Cell-cycle-specific activators of the Mec1/ATR checkpoint kinase

Vasundhara M. Navadgi-Patil and Peter M. Burgers[1]
Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, MO 63110, U.S.A.

Abstract

Mec1 [ATR (ataxia telangiectasia mutated- and Rad3-related) in humans] is the principle kinase responsible for checkpoint activation in response to replication stress and DNA damage in *Saccharomyces cerevisiae*. The heterotrimeric checkpoint clamp, 9-1-1 (checkpoint clamp of Rad9, Rad1 and Hus1 in humans and Ddc1, Rad17 and Mec3 in *S. cerevisiae*; Ddc1-Mec3-Rad17) and the DNA replication initiation factor Dpb11 (human TopBP1) are the two known activators of Mec1. The 9-1-1 clamp functions in checkpoint activation in G_1- and G_2-phase, but its employment differs between these two phases of the cell cycle. The Ddc1 (human Rad9) subunit of the clamp directly activates Mec1 in G_1-phase, an activity identified only in *S. cerevisiae* so far. However, in G_2-phase, the 9-1-1 clamp activates the checkpoint by two mechanisms. One mechanism includes direct activation of Mec1 by the unstructured C-terminal tail of Ddc1. The second mechanism involves the recruitment of Dpb11 by the phosphorylated C-terminal tail of Ddc1. The latter mechanism is highly conserved and also functions in response to replication stress in higher eukaryotes. In *S. cerevisiae*, however, both the 9-1-1 clamp and the Dpb11 are partially redundant for checkpoint activation in response to replication stress, suggesting the existence of additional activators of Mec1.

Introduction

Faithful replication of DNA is crucial for maintaining genomic integrity. In addition to the presence of high-fidelity DNA polymerases to replicate the DNA, cells also have to ensure that the integrity of the genome is maintained in the face of constantly recurring DNA damage. Various repair mechanisms exist to deal with different kinds of damage in the DNA. In addition, various checkpoint machineries exist, which stop or slow down cell cycle progression until the damage is rectified. These DNA replication, repair and checkpoint activation pathways are highly regulated and coordinated. Defects in any of these functions lead to genomic instability. Initial processing of the DNA damage by damage sensors is required to activate the checkpoint. Although DNA can be damaged in many different ways, many forms of damage are processed to generate ssDNA (single-stranded DNA). Binding of the single-stranded-binding protein RPA (replication protein A) to ssDNA appears to be the common signal to activate the DNA damage checkpoint. For instance, UV-damaged DNA is repaired by the NER (nucleotide excision repair) pathway. Initial processing of the damage is required for generating the ssDNA in order to recruit the 9-1-1 clamp (checkpoint clamp of Rad9, Rad1 and Hus1 in humans and Ddc1, Rad17 and Mec3 in *Saccharomyces cerevisiae*) [1], which is an initial event in the activation of the DNA damage checkpoint. Similarly, DNA double-strand breaks have to be processed to generate 3′-ssDNA tails for repair by homologous recombination, but this process also serves to recruit the checkpoint machinery [2,3]. Replication stress has been shown to generate long stretches of ssDNA, most likely because of uncoupling of the DNA polymerase and helicase activities at the stalled replication fork. These stretches of ssDNA are coated with RPA and form a signal for the recruitment of the replication checkpoint machinery [4]. ssDNA generated at telomeres by the loss of the telomere ssDNA-binding protein Cdc13 (cell division cycle 13) also activates the checkpoint [5]. However, DNA damage that is repaired by the Base Excision Repair machinery does not generate long stretches of ssDNA and hence the damage does not appear to elicit checkpoint responses in G_1- and G_2-phase, but the checkpoint is activated in S-phase once the replication fork stalls at these damaged sites [6,7]. Defects in the Base Excision Repair machinery lead to checkpoint activation in both G_1- and G_2-phase, suggesting that other pathways such as NER process the damage and generate ssDNA in the process [6].

