PERSPECTIVE

Four pillars of the S-phase checkpoint

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The yeast Mec1 kinase is a key regulator of the DNA damage response (DDR). In this issue of *Genes & Development*, Kumar and Burgers (pp. 313–321) report that Ddc1, Dpb11, and Dna2 function in concert to activate Mec1 during S phase of the cell cycle. Furthermore, the Tel1 kinase also contributes to the DDR in S phase when Mec1 activation is compromised.

The DNA damage signaling pathway, which is often referred to as the checkpoint, is crucial for genomic stability in all eukaryotes. In human cells, the ATR (ataxia telangiectasia and Rad3-related) kinase is a master regulator of DNA damage signaling (Cimprich and Cortez 2008; Flynn and Zou 2011). In the budding yeast *Saccharomyces cerevisiae*, Mec1, the ortholog of human ATR, is a key initiator of DNA damage signaling (Melo and Toczyski 2002). During the cell cycle, Mec1 can be activated by a broad spectrum of DNA damage in the G1, S, and G2 phases. In addition, Mec1 can also be activated during S phase by DNA replication stress, such as inhibition of DNA polymerases or depletion of dNTP pools. The activation of Mec1 is multistep process that involves (1) recognition of the DNA structures induced by DNA damage and replication stress, (2) recruitment of Mec1 to sites of DNA damage or stressed replication forks, and (3) stimulation of the kinase activity of Mec1 (Flynn and Zou 2011). Two of the Mec1 regulators, Ddc1 and Dpb11, are capable of stimulating the kinase activity of Mec1 in vitro (Majka et al. 2006; Mordes et al. 2008b; Navadgi-Patil and Burgers 2008). Interestingly, Ddc1 and Dpb11 have distinct roles in Mec1 activation during G1 and G2. Ddc1 is the predominant activator of Mec1 in G1, whereas Ddc1 and Dpb11 act redundantly to activate Mec1 in G2 (Navadgi-Patil and Burgers 2009; Zou 2009). Surprisingly, when the functions of Ddc1 and Dpb11 in Mec1 activation are both disrupted, Mec1 activation still occurs in S phase, suggesting the existence of an additional mechanism for Mec1 activation. In the study by Kumar and Burgers (2013) in this issue of *Genes & Development*, they identify Dna2 as the third protein that stimulates Mec1 in vitro. Furthermore, they demonstrate

that during S phase, Dna2 functions in concert with Ddc1 and Dpb11 to activate Mec1 in vivo and that Tel1 contributes to DNA damage signaling when Mec1 activation is compromised. These findings present a clear picture of how DNA damage signaling is initiated during S phase in budding yeast.

The activation of Mec1 in G1 and G2

Previous genetic and biochemical studies in yeast and other organisms have revealed a number of regulators of ATR/Mec1 (Cimprich and Cortez 2008; Flynn and Zou 2011). In vertebrates, ATR functions in a complex with its regulatory partner, ATRIP. Similarly, the budding yeast Mec1 and Ddc2 (the ortholog of ATRIP) exist and function as a complex. The ssDNA-binding protein RPA (replication protein A) is an important regulator of ATR/Mec1 in both vertebrates and yeast. RPA-coated ssDNA (RPA-ssDNA) is a common intermediate of DNA repair and is induced by DNA replication stress. Both ATRIP and Ddc2 are able to recognize RPA-ssDNA directly, providing a key mechanism to target ATR and Mec1 to sites of DNA damage and stressed replication forks. In addition to RPA, the PCNA-like checkpoint clamp and the RFC-like checkpoint clamp loader are also important to ATR/Mec1 activation. In vitro, the human RFC-like Rad17–RFC complex is able to recognize the 5' junctions of ssDNA and dsDNA and recruit the human PCNA-like 9-1-1 (Rad9–Rad1–Hus1) complex onto dsDNA. Similarly, the budding yeast Rad24–RFC complex also recognizes ssDNA–dsDNA junctions and functions as a loader of the yeast 9-1-1 (Ddc1–Mec3–Rad17) complex. Together, RPA, Rad17/Rad24–RFC, and 9-1-1 provide a unique mechanism to sense resected DNA breaks, ssDNA gaps generated by DNA repair, and ssDNA gaps at or behind stressed replication forks (Zou 2007).

