

Lagging strand maturation factor Dna2 is a component of the replication checkpoint initiation machinery

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Initiation of the DNA replication checkpoint in yeast is mainly mediated by Mec1 protein kinase, the ortholog of human ATR, while its homolog Tel1, the ortholog of human ATM, has a minor replication checkpoint function. Checkpoint initiation requires stimulation of Mec1 kinase activity by specific activators. *Saccharomyces cerevisiae* Dna2, a nuclease-helicase that is essential for Okazaki fragment maturation, is employed specifically during S phase to stimulate Mec1 kinase and initiate the replication checkpoint. Mutations (*W128A* and *Y130A*) in the unstructured N terminus of Dna2 abrogate its checkpoint function in vitro and in vivo. Dna2 shows partial redundancy for the replication checkpoint with checkpoint initiators 9-1-1 (*S. cerevisiae* Ddc1–Mec3–Rad17 and human Rad9–Rad1–Hus1) and Dpb11, the ortholog of human TopBP1. A triple mutant that eliminates the checkpoint functions of all three initiators abrogates the Mec1-dependent checkpoint.

[**Keywords:** DNA replication; cell cycle checkpoint; 9-1-1; ATR; ATM]

Supplemental material is available for this article.

Received August 30, 2012; revised version accepted December 26, 2012.

DNA damage resulting from internal or external insult constantly challenges cellular genome integrity. Analogous challenges are presented during DNA replication due to the presence of structural blocks or to replisome dysfunction. Eukaryotes have evolved several checkpoints that ensure an arrest of the cell cycle in order to provide an appropriate time frame for DNA repair or the completion of genome duplication (Hartwell and Weinert 1989). Checkpoints are initiated by the activation of two large protein kinases that belong to the phosphatidylinositol 3-kinase-related protein kinase (PIKK) family. Classically, checkpoints are grouped into two separate pathways: one that responds to dsDNA breaks, which activates Tel1 kinase (human ATM) (Morrow et al. 1995; Bakkenist and Kastan 2003), and one that responds to stretches of ssDNA coated with RPA (ssDNA-binding protein), which activates Mec1 kinase (human ATR) (Sanchez et al. 1996; Zou and Elledge 2003). However, some redundancy exists for Mec1 and Tel1 checkpoint function. Of particular importance for this study, we note that stalled replication forks in yeast signal with partial redundancy to both Mec1 and Tel1 kinase, although Mec1 is the predominant kinase in this pathway (Morrow et al. 1995; Mallory and Petes 2000), and dominant mutations in *TEL1* can further

enhance the phenotypic suppression of *mec1Δ* mutants (Baldo et al. 2008).

Mec1 is constitutively associated with Ddc2 (human ATRIP), which helps in its localization to ssDNA regions by interacting with RPA (Rouse and Jackson 2002; Zou and Elledge 2003). However, localization of Mec1–Ddc2 to sites of DNA damage is not sufficient to convert it into a catalytically active complex. Specific sensors transduce the DNA damage response signal to Mec1 and stimulate its kinase activity. Two sensor proteins identified to date are the PCNA-like heterotrimeric checkpoint clamp 9-1-1 (*Saccharomyces cerevisiae* Ddc1–Mec3–Rad17 and human Rad9–Rad1–Hus1) (Majka et al. 2006a) and the replication initiation factor Dpb11 (human TopBP1) (Kumagai et al. 2006; Choi et al. 2007; Mordes et al. 2008; Navadgi-Patil et al. 2011). These factors stimulate Mec1/ATR to phosphorylate many downstream proteins, including the principal downstream effector kinase Rad53 (the functional homolog of human Chk1), which propagates the checkpoint pathway (Sanchez et al. 1996).

Previous studies by us and others have demonstrated that yeast 9-1-1 and Dpb11 perform critical checkpoint functions by stimulating the catalytic activity of Mec1 during the G1 and G2 phases of the cell cycle (Puddu et al. 2008; Navadgi-Patil and Burgers 2009; Pfander and Diffley 2011). The G1-phase DNA damage checkpoint is mediated by the Ddc1 subunit of 9-1-1, while the function of Dpb11 is dispensable during G1 (Navadgi-Patil and Burgers 2009; Pfander and Diffley 2011). Mutation of two conserved

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Article published online ahead of print. Article and publication date are online at <http://www.genesdev.org/cgi/doi/10.1101/gad.204750.112>.

