

Dpb11 coordinates Mec1 kinase activation with cell cycle-regulated Rad9 recruitment

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Eukaryotic cells respond to DNA damage by activating checkpoint signalling pathways. Checkpoint signals are transduced by a protein kinase cascade that also requires non-kinase mediator proteins. One such mediator is the *Saccharomyces cerevisiae* Dpb11 protein, which binds to and activates the apical checkpoint kinase, Mec1. Here, we show that a ternary complex of Dpb11, Mec1 and another key mediator protein Rad9 is required for efficient Rad9 phosphorylation by Mec1 *in vitro*, and for checkpoint activation *in vivo*. Phosphorylation of Rad9 by cyclin-dependent kinase (CDK) on two key residues generates a binding site for tandem BRCT repeats of Dpb11, and is thereby required for Rad9 recruitment into the ternary complex. Checkpoint signalling via Dpb11, therefore, does not efficiently occur during G1 phase when CDK is inactive. Thus, Dpb11 coordinates checkpoint signal transduction both temporally and spatially, ensuring the initiator kinase is specifically activated in proximity of one of its critical substrates.

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Introduction

Lesions in DNA arising from extrinsic and intrinsic sources can compromise the integrity of genetic information and cause cell death. In eukaryotes, the DNA damage checkpoint modulates many aspects of the cellular program in response to DNA lesions (Melo and Toczyski, 2002; Harrison and Haber, 2006). Checkpoint signalling involves a protein kinase cascade initiated by one of the two apical kinases of the phosphoinositide 3 kinase-related kinases (PIKK) family. In *Saccharomyces cerevisiae*, these kinases are Mec1 and Tel1 (homologous to vertebrate ATR and ATM, respectively). They phosphorylate and activate the effector kinases Rad53 (in vertebrates, Chk2) and Chk1. Conserved, non-kinase mediator proteins of the DNA damage checkpoint pathway include the BRCT domain-containing Rad9 and Dpb11 pro-

teins and the PCNA-like Ddc1–Mec3–Rad17 (9-1-1) complex (Parrilla-Castellar *et al*, 2004; Garcia *et al*, 2005; FitzGerald *et al*, 2009).

The DNA damage checkpoint must respond to a very wide variety of DNA lesions. The apical kinases, however, do not sense lesions directly, but are recruited via interactions with other proteins that either bind directly to lesions or to processed intermediates. Mec1 is recruited to ssDNA at stalled replication forks or resected DSBs by interactions with RPA (Rouse and Jackson, 2002; Zou and Elledge, 2003; Ball *et al*, 2005, 2007). Separately, the 9-1-1 complex is loaded at ss-ds-DNA junctions by the Rad24-clamp loader complex (Ellison and Stillman, 2003; Majka and Burgers, 2003; Majka *et al*, 2006a) where it acts as a co-sensor of DNA damage (Bonilla *et al*, 2008).

Mediators are recruited to sites of DNA damage by a complex network of interactions. The 9-1-1 complex plays a role in recruiting Dpb11 and its orthologues to sites of DNA damage (Furuya *et al*, 2004; Delacroix *et al*, 2007; Lee *et al*, 2007; Puddu *et al*, 2008). In budding yeast, this recruitment involves Mec1 phosphorylation of the 9-1-1 complex subunit Ddc1, which generates a binding site for the second pair of phospho-protein binding BRCT repeats (BRCT3&4) of Dpb11 (Wang and Elledge, 2002; Puddu *et al*, 2008).

Recruitment of Rad9 to sites of DNA damage involves multiple interactions. One pathway depends on histone modifications: Rad9 can bind via its BRCT repeat domain to histone H2A that has been phosphorylated by Mec1 or Tel1 (γ H2A) (Hammet *et al*, 2007). It also binds lysine 79 methylated histone H3 via its TUDOR domain (Grenon *et al*, 2007). This modification is catalysed by the Dot1 methyltransferase (Ng *et al*, 2002; van Leeuwen *et al*, 2002), but currently there is no evidence that this modification is regulated in response to DNA damage. Both unphosphorylatable H2A mutants and *dot1Δ* mutants show defects in checkpoint activation during G1 phase (Giannattasio *et al*, 2005; Wysocki *et al*, 2005; Grenon *et al*, 2007; Hammet *et al*, 2007).

In contrast to G1 checkpoint activation, G2/M checkpoint activation occurs in *dot1Δ* cells (Giannattasio *et al*, 2005). However, checkpoint activation is abolished in *dot1Δ dpb11-1* double mutants suggesting that an additional, alternative mode of Rad9 recruitment may involve Dpb11 (Puddu *et al*, 2008). This idea is supported by the finding that Crb2, the fission yeast homologue of Rad9, interacts with Cut5/Rad4, the homologue of Dpb11 (Mochida *et al*, 2004; Du *et al*, 2006). This interaction is regulated by cyclin-dependent kinase (CDK) phosphorylation of Crb2 and facilitates histone-independent Crb2 recruitment to DNA damage foci (Esashi and Yanagida, 1999; Nakamura *et al*, 2004; Du *et al*, 2006). After phosphorylation by Mec1 Rad9 creates a platform for the recruitment of Rad53 (Emili, 1998; Sun *et al*, 1998; Vialard *et al*, 1998; Schwartz *et al*, 2002).

Another feature of checkpoint signalling is the activation of the apical kinase by activator proteins. Mec1 and its homologue ATR are stimulated by binding to Dpb11 and

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TopBP1, respectively (Kumagai *et al.*, 2006; Mordes *et al.*, 2008; Navadgi-Patil and Burgers, 2008). Mec1 is also stimulated by binding to the Ddc1 subunit of 9-1-1 (Majka *et al.*, 2006b). It is not sufficiently understood how relevant this stimulation is for checkpoint signalling *in vivo* and especially where in the signalling cascade these activators become important. In this paper, we show that Dpb11 plays an important role in three aspects of checkpoint signalling: cell-cycle regulation, mediator recruitment and Mec1 kinase activation. Dpb11 integrates these functions through formation of a ternary checkpoint complex.

Results

Interactions between Dpb11 and checkpoint proteins

To identify Dpb11 interacting proteins, we purified recombinant, full-length, His-tagged Dpb11 via Ni²⁺-NTA agarose beads after incubation with whole cell extracts from asynchronous yeast cultures. We identified Mec1, Ddc2 and Rad9 as specific Dpb11 interactors by mass spectrometry (Figure 1A). We next repeated the pull-down experiment with different domains of Dpb11 fused to GST (see Figure 1D). Figure 1B shows that Rad9 from yeast extracts binds to N-terminal BRCT1&2 domain (aa 1–275) but not to BRCT3&4 (aa 276–600) or the C-terminal fragment of Dpb11 (aa 556–764) (Figure 1B). The Rad9 and Dpb11 orthologues in fission yeast have previously been found to interact (Mochida *et al.*, 2004; Du *et al.*, 2006), which suggests evolutionary conservation.

Similarly to results published by the Burgers and Cortez laboratories (Mordes *et al.*, 2008; Navadgi-Patil and Burgers, 2008), we observe that the C-terminal domain of Dpb11 is both necessary and sufficient to mediate the binding to Mec1 and Ddc2 (Figure 1C). Consistent with the fact that BRCT repeats are not involved, the interaction does not depend on phosphorylation of Mec1–Ddc2 and appears not to be regulated during the cell cycle or in response to DNA damage (Supplementary Figure S1). Together with previous work showing that Dpb11 also interacts with Mec1-phosphorylated Ddc1 via BRCT3&4 (Wang and Elledge, 2002; Puddu *et al.*, 2008), Figure 1D summarizes interactions between Dpb11 and DNA damage checkpoint proteins.

The C-terminus of Dpb11 affects Mec1 signalling in vitro and in vivo via conserved aromatic residues

Until now checkpoint studies have generally used the *dpb11-1* allele, which introduces a STOP codon in place of W583. However, in addition to defects in interacting with Mec1–Ddc2, this mutant is also thermosensitive and shows strongly reduced binding of the BRCT3&4 interactors Sld2 and Ddc1 (Kamimura *et al.*, 1998; Wang and Elledge, 2002). Since the Sld2–Dpb11 interaction is required for replication initiation and a reduced initiation frequency can mimic a checkpoint defect (Shimada *et al.*, 2002; Tercero *et al.*, 2003), we felt it was important to generate true separation of function mutants. Constructs truncated upstream of W583 were lethal; however, a truncation at amino acid 600 (*dpb11ΔC*) in the endogenous *DPB11* gene resulted in a viable haploid strain. FACS analysis of *WT* and *dpb11ΔC* cells released from α -factor arrest showed very similar replication profiles (Figure 2A). Furthermore, in contrast to *dpb11-1*, we did not observe any temperature sensitivity associated with *dpb11ΔC* (Figure 2B).

