

# Dpb11 activates the Mec1–Ddc2 complex

Daniel A. Mordes, Edward A. Nam, and David Cortez<sup>1</sup>

Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232

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**The *Saccharomyces cerevisiae* Mec1–Ddc2 checkpoint kinase complex (the ortholog to human ATR–ATRIP) is an essential regulator of genomic integrity. The *S. cerevisiae* BRCT repeat protein Dpb11 functions in the initiation of both DNA replication and cell cycle checkpoints. Here, we report a genetic and physical interaction between Dpb11 and Mec1–Ddc2. A C-terminal domain of Dpb11 is sufficient to associate with Mec1–Ddc2 and strongly stimulates the kinase activity of Mec1 in a Ddc2-dependent manner. Furthermore, Mec1 phosphorylates Dpb11 and thereby amplifies the stimulating effect of Dpb11 on Mec1–Ddc2 kinase activity. Thus, Dpb11 is a functional ortholog of human TopBP1, and the Mec1/ATR activation mechanism is conserved from yeast to humans.**

ATR | checkpoint | DNA damage | TopBP1

Eukaryotic cells have elaborate mechanisms to ensure the faithful maintenance and replication of the genome. Genotoxic stress activates a signal transduction pathway called the DNA damage response (DDR) that coordinates cell cycle transitions, DNA replication, transcription, apoptosis, and DNA repair (1). In *Saccharomyces cerevisiae*, the PIKK (Phosphatidylinositol 3-kinase-related kinase) kinase Mec1, the mammalian ATR ortholog, senses DNA damage and replication stress and initiates the DNA damage response (2). Mec1 phosphorylates substrates involved in DNA replication and repair, cell cycle checkpoints, RNA metabolism, and transcription (3).

Activation of the DNA damage response requires the colocalization of 2 checkpoint complexes to damaged chromatin. Mec1 is localized to DNA damage through its associated partner, Ddc2, which binds to RPA-coated ssDNA (4–7). Independently of Mec1–Ddc2, the 9-1-1 checkpoint clamp complex composed of Ddc1–Mec3–Rad17 (Rad9–Hus1–Rad1 in mammalian cells) also localizes to sites of DNA damage (8–10). Forced colocalization of the Mec1–Ddc2 complex and the 9-1-1 complex to chromatin can trigger the DNA damage response in the absence of a DNA lesion (11). Furthermore, colocalization of only the Ddc1 subunit of the 9-1-1 complex with Mec1–Ddc2 is sufficient to activate to the DNA damage response (11). This suggests that Ddc1 and/or a Ddc1-interacting protein may function as a direct activator of Mec1. In fact, Ddc1 purified from yeast has been shown to modestly stimulate the kinase activity of Mec1 in vitro under low-salt conditions (12).

Ddc1 interacts with another checkpoint protein Dpb11 (13). Dpb11 and its sequence homologs are essential for the initiation of DNA replication in eukaryotic organisms (14, 15). Specifically, Dpb11 acts as a molecular bridge between the Sld3–Cdc45–MCM helicase complex and the Sld2–DNA polymerase  $\epsilon$  complex (16, 17). Dpb11 also has a cell cycle checkpoint function and *dpb11* mutants exhibit sensitivity to DNA damaging agents and replication stress (14, 18). In metazoans, the Dpb11 homolog TopBP1 is a general activator of ATR. A region between the sixth and seventh BRCT (BRCA1 C-terminal) domains of TopBP1 called the ATR activation domain (AAD) is sufficient to activate ATR–ATRIP in vitro and in cells (19). However, it is not clear whether Mec1 is regulated in the same manner as ATR because Dpb11 lacks sequence homology to the AAD of TopBP1 and has not been shown to interact with the Mec1–Ddc2 complex.