While this review focuses on the initial steps of the checkpoint pathway, i.e. the activation of Mec1 kinase, it is useful to briefly view this activation in the context of the entire pathway, as outlined in Figure 1. Damage and stress that result in the generation of RPA-coated ssDNA leads to the independent recruitment of both Mec1 and 9-1-1 to these sites. Mec1 [ATR (ataxia telangiectasia mutated- and Rad3-related) in humans] is required for checkpoint activation

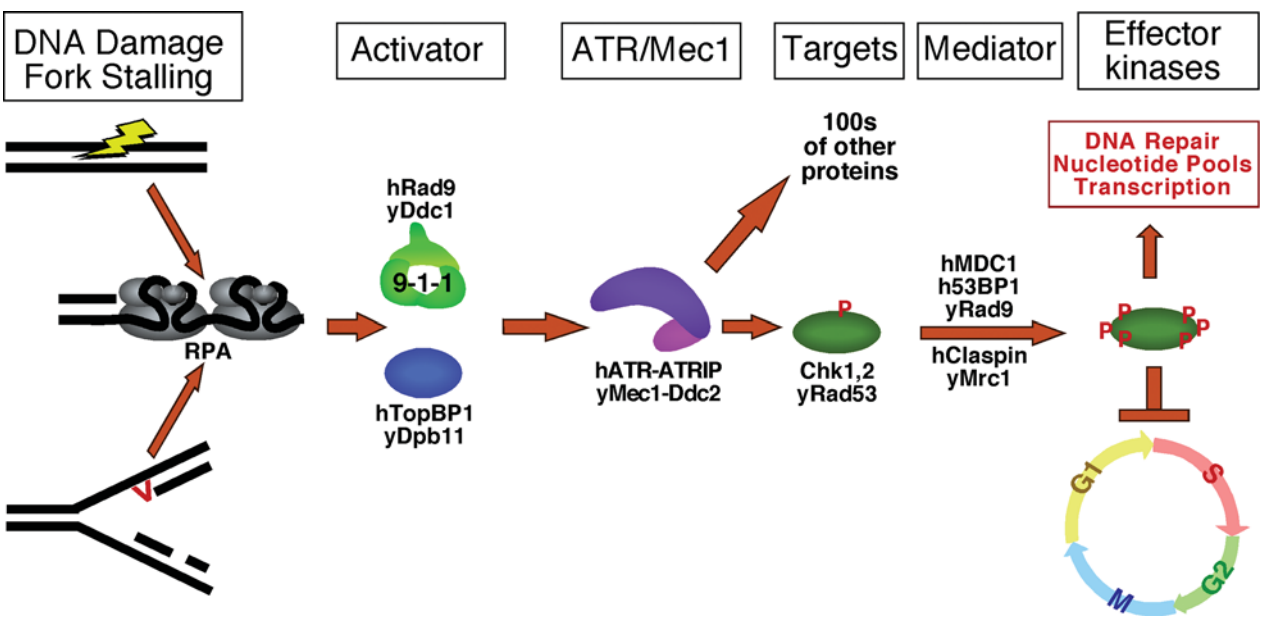
Key words: ataxia telangiectasia mutated- and Rad3-related (ATR), cell cycle, checkpoint, DNA damage, DNA replication, Mec1.

Abbreviations used: ATR, ataxia telangiectasia mutated- and Rad3-related; ATRIP, ATR-interacting protein; BRCT, BRCA1 C-terminal domain; Chk, checkpoint kinase; dsDNA, double-stranded DNA; HU, hydroxyurea; MMS, methyl methanesulfonate; NER, nucleotide excision repair; PCNA, proliferating-cell nuclear antigen; RFC, replication factor C; RPA, replication protein A; ssDNA, single-stranded DNA.

[1]To whom correspondence should be addressed (email burgers@biochem.wustl.edu).