In addition to the DNA damage sensors that recognize specific DNA structures, ATR/Mec1 activation requires additional factors that stimulate their kinase activities. In vertebrates, a protein called TopBP1 is able to directly stimulate the ATR–ATRIP kinase complex in the absence of DNA and other proteins (Kumagai et al. 2006). Furthermore, TopBP1 is crucial for the phosphorylation of ATR substrates in human cells (Yamane et al. 2003; Liu et al. 2006; Mordes et al. 2008a). Similar to TopBP1, its budding yeast ortholog, Dpb11, also stimulates the Mec1–Ddc2 kinase directly (Mordes et al. 2008b; Navadgi-Patil

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and Burgers 2008). Furthermore, Ddc1, a component of the yeast 9-1-1 complex, is able to stimulate Mec1–Ddc2 in vitro under low-salt conditions (Majka et al. 2006). At a physiologically relevant salt concentration, only the yeast 9-1-1 complex but not Ddc1 alone stimulates Mec1–Ddc2. Importantly, only when the 9-1-1 complex is loaded onto DNA structures containing ssDNA–dsDNA junctions is it able to stimulate Mec1–Ddc2, suggesting that this function of 9-1-1 is regulated by DNA damage (Majka et al. 2006). These findings in budding yeast suggest that Mec1 could be stimulated by multiple factors, raising the question of how Mec1 activation is regulated by these factors in vivo.

While both Ddc1 and Dpb11 are implicated in Mec1 activation genetically, proving them as the Mec1 activators in vivo is a challenging task because they are present in complexes with other checkpoint proteins. Furthermore, Dpb11 plays an important role in the initiation of DNA replication at origins, which may affect Mec1 activation indirectly. A critical step toward solving this important problem was made by the Burgers laboratory (Navadgi-Patil and Burgers 2009). In a previous study, Navadgi-Patil and Burgers (2009) carefully analyzed the Mec1 activation domain of Ddc1 and found that an unstructured region near the C terminus of Ddc1 is both necessary and sufficient for Mec1 activation in vitro.

Inspired by a study on the *Xenopus* TopBP1 (Kumagai et al. 2006), they mutated two aromatic residues (W352 and W544) in this unstructured region and showed that the resulting Ddc1-2W2A mutant is defective for Mec1 activation in vitro. Importantly, although Ddc1-2W2A is unable to stimulate Mec1 in vitro, it retains the ability to form the 9-1-1 complex and interact with Dpb11. When the *ddc1-2W2A* mutant cells were arrested in G1 and treated with the DNA-damaging agent 4-nitroquinoline-1-oxide (4-NQO), the phosphorylation of Rad53, a key effector kinase of Mec1, was virtually abolished. This result demonstrates that in G1, Ddc1 is indeed the key activator of Mec1 in vivo (Fig. 1).

Compared with that in G1, the activation of Mec1 in G2 is more complicated. While defective for Rad53 phosphorylation in G1, Ddc1-2W2A is able to support Rad53 phosphorylation in G2. Importantly, the Rad53 phosphorylation in G2 *ddc1-2W2A* cells is dependent on Dpb11. Dpb11 is known to interact with Ddc1 through the phosphorylated residue T602 (Puddu et al. 2008). The Ddc1-2W2A, T602A triple mutant, which is unable to stimulate Mec1 and interact with Dpb11, is completely defective for Rad53 phosphorylation in G2 cells. Thus, Ddc1 contributes to Mec1 activation in two distinct ways in G2. First, Ddc1 directly stimulates Mec1 as a component of the 9-1-1 complex. Second, Ddc1 interacts with