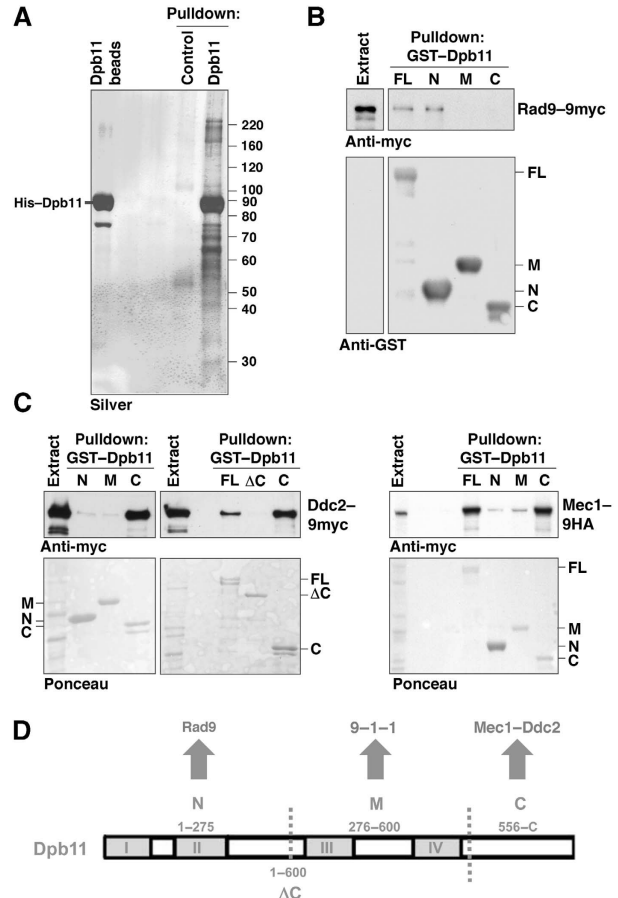


Figure 1 Dpb11 physically interacts with the DNA damage checkpoint proteins Mec1–Ddc2 and Rad9. **(A)** Pull-down of Dpb11 protein interactors with purified His-Dpb11 and cell lysates of asynchronously dividing yeast. MS analysis showed the presence of Mec1 (>220 kDa), Rad9 (>160 kDa) and Ddc2 (=90 kDa). **(B)** Rad9 interacts with the N-terminus of Dpb11. Pull-down experiment with immobilized GST-Dpb11 or GST-Dpb11 fragments (N = aa 1–275, M = aa 276–600, C = 556–764; see **D**) and whole cell extracts of yeast containing Rad9-9myc. **(C)** The C-terminus of Dpb11 contains an interaction site for Mec1–Ddc2. Pull-down with GST-Dpb11 or GST-Dpb11 fragments (see **D**) and extracts containing Ddc2-9myc or Mec1-9HA. **(D)** Schematic diagram of Dpb11 domains and interactors involved in the DNA damage checkpoint. BRCT repeat domains (I–IV) are marked as grey boxes.

Therefore, *dpb11ΔC* appears to be functional for DNA replication.

We next examined checkpoint responses in cells expressing the *dpb11ΔC* mutant treated with phleomycin. Figure 2C shows that these cells, synchronized in G2/M phase, showed no significant defect in either cell survival or Rad53 activation (Figure 2C). Previous work has shown that *dpb11-1* has a G2/M checkpoint defect only when combined with deletion of *DOT1* (Puddu *et al.*, 2008). When *dpb11ΔC* was combined with *dot1Δ*, we observed a deficient G2/M checkpoint as indicated by reduced phosphorylation of Rad53 and survival after DNA damage treatment (Figure 2C and D). These defects are not as severe as the defect seen in a *rad9Δ* mutant (Figure 2C; Supplementary Figure S2A). The checkpoint defect in *dpb11ΔC dot1Δ* is also less severe than that seen in the *dpb11-1 dot1Δ* double mutant at the permissive temperature for *dpb11-1* (Supplementary Figure S2C and D). This