We recently reported that a *ddc2* mutation, termed *ddc2-top*, causes sensitivity to DNA damage and replication stress and defects in Mec1 checkpoint signaling (20). Here, we took advantage of the *ddc2-top* mutant to search for a Mec1 activator protein. We found that Ddc2 interacts genetically and physically with Dpb11. Moreover, we discovered a domain of Dpb11 that is sufficient to strongly stimulate the kinase activity of Mec1. Mec1 phosphorylates Dpb11 and this phosphorylation further enhances the ability of Dpb11 to serve as a Mec1 activator. These data demonstrate that Dpb11 is a Mec1 activator linking the Mec1–Ddc2 and 9-1-1 checkpoint complexes.

## Results

**Dpb11 Suppresses the HU Sensitivity of *ddc2-top*.** We hypothesized that the *ddc2-top* mutation disrupted an interaction between Ddc2 and a Mec1–Ddc2 activator. If so, overexpression of that protein might be expected to suppress the phenotype of *ddc2-top* yeast. To search for this protein, we overexpressed the 2 likely candidates, Ddc1 and Dpb11, in *ddc2-top* yeast. Dpb11 but not Ddc1 partially suppressed the hydroxyurea (HU) sensitivity caused by the *ddc2-top* mutation. This effect was greater when Dpb11 was expressed on a high-copy (2 $\mu$ ) plasmid compared with a low-copy (cen) plasmid (Fig. 1A). Overexpression of Dpb11 did not restore the viability of  $\Delta$ *ddc2* yeast in hydroxyurea, demonstrating that the observed suppression was Ddc2-dependent (Fig. 1B). *dpb11-1* yeast are sensitive to hydroxyurea and DNA damaging agents and are defective in S-phase checkpoint signaling (14, 18), similar to the *ddc2-top* phenotype (20). Overexpression of the Dpb11–1 mutant did not suppress the HU sensitivity of *ddc2-top* yeast indicating allele-specific suppression that is consistent with a direct protein–protein interaction (Fig. 1C).

**Dpb11 Associates with Mec1–Ddc2.** Dpb11 contains four BRCT repeats, which function in tandem as phosphoprotein-interacting domains. The N-terminal pair and C-terminal pair of BRCT domains bind to CDK-phosphorylated residues of Sld3 and Sld2, respectively (16, 17). *dpb11-1* encodes a nonsense mutation at residue 583, resulting in a truncation C-terminal to the BRCT domains (21) (Fig. 2A). Given that *dpb11-1* is unable to suppress to the HU sensitivity of *ddc2-top* yeast, we suspected that the C-terminal region of Dpb11 might be responsible for an interaction with Ddc2. To determine whether there is a physical interaction between Dpb11 and Ddc2, we incubated yeast protein lysates with recombinant Dpb11 fragments encoding full-length Dpb11, the Dpb11–1 mutant, or the Dpb11 C-terminal domain, Dpb11-C (amino acid 571–764). Both Dpb11 and Dpb11-C bind to Mec1–Ddc2 but neither GST alone nor the Dpb11–1 protein bind indicating that the C terminus of Dpb11 is both necessary and sufficient to interact with Mec1–Ddc2 (Fig. 2B).

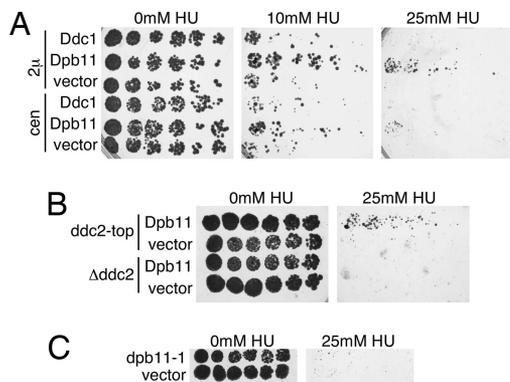
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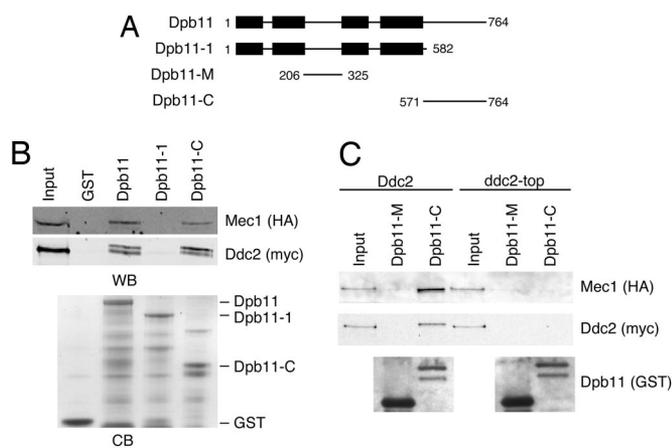
<sup>1</sup>To whom correspondence should be addressed at Department of Biochemistry, Vanderbilt University, 613 Light Hall, Nashville, TN 37232. E-mail: david.cortez@vanderbilt.edu.