Figure 1. The initiation of DNA damage signaling in budding yeast during the cell cycle. In G1, the Mec1–Ddc2 kinase recruited by RPA–ssDNA is primarily activated by the Ddc1 in the 9-1-1 complex at ssDNA/dsDNA junctions. The aromatic residues of Ddc1 critical for Mec1 activation are depicted as red patches. During S phase, DNA damage signaling can be initiated by four distinct mechanisms: (1) Mec1–Ddc2 is activated by the Ddc1 in the 9-1-1 complex, (2) Mec1–Ddc2 is activated by the Dpb11 associated with Ddc1, (3) Mec1–Ddc2 is activated by Dna2 (the question mark indicates the possibility that Dna2 recognizes RPA-coated 5' flap in this process), and (4) Tel1 is activated by DNA damage when Mec1 activation is compromised (the question mark indicates that the DNA structures activating Tel1 is still unclear). In G2, Mec1–Ddc2 is activated by either Ddc1 or Dpb11.

Dpb11 via phosphorylated T602 and enables Dpb11 to stimulate Mec1 (Fig. 1). Similar to Ddc1, Dpb11 stimulates Mec1–Ddc2 using two aromatic residues (W700 and Y735) in an unstructured region (Navadgi-Patil et al. 2011). The Dpb11-W700A, Y735A mutant is proficient for DNA replication but fails to support Rad53 phosphorylation in G2 *ddc1-2W2A* mutant cells. Together, these results show that Ddc1 and Dpb11 act redundantly in G2 to stimulate Mec1 in vivo (Fig. 1).

Why Mec1 is activated by distinct mechanisms in G1 and G2 is still unclear. Since Dpb11 is needed for the initiation of DNA replication at origins, it may be necessary to prevent Dpb11 from engaging in the DNA damage response (DDR) in G1. Several proteins involved in Mec1 activation are phosphorylated by CDK and other cell cycle-regulated kinases in the S and G2 phases. In addition, as exemplified by the resection of DNA breaks, the processing of DNA damage could be differentially regulated in G1 and G2. Regardless of how the mechanisms of Mec1 activation differ between G1 and G2, the activator functions of Ddc1 and Dpb11 are sufficient to explain how Mec1 is stimulated in these situations.

The activation of Mec1 in S phase

During S phase, the activation of Mec1 gets even more complicated than that in G2. The *ddc1-2W2A*, *T602A* mutant, which is completely defective for Rad53 phosphorylation in G2, is still able to support Rad53 phosphorylation in S phase. In fact, Rad53 phosphorylation still occurs in *ddc1Δ* cells during S phase, suggesting that Mec1 is activated by a Ddc1- and Dpb11-independent mechanism.

To identify the “missing” Mec1 activator in S phase, Kumar and Burgers (2013) biochemically tested 20 protein complexes (39 proteins) involved in DNA replication for the ability to stimulate Mec1–Ddc2 in vitro. Only one of these proteins, Dna2, directly stimulates Mec1–Ddc2 like Ddc1 and Dpb11. Dna2 is a protein involved in lagging strand maturation during DNA replication (see the next section). Similar to Ddc1 and Dpb11, Dna2 also stimulates Mec1–Ddc2 using two aromatic residues (W128 and Y130) in an unstructured region. When the W128 and Y130 of Dna2 were mutated to alanines, the resulting Dna2-WY-AA mutant failed to stimulate Mec1 in vitro but was still proficient for DNA replication in cells. Surprisingly, however, even in the *ddc1Δ dna2-WY-AA* double mutant, Rad53 and another Mec1 substrate, Mrc1, were still phosphorylated in S phase, suggesting yet another mechanism to phosphorylate these proteins (Fig. 1).

In addition to Mec1, budding yeast possesses another PI3K-like protein kinase (PIKK): Tel1. Although Tel1 shares sequence homology with the human ATM kinase, it does not play a major role in DNA damage signaling in the presence of Mec1. However, in the absence of Mec1, Tel1 has been shown to phosphorylate some of the Mec1 substrates (Sanchez et al. 1996). Indeed, when Tel1 was deleted from the *dna2-WY-AA* mutant, the phosphorylation of Rad53 and Mrc1 was partially reduced in S phase (Kumar and Burgers 2013). Furthermore, in the *tel1Δ*