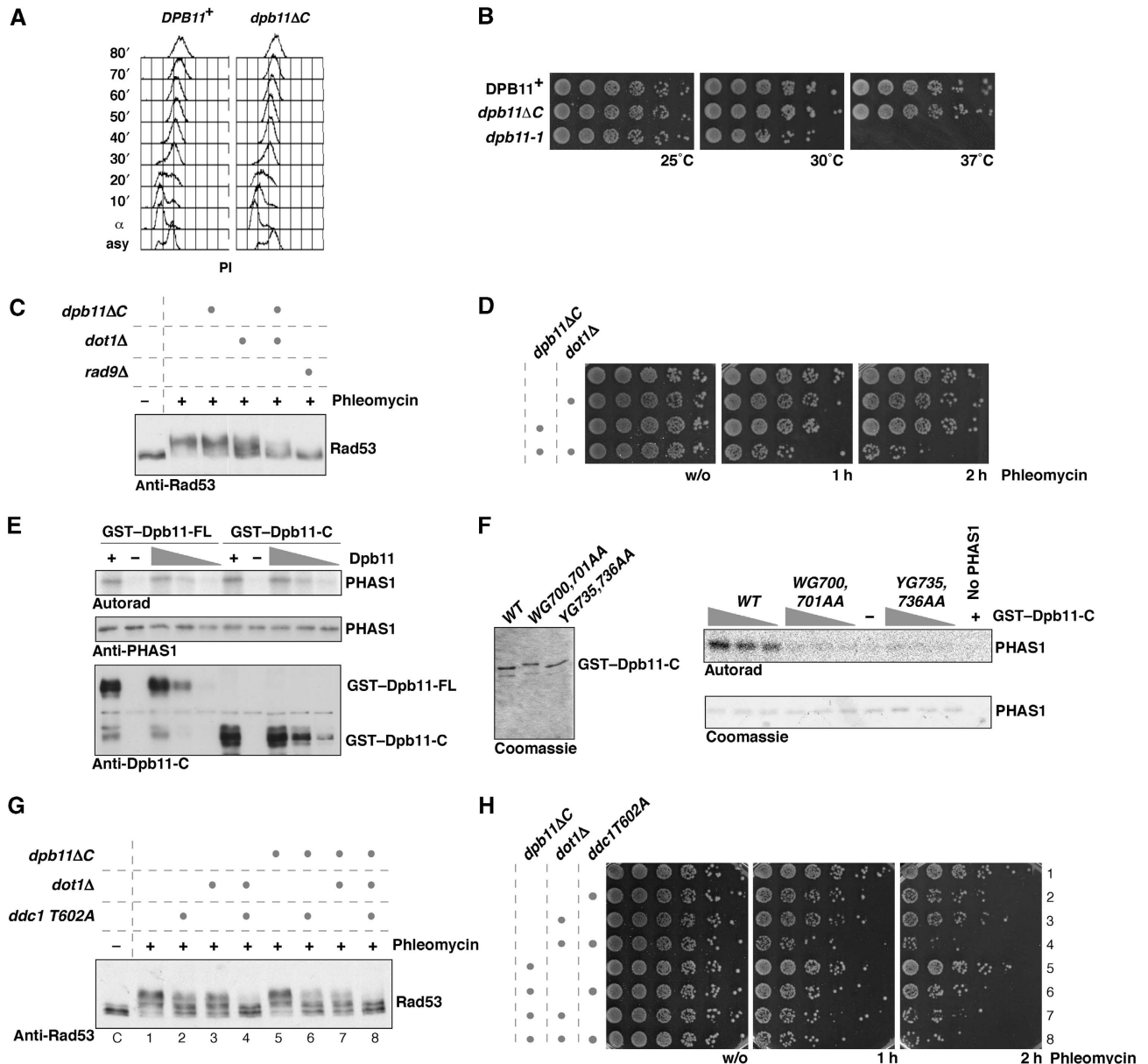


Figure 2 The C-terminal domain of Dpb11 is dispensable for DNA replication, but in the absence of *DOT1* is required for the G2/M DNA damage checkpoint *in vivo* and stimulates the Mec1 kinase via two W/YG motifs *in vitro*. (A) The C-terminus of Dpb11 is not required for DNA replication, since *WT* and *dpb11ΔC* cells synchronously released from G1 arrest (α) show identical replication profiles. (B) The *dpb11ΔC* mutant, in contrast to *dpb11-1*, is not temperature sensitive. (C, D) The *dpb11ΔC* mutation results in a G2/M DNA damage checkpoint defect in the absence of *DOT1*, as indicated by reduced Rad53 phosphorylation (C) and loss of viability (D) after treatment with phleomycin (50 μ g/ml). (C) Samples were taken before (–) or 30' after (+) addition of phleomycin. (E) The C-terminal domain of Dpb11 is sufficient for stimulation of Mec1 kinase *in vitro*. Mec1–18myc–Ddc2 phosphorylation towards the model substrate PHAS1 is stimulated by GST–Dpb11 or GST–Dpb11-C (555–764). (F) Stimulation of Mec1 kinase requires two W/YG motifs. GST–Dpb11-C (555–764) and mutant versions *WG700,701AA* and *YG735,736AA* of comparable amount and purity (left) were used to activate Mec1 kinase *in vitro* (right). (G, H) The checkpoint phenotypes of the *dpb11ΔC* and *ddc1-T602A* mutants are epistatic. Strains harbouring indicated combinations of mutations were analysed as in (C) and (D).

may be due to defects in DNA replication in the *dpb11-1* mutant that may exacerbate defects in checkpoint activation. Alternatively, this may be because Dpb11-1 is unable to bind both Ddc1 and Mec1–Ddc2 (Wang and Elledge, 2002; Mordes *et al*, 2008; Navadgi-Patil and Burgers, 2008), while Dpb11ΔC is only deficient in Mec1–Ddc2 interaction (Figure 1C).

Dpb11 and TopBP1 can stimulate the kinase activities of Mec1 and ATR, respectively, *in vitro* (Kumagai *et al*, 2006; Mordes *et al*, 2008; Navadgi-Patil and Burgers, 2008). As shown in Figure 2E, we also observed significant stimulation

of immunopurified Mec1–Ddc2 kinase activity by recombinant Dpb11, and the C-terminal domain of Dpb11 is sufficient for this activation (Figure 2E; Mordes *et al*, 2008). Although it has been reported that Dpb11 does not contain sequences related to the ATR activation domain (AAD) of TopBP1 (Mordes *et al*, 2008), we noticed that sequence homology among Dpb11 C-termini from *Saccharomyces sensu lato* was restricted to two patches of amino acids surrounding conserved W/YG motifs (Supplementary Figure S2B). Because a tryptophan residue is critically important in the TopBP1 AAD

(Kumagai *et al.*, 2006), we examined the effect of mutating these residues on the interaction with and activation of Mec1–Ddc2. Both Dpb11-WG700,701AA and Dpb11-W700A were unable to interact with Mec1–Ddc2 and Dpb11 YG735,736AA showed a reduced interaction (Supplementary Figure S2E). Moreover, individual mutation of the W/YG motifs strongly reduced the stimulatory effect of Dpb11 on the kinase activity of Mec1–Ddc2 (Figure 2F). Supplementary Figure S2F shows that *dpb11 WG700,701AA* point mutant is as defective as *dpb11ΔC* in checkpoint activation and the inability to activate Mec1, therefore, correlates which a deficiency to support checkpoint signalling.

The interaction between the 9-1-1 complex and Dpb11 or their orthologues is thought to recruit Dpb11 orthologues to sites of DNA damage (Furuya *et al.*, 2004; Delacroix *et al.*, 2007; Puddu *et al.*, 2008). We, therefore, tested a specific Mec1-phosphorylation site mutant of Ddc1 (*ddc1 T602A*), which prevents binding to Dpb11 (Puddu *et al.*, 2008). In *ddc1 T602A* or *ddc1 T602A dot1Δ* mutant strains, addition of the *dpb11ΔC* mutation did not result in increased checkpoint defects as measured by Rad53 phosphorylation and survival after phleomycin treatment (Figure 2G and H compare sample 2 with 6; and 4 with 8). This suggests that the Dpb11–Mec1 interaction is functionally dependent on the Dpb11–Ddc1 interaction, consistent with 9-1-1-dependent recruitment of Dpb11. Defects in the Dpb11–Ddc1 module (i.e., *ddc1 T602A*) cause more severe phenotypes than defects in the Dpb11–Mec1–Ddc2 module (i.e., *dpb11ΔC*, Figure 2G and H, compare sample 4 with 7), suggesting that at least in this mutant background Dpb11 has a function in checkpoint signalling independent of its ability to activate Mec1–Ddc2.

CDK phosphorylation of Rad9 regulates binding to Dpb11

Although the Dpb11–Rad9 interaction could occur in the absence of exogenous DNA damage (Figure 1B), Figure 3A shows that the Rad9–Dpb11 interaction was cell-cycle regulated: it was detected in extracts from G2/M-arrested cells, but not in extracts from G1-arrested cells (Figure 3A). Moreover, the interaction was lost in extracts from G2/M-arrested cells in which a stable version of the CDK inhibitor Sic1 (Sic1ΔN) was overexpressed (Figure 3B). To test whether this cell cycle-regulated interaction was directly mediated by CDK phosphorylation, we expressed and purified recombinant MBP–Rad9 and phosphorylated it with recombinant CDK. Figure 3C shows that CDK phosphorylation strongly stimulated the binding of MBP–Rad9 to either full-length GST–Dpb11 or GST–Dpb11-N, which contains just BRCT1&2.

Rad9 has previously been shown to be a CDK substrate (Ubersax *et al.*, 2003) and in the fission yeast orthologue Crb2 the CDK site T215 was found to be important for binding of the Dpb11 orthologue Cut5/Rad4 (Esashi and Yanagida, 1999; Du *et al.*, 2006). Homologues of Rad9 that contain conserved C-terminal BRCT and TUDOR domains can be found across the eukaryotic kingdom, but upstream of these domains they differ in length and sequence (Supplementary Figure S5B). Since the majority of putative CDK phosphorylation sites can be found in this region (Supplementary Figure S5B), we decided to unambiguously map the phosphorylation site that regulates the interaction with Dpb11. We generated N- and C-terminal truncations of Rad9 *in vivo* and tested their ability to bind GST–Dpb11-N in extracts from G2/M-arrested

cells. We found that versions of Rad9 harbouring significant N- or C-terminal truncations were still able to interact with Dpb11, but the interaction was abolished when a region between aa 451 and 540 of Rad9 was deleted (Figure 3D). This region of Rad9 contains a cluster of four S/TP sites (S462, T474, S494 and T507; compare Supplementary Figure S3A). In order to test whether any of these are sufficient to mediate phosphorylation-specific binding to Dpb11, we constructed four independent biotinylated 35mer peptides, each harbouring one phosphorylated CDK site at the same position (26) in the peptide, and tested their binding to Dpb11 BRCT1&2 by streptavidin bead pulldown. Figure 3E shows that phospho-S462 and phospho-T474 peptides exhibited phosphorylation-dependent binding to Dpb11, comparable to the Dpb11-binding peptide from Sld3 (Figure 3E; Supplementary Figure S3B). Notably, out of 12 Rad9 peptides tested, which covered 12 out of 16 conserved S/TP sites, only Rad9 pS462 and Rad9 pT474 were able to bind Dpb11 (Supplementary Figure S3A). Among these, we did not see significant Dpb11 binding to peptides containing phosphorylated Ser11, a residue recently implicated in Dpb11–Rad9 interaction (Granata *et al.*, 2010). Sld3 and its mammalian orthologue Treslin/ticrr also utilize two phosphorylated residues to interact with BRCT1&2 of Dpb11/TopBP1 (Tanaka *et al.*, 2007; Zegerman and Diffley, 2007; Boos *et al.*, 2011). We were able to detect limited conservation of sequences surrounding the two phosphorylation sites of Sld3 and Rad9, using the sequences of different *Saccharomyces sensu lato* species (Supplementary Figure S5A), suggesting that both proteins interact with Dpb11 in a similar fashion.