aromatic amino acids in Ddc1 (*ddc1-WW352,544AA*) abrogates Mec1 kinase stimulation in vitro and the G1 DNA damage checkpoint. On the other hand, a fully active G2/M DNA damage checkpoint requires the functions of both 9-1-1 and Dpb11. In *S. cerevisiae*, 9-1-1 functions in two pathways: (1) It directly activates Mec1, dependent on the two conserved aromatic amino acids in Ddc1 (W352 and W544), and (2) it recruits Dpb11 to Mec1 through conserved phosphorylation of T602. The triple *ddc1-WWT352,544,602AAA* mutant is completely defective for the G2/M checkpoint because 9-1-1 fails to both stimulate Mec1 kinase and recruit Dpb11. To date, direct Mec1/ATR kinase stimulation by 9-1-1 has only been demonstrated in *S. cerevisiae*. However, the recruitment function of Ddc1/Rad9 is conserved in *Schizosaccharomyces pombe* and in metazoan (Furuya et al. 2004; Delacroix et al. 2007; Lee et al. 2007). Fusion to PCNA of the activation domain of human TopBP1, the ortholog of Dpb11, bypasses the requirement of 9-1-1 for establishing the DNA damage checkpoint (Delacroix et al. 2007).

Furthermore, mutants lacking the Mec1 stimulatory activity of *DDC1* and *DPB11* that completely abrogated the G1 and G2/M DNA damage checkpoints still showed the presence of a robust replication and S-phase DNA damage checkpoint (Navadgi-Patil et al. 2011). Even after potential contributions by Tel1 were eliminated (this study), a replication checkpoint remained, suggesting the presence of another Mec1 stimulatory factor that is specific for the S phase of the cell cycle.

Here, we show that Dna2 activates Mec1 kinase activity both in vitro and in vivo. Dna2 has both ssDNA exonuclease and 5'–3' DNA helicase activities (Budd et al. 1995; Bae et al. 1998). It plays an important role in Okazaki fragment maturation (Ayyagari et al. 2003), DNA resection during dsDNA break repair (Zhu et al. 2008; Mimitou and Symington 2009), and mitochondrial DNA maintenance (Zheng et al. 2008; Duxin et al. 2009). The nuclease activity of Dna2 also aids in stabilizing stalled DNA replication forks from collapsing (Hu et al. 2012). Thus, Dna2 is a major contributor to genomic stability. A domain analysis of Dna2 has ascribed critical genome stability functions to each of its three domains: the unstructured N-terminal domain (NTD), the nuclease domain, and the helicase domain (Fig. 1A; Bae et al. 2001). Here, we show that Mec1 activation by Dna2 does not require its nuclease or helicase activity, but the activity resides in the unstructured NTD. Mutation of two aromatic residues in the NTD (W128A and Y130A) abrogates its checkpoint function. Also, the three checkpoint initiators known to date (Dna2, Dpb11, and the Ddc1/Rad9 subunit of 9-1-1) employ a similar strategy to activate Mec1 kinase.

Results

Dna2 specifically stimulates kinase activity of Mec1 in vitro

Our analysis of Dpb11 and the Ddc1 subunit of 9-1-1, both of which stimulate Mec1 kinase, showed that