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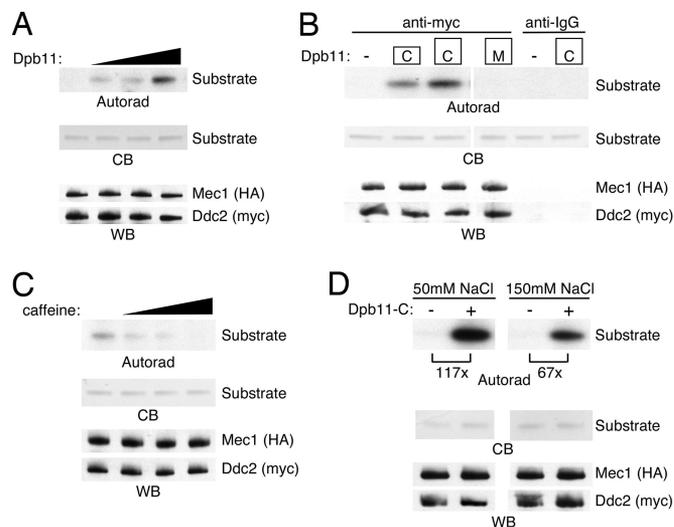


**Fig. 1.** Overexpression of Dpb11 but not Ddc1 suppresses the HU sensitivity of *ddc2-top*. (A) YDM003, a  $\Delta ddc2 \Delta sml1$  yeast strain carrying pDM158 (a *URA3* *CEN* plasmid expressing *ddc2-top* under its endogenous promoter) was transformed with galactose-inducible Dpb11 or Ddc1 high-copy (2 $\mu$ ) or low-copy (*cen*) expression vectors or an empty vector control. Cells were grown to mid-log phase and serial dilutions were spotted onto galactose plates with the indicated concentration of hydroxyurea (HU) and incubated at 30 °C. (B and C) The HU sensitivity of  $\Delta ddc2 \Delta sml1$  or *ddc2-top* yeast strains containing galactose-inducible Dpb11, Dpb11-1, or an empty *TRP1* plasmid as indicated were compared on galactose plates as in A.

We then examined whether the Dpb11 interaction with Mec1–Ddc2 is disrupted by the *ddc2-top* mutation as suggested by the genetic suppression results. Yeast protein lysates from Ddc2 or *ddc2-top* cells were incubated with Dpb11-C or the other non-BRCT region of Dpb11, Dpb11-M (amino acids 206–325) as a control. Unlike wild-type Ddc2, Ddc2-top does not associate with Dpb11-C (Fig. 2C). Furthermore, in the presence of Ddc2-top, Mec1 is no longer able to associate with Dpb11, indicating that the observed association of Mec1 with Dpb11 is Ddc2-dependent. We conclude that Ddc2 contains a binding site



**Fig. 2.** The C terminus of Dpb11 interacts with Mec1–Ddc2. (A) Schematic of wild-type Dpb11, the Dpb11-1, Dpb11-M, and Dpb11-C proteins used in this study. Boxes indicate BRCT domains. (B) Dpb11 and Dpb11-C interact with Mec1–Ddc2. Recombinant GST, Dpb11, Dpb11-1, or Dpb11-C proteins bound to glutathione beads were incubated with yeast protein extracts. After extensive washing, the proteins bound to the beads were eluted, separated by SDS/PAGE and immunoblotted with antibodies to detect Mec1 and Ddc2 (WB). The GST-tagged recombinant proteins used in this experiment were separated by SDS/PAGE and visualized with Coomassie blue staining (CB). (C) Yeast protein extracts from cells expressing Ddc2 or Ddc2-top were incubated with recombinant GST-tagged Dpb11 fragments bound to glutathione beads. Proteins bound to the beads were eluted, separated by SDS/PAGE, and immunoblotted with antibodies to detect Mec1, Ddc2, and the GST-tagged Dpb11 fragments. Input represents 10% of the lysate loaded in the binding reactions.

