major deleterious effect of Rad53 hyperactivation upon MMS treatment is the mis-regulation of the Mus81 nuclease, which has a prominent role in the resolution of MMS-induced JMs (Saugar *et al*, 2013; Szakal & Branzei, 2013). Indeed, DDC has been shown to inhibit Mus81 action, with the prevailing model that it negatively affects the ability of the Cdc5 polo-like kinase to phosphorylate Mms4, a cofactor of Mus81, and activate the nuclease activity of Mus81. In this scenario, the DAMP mechanism would facilitate Mus81 action by alleviating the inhibitory action of DDC on Cdc5. Recently, it has been shown that Dpb11 can interact with Mms4 specifically in G2/M phase (Gritenaite *et al*, 2014), suggesting an additional regulatory mechanism by which the Dpb11-Slx4 interaction may help promote the resolution of JMs following DNA replication. Here, we showed that the DAMP mechanism is independent of Mus81 as MBD can down-regulate Rad53

activation even in *mus81Δ* cells (Fig 5D). However, we also detected an interaction between MBD and Mms4 (Fig 5C), supporting that Mus81-Mms4 may also be regulated by an interaction with Dpb11. Nonetheless, the extent by which the Dpb11-Mms4 interaction contributes to promoting Mus81 action is currently unclear, as a separation of function mutant of Mms4 that cannot interact with Dpb11 has not been generated. Furthermore, it remains to be defined whether BRCT 3/4 of Dpb11 is directly recognizing phosphorylated motifs in Mms4 or is actually binding Mms4 via another protein.

Coordination of DDC signalling and DNA repair via combinatorial Dpb11 interactions

Collectively, the findings reported here provide insights on how Dpb11 functions in the coordination of DDC and DNA repair. By defining which pairs of BRCT domains of Dpb11 recognize the Slx4-Rtt107 and the Mus81-Mms4 complexes, our work reveals the architecture of distinct Dpb11-mediated complexes (Fig 7). Interestingly, our findings imply that several Dpb11 interactions are mutually exclusive and we speculate that this feature is important for Dpb11 to ensure that distinct cellular events, such as checkpoint signalling and Mus81-mediated JM resolution, are executed in a mutually exclusive manner. As shown here, Dpb11 binds Rad9 and Slx4 through the same pair of BRCT domains, preventing Slx4 from participating in Rad53 activation. In this scenario, the Dpb11-Slx4 interaction establishes checkpoint dampening in preparation for JM resolution. It is tempting to further speculate that the mutually exclusive Ddc1-Dpb11 and Mms4-Dpb11 interactions (established via BRCT 3/4) also provide an additional safeguard mechanism to spatio-temporally separate checkpoint signalling from JM resolution. Such separation would be important to prevent Mus81 from prematurely acting on replication forks structures or DNA repair intermediates during S-phase, as recently proposed (Szakal & Branzei, 2013; Gritenaite *et al*, 2014). Furthermore, Dpb11 has been previously shown to interact with Sld3 and Sld2 via BRCT domains 1/2 and 3/4, respectively, to initiate replication (Tak *et al*, 2006; Tanaka *et al*, 2007; Zegerman & Diffley, 2007). Therefore, it is also conceivable that mutually exclusive Dpb11-Sld3 and Dpb11-Rad9 interactions, established via BRCT 1/2, play a role in ensuring that origin firing and checkpoint activation occur in a mutually exclusive manner. Interestingly, the Dpb11 ortholog in mammals, TOPBP1, is also involved in replication initiation and checkpoint signalling (Wardlaw *et al*, 2014), and the TOPBP1-SLX4 interaction was recently found to be conserved in humans (Gritenaite *et al*, 2014). It will be interesting to extend the findings from our work to understand how TOPBP1 operates in the coordination of replication initiation, DDC and DNA repair in mammals. Finally, the implications of this work extend beyond TOPBP1, as the results could help understand the mechanism for spatio-temporal control of other multi-BRCT complexes.