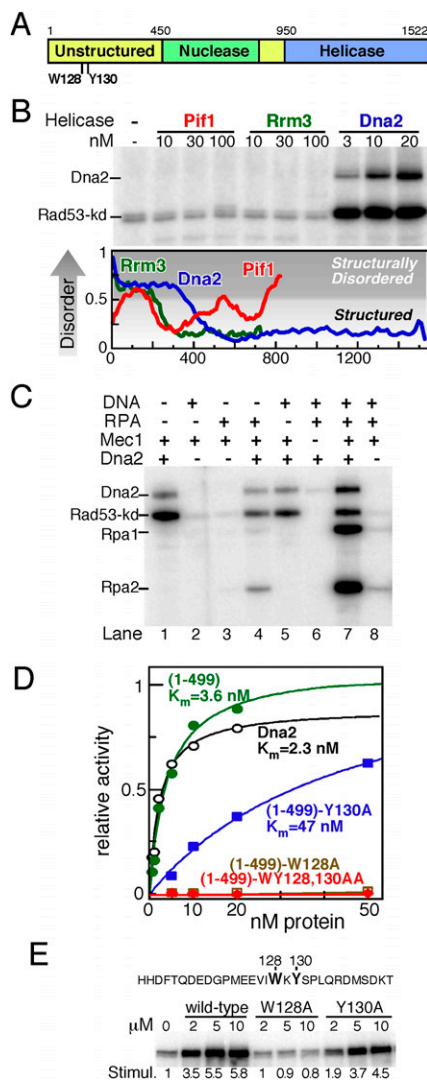


Figure 1. Dna2 activates Mec1 kinase in vitro. (A) Domain map of Dna2 depicting the unstructured domain and the nuclease and DNA helicase domains. Trp128 and Tyr130, required for Mec1 activation, are indicated. (B, top panel) Standard Mec1 kinase stimulation assays contain the indicated concentrations of Pif1, Rrm3, and Dna2 (see the Materials and Methods). (Bottom panel) Disorder prediction for Pif1, Rrm3, and Dna2. Disorder prediction programs IUPRED, PONDR, and PrDOS were used, and their output was averaged. Unstructured regions of proteins have a value >0.5. (C) Standard Mec1 kinase stimulation assays were carried out with the indicated factors. (D) Michaelis-Menten analysis of the rate of Rad53-kd phosphorylation as a function of Dna2 or Dna2(1-499) wild-type or mutant concentration. (E) Sequence of a 30mer Dna2-derived peptide (amino acids 112–141) spanning the critical W128 and Y130. Mec1 activation was carried out with the indicated concentrations of either wild-type or mutant peptide. Values at the bottom represent fold stimulation of Mec1 activity.

stimulatory activity resides in an extended unstructured domain of these proteins (more than ~40 amino acids by phylogenetic analysis), containing at least two essential aromatic amino acids (Navadgi-Patil and Burgers 2009; Navadgi-Patil et al. 2011). Many replication/repair proteins

Dna2 activates Mec1 during S phase

contain such unstructured regions. Therefore, we tested purified proteins available in our laboratory for their ability to stimulate Mec1 activity, using Rad53-kd (Rad53-K227A; kinase-dead) as a physiological phosphorylation target. Out of 20 protein complexes surveyed (39 unique polypeptides), only Dna2 was shown to stimulate Mec1 kinase activity (Supplemental Fig. 1A,B).

Dna2 has an extended unstructured NTD (Fig. 1A). Many DNA helicases possess unstructured NTDs, as exemplified by the Pif1 and Rrm3 DNA helicases (Fig. 1B). However, even though both Pif1 and Rrm3 have several aromatic amino acids in their unstructured NTDs, they failed to stimulate Mec1 protein kinase activity (Fig. 1B). Therefore, while an unstructured domain with aromatic amino acids appears to be a necessary determinant for Mec1 activation, it is not sufficient.

All kinase activity is Mec1-dependent (Fig. 1C, lane 6). As previously observed for Dpb11-mediated activation of Mec1 (Mordes et al. 2008; Navadgi-Patil et al. 2011), DNA is not required for Dna2 to stimulate Mec1 kinase (Fig. 1C, cf. lanes 1 and 5; Supplemental Fig. 1C). However, Mec1-mediated phosphorylation of the Rpa1 and Rpa2 subunits of RPA required that the RPA is bound to ssDNA (Fig. 1C, cf. lanes 4 and 7). Neither the nuclease nor helicase activities were required for Mec1 kinase stimulation (Supplemental Fig. 1D,E). This is consistent with the observation that the unstructured NTD (amino acids 1–499) of Dna2 is fully functional in Mec1 kinase stimulation (Fig. 1D). We noted the presence of several aromatic amino acids in the NTD, but only mutation to alanine of W128 and Y130 significantly affected the Mec1 stimulatory activity. The Y130A mutant reduced the apparent affinity of Dna2(1–499) for Mec1 by ~15-fold, whereas the W128A mutant showed very low kinase activation (~2% of wild type). The double mutant *dna2-WY128,130AA*, hereafter called *dna2-WY-AA*, was completely defective (Fig. 1D; see also Supplemental Fig. 1F). Consistent with these results, we found that a small Dna2-derived oligopeptide containing the Trp128–Tyr130 motif displayed Mec1 stimulatory activity, and this activity was reduced in the Y130A mutant peptide and abrogated in the W128A mutant peptide (Fig. 1E).