ddc1Δ dna2-WY-AA triple mutant, the 4-NQO-induced phosphorylation of Rad53 and Mrc1 is virtually abolished in S phase. The hydroxyurea (HU)-induced Rad53 phosphorylation is also eliminated in the triple mutant. Together, these results suggest that the initiation of DNA damage signaling can take place in S phase through four distinct mechanisms: (1) stimulation of Mec1 by Ddc1, (2) stimulation of Mec1 by the Dpb11 associated with Ddc1, (3) stimulation of Mec1 by Dna2, and (4) the function of Tel1 as a backup kinase of Mec1 (Fig. 1). Only when all four mechanisms are eliminated together is DNA damage signaling abolished in S phase.

How does Dna2 activate Mec1?

Dna2 is an essential protein that possesses ssDNA nuclease and 5′–3′ DNA helicase activities (Kang et al. 2010). The nuclease activity of Dna2, rather than its helicase activity, is essential for cell survival (Budd et al. 2000; Lee et al. 2000). During DNA replication, the DNA synthesis on the lagging strand is carried out discontinuously as Okazaki fragments. For the synthesis of each Okazaki fragment, a RNA–DNA primer is first generated by the DNA polymerase α (Pol α)-primase, and then extended by the DNA polymerase δ (Pol δ) (Waga and Stillman 1994). When Pol δ reaches the 5′ end of the previous Okazaki fragment, it displaces the 5′ end and generates a 5′ flap. If the flap is short (1–2 nucleotides [nt]), it is cleaved by the FEN1 nuclease. If the flap is long (>25 nt) and coated by RPA, it cannot be cut by FEN1 directly. In this situation, the long flap is first cut by Dna2 to generate a short flap, which is subsequently removed by FEN1 (Bae et al. 2001; Rossi and Bambara 2006). Together, Pol δ , FEN1, and Dna2 act in concert to remove the primer of the previous Okazaki fragment, allowing ligation of the two Okazaki fragments by the DNA ligase.

In addition to its role in lagging strand maturation, the nuclease activity of Dna2 is involved in resection of double-stranded DNA breaks (DSBs). In budding yeast, the 5′–3′ resection of DSBs is initiated by the MRX (Mre11–Rad50–Xrs2) complex and Sae2 and extended by an Exo1-mediated mechanism or a Dna2–Sgs2–RPA-mediated mechanism (Mimitou and Symington 2008; Zhu et al. 2008). During this process, the Sgs2 helicase unwinds DSB ends and generates 5′ flaps, which are recognized by RPA and subsequently removed by Dna2 (Cejka et al. 2010; Niu et al. 2010). In a recent fission yeast study, the endonuclease activity of Dna2 was suggested to prevent accumulation of the “chicken foot” structures at stalled replication forks (Hu et al. 2012). Thus, the nuclease activity of Dna2 is implicated in multiple processes important for genomic stability.

Guided by the previous studies on TopBP1, Dpb11, and Ddc1, Kumar and Burgers (2013) postulated that Dna2 might use an unstructured region to activate Mec1. Indeed, the N terminal region of Dna2 is predicted to be unstructured and does not overlap with the nuclease and helicase domains of Dna2. When tested in vitro, the N-terminal region of Dna2 is sufficient to activate Mec1–Ddc2 as efficiently as the full-length Dna2 (Kumar and

Burgers 2013). Furthermore, similar to the other ATR/Mec1 activators, Dna2 relies on two aromatic residues (W128 and Y130) in the unstructured region to stimulate Mec1. These results strengthen the hypothesis that a long unstructured region with “bipartite” aromatic residues is a common feature of the ATR/Mec1 activation domains. However, among the known ATR/Mec1 activation domains, the distances between the key aromatic residues vary significantly (Kumar and Burgers 2013). Moreover, several other proteins involved in DNA replication, such as Pif1 and Rrm3, also contain long unstructured regions with aromatic residues but lack the ability to stimulate Mec1. These findings suggest that the ATR/Mec1 activation domains may possess additional features important for their functions.