To assess the importance of S462 and/or T474 phosphorylation to the Dpb11 interaction, we introduced point mutations into full-length Rad9 fused to MBP and examined CDK-dependent binding to Dpb11-N *in vitro*. Mutation of these two phosphorylation sites, but not mutation of two neighbouring sites (*ST494,507AA*), greatly reduced the CDK-dependent interaction with Dpb11 *in vitro* (Figure 3F) indicating that, even in the presence of the other 18 potential CDK sites, S462 and T474 are critical for efficient CDK-dependent Dpb11 interaction. We generated phospho-specific antibodies to these two sites to determine whether S462 and T474 are phosphorylated *in vivo* (Supplementary Figure S4). Figure 3G shows that both of these antibodies detect wild-type Rad9–3Flag after pulldown of Rad9 with anti-FLAG antibody from G2/M-arrested cells, but not from G1-arrested cells. Moreover, the *ST462,474AA* mutant was not detected from either G1- or G2/M-arrested cell extracts with these antibodies. Therefore, these sites are phosphorylated *in vivo* in a cell cycle-dependent manner. Finally, we employed the two-hybrid assay to analyse the requirements of the Dpb11–Rad9 interaction *in vivo* and found that the *T474A* mutation reduced the interaction with Dpb11 and *S462A* or *ST462,474AA* mutations appeared to abolish it completely (Figure 3H). Taken together, these results show that phosphorylation of S462 and T474 is necessary and sufficient for CDK-dependent, cell cycle-regulated interaction between Dpb11 and Rad9.

Using a degenerate Dpb11 BRCT binding consensus derived from the Rad9 and Sld3 sequences, we were able to detect potential CDK phosphorylation-dependent Dpb11 binding sites in the N-termini of different Rad9 fungal orthologues (Supplementary Figure S5C). The alignment suggests that

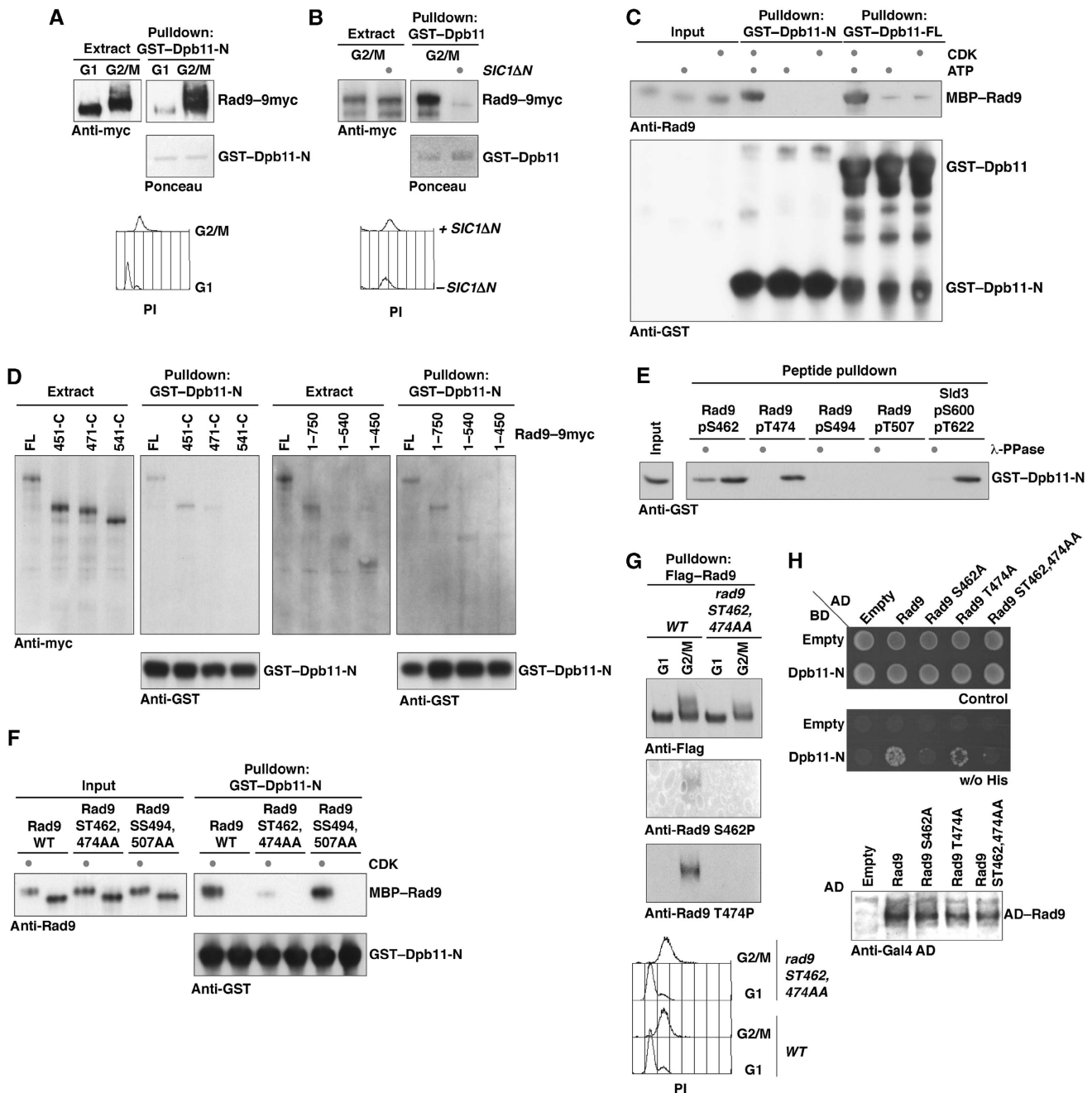


Figure 3 The Rad9-Dpb11 interaction is cell-cycle regulated through direct binding of Dpb11 to Rad9 CDK sites S462 and T474. (A) The Rad9-Dpb11 interaction is cell-cycle regulated. Rad9-9myc from G1- or G2/M-arrested cells was tested for binding to GST-Dpb11-N in pull-down. (B) Overexpression of a stable version of Sic1 (*Sic1ΔN*; Desdouets *et al*, 1998) inhibits the Rad9-Dpb11 interaction in G2/M-arrested cells. (C) Recombinant, purified MBP-Rad9 specifically interacts with GST-Dpb11 or GST-Dpb11-N after *in vitro* phosphorylation of Rad9 by CDK (Cyclin AΔN170-Cdk2; Brown *et al*, 1995). (D) N- and C-terminal truncations of endogenous Rad9 from lysates of G2/M-arrested cells were analysed by GST-Dpb11 pull-downs. A central region of Rad9, which contains a cluster of CDK sites, is required for interaction with Dpb11. (E) Phosphorylated Serine 462 and Threonine 474 peptides of Rad9 pull down GST-Dpb11-N. λ-Phosphatase treatment demonstrated phosphorylation specificity. The Dpb11-N interacting peptide from Sld3 served as a positive control (Zegerman and Diffley, 2007). (F) Rad9 ST462,474AA is deficient for *in vitro* binding to GST-Dpb11-N after CDK treatment. Experiment as in (B) but with mutant versions of MBP-Rad9. (G) Rad9 is phosphorylated at CDK sites Serine 462 and Threonine 474 *in vivo*. Phospho-specific antibodies (Supplementary Figure S4) were used to probe pull-downs of Flag-Rad9 or Flag-Rad9 ST462,474AA from G1- or G2/M-arrested cells. (H) Rad9 CDK sites are required for the interaction with Dpb11 *in vivo*. Gal4-BD-Dpb11-N and Gal4-AD-Rad9 (WT and S462A, T474A, ST462,474AA mutants) fusions were used to test the Rad9-Dpb11 interaction in the two-hybrid system. Expression of Rad9-fusion constructs was confirmed by Gal4-AD-westerns.

T215 (Esashi and Yanagida, 1999; Du *et al*, 2006) and perhaps T235 or T252 of *Schizosaccharomyces pombe* Crb2 are homologous to S462 and T474. Thus, in addition to the Dpb11-

Ddc1 and the Dpb11-Mec1-Ddc2 interactions, the Dpb11-Rad9 interaction is also a conserved feature of Dpb11/TopBP1 function.