Figure 1 | **Overview of the Mec1/ATR checkpoint**

Damaged DNA is processed by the DNA repair machinery, which results in the formation of the RPA-coated ssDNA region. The 9-1-1 checkpoint clamp is loaded on to this DNA, which may lead to the recruitment of Dpb11. These factors bind and activate Mec1/ATR that is independently localized to the RPA-coated ssDNA. Among the many targets of Mec1/ATR are the effector kinases Rad53/Chk1,2 that subsequently undergo *trans*-autophosphorylation with the aid of a mediator scaffold Rad9/MDC1/53BP1 (for DNA damage) or Mrc1/Claspin (for replication stress). Hyperphosphorylated and activated Rad53/Chk1,2 regulates downstream pathways including cell cycle arrest.



in all stages of the cell cycle in response to various kinds of DNA lesions and following replication stress [8]. The kinase activity of Mec1 is highly regulated and is activated only during genotoxic stress. Mec1 is the catalytic subunit and Ddc2 [ATRIP (ATR-interacting protein) in humans] is the regulatory subunit of a heterodimeric complex. Ddc2 (ATRIP) regulates binding of the Mec1–Ddc2 complex to DNA [9,10]. Ddc1, Rad17 and Mec3 (Rad9-Rad1-Hus1) form a heterotrimeric checkpoint clamp (9-1-1) that is similar to the replication clamp PCNA (proliferating-cell nuclear antigen) [11,12]. Rad24–RFC (replication factor C) (human Rad17–RFC) is the checkpoint clamp loader that loads the clamp on DNA [13–15]. The yeast checkpoint clamp is essential for checkpoint activation in G_1- and G_2-phases, but is dispensable for S-phase checkpoint activation [16–18]. Activation of Mec1 causes phosphorylation of a large number of proteins in the cell, including the effector kinases. Chk (checkpoint kinase) 1 and Rad53 are the two effector kinases in *S. cerevisiae*. Chk1 is designated as such in all organisms, whereas hChk2 (human Chk2) is the sequence homologue of Rad53. The actual employment of these two kinases in checkpoint signalling has evolved somewhat differently in different eukaryotes (reviewed in [19,20]). *S. cerevisiae* Rad53 is required for the checkpoint in response to DNA damage during all phases of the cell cycle and in response to replication fork stalling, whereas scChk1 (*S. cerevisiae* Chk1) is primarily required for the DNA damage checkpoint in G_2/M-phase. In human cells, however, many of the Rad53 functions, including the replication checkpoint, are assumed by Chk1. This is somewhat an oversimplification as the two effector kinases show partial redundancy in most pathways.

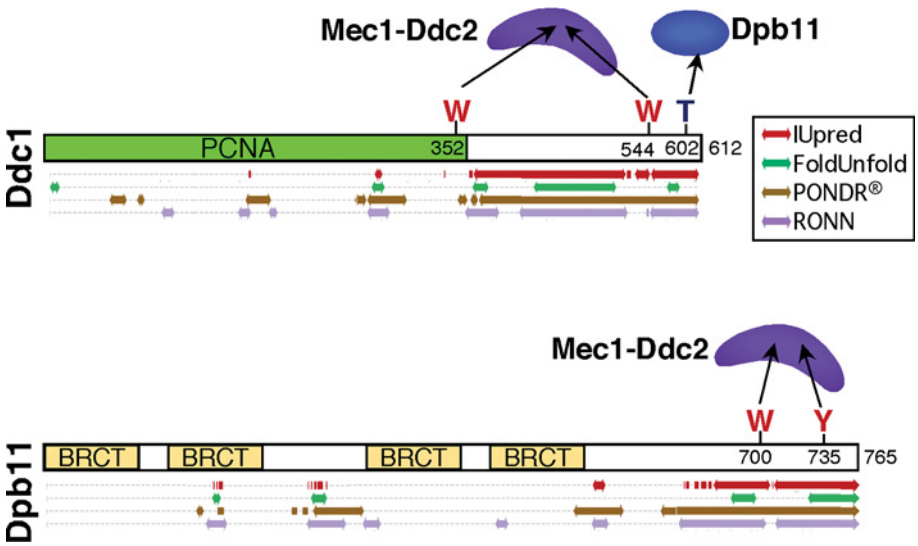
Following initial phosphorylation of Rad53, complete activation involves an autotransphorylation cascade of Rad53 that is mediated in the scaffold by a mediator protein, Rad9 or Mrc1. These proteins also need to be phosphorylated in order to function as mediators, and they are phosphorylated by activated Mec1. In the final steps of this signal transduction cascade, hyperphosphorylated Rad53 is active as a protein kinase and phosphorylates key downstream targets in order to activate many downstream pathways including cell cycle arrest. Other than causing the cell cycle delay in response to genotoxic stress, Mec1 and Rad53 are also important for normal DNA replication, fork stability and regulation of late origin firing in the presence of replication stress (reviewed in [21]).