Is the function of Dna2 as a Mec1 activator coupled with its other functions? The temperature-sensitive growth defects of a *dna2* mutant are suppressed by FEN1 overexpression (Budd and Campbell 1997), indicating that enhanced FEN1 nuclease function is able to bypass the essential nuclease function of Dna2. However, FEN1 overexpression fails to suppress the growth defects of the *tel1Δ ddc1Δ dna2-WY-AA* triple mutant, suggesting that Dna2-WY-AA is not defective in the nuclease function. Interestingly, although the Mec1 activation domain of Dna2 is sufficient to stimulate Mec1-Ddc2 in vitro, its role in Mec1 activation in vivo is restricted to S phase. When the Mec1 activation domain of Dna2 was fused with Ddc1, it substituted the Mec1 activation domain of Ddc1 and supported Rad53 phosphorylation in G1 (Kumar and Burgers 2013). These results clearly demonstrate that the Mec1 activation domain of Dna2 cannot function alone in vivo. The function of Dna2 as a Mec1 activator requires not only the Mec1 activation domain, but also the ability of Dna2 to localize to replication forks and/or sites of DNA damage.

Is Dna2 a sensor of DNA damage at replication forks?

To understand how Dna2 stimulates Mec1 in S phase, it is important to determine how Dna2 is localized to replication forks and/or sites of DNA damage. Although Dna2 acts in concert with Ddc1 and Dpb11 to stimulate Mec1 in S phase, the functions of these Mec1 activators are not identical. For example, the *ddc1Δ* mutant is partially defective in Rad9 and Rad53 phosphorylation during S phase, but the *dna2-WY-AA* mutant is not. Furthermore, the effects of Dna2-WY-AA on Rad53 and Mrc1 phosphorylation are most evident in the absence of Ddc1 and Tel1, suggesting that Dna2 may primarily function as a backup activator of Mec1 when the other mechanisms fail. As mentioned above, 9-1-1 is loaded onto dsDNA by the Rad24-RFC complex at ssDNA-dsDNA junctions. This DNA structural specificity of 9-1-1 provides the critical DNA damage regulation to Ddc1 and Dpb11. If the DNA damage regulation of Dna2 is distinct from Ddc1 and Dpb11, then how is Dna2 regulated?

Dna2 may recognize specific DNA structures at replication forks that are induced by DNA damage or replication stress. Dna2 is known to act on long 5' flaps during

lagging strand maturation. In budding yeast, deletion of the Pif1 helicase suppresses the lethality of the *dna2Δ* mutant, implicating Pif1 in the generation of the long 5' flaps that are processed by Dna2 (Ryu et al. 2004; Budd et al. 2006). When DNA polymerases are impeded on the lagging strand, the 5' ends of the previous Okazaki fragments may be persistently exposed. These 5' ends may be initially recognized by the Rad24-RFC and 9-1-1 complexes. However, in the absence of 9-1-1, Pif1 may gain increased access to these 5' ends and convert them into long 5' flaps, which would facilitate the accumulation of Dna2 at the stressed forks and the Dna2-mediated Mec1 activation (Fig. 1).

Pif1 may not be the only way to generate long 5' flaps in S phase. The contribution of Tel1 to Rad53 phosphorylation in S phase indicates that DSBs are generated during the response to HU and 4-NQO. Long 5' flaps may be present at some of the DSBs generated at collapsed replication forks. Some of the DSBs generated at collapsed forks may be recognized by different resection factors (Cejka et al. 2010; Niu et al. 2010). Furthermore, if the “chicken foot” structures are formed at stalled replication forks, they may present DSB-like dsDNA ends. These DSBs or related structures, once engaged by the Dna2-Sgs2-RPA pathway, may provide a mechanism to recruit both Dna2 and Mec1-Ddc2, allowing Dna2 to stimulate Mec1.