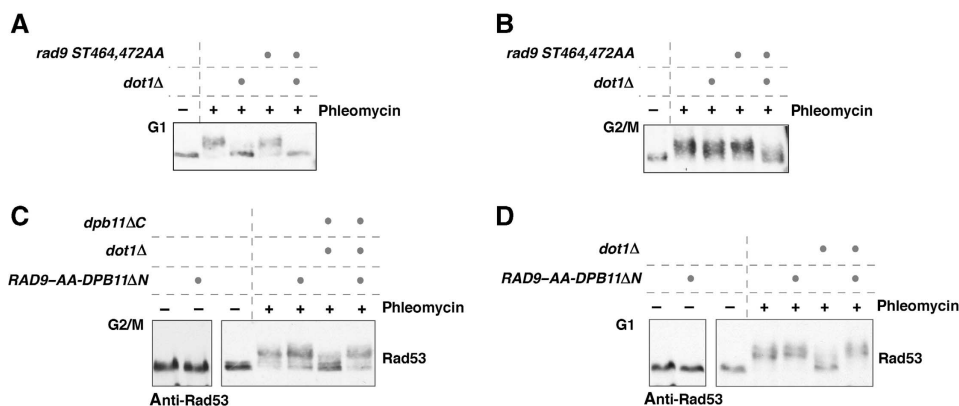


Figure 4 CDK regulation of the Rad9–Dpb11 interaction constitutes the essential cell-cycle regulation of checkpoint signalling. **(A)** The *rad9 ST462,474AA* allele is proficient for the G1 checkpoint. Rad53 phosphorylation was determined in samples of G1-arrested cells, before (–) or after (+) 30' treatment with phleomycin (50 µg/ml). **(B)** *rad9 ST462,474AA dot1Δ* cells show defects in DNA damage-induced Rad53 phosphorylation in G2/M-arrested cells. Experiment as in **(A)** but with G2/M-arrested cells. **(C)** A fusion protein consisting of the two checkpoint deficient alleles *rad9 ST462,474AA* and *dpb11ΔN* (276–C) can support checkpoint activation in G2/M. The *RAD9-AA-DPB11ΔN* fusion was ectopically expressed from the *DPB11* promoter as only cellular copy of *RAD9* in *WT* or *dot1 dpb11ΔC* strains. **(D)** The *RAD9-AA-DPB11ΔN* fusion restores checkpoint activation in G1-arrested *dot1Δ* cells.

CDK regulation of the Rad9–Dpb11 interaction determines cell-cycle regulation of the DNA damage checkpoint

The *rad9 ST462,474AA* mutant, which is defective in Dpb11 interaction, is fully able to activate the checkpoint in G1 as well as in G2/M in otherwise wild-type cells (Figure 4A and B). However, when this mutant was combined with *dot1Δ*, it was defective in G2/M checkpoint activation (Figure 4B) similar to the *dpb11ΔC* mutant (Figure 2C). The *rad9 ST462,474AA* mutation did not lead to an increase of the phenotype of the *ddc1 T602A* mutant (Supplementary Figure S6A), which completely abolishes the checkpoint function of Dpb11 providing additional evidence that the *rad9 ST462,474AA* phenotype is *DPB11* dependent. Analysis of the Mec1-dependent phosphorylation of Dpb11 suggests that the Rad9–Dpb11 is not involved in recruitment of Dpb11 to DNA damage sites (Supplementary Figure S6B). We observed a similar checkpoint defect for *rad9 ST462,474AA dot1Δ* and *dpb11ΔC dot1Δ* mutants, but the *rad9 ST462,474AA dpb11ΔC dot1Δ* triple mutant showed a slightly stronger phenotype (Supplementary Figure S6C). This suggests that the Rad9–Dpb11 interaction may be partially independent of the Dpb11–Mec1–Ddc2 interaction at least in these mutant backgrounds.

The results shown in Figure 4A and B are consistent with the hypothesis that Dot1 and Dpb11 act redundantly in G2/M but the Dpb11 pathway does not function during G1 phase because Rad9 cannot be phosphorylated by CDK. To test whether the Rad9–Dpb11 interaction is sufficient to explain cell cycle-regulated checkpoint signalling, we constructed a covalent fusion of Rad9 ST462,474AA and Dpb11 lacking the N-terminal BRCT1&2 repeat domain (*RAD9-AA-DPB11ΔN* fusion). This fusion is exactly analogous to the fusion we previously used to show that phosphorylation of Sld3 by CDK generates a binding site for Dpb11 during replication initiation (Zegerman and Diffley, 2007). Expression of *RAD9-AA-DPB11ΔN* appears not to negatively influence DNA replication (Supplementary Figure S7A). Neither *rad9-ST462,474AA* nor *dpb11ΔN* alone is able to support checkpoint signalling in a *dot1Δ* background (see Figures 4B and 5D). Figure 4C shows that the *RAD9-AA-DPB11ΔN* fusion was able to

restore phleomycin-induced Rad53 activation to WT levels during G2/M phase in a *dot1Δ, dpb11ΔC, rad9Δ* background. Indeed, the *RAD9-AA-DPB11ΔN* fusion appears to be a gain-of-function mutant, since the checkpoint was dominantly activated even at lower phleomycin concentrations compared with wild-type cells and also the fusion protein could be recruited in a Ddc1 T602-independent way (Supplementary Figure S7B–D).

Figure 4D shows that the fusion was also able to restore phleomycin-induced Rad53 phosphorylation in G1-arrested cells in the absence of Dot1 and the requirement for Dot1 in the G1 checkpoint is therefore bypassed. Taken together, these results show that CDK phosphorylation of Rad9 is required to induce interaction between Dpb11 and Rad9 and that lack of this interaction in G1 phase is sufficient to explain the cell-cycle regulation of checkpoint signalling.

Dpb11 specifically induces Rad9 phosphorylation by Mec1 in a ternary Rad9–Dpb11–Mec1–Ddc2 complex in vitro

If Dpb11 operates as a molecular scaffold in the DNA damage response, it should be able to simultaneously interact with different checkpoint proteins, for example, Rad9 and Mec1–Ddc2. To test this, we examined the ability of Dpb11 to bridge an interaction between Mec1–Ddc2 and Rad9. Figure 5A shows that, in the presence of full-length Dpb11 but not a C-terminal fragment of Dpb11 (Dpb11-C), CDK-phosphorylated Rad9 was specifically co-immunoprecipitated with Mec1 in a Mec1 pull-down.

To integrate the scaffolding function and the Mec1–Ddc2 activation function of Dpb11 into a mechanistic model, we hypothesized that Dpb11 may work by activating Mec1–Ddc2 and bringing active Mec1–Ddc2 into proximity with one of its key downstream targets, Rad9. We examined the ability of Dpb11 full length and Dpb11-C to activate Mec1–Ddc2 towards the non-specific PHAS1 substrate and towards Rad9. Figure 5B shows that both full-length Dpb11 and Dpb11-C activate Mec1–Ddc2 to a very similar extent when PHAS1 is used as a substrate. However, when we used Rad9 that had been pre-phosphorylated with CDK and repurified as a substrate (Supplementary Figure S8A and B), we found that the