In vitro activation of Mec1 by the 9-1-1 clamp

The heterotrimeric 9-1-1 clamp is loaded on ssDNA–dsDNA (double-stranded DNA) junctions by the Rad24–RFC clamp loader in an ATP-driven reaction. RPA restricts the clamp loading specifically to the 5′ ssDNA–dsDNA junctions [22]. To date there is no other function known for Rad24–RFC other than loading the 9-1-1 clamp, and the clamp

Figure 2 | **Domain structures of Ddc1 and Dpb11**

Structurally disordered regions, according to four different prediction programs, are indicated by coloured bars below the linear representation of each protein. They were generated using the MeDor metaserver (http://www.afmb.univ-mrs.fr). Indicated are the anchoring aromatic amino acids in the motifs that are critical for Mec1 activation. Also shown, Ddc1 Thr^{602} that mediates binding of Dpb11 upon phosphorylation.



subunits Ddc1, Rad17 and Mec3 are only known to act as a heterotrimeric clamp and not as individual subunits. The 9-1-1 clamp stimulates the kinase activity of Mec1 towards all its physiological targets such as Rad53, RPA, Rad24, Ddc1, Mec3 and also a common PIKK (phosphoinositide 3-kinase-related kinase) target such as PHAS-I (phosphorylated heat- and acid-stable protein regulated by insulin 1) [23]. Under physiological conditions, activation of Mec1 critically depends on the loading of 9-1-1 by Rad24–RFC on to the appropriate DNA substrate. However, at low-salt conditions, the Ddc1 subunit of the 9-1-1 clamp is able to interact with Mec1 and activates its kinase activity in the absence of DNA, clamp loader or any other clamp subunits, suggesting that the critical motifs for activating Mec1 reside in Ddc1 [23]. Remarkably, this hypothesis was validated by the artificial co-localization of Ddc1 with Mec1, via its Ddc2 subunits. This co-localization serves to activate the checkpoint even in the absence of DNA damage [24]. These studies suggested that the minimal requirement for checkpoint activation is the interaction of the Ddc1 subunit with the Mec1–Ddc2 kinase. The N-terminal tail of Ddc1 has a PCNA-like domain and is involved in the 9-1-1 clamp formation. The C-terminal tail is unstructured and is dispensable for the clamp formation and loading, but it is essential for Mec1 activation *in vitro* and checkpoint function *in vivo* [18]. The Ddc1 (Rad9) homologues have C-terminal tails of various lengths and very poor sequence conservation. Ddc1 has a bipartite Mec1 activation domain, with one motif near the C-terminal end of the PCNA-like domain and the other motif approx. 200 amino acids further down in the unstructured C-terminal tail (Figure 2). Each motif is loosely characterized by a tryptophan residue surrounded by 1 or 2 hydrophobic amino acids. Mutation of the two critical aromatic residues, Trp^{352} and Trp^{544}, in these motifs of Ddc1 (*ddc1–2W2A*) leads to the failure of Mec1 activation *in vitro* and G_1 checkpoint activation *in vivo* [18]. In *Schizosaccharomyces pombe*, these two motifs are separated by only 40 amino acids. These observations suggest that conformational flexibility of the unstructured region separating the two motifs permits a high degree of length variability of this linker region. And indeed, a small 30-mer peptide sequence consisting of the two motifs of Ddc1 was sufficient to activate Mec1 *in vitro*, and mutation of either tryptophan residue completely abrogated activity of the peptide. Moreover, a similar peptide consisting of the putative Mec1 activation motifs in *S. pombe* Rad9, also activated *S. cerevisiae* Mec1 (V.M. Navadgi-Patil and P.M. Burgers, unpublished work), suggesting that both the activation motifs and the activation mechanism may be conserved.