In addition to long 5' flaps, Dna2 may directly recognize other forms of ssDNA, such as ssDNA gaps and 3' flaps. Like ATRIP, Dna2 is known to interact with RPA through multiple contacts (Bae et al. 2003; Zou and Elledge 2003; Namiki and Zou 2006). During lagging strand maturation and DSB resection, RPA plays an important role in specifying and stimulating Dna2's nuclease activity toward 5' flaps (Kao et al. 2004; Masuda-Sasa et al. 2008; Cejka et al. 2010; Niu et al. 2010). The ability of Dna2 to bind RPA may enable it to recognize different forms of ssDNA but limit its nuclease activity to 5' flaps. If RPA-ssDNA is directly recognized by both Mec1-Ddc2 and Dna2, it may serve as a platform for Dna2 to stimulate Mec1.

Even if Dna2 does not recognize stress-induced DNA structures at replication forks, it may travel with the forks through its interactions with other replication proteins. In addition to FEN1 and RPA, Dna2 is known to interact with several other replisome components, such as Pol δ, Pol α-primase, and Ctf4 (Budd et al. 2005). The ability of Dna2 to travel with the forks may poise it for Mec1 activation during DNA synthesis, resembling the involvements of the replisome components Mrc1, Tof1, and Csm3 in DNA damage signaling.

Why is the checkpoint activated by four distinct mechanisms in S phase?

The discovery of four distinct mechanisms for checkpoint activation in S phase raises an important question as to why such an extraordinary functional redundancy is necessary. In budding yeast, Mec1 is essential for cell survival unless the dNTP pools are increased by Sml1

deletion (Zhao et al. 1998). Even in the *sml1Δ* background, deletion of both Mec1 and Tel1 leads to severe growth defects (Myung et al. 2001; Craven et al. 2002). Similar to the *sml1Δ mec1Δ tel1Δ* mutant, the *sml1Δ tel1Δ ddc1Δ dna2* WY-AA quadruple mutant displays severe growth defects (Kumar and Burgers 2013). The poor growth of the quadruple mutant is in marked contrast to the normal growth of the *ddc1Δ* mutant, which is defective for Mec1 activation in G1 and G2. These results strongly argue that the ability to initiate DNA damage signaling in S phase is critical to cell survival.

Indeed, even during an unperturbed S phase, Ddc2 and RPA are transiently phosphorylated by Mec1 (Brush et al. 1996; Paciotti et al. 2000), suggesting that Mec1 is elicited by intrinsic stress during DNA replication. When Mec1's function is compromised in the absence of extrinsic stress, chromosomes fail to complete replication with normal kinetics in S phase and are increasingly fragmented in specific replication slow zones (RSZs) and at compromised early origins (CEOs) (Cha and Kleckner 2002; Raveendranathan et al. 2006). Consistent with these previous findings, the *sml1Δ tel1Δ ddc1Δ dna2*-WY-AA quadruple mutant, which lacks all four mechanisms to initiate DNA damage signaling in S phase, fails to complete DNA replication efficiently even in the absence of HU (Kumar and Burgers 2013). In contrast to the quadruple mutant, the *sml1Δ tel1Δ ddc1Δ* triple mutant that contains wild-type Dna2 progresses through S phase without an obvious delay, suggesting that Dna2 alone is sufficient to support the critical function of Mec1 in DNA replication. Furthermore, the *sml1Δ tel1Δ ddc1Δ* triple mutant progresses through S phase much more efficiently than the *sml1Δ tel1Δ ddc1Δ dna2*-WY-AA quadruple mutant in the presence of HU, showing that even one of the four mechanisms to initiate DNA damage signaling (Dna2-mediated Mec1 activation) is able to cope with HU-induced replication stress. Whether the different mechanisms to initiate DNA damage signaling in S phase are important for the response to different cellular stresses remains to be tested. The multiple mechanisms to initiate DNA damage signaling in S phase may not only provide a safety net for DNA replication, but also afford cells the ability to cope with a wide range of cellular stress.