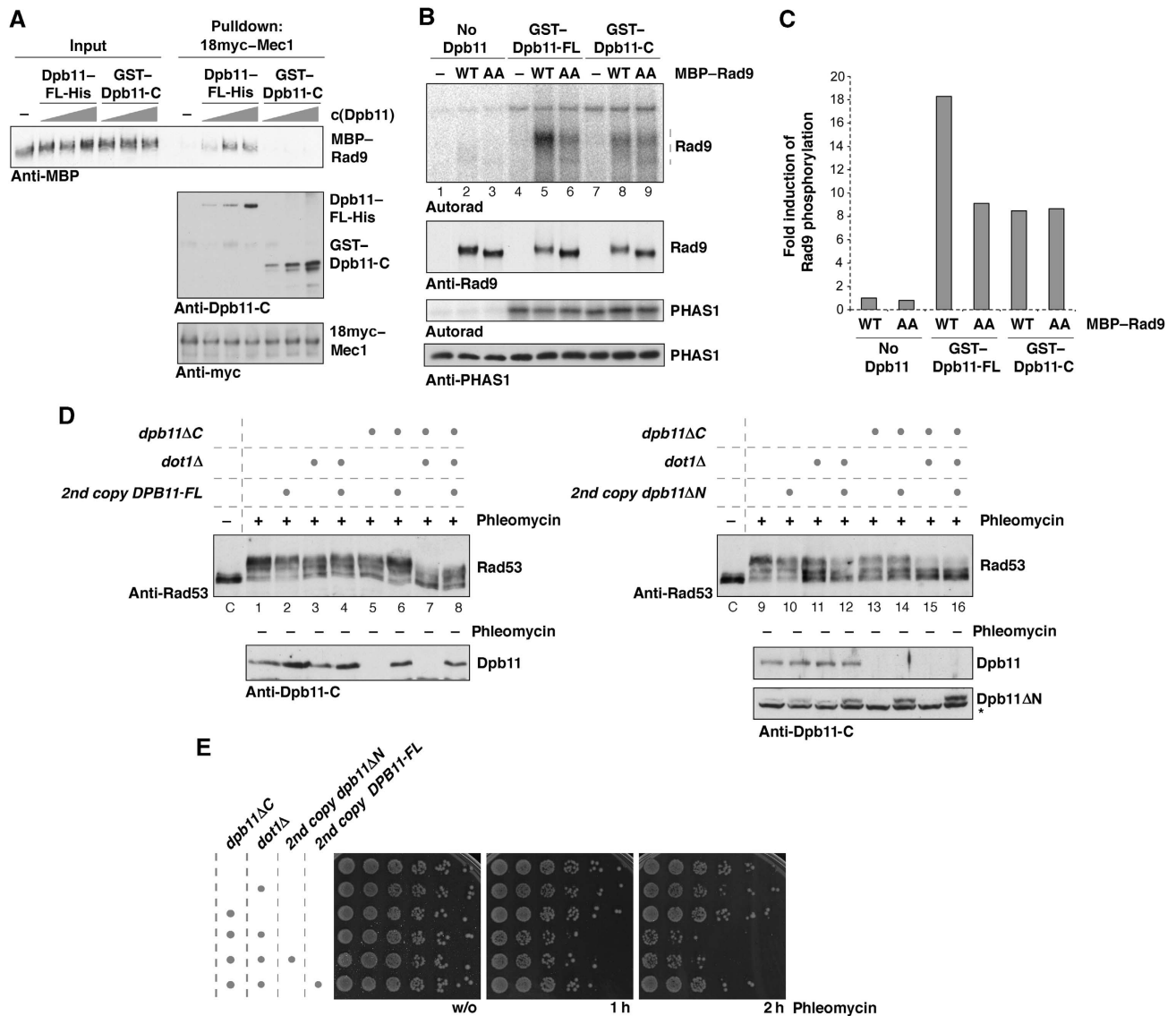


Figure 5 Dpb11 forms a ternary complex with Rad9 and Mec1-Ddc2, which is critical for phosphorylation by Mec1 *in vitro* and checkpoint activation *in vivo*. (A) Mec1-18myc can pull down CDK-phosphorylated MBP-Rad9 in the presence of increasing amounts of full-length Dpb11-His but not the isolated C-terminal domain (GST-Dpb11-C), demonstrating formation of a ternary Rad9-Dpb11-Mec1-Ddc2 complex *in vitro*. (B) *In vitro* phosphorylation of Rad9 by Mec1 is specifically enhanced by a Rad9-Dpb11-Mec1-Ddc2 complex. Recombinant, purified MBP-Rad9 or MBP-Rad9 ST462,474AA is quantitatively phosphorylated by CDK, repurified (see Supplementary Figure S8A) and used as substrate in Mec1 kinase assays. Mec1 was activated with GST-Dpb11 or GST-Dpb11-C at equimolar concentration as MBP-Rad9. The PIKK substrate PHAS1 was used as a specificity control. (C) Quantification of Dpb11-dependent stimulation of Mec1 phosphorylation of Rad9. Signals are normalized to phosphorylation of WT Rad9 in the absence of Dpb11. (D, E) Dpb11ΔN fails to rescue the checkpoint defect of *dot1Δ dpb11ΔC* cells. Dpb11 or Dpb11ΔN was ectopically expressed from the endogenous promoter as a second copy of *DPB11*. G2/M DNA damage checkpoint activation was measured by Rad53 phosphorylation (D) or survival (E) after phleomycin treatment (50 μg/ml). (D) Samples were taken before (–) or 30' after (+) addition of phleomycin.

full-length Dpb11 promoted ~2-fold more Rad9 phosphorylation than Dpb11-C (Figure 5B and C). This stimulation was not seen when we used Rad9 ST462,474AA (AA) as substrate, indicating that recruitment of Rad9 to Dpb11 was critical for the stimulation. This stimulation by Dpb11 was maximal at approximately equimolar concentrations of Rad9 and Dpb11, while a further increase of the Rad9 concentration increased overall Rad9 phosphorylation but reduced the stimulatory effect of Dpb11 (Supplementary Figure S8C and D). Taken together, these results support the hypothesis that Dpb11 acts both to activate Mec1-Ddc2 and to bring the active kinase together with CDK-phosphorylated Rad9 protein. When we compared the efficiency of phleomycin-induced Rad9 and

Rad53 phosphorylation *in vivo*, we noticed that in the *dpb11ΔC dot1Δ* mutant Rad53 phosphorylation was affected more strongly than phosphorylation of Rad9 (Figure 2C; Supplementary Figure S9A). This suggests that activation of Mec1 by Dpb11 may be more important for the efficient phosphorylation of Rad53.

Simultaneous interaction of Dpb11 with Rad9 and Mec1-Ddc2 is required for efficient checkpoint activation *in vivo*

This model predicts that simultaneous interaction of Dpb11 with Rad9 and Mec1-Ddc2 should be required for checkpoint activation. Alternatively, if the Rad9-Dpb11 and Dpb11-

Mec1–Ddc2 interactions were independent of each other, checkpoint signalling should be functional in cells expressing two versions of Dpb11, one able to support the interactions with the 9-1-1 and Mec1–Ddc2 but defective in the Rad9 interaction (*dpb11ΔN*), and the other able to support interactions with Rad9 and the 9-1-1 complex but not with Mec1–Ddc2 (*dpb11ΔC*). To address this, we asked whether the *dpb11ΔN* and *dpb11ΔC* mutants, when expressed together, could support checkpoint activation. Figure 5D and E show, however, that both Rad53 activation and cell survival are compromised in *dpb11ΔC dpb11ΔN dot1Δ* cells (compare lanes 15 and 16 in Figure 5D and E). In contrast, if full-length DPB11 is expressed as a second copy in *dpb11ΔC dot1Δ* cells, the checkpoint defect is rescued to levels comparable to *WT* or *dot1* strains (Figure 5D, compare lanes 7 and 8; Figure 5E). The *dpb11ΔC* protein is likely to be functional because it can fully support the replicative function of Dpb11 (Figure 2A), which requires functional N-terminal and central BRCT repeat domains. The Dpb11ΔN protein is expressed at a similar level to that of the full-length protein (Figure 5D). Moreover Dpb11ΔN must be able to bind to the 9-1-1 complex and Mec1–Ddc2, because it is able to support residual checkpoint activation in G1, which can be observed under high concentrations of phleomycin (Supplementary Figure S10). This checkpoint activation is independent of the Rad9–Dpb11 interaction, but requires Dpb11 to bind to the 9-1-1 complex and Mec1–Ddc2 (Supplementary Figure S10). As a final test, we examined the effect of a single point mutation in BRCT1, *dpb11-T12A*. Supplementary Figure S11 shows that *dpb11-T12A* also fails to complement *dpb11ΔC* in the checkpoint, similar to *dpb11ΔN* (Supplementary Figure S11). Taken together, these results indicate that Dpb11 must simultaneously interact with Rad9 and Mec1–Ddc2 for efficient checkpoint signalling.

Discussion

Our work, taken with previous work in budding and fission yeast as well as *Xenopus*, argues that Dpb11 plays a crucial role in integrating cell cycle and DNA damage signals to ensure correct spatial and temporal checkpoint activation. Dpb11 accomplishes this by providing a scaffolding function for Rad9, 9-1-1 and Mec1–Ddc2 and activating the Mec1–Ddc2 kinase specifically in this context (Figure 6A).