Materials and Methods

Yeast strains and plasmids

Strains generated in this study were derived either from MBS164 or MBS191 (both congenic to S288C), BY4741 or W303 (where

indicated). All tags were inserted at the C-terminus of the corresponding genes by homologous recombination at the genomic locus and were verified by Western blotting. Tagged strains were assayed for sensitivity to MMS to ensure they behaved similarly to the wild-type strain. Standard cloning methods were used to generate the plasmids for this study. *DPB11* or *MBD* constructs containing a *CPY* or an *ADH1* promoter were generated by fusing the respective promoters (800 base pairs upstream of the start codon) to the corresponding open reading frame. The resulting PCR products were subsequently cloned into the *pRS416* vector (pMBS148). All point mutations were generated by site-directed mutagenesis using either the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) or the Primestart[®] Max DNA Polymerase (Takara). All yeast strains and plasmids used in this study are described in Supplementary Tables S1 and S2, respectively.

Western blot analysis

Rad53 and epitope-tagged proteins were probed using specific monoclonal antibodies: anti-Rad53 (EL7, gift from Dr. Achille Pelliccioli), anti-FLAG (M2; Sigma), anti-HA (12CA5; Roche), anti-Myc (9E10; Santa Cruz Biotechnology), ECL HRP-Linked Secondary Antibodies (NA931-GE).

Cell culture and pull-down procedure for co-immunoprecipitation experiments

Cells with the indicated epitope tags were grown until log phase in YPD and treated with MMS (concentration and incubation time used for each experiment are described in the figure legends). In case of strains carrying the *pRS416* plasmids, cells were grown in synthetic complete medium lacking uracil (SC –URA). After centrifugation, pellets were washed with TE + PMSF and kept at –80°C prior to cell lysis. Approximately 0.1 g of cell pellet of each strain was lysed by bead beating at 4°C in 1 ml of lysis buffer [50 mM Tris–HCl pH 7.5, 0.2% Tergitol, 150 mM NaCl, 5 mM EDTA, Complete EDTA-free protease inhibitor cocktail (Roche), 5 mM sodium fluoride, 10 mM B-glycerol-phosphate]. Samples were normalized by protein concentration, and lysates were incubated with either anti-HA, anti-Flag or anti-Myc agarose resin (Sigma) for 2 h at 4°C. After three washes with lysis buffer, bound proteins were eluted with three resin volumes of elution buffer (for anti-HA and anti-Myc: 100 mM Tris–HCl pH 8.0, 1% SDS; for anti-FLAG: 0.5 µg/ml of FLAG peptide in 100 mM Tris, 0.2% Tergitol).

5-FOA counter-selection

Cells carrying a plasmid containing the *URA3* auxotrophic marker were grown in SC –URA media until saturation. Cells were then normalized by OD, streaked onto SC + 5-FOA plates (1 mg/ml, Zymo Research F9001-1) and allowed to grow for 48 h at 30°C.

Cell cycle synchronization

Cells were grown in YPD or SC –URA media at 30°C until log phase. For arrest of cells in G1, α -factor (Zymo Research) was added to a final concentration of 30 ng/ml (for *bar1Δ* background strains) and incubated for 2 h. To release cells from G1 arrest, cells were

centrifuged and resuspended in fresh media in the presence of pronase (50 µg/ml). For arrest of cells in G2/M, nocodazole (Calbiochem #487928) was added to a final concentration of 7.5 µg/ml and cells were incubated for 2 h. To attest whether the arrest was successful, flow cytometry (FACS) analysis of yeast cell DNA content were performed using a C6 Flow Cytometer[®] (BD Biosciences).

Pulse-field gel electrophoresis

Cells were transformed with either an empty vector [*pRS416* (pMBS148)], *pSLX4* (pMBS213) or *pMBD* (pMBS798) plasmids and allowed to reach log phase in SC –URA media. An untreated, asynchronous sample (ASY) was taken for control. Cells were then treated with 0.033% MMS for 2 h and then recovered in MMS-free media for up to 6 h. At each indicated time point, 50 mg of cells were collected. Pulse-field gel electrophoresis (PFGE) analysis was adapted from Argueso *et al* (2008).

For more details of PFGE, confocal fluorescence microscope analysis and pull-down of recombinant BRCT domains, please see Supplementary Materials and Methods.

Supplementary information for this article is available online: <http://emboj.emboipress.org>

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Author contributions

MBS and JRC conceived and designed experiments. JRC, CMJ and AY performed the experiments. MBS, JRC and GWB analysed the data. MBS and JRC wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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