The unstructured NTD of Dna2 can activate the kinase activity of Mec1 in vivo

Next, we asked whether the Dna2 NTD could function as a Mec1 activator in vivo by fusing this domain to an activation-defective 9-1-1 clamp (Fig. 2A). The 9-1-1 clamp is a heterotrimer of Ddc1, Rad17, and Mec3 (human Rad9, Hus1, and Rad1, respectively) (Parrilla-Castellar et al. 2004). While the entire complex and the clamp loader are essential for its checkpoint function, all Mec1 stimulatory activity resides in the C terminus of Ddc1/Rad9 (Navadgi-Patil and Burgers 2009). In fact, the artificial colocalization in *S. cerevisiae* of just Ddc1 with Mec1 causes gratuitous checkpoint activation (Bonilla et al. 2008). A *DDC1* mutant [*ddc1-(1–404^{WA})*] that lacks its unstructured C-terminal tail and carries an additional W352A mutation in the PCNA-like domain is defective

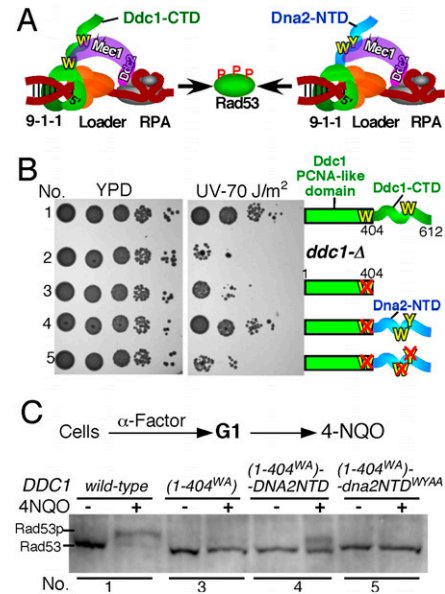


Figure 2. The Dna2 NTD is functional in vivo. (A) Cartoon diagram depicting activation of Mec1 by the 9-1-1 clamp, loaded onto DNA by the checkpoint clamp loader (Majka et al. 2006b). Activation is either through interaction between the Ddc1 C-terminal domain (CTD) (left) or with the Dna2 NTD fused to a tail-less form of Ddc1 (right). (B) Serial dilutions of a *ddc1Δ* strain (YLL244) transformed with a plasmid containing either wild-type *DDC1*, empty vector, *ddc1-(1–404^{W352A})*, *ddc1-(1–404^{W352A})-DNA2(41–243)*, or *ddc1-(1–404^{W352A})-dna2NTD^{WY-AA}* were tested for sensitivity to UV₂₅₄ irradiation (70 J/m²). (C) Western analysis of Rad53 phosphorylation in cells arrested in G1 with α factor (5 μ g/mL) and treated with 4-NQO (2 μ g/mL) for 45 min. The numbers below the figure refer to the same specific strain as used in B.

for the G1 and G2/M checkpoint (Navadgi-Patil and Burgers 2009) and shows profound sensitivity to UV (Fig. 2B, row 3). We fused the active region of the Dna2 NTD (amino acids 41–243) to the C terminus of this mutant Ddc1 truncation, and this form of 9-1-1 was efficiently loaded onto DNA by the checkpoint clamp loader and stimulated Mec1 kinase-like wild-type 9-1-1 (Supplemental Fig. 2). The fusion gene also restored UV resistance of the *ddc1* mutant (Fig. 2B, row 4). However, point mutations in the two aromatic amino acids that abrogated the stimulatory activity of Dna2 (Fig. 1D) also eliminated UV resistance (Fig. 2B, row 5). The observed UV resistance is likely a direct consequence of a functional checkpoint in cells containing the fusion gene. The G1 DNA damage checkpoint is defective in the *DDC1* truncation mutant (Fig. 2C, No. 3), is restored to significant levels in cells with the fusion gene (Fig. 2C, No. 4), and is again inactivated when this fusion gene is mutated at the amino acids critical for Dna2's checkpoint activity (WY-AA) (Fig. 2C, No. 5). These data show that the NTD of Dna2 can function as a G1 checkpoint factor, but only because it is brought into conjunction with Mec1 onto damaged chromatin through loading of the 9-1-1 clamp. Dna2 itself does not function independently as a check-

point protein in G1 or G2/M because (1) the stimulation-defective mutant *dna2-WY-AA* showed no defect in either the G1 or G2/M DNA damage checkpoint (Fig. 3A), and (2) deletion of any of the 9-1-1 genes shows a complete checkpoint defect in G1 and G2/M, excluding a significant compensatory function by Dna2 (Fig. 3A; Longhese et al. 1997; Kondo et al. 1999; Navadgi-Patil and Burgers 2009). We therefore investigated a potential checkpoint function for Dna2 during the S phase of the cell cycle.