The crystal structures of the human 9-1-1 clamp reveal a strong structural similarity with the replication clamp PCNA [11,12]. His^{239} of hRad9, analogous to the critical Trp^{352} of Ddc1 is solvent-exposed on the outer surface of the clamp allowing for a possible interaction with ATR/Mec1. Remarkably, a H239R mutation in the human Rad9 is associated with increased incidence of lung adenocarcinoma [25]. Interestingly when the Trp^{352} was replaced by a histidine residue in the Ddc1 bipartite activation peptide, it still retained its ability to activate Mec1 while a mutation to arginine residue completely inactivated it [18]. Although there is no evidence as yet that the 9-1-1 clamp from other eukaryotes activate the ATR kinase, these studies hint at such a possibility.

In vitro activation of Mec1 by Dpb11/TopBP1

S. cerevisiae Dpb11, the orthologue of vertebrate TopBP1 and *S. pombe* Cut5, is an essential replication protein with several BRCT (BRCA1 C-terminal domain) motifs. Binding of phosphorylated replication initiation factors to these BRCT domains defines the CDK (cyclin-dependent kinase)-dependent step in replisome biogenesis [26,27]. Following initial studies in *Xenopus* that TopBP1 can activate ATR [28], similar studies in human cells and in *S. cerevisiae* showed that this mechanism of activation is highly conserved [29–31]. Although the studies in *Xenopus* and in yeast failed to establish a DNA dependence of this Mec1/ATR activation mechanism, recent studies with the purified reconstituted human checkpoint system show that the presence of RPA-coated ssDNA significantly stimulates TopBP1 in the activation of ATR [32]. Moreover, this stimulation is dependent on the interaction between TopBP1 and RPA.

Mec1 activation by Dpb11, and ATR by TopBP1, is similar to that of the Ddc1 subunit of 9-1-1. First, activation of Mec1 by Dpb11 or by Ddc1 leads to enhanced phosphorylation of all its substrates. Secondly, activation by Dpb11 and by Ddc1 requires their unstructured C-terminal tails [18,30,31] (Figure 2). Thirdly, as with Ddc1, a bipartite domain in the unstructured tail of Dpb11 mediates Mec1 activation. Similarly, each motif is anchored by an aromatic amino acid, and mutation of the critical aromatic amino acid in either motif leads to a defect in Mec1 activation (Figure 2; V.M. Navadgi-Patil and P.M. Burgers, unpublished work). Although, no sequence similarity can be found between the activation domain of yeast Dpb11 and vertebrate TopBP1, the vertebrate activation domain is also unstructured, and mutation of a conserved (in vertebrates) tryptophan residue also decreased ATR activation [28]. Furthermore, the activation domain of *Xenopus* TopBP1 efficiently activated *S. cerevisiae* Mec1, attesting to the strong evolutionary conservation of activation mechanisms (V.M. Navadgi-Patil and P.M. Burgers, unpublished work).

Considering the analogous bipartite motif structure of the Ddc1 and Dpb11 activators, it is likely that both activators act on similar sites of Mec1–Ddc2. However, the 9-1-1 clamp and Dpb11 display synergism in Mec1 activation [30]. The 9-1-1 clamp with an activation-defective *ddc1-2W2A* mutant that is still loaded appropriately on to DNA, retains interaction with Dpb11 and is able to enhance the activation of Mec1 by Dpb11, most likely through a facilitated recruitment mechanism [18].