Further questions about Dna2 and ATR activation

The discovery of Dna2 as an S-phase-specific activator of Mec1 in budding yeast raises a number of interesting questions for future studies. One of these questions is how the function of Dna2 in Mec1 activation is restricted to S phase. Interestingly, in budding yeast, the bulk of Dna2 is localized to telomeres in G1 and G2 (Choe et al. 2002). Furthermore, Dna2 is a substrate of CDK (Chen et al. 2011). In fission yeast, Cds1, an effector kinase of ATR/Rad3, phosphorylates Dna2 and promotes its retention at stalled replication forks (Hu et al. 2012). In addition to the regulation of Dna2 itself, the function of Dna2 in Mec1 activation may be controlled by specific DNA structures during S phase. In the presence of DNA damage or replication stress, the DNA structures that

regulate Dna2 could be generated by proteins that function at replication forks or by processing factors that are activated during S phase. These regulatory mechanisms of Dna2 may define a cell cycle window in which Dna2 is able to activate Mec1 efficiently.

Another question about the role of Dna2 in Mec1 activation is whether it has a unique specificity for DNA damage. Although the functional redundancy among Ddc1, Dpb11, and Dna2 is clear, these Mec1 activators are likely regulated by distinct mechanisms. Elucidating how Dna2 activates Mec1 on DNA and how the role of Dna2 is different from those of Ddc1 and Dpb11 will help us understand how these Mec1 activators function in concert during S phase. The potential function of Dna2 in Mec1 activation outside of S phase is also worth exploring. The Dna2-Sgs2-RPA pathway may actively engage in DSB resection in both S and G2 phases. It would be interesting to determine whether Dna2 is able to stimulate Mec1 in the context of resected DSBs in G2.

The study by Kumar and Burgers (2013) also raises the important question of whether the vertebrate ATR kinase can be stimulated by multiple activators. Whether the human 9-1-1 complex and Dna2 can stimulate the ATR-ATRIP kinase is still unknown. In asynchronously growing human cells, ATR phosphorylates its effector kinase, Chk1, in response to DNA damage in a TopBP1-dependent manner (Yamane et al. 2003; Liu et al. 2006). In *Xenopus* egg extracts, the phosphorylation of Chk1 by ATR in response to replication inhibition and DSBs is also dependent on TopBP1 (Hashimoto et al. 2006; Kumagai et al. 2006). These findings suggest that TopBP1 may be the predominant activator of ATR in vertebrates. Interestingly, in human cells, the levels of TopBP1 are low outside of S phase (Yamane et al. 2002). However, ATR-dependent H2AX phosphorylation was detected in nonproliferating cells (O'Driscoll et al. 2003), and ATR-mediated checkpoint responses were observed in G2 (Stiff et al. 2008). In addition, the DNA damage-induced ATR autophosphorylation at T1989 is independent of TopBP1 (Liu et al. 2011). Although it is still unclear whether these ATR-mediated events are dependent on the stimulation of ATR kinase activity, they raise the possibility that ATR may perform some of its functions independently of TopBP1.

The finding of multiple mechanisms to initiate DNA damage signaling in S phase highlights the importance of the DNA damage signaling pathway in DNA replication. In vertebrates, although it is still unclear whether ATR can be activated by multiple mechanisms, the functional redundancy among the different PIKKs is evident. Unlike the yeast Tel1, the human ATM kinase plays a crucial role in the response to DSBs throughout the cell cycle. ATM not only phosphorylates many important substrates directly, it also promotes the activation of ATR by DSBs in the S and G2 phases (Jazayeri et al. 2006; Myers and Cortez 2006). In *Xenopus* extracts, both ATM and ATR are important for preventing accumulation of DSBs during DNA replication (Trenz et al. 2006). In human cells, DNA-PKcs, a PIKK that is absent in yeast, also contributes to DNA damage signaling. Furthermore, recent studies have implicated DNA-PKcs in the

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replication stress response (Yajima et al. 2009; Liu et al. 2012). Thus, although ATR, ATM, and DNA-PKcs each have unique functions, they also work as a group to initiate DNA damage signaling in S phase. To better understand how DNA damage signaling is initiated during S phase in human cells, it would be important to elucidate how ATR, ATM, and DNA-PKcs are activated and how they act in concert in S phase. In addition, it will be a formidable challenge for future studies to reveal how the DNA damage signaling pathway protects the genome during DNA replication under different cellular stresses.

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