Dna2 is a specific checkpoint activator for Mec1 during S phase

The replication checkpoint is initiated in response to treatment of cells with hydroxyurea, an inhibitor of ribonucleotide reductase. The replication checkpoint is more complex than the G1 and G2 checkpoints because partial redundancies exist for the two apical kinases Mec1 and Tel1 (Morrow et al. 1995; Mallory and Petes 2000; Myung and Kolodner 2002). In addition, we anticipated that complete abrogation of the replication checkpoint might result in lethality, since the *MEC1* deletion itself confers lethality. Lethality of *mec1Δ* is suppressed by deletion of *SML1*, a gene that negatively regulates ribonucleotide reductase (Zhao et al. 1998). Therefore all of our checkpoint studies were carried out in a *sml1Δ* background.

We used a set of isogenic strains that were all derived from heterozygous diploid strain PY301. The diploid was sporulated, and the desired mutations were identified in the spores by marker selection and PCR genotyping. Subsequently, the *DNA2* mutations were introduced by a plasmid shuffle in which chromosomal *dna2Δ* cells containing *DNA2* on a *URA3* plasmid were transformed with a *TRP1* plasmid containing either wild-type or mutant *DNA2* or empty vector, and subsequently, the strains were plated on 5-fluoroorotic acid-containing medium in order to evict the complementing *URA3-DNA2* plasmid. The resulting strains were immediately analyzed for growth and checkpoint phenotypes. This approach limits the prolonged propagation of poorly growing mutants and the associated generation of suppressors that occur readily due to the extremely high genome instability of fully checkpoint-defective yeast strains (Myung and Kolodner 2002). All strains were *sml1Δ* and either *ddc1Δ* and/or *tel1Δ*. Since the Mec1 stimulatory activity of Dpb11 is absolutely dependent on its interaction with the phosphorylated tail of Ddc1 (Furuya et al. 2004; Navadgi-Patil and Burgers 2009), using a *ddc1Δ* mutant ensured that checkpoint contributions by Dpb11 were also eliminated. In these mutant backgrounds, we probed whether the Mec1 stimulatory activity of Dna2 contributed to the replication checkpoint and the S-phase DNA damage checkpoint.

During checkpoint activation, Rad53 is initially phosphorylated by Mec1 and/or Tel1 kinase. Further propagation of the checkpoint and full hyperphosphorylation of Rad53 requires either the Rad9 or Mrc1 mediator (Alcasabas et al. 2001; Osborn and Elledge 2003). Rad9 is the putative