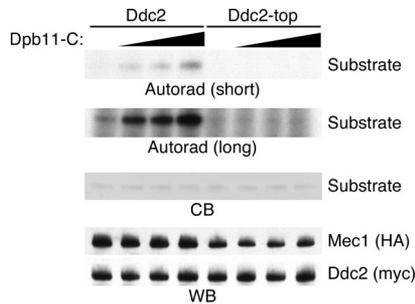


**Fig. 3.** Dpb11 activates Mec1–Ddc2. (A) HA-Mec1-myc-Ddc2 complexes were immunopurified from yeast lysates and were incubated with recombinant Dpb11, substrate, and  $\gamma$ -<sup>32</sup>P ATP. Kinase reactions were separated by SDS/PAGE, stained with Coomassie blue (CB) to visualize the amount of substrate, and exposed to film (autorad). A duplicate gel was immunoblotted with anti-HA and anti-myc antibodies to detect Mec1 and Ddc2, respectively (WB). (B) Mec1–Ddc2 complex kinase reactions were performed in the absence (–) or presence of recombinant Dpb11-M or Dpb11-C fragments. Anti-IgG antibody was used as a control for the immunopurifications. (C) Mec1–Ddc2 complex kinase reactions containing Dpb11-C were performed in the presence of 0, 0.2, 1, and 5 mM caffeine. (D) Mec1–Ddc2 complex kinase reactions were performed with buffer containing 50 mM or 150 mM NaCl. Substrate phosphorylation was quantitated by using a PhosphorImager.

for Dpb11 that is necessary for the interaction of the Mec1–Ddc2 complex with the C terminus of Dpb11.

**Dpb11 Stimulates the Kinase Activity of Mec1–Ddc2.** We next tested whether Dpb11 could function as a Mec1 activator. We immunopurified Mec1–Ddc2 from yeast lysates and incubated the complexes with recombinant Dpb11 and an established substrate, MCM2 (22, 23). Addition of Dpb11 strongly stimulated the kinase activity of Mec1 in a dose-dependent manner (Fig. 3A). Dpb11-C also stimulated Mec1 kinase activity, whereas Dpb11-M did not (Fig. 3B). Thus, the Dpb11 C-terminal domain is sufficient to activate Mec1. Dpb11-C did not produce an increase in substrate phosphorylation in control immunoprecipitation reactions lacking Mec1 (Fig. 3B). To confirm that the observed kinase activity was due to Mec1, we carried out the Mec1 kinase assay in the presence of Dpb11-C and increasing amounts of caffeine, an inhibitor of PIK kinases (24). Caffeine effectively inhibited the kinase activity of the activated Mec1 (Fig. 3C). These Mec1 kinase assays were performed in low-salt (50 mM NaCl) conditions. Under these conditions, Dpb11 stimulated the kinase activity of Mec1 >100-fold. We wanted to determine whether Dpb11 could stimulate Mec1 in the presence of a physiological salt concentration, so the Mec1 kinase assay was performed in the presence of 150 mM NaCl. In this case, the basal Mec1 kinase activity and the stimulated Mec1 kinase activity were both slightly lower, yet the degree of Dpb11-dependent activation was still very large (67-fold) (Fig. 3D). Thus, Dpb11 is a potent Mec1 activator.