Direct activation of Mec1 by the 9-1-1 clamp in G_1-phase

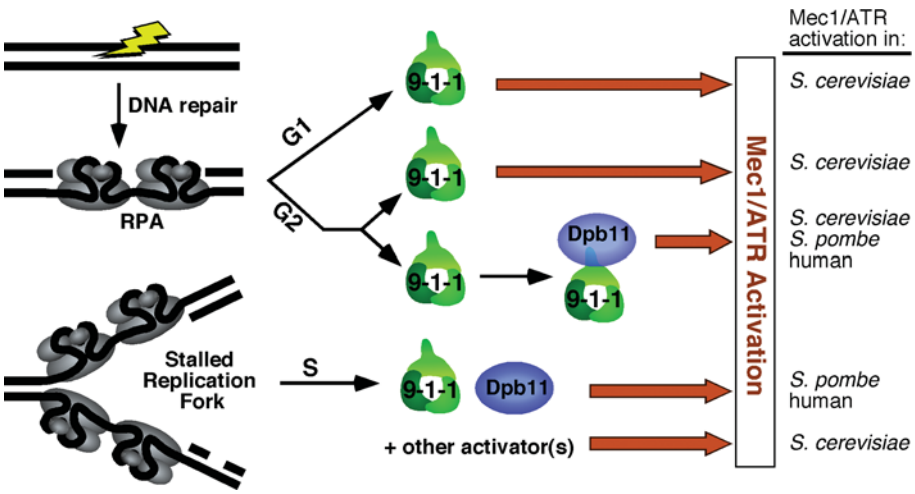
The activation of ATR in *S. pombe* and higher eukaryotes appears to involve a single pathway in which both the 9-1-1 clamp and the TopBP1/Cut5 function [33]. TopBP1/Cut5 interacts with Rad9 subunit of the 9-1-1 clamp and the interaction is dependent on the phosphorylation of the Rad9 C-terminal tail. The 9-1-1 clamp acts to recruit TopBP1/Cut5 to stalled replication forks and damage sites [34–36]. Whereas TopBP1 has been shown to activate ATR [28,32], evidence that 9-1-1 activates ATR in *S. pombe* or in higher eukaryotes is lacking. In contrast, although *S. cerevisiae* Mec1 is responsible for checkpoint activation in all stages of the cell cycle, its kinase is activated and regulated differently dependent on the specific phase of the cell cycle.

In the G_1-phase of the cell cycle, checkpoint activation is dependent on the 9-1-1 clamp subunits [17]. The genes coding for the checkpoint clamp and clamp loader belong to the same epistasis group and hence deletion of any single gene disrupts DNA loading of the checkpoint clamp [37,38]. A 9-1-1 clamp with a mutant Ddc1–2W2A subunit is completely defective for Mec1 activation *in vitro*, and for the G_1 checkpoint *in vivo* [18]. However, a robust G_1 checkpoint is observed in *DDC1* mutants that cannot be phosphorylated by Mec1, which is critical for recruitment of Dpb11 to sites of DNA damage [18,39]. This suggests that Dpb11 is not required for the G_1 checkpoint. Indeed, *DPB11* mutants that are defective for activation of Mec1 *in vitro* show no appreciable G_1 checkpoint defect. All these data point to a simple G_1 checkpoint in *S. cerevisiae*, in which mere loading of 9-1-1 to RPA-coated DNA suffices for the activation of Mec1 (Figure 3).

Both the 9-1-1 clamp and Dpb11 activate Mec1 in G_2-phase

The 9-1-1 clamp is also required for Mec1 activation in G_2-phase [16]. Mutational studies of the Ddc1 subunit revealed that the 9-1-1 clamp activates Mec1 by two mechanisms in the G_2-phase [18]. The activation-defective *ddc1–2W2A* mutant that is completely defective in G_1 checkpoint activation, still maintains Rad53 phosphorylation during G_2-phase in response to DNA damage, albeit to a reduced extent of approx. 50%. This *ddc1–2W2A* mutant still retains its interaction with Dpb11 and is able to stimulate the activation of Mec1 by Dpb11. On the other hand phosphorylation-defective mutants of *DDC1* also result in ~2-fold reduced checkpoint activity in G_2-phase, as measured by phosphorylation of Rad53 after treatment with DNA-damaging agents. The same residual activity was observed regardless of whether all five putative serine/threonine residues in Ddc1 were mutated to alanine or just Thr^{602}. As phosphorylation of Thr^{602} is known to mediate recruitment of Dpb11 [39], it is plausible that the defect in Dpb11 recruitment results in a partially defective G_2 checkpoint. These studies indicate a model in which G_2 checkpoint is in part mediated through activation of Mec1 by Ddc1, and in part through activation by Dpb11 following its recruitment to sites of damage by phosphorylated Ddc1 (Figure 3). Consistent with this model, the *ddc1–2W2A, T602A* mutant that is both activation and recruitment-defective, abrogates the G_2 checkpoint. Similarly, a *ddc1–2W2A dpb11–1* double mutant in which both Mec1 activation domains are defective, also lacks a G_2 DNA damage checkpoint response.