Our data indicate that Rad9 specifically interacts with Dpb11 after phosphorylation by CDK, thus promoting interaction during S, G2 and M phases of the cell cycle. We identified two key CDK phosphorylation sites on Rad9 (S462 and T474) that bind directly to the N-terminal BRCT repeats 1 and 2 of Dpb11 and are necessary and sufficient for Rad9–Dpb11 interaction. Fusion of Rad9 to Dpb11 bypassed the requirement for CDK phosphorylation of Rad9 and supported Dot1-independent checkpoint signalling even during G1 phase, when CDK is inactive. Thus, CDK phosphorylation of Rad9 is crucial for coordinating DNA damage signalling with the cell cycle. CDK phosphorylation of S11 of Rad9 has recently been implicated in the Dpb11 interaction (Granata *et al.*, 2010). However, peptides containing phospho S11 did not interact with Dpb11 (Supplementary Figure S3) and *rad9* mutants in which the N-terminal cluster of CDK sites has been deleted can still interact with Dpb11 (Figure 3D) while full-length Rad9 ST462,474AA cannot interact with Dpb11

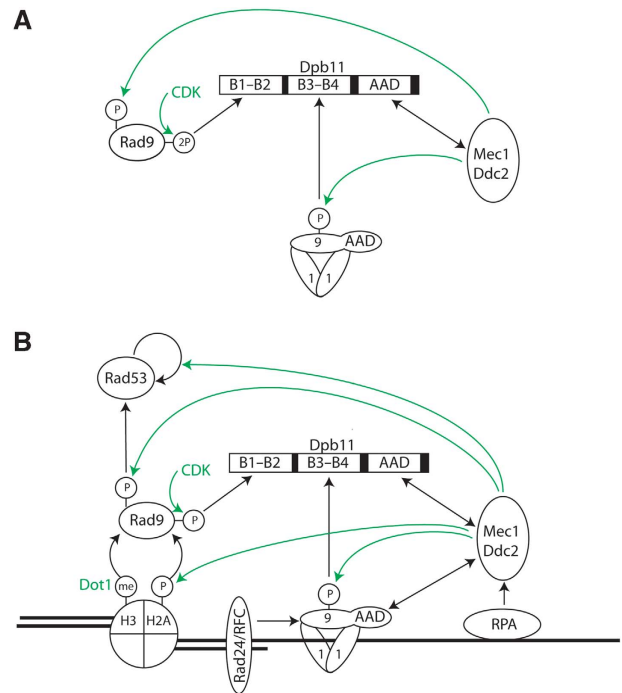


Figure 6 Model of Dpb11 function in the checkpoint response. **(A)** The Dpb11 module of checkpoint signalling: Dpb11 engages three protein domains to form a checkpoint signalling complex with Rad9, 9-1-1 and Mec1–Ddc2. In this assembly, Dpb11 coordinates specific activation of Mec1 with cell cycle-regulated recruitment of the Mec1 substrate Rad9. This signalling complex is presumably localized to DNA damage sites by the loaded 9-1-1 complex and RPA-bound Mec1–Ddc2. **(B)** Holistic view of DNA damage checkpoint signalling upstream of Rad53 activation. The Dpb11 module is integrated with cell cycle- and Dpb11-independent modules: Binding of Rad9 to histones, which are H3-K79 methylated and H2a-S129 phosphorylated can localize Rad9 to DNA damage sites. The Mec1 kinase can be activated Dpb11-independently by the Ddc1 subunit of the 9-1-1 complex. It remains to be clarified, if crosstalk of Dpb11-dependent and -independent modules exists in *WT* cells.

(Figure 3F and H). Consequently, phosphorylation of S11 appears to play no direct role in Dpb11 binding. S11 phosphorylation may, of course, contribute in some way indirectly, similar to Sld2 where phosphorylation of a cluster of CDK sites is required for the phosphorylation of T84, which constitutes the direct Dpb11 binding site (Tak *et al.*, 2006). The region around S462 in Rad9 aligns well with T215 of the *Schizosaccharomyces pombe* Rad9 orthologue, Crb2 (Supplementary Figure S5C), which has previously been implicated in binding of the fission yeast Dpb11 orthologue, Cut5/Rad4 (Du *et al.*, 2006). There are two potential CDK sites in Crb2 downstream of T215 which have some homology with T474 of Rad9 and it will be interesting to know if they also contribute to Cut5/Rad4 binding. Both Rad9 and Sld3 contain two CDK phospho-sites required for efficient interaction with BRCT1&2 of Dpb11. The recent crystal structure of TopBP1 shows that BRCT repeats 1 and 2 each have potential phosphopeptide binding sites (Rappas *et al.*, 2010). We speculate that these two phosphorylation sites may each bind to different BRCT repeats, though further work is required to verify this.

Consistent with previous work, we found a domain of Dpb11 distal to the last BRCT repeat that binds to and activates the Mec1/Ddc2 kinase. While there is very little

sequence similarity between this domain and the ATR activating domain (AAD) from TopBP1, we identified two motifs that contain conserved aromatic residues, which are critical for both interaction and activation, similar to the AAD in *Xenopus* TopBP1. Both Mec1 and Ddc2 are required for interaction with Dpb11, suggesting that either interaction occurs near the interface between Mec1 and Ddc2 or conformation changes that occur when Mec1 and Ddc2 interact are required to generate a Dpb11 binding site on either Mec1 or Ddc2. How the Dpb11 AAD activates Mec1–Ddc2 is unknown, but the presence of conserved aromatic residues in the AADs of Dpb11, TopBP1 and Ddc1 suggests a common mechanism (Kumagai *et al.*, 2006; Navadgi-Patil and Burgers, 2009). Interestingly, a Mec1-phosphorylation site (T731) in Dpb11 has been identified that is required for activation of Mec1 (Mordes *et al.*, 2008) and might be part of the AAD motif. This suggests that AADs may not work constitutively, but may be regulated by post-translational modifications.

Because *dpb11ΔC* mutants are viable at all temperatures tested, our results demonstrate that the C-terminus of Dpb11 including the AAD is not required for viability. Thus, while Mec1 and Ddc2 are essential, their activation by Dpb11 is not essential. Because deletion of the other AAD-containing protein, Ddc1, is not lethal in combination with *dpb11ΔC*, either Mec1 activation is not essential or there are other as yet unidentified Mec1-activating proteins in yeast. We favour this latter possibility because the *dpb11ΔC ddc1WW352,544AA* double mutant is not as sensitive to phleomycin as the *rad9Δ* mutant and because Rad53 activation is only partly defective in this double mutant (Supplementary Figure S9B). The viability of *dpb11ΔC* also shows that the C-terminus of Dpb11 does not have an essential role in DNA replication at any temperature.

Our biochemical and genetic data indicate that Dpb11 must be able to interact with both CDK-phosphorylated Rad9 and AAD-activated Mec1–Ddc2 for efficient Rad9 phosphorylation by Mec1–Ddc2 and efficient checkpoint activation. In this regard, Dpb11 appears to act analogously to the archetypal scaffold protein, Ste5, which provides a scaffold for mating type signalling and also specifically activates the bound Fus3 kinase (Choi *et al.*, 1994; Bhattacharyya *et al.*, 2006; Hao *et al.*, 2008; Good *et al.*, 2009). Dpb11 also acts as a scaffold protein in DNA replication, bridging interactions between Sld3 and Sld2 (Tanaka *et al.*, 2007; Zegerman and Diffley, 2007). Although the C-terminal AAD is not required for replication, it is interesting to consider that Dpb11 may, by analogy to its role in checkpoints, play roles in the initiation process beyond simple scaffolding.

Figure 6B considers our results in the broader context of checkpoint activation. After ds break formation and resection, RPA binds to ssDNA and recruits Mec1–Ddc2 (Rouse and Jackson, 2002; Zou and Elledge, 2003; Ball *et al.*, 2007). And separately, the 9-1-1 complex is loaded onto the recessed 5' end by the Rad24/RFC complex (Ellison and Stillman, 2003; Majka and Burgers, 2003; Zou *et al.*, 2003; Majka *et al.*, 2006a). The phosphorylation of the Ddc1 subunit of the 9-1-1 complex is critical for the function of Dpb11 in checkpoint signalling since the *ddc1 T602A* mutant is epistatic to both *dpb11ΔC* and *rad9 ST462,474AA*. This, however, creates an apparent paradox: Mec1–Ddc2 activation requires the AAD of Dpb11, yet Dpb11 cannot be recruited to ds breaks until Ddc1 is phosphorylated by Mec1–Ddc2. It is

possible that direct recruitment of Dpb11 to Mec1/Ddc2 via interaction with the AAD results in sufficient activation of Mec1–Ddc2 to promote Ddc1 phosphorylation, which supports further Dpb11 recruitment and Mec1–Ddc2 activation. Alternatively, the AAD of Ddc1 (or some, as yet unidentified AAD) may play some priming role in Mec1–Ddc2 activation, allowing subsequent Ddc1 phosphorylation and Dpb11 recruitment. Furthermore, the ATM orthologue Tel1 may be involved in this phosphorylation event thus potentially establishing crosstalk between the two apical checkpoint kinases. Finally, Mec1–Ddc2, recruited via RPA, may have sufficient activity prior to AAD interaction to phosphorylate Ddc1, promoting Dpb11 recruitment and complete activation. Further work is required to resolve this issue.