**Dpb11-Dependent Mec1 Activation Requires a Ddc2-Dpb11 Interaction.** We previously reported that *ddc2-top* yeast have a defect in checkpoint signaling after replication stress and DNA damage (20). To determine whether this defect is due to an inability of Mec1–Ddc2-top complexes to be activated by Dpb11, Mec1–



**Fig. 4.** Ddc2-top does not support Mec1 activation by Dpb11. Mec1–Ddc2 or Mec1–Ddc2-top complexes were purified and incubated with increasing amounts of Dpb11-C, substrate, and  $\gamma$ - $^{32}$ P ATP. Kinase reactions were separated by SDS/PAGE, stained with Coomassie blue (CB) to visualize the substrate, and exposed to film (autorad). Both long and short exposures of the autoradiogram are shown. A duplicate gel was blotted and probed with anti-HA and anti-myc antibodies to detect Mec1 and Ddc2, respectively (WB).

Ddc2 and Mec1–Ddc2-top complexes were isolated and incubated with increasing amounts of the Dpb11-C fragment. In the presence of wild-type Ddc2, we observed increased Mec1 kinase activity with increasing amounts of Dpb11-C. However, even high concentrations of Dpb11-C did not stimulate Mec1–Ddc2-top complexes (Fig. 4). Therefore, an interaction between Ddc2 and Dpb11 is required for Dpb11 to activate Mec1. After the high-stringency washes used for the kinase assays, we did note that the Ddc2-top mutant samples contained slightly less associated Mec1 suggesting the mutation may slightly alter the affinity of Mec1 for Ddc2 (Fig. 4).

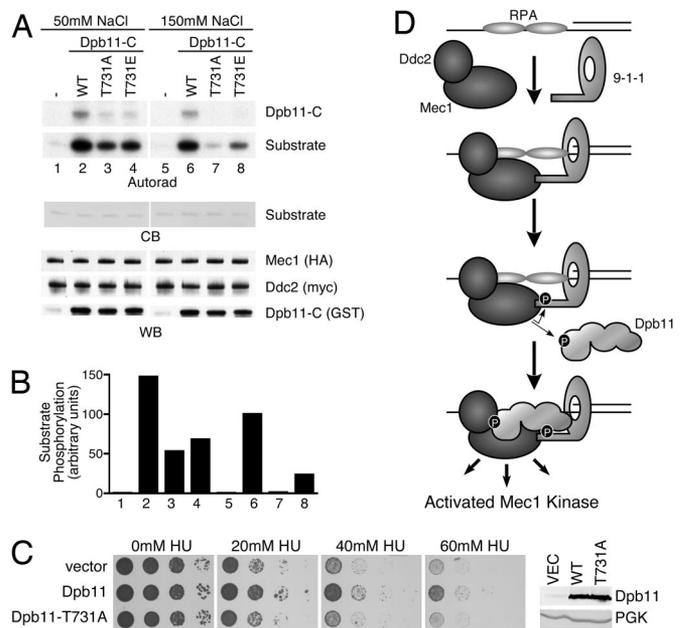
**Dpb11 Phosphorylation Potentiates Mec1 Activation.** Mec1 targets its substrates at SQ/TQ motifs (3, 25). In the course of our kinase assays, we noticed that Dpb11-C itself served as a Mec1 substrate. This region of Dpb11 contains a single Mec1 consensus site, threonine 731. Mutation of T731 to alanine largely abolished the phosphorylation of Dpb11 by Mec1 (Fig. 5A).

To assess the functional significance of this Dpb11 phosphorylation, we tested whether T731 phosphorylation regulates the ability of Dpb11 to activate Mec1. In low-salt (50 mM) conditions, Dpb11-C T731A stimulated Mec1 kinase activity  $\approx$ 3-fold less efficiently than wild-type Dpb11-C (Fig. 5A and B). However, at more physiologically relevant salt concentrations, Dpb11-C T731A is 100-fold less efficient at activating Mec1 compared with wild-type Dpb11-C (Fig. 5A and B). These data suggest that Mec1-catalyzed Dpb11 T731 phosphorylation increases the ability of Dpb11 to activate the Mec1–Ddc2 complex. We also created a Dpb11-C phosphorylation-mimetic mutant at this site. The Dpb11-C T731E mutant partially restored Mec1 activation compared with the T731A mutant at the physiological salt concentration (Fig. 5A and B), supporting the idea that T731 phosphorylation promotes the ability of Dpb11 to activate Mec1.