Figure 3 | **Cell cycle-dependent activation pathways in *S. cerevisiae***
See the text for details.



Currently, it is not clear why the G_1 and G_2 checkpoint responses are different. Possibly, the 9-1-1 clamp is unable to recruit Dpb11 during the G_1-phase. Dpb11 protein is present throughout the cell cycle, and it participates in replisome assembly during G_1-phase [26,27]. However, it appears that Ddc1 is not phosphorylated in response to DNA damage in G_1-phase, and therefore would be unable to recruit Dpb11 to sites of damage [16]. A second matter of uncertainty regarding the G_2/M checkpoint is how Mec1 activation by 9-1-1 and activation by Dpb11 are distinct, if they are. Do both mechanisms mediate a checkpoint response to all types of DNA damage? This would suggest partial redundancy of these pathways. Alternatively, different types of DNA damage could result in different types of effectors for loading 9-1-1, and some DNA–clamp complexes would be effective in activating Mec1, and others would require recruitment of Dpb11 for appropriate Mec1 activation. One logical consequence of the latter model would be that damage in G_1-phase would be processed to give activation-proficient DNA–clamp complexes. Finally, downstream factors such as the checkpoint mediator Rad9 and chromatin modification factors such as Dot1 could also influence the efficiency of signal transduction of initial checkpoint complexes with or without Dpb11 [39].

Activation of Mec1 during the replication checkpoint

S. pombe Cut5 or vertebrate TopBP1, as well as their 9-1-1 clamps are absolutely required for checkpoint activation in response to stalled replication forks [34–36]. In contrast, in *S. cerevisiae*, the mechanism of checkpoint activation in response to replication stress is far from clear. *ddc1Δ* mutants are sensitive to UV and MMS (methyl methanesulfonate), but not to the replication inhibitor HU (hydroxyurea) [16]. This suggested that 9-1-1 clamp is required in response to DNA damage but not replication stress. The 9-1-1 clamp is, however, required for slowing of the S-phase in response to DNA damage caused by MMS [40]. In a *dpb11-1* mutant lacking the Dpb11 C-terminal domain required for Mec1 activation, Rad53 is still phosphorylated in response to HU treatment [18]. This suggests that Dpb11 is dispensable for Mec1 activation in response to replication stress. Moreover, double mutants eliminating the activation functions of both Ddc1 and Dpb11 still show a checkpoint response after replication stress, albeit somewhat reduced in efficiency [18]. These results suggest that another activator of Mec1 must exist, and this activator is specific for mounting a signal in response to replication stress. The components of replication machinery such as Pol2, Mrc1 and the Sgs1 helicase modulate the activity of Mec1, leading to Rad53 activation during the replication checkpoint. Certain mutants in *POL2*, *SGS1* and *MRC1* have functional G_1/S and G_2/M checkpoints but are defective in S-phase checkpoint regulation [41–43]. Possibly, one or more of these factors, or proteins associated for these factors, may be responsible for activating Mec1 during S-phase. Some calculated predictions with regard to the structure of this putative factor can be made with some confidence. Since the Ddc1 and Dpb11 activators have certain structural characteristics in common (Figure 2), even though they lack recognizable sequence conservation, database searches that identify proteins with these characteristics, combined with biochemical and genetic studies should make the identification of additional Mec1 activator(s) feasible.

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