The role of Ddc1 in checkpoint activation is, however, more complex. The Ddc1 AAD mutant (*ddc1WW352,544AA*) is epistatic to *dot1Δ*, not to *dpb11ΔC* (Supplementary Figure S9B), suggesting that the Ddc1 AAD acts primarily within the Dot1 module of the pathway. This suggests that Ddc1 plays different roles in the two pathways: it is required to recruit Dpb11 via Ddc1 phosphorylation in the Dpb11 pathway, but it is required to directly activate Mec1–Ddc2 in the Dot1 pathway. Currently we can only speculate, why Ddc1 cannot simultaneously bind to Dpb11 and activate Mec1: both interactions involve the C-terminal domain of Ddc1 and may be due to steric constraints.

There is evidence for alternative routes to checkpoint activation, at least in mutant backgrounds. For example, at high concentrations of phleomycin, G1 cells can activate Rad53 even in *dot1Δ* mutants (Supplementary Figure S10). This activation requires the AAD of Dpb11 but not the CDK phosphorylation sites in Rad9, and is therefore most likely mediated by a Dpb11 subcomplex. It is currently unclear whether such subcomplexes (Rad9–Dpb11–9-1-1 or 9-1-1–Dpb11–Mec1–Ddc2) are relevant for checkpoint activation in wild-type cells, since their function can only be observed in mutant backgrounds and/or at high amounts of DNA damage and results only in partial checkpoint activation.

Once Rad9 is recruited, Mec1–Ddc2 phosphorylates it, which promotes both oligomerization of Rad9 via its BRCT domain and recruitment of Rad53 via its FHA domains (Emili, 1998; Sun *et al.*, 1998; Vialard *et al.*, 1998; Soulier and Lowndes, 1999; Sweeney *et al.*, 2005; Usui *et al.*, 2009). Mec1–Ddc2-dependent phosphorylation of Rad53 allows further oligomerization and autoactivation (Sanchez *et al.*, 1996; Sun *et al.*, 1996). Understanding in detail how Rad53 gets activated will ultimately require reconstitution of this complex network with purified proteins. Our partial reconstitution of the Dpb11 module will hopefully contribute towards this goal.

Materials and methods

Strains, plasmids, antibodies and proteins

All yeast strains are based on W303; details are listed in Supplementary Table 1. N-terminal truncations (451–1309, 471–1309 and 541–1309) of Rad9 were created by deletion of the corresponding sequence from the endogenous gene and integration of the RAD9 promoter and new start codon at the site of truncation. Additionally, a 9myc tag was integrated at the C-terminus. C-terminal truncations (1–750, 1–540 and 1–450) of Rad9 were generated through deletion and integration of the 9myc tag at the position of truncation. Unless specifically indicated yeast strains were grown in rich medium at 30°C. Details on plasmid constructs

can be found in Supplementary Table 2. Antibodies used in this study are listed in Supplementary Table 3. Purification protocols for proteins used in this study can be found in Supplementary data.

Protein interaction techniques

The initial pull-down was performed with ~1 nmol Dpb11-His bound to Ni-NTA magnetic beads (Qiagen) and 6 ml of DNase treated (1500 U, 30', 4°C) yeast extract (300 mM KOAc, 5 mM Mg₂SO₄, 1 mM CaCl₂, 25 mM Hepes pH 7.6, 10% glycerol, 0.02% NP-40, 2 mM β-Me, protease inhibitors) corresponding to 5 × 10¹⁰ asynchronously dividing cells. Bound proteins were eluted with 500 mM imidazole and concentrated by TCA precipitation. 10% of the bound sample was loaded on 4–12% NuPAGE (Figure 1A), which was silver stained. 90% of the sample was run under identical conditions and stained with colloidal Coomassie. The gel was cut into 30 slices and after trypsin digestion bound proteins were identified by ESI ion trap MS.

For small-scale pull-downs, GST-Dpb11 or GST-tagged protein fragments were immobilized on glutathione sepharose 4B (GE Healthcare) and incubated with 600 μl ammonium sulphate precipitated (57%) cell extracts (buffer for binding experiments with Ddc2: 300 mM KOAc, 25 mM Hepes pH 7.6, 10% glycerol, 0.02% NP-40, 2 mM β-Me, protease inhibitors; for binding experiments with Rad9: 200 mM KOAc, 100 mM Hepes pH 7.6, 10% glycerol, 0.02% NP-40, 2 mM β-Me, 20 mM β-glycerophosphate, 10 mM NaF, 100 μM okadaic acid, protease inhibitors) corresponding to 1 × 10⁹ cells.

For pull-downs with Dpb11 and purified Rad9, the procedure was as described above, but instead of cell extract GST-Dpb11 beads were incubated with 6 pmol MBP-Rad9, which was quantitatively pre-phosphorylated with btCyclin AAN170-hsCdk2 (Brown *et al.*, 1995).

In order to test ternary complex formation, immunopurified Mec1-18myc-Ddc2 (protocol in Supplementary data) was used to pull down 0.3 pmol MBP-Rad9 and 0, 1, 3, 10 pmol Dpb11 FL-His or GST-Dpb11-C in buffer containing 500 mM KOAc, 100 mM Hepes pH 7.6, 10% glycerol, 0.02% NP-40, 2 mM β-Me, 20 mM β-glycerophosphate, 10 mM NaF, 100 μM okadaic acid and protease inhibitors.

Peptide binding was investigated with 35mer peptides corresponding to endogenous Rad9 sequence, harbouring a phosphorylated amino acid at position 26, an N-terminal EAhx-linker and biotin tag. 75 μl Streptavidin Dynabeads (Dyna) were saturated with peptide, treated with λ-phosphatase or left untreated, and used to pull down 40 pmol GST-Dpb11-N (aa 1–276) in buffer containing 500 mM KOAc, 100 mM Hepes pH 7.6, 10% glycerol, 0.02% NP-40 and 2 mM β-Me.

In vivo checkpoint assays

To investigate checkpoint activation *in vivo*, yeast strains were arrested in G1 with α-factor or in G2/M with nocodazole. Phleomycin was added to 50 μg/ml (or the indicated concentration)

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and samples corresponding to 2 × 10⁷ cells were taken at indicated time points and subjected to TCA precipitation. Rad53 activation was measured by its phosphorylation-dependent shift in electrophoretic mobility. For determination of survival, cells were released from cell-cycle block simultaneously with addition of phleomycin. At indicated time points, cells were diluted to 2 × 10⁶ cells/ml. This solution as well as five 1:5 dilutions were spotted on plates without drugs and incubated for 2 days.

In vitro kinase assays

Mec1 kinase assays were performed as described (Mordes *et al.*, 2008), with small modifications. A protocol of the Mec1-18myc-Ddc2 preparation can be found in Supplementary data. 10 μl of bead-bound Mec1-18myc-Ddc2 was incubated for 30' at 30°C with ~0.5 pmol CDK pre-phosphorylated MBP-Rad9 or MBP-Rad9-AA and 0.75 pmol GST-Dpb11 or GST-Dpb11-C (0.75, 0.25 or 0.075 pmol in Figure 2E) and/or 15 pmol PHAS1 (60 pmol in Figure 2F) in 40 μl kinase reactions (100 mM KOAc, 10 mM Hepes pH 7.6, 50 mM β-glycerophosphate, 10 mM MgCl₂, 10% glycerol, 2 mM β-Me, 10 mM ATP, 5 μCi γP³²-ATP). Phosphorylation of Rad9 and PHAS1 was visualized by autoradiography; equal loading of the substrates was confirmed by western blots (Coomassie stain in Figure 2F).

Supplementary data

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

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Author contributions: BP and JFXD conceived and designed experiments, analysed the data and wrote the manuscript. BP performed all the experiments.

Conflict of interest

The authors declare that they have no conflict of interest.

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