Finally, to determine the effect of the T731 mutation on Mec1–Ddc2 function in vivo we asked whether the T731A protein could function like wild-type Dpb11 to suppress the HU hypersensitivity of the *ddc2-top* yeast. In contrast to wild-type Dpb11, overexpression of Dpb11 T731A does not reduce the HU sensitivity of the *ddc2-top* yeast (Fig. 5C). Thus, phosphorylation of Dpb11 is important to activate Mec1–Ddc2 complexes in vitro and regulate Mec1–Ddc2 activity in vivo.

## Discussion

The Mec1/ATR kinase is an essential regulator of genome integrity (2). In this study, we examined how the kinase activity of Mec1 is regulated and discovered that an essential replication and checkpoint protein, Dpb11, functions as a direct Mec1 activator. Just as Dpb11 bridges the helicase and polymerase



**Fig. 5.** Dpb11 T731 phosphorylation potentiates Mec1 activation. (A) Mec1–Ddc2 complexes were purified and incubated with wild-type or mutant forms of Dpb11-C, substrate, and  $\gamma$ - $^{32}$ P ATP. Kinase reactions were separated by SDS/PAGE, stained with Coomassie blue (CB) to visualize the substrate, and exposed to film (autorad). A duplicate gel was blotted and probed with anti-HA and anti-myc antibodies to detect Mec1 and Ddc2, respectively. An anti-GST antibody was used to detect Dpb11-C proteins (WB). (B) Quantification of substrate phosphorylation corresponding to the numbered lanes in A. (C) YDM003, a  $\Delta ddc2 \Delta sm1$  yeast strain carrying pDM158 (a *URA3 CEN* plasmid expressing *ddc2-top* under its endogenous promoter) was transformed with galactose-inducible Dpb11 or Dpb11 T731A ( $2\mu$ ) expression vectors or an empty vector control. Cells were grown to mid-log phase and serial dilutions were spotted onto galactose plates with the indicated concentration of hydroxyurea (HU) and incubated at 30 °C. The expression level of the wild-type and T731A Dpb11 proteins was confirmed to be equivalent by Western blotting. Anti-PGK antibodies were used as a loading control. (D) Simplified model of Mec1–Ddc2 activation described in Discussion. In this model, we propose that Dpb11 functions downstream of 9-1-1. Our data do not exclude the possibility that Dpb11 could function upstream of 9-1-1 in some circumstances. In this alternative scenario, an initial weak Dpb11–Ddc2 interaction could promote partial Mec1 activation, leading to Ddc1 phosphorylation. Phosphorylated Ddc1 could then stabilize the Dpb11 protein at the damaged site and allow further amplification of Mec1 signaling.

complexes during the initiation of replication, our data suggests that Dpb11 also serves as a link between the Mec1–Ddc2 kinase complex and 9-1-1 checkpoint clamp complex in the DNA damage response. Significantly, phosphorylation of Dpb11 by Mec1 increases its ability to serve as an activator providing a means of signal amplification.

The Mec1 activation domain of Dpb11 is located C-terminal to its BRCT domains. This Dpb11-C domain appears to be functionally equivalent to the TopBP1 ATR activation domain (AAD) (19) despite the lack of sequence conservation. The TopBP1 AAD binds the ATR–ATRIP complex through interactions with ATRIP and the ATR PIKK regulatory domain (PRD) (20). An *ATRIP* allele (*-top*) that eliminates TopBP1 binding to ATR–ATRIP cannot be activated. Similarly, we found an equivalent mutation in *Ddc2* (*-top*) also cannot be activated by Dpb11. Given that very little sequence homology exists between Ddc2 and ATRIP, it is not surprising that the sequence of the TopBP1 AAD is not similar to the Dpb11-C domain. Although, given the similar mechanisms of ATR and Mec1 activation, we expect that Dpb11-C and the TopBP1 AAD proteins adopt a similar tertiary structure.



1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride]. Bound proteins were eluted in 2× SDS sample buffer, and processed for SDS/PAGE.

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