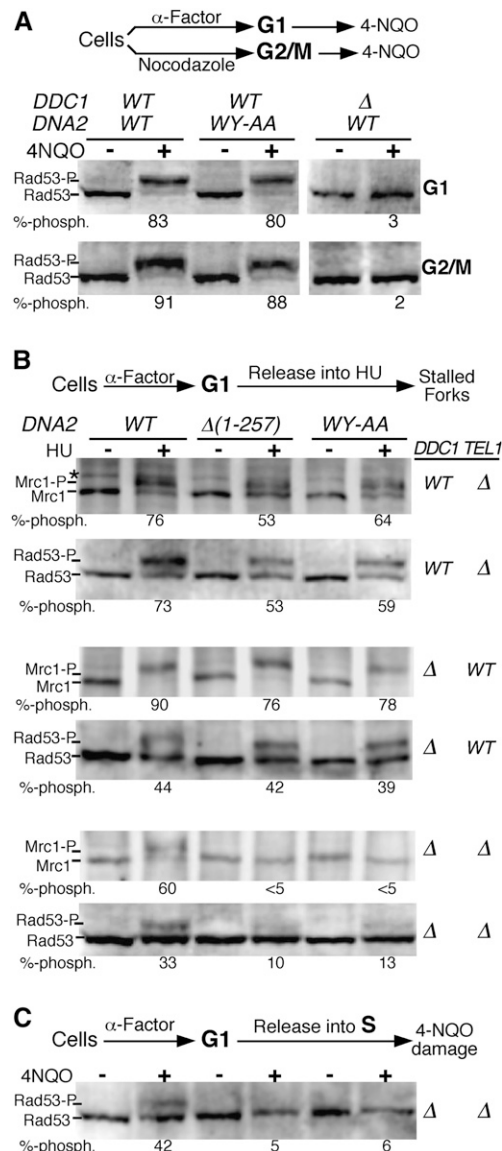


Figure 3. Dna2 activates Mec1 specifically during S phase. (A) Yeast strains PY288 and PY282, with the indicated genotypes, were arrested in G1 phase with α factor or in G2 phase with nocodazole (20 μ g/mL; bottom half) and then treated with 4NQO for 30 min. Western analysis of Rad53 phosphorylation was carried out as described in the Materials and Methods. Percentage of phosphorylated Rad53 is indicated. (B) Strains PY290, PY282, and PY286, with the indicated genotypes and with either wild-type *DNA2* or the indicated *dna2* mutants, were synchronized in G1 with α factor and released into fresh YPD with 200 mM hydroxyurea for 90 min. Cell extracts were probed with anti-Mrc1 or anti-Rad53 antibodies. (C) PY286 was synchronized as described above and released into fresh YPD with 4-NQO for 45 min. Analysis for Rad53 phosphorylation was as under B. (*) The appearance of a nonspecific band.

ortholog of human 53BP1 and primarily transduces the checkpoint in response to DNA damage, while Mrc1 is the ortholog of human claspin and responds to both damage and replication stress (for review, see Branzei and Foiani

2009). The function of either mediator requires phosphorylation by Mec1 and/or Tel1. Therefore, as phenotypic readouts of checkpoint activity, we measured the extent of hyperphosphorylation of Rad53, Mrc1, and Rad9 in response to replication fork stalling by hydroxyurea and in response to DNA damage caused by 4-nitroquinoline-1-oxide (4-NQO), a UV-mimetic drug.

We determined the phenotypes of two *DNA2* mutants: the *dna2-WY-AA* mutant that fails to stimulate Mec1 in vitro (Fig. 1D) and that of a *dna2-Δ(1–257)* mutant that deletes the entire relevant region of the NTD (Chen et al. 2011). In all checkpoint assays, the phenotype of *dna2-WY-AA* was comparable with that of *dna2-Δ(1–257)* (Fig. 3B,C; Supplemental Figs. 3, 4). Furthermore, neither *DNA2* mutation caused a detectable growth defect or checkpoint phenotype by itself; i.e., in an otherwise wild-type strain (Figs. 3A, 5A [below]; Supplemental Fig. 3).

A robust replication checkpoint in response to hydroxyurea treatment was observed in a wild-type cell (Supplemental Fig. 3A) and in a *tel1Δ* mutant (Fig. 3B). This is evident from the strong hyperphosphorylation of both Mrc1 and Rad53. Rad9 does not mediate the replication checkpoint, and in agreement with other studies (Alcasabas et al. 2001; Osborn and Elledge 2003), we did not observe significant Rad9 phosphorylation in either wild-type or mutant strains upon hydroxyurea treatment (Supplemental Fig. 3A). Introduction of the *dna2-WY-AA* or *dna2-Δ(1–257)* mutation into the *tel1Δ* strain significantly reduced the extent of both Mrc1 and Rad53 phosphorylation (Fig. 3B). Similar results were obtained when we introduced the *DNA2* mutations into the single *ddc1Δ* strain; a minor reduction in Mrc1 and Rad53 phosphorylation was observed in the double mutants (Fig. 3B).

Next, we examined the phenotype of *DNA2* mutations in the *ddc1Δ tel1Δ* double mutant. In a wild-type *DNA2* background, the *ddc1Δ tel1Δ* double mutant still showed considerable Mrc1 phosphorylation in response to hydroxyurea treatment, although less robust than either the *ddc1Δ* or *tel1Δ* single mutant. However, strikingly, this phosphorylation signal was abrogated when, in addition, the Mec1 stimulatory function of *DNA2* was mutated. Consistent with previous observations that Mrc1 phosphorylation is required in order to transduce the replication checkpoint signal to Rad53 (Alcasabas et al. 2001), negligible Rad53 phosphorylation was also detected in the triple mutants after hydroxyurea treatment. Thus, the replication checkpoint is essentially eliminated in the triple mutants (Fig. 3B).

In order to measure the S-phase DNA damage checkpoint, cells were synchronized in G1 phase with α factor, then allowed to proceed into S phase in fresh growth medium and treated with 4-NQO. Robust phosphorylation of both Rad9 and Mrc1 was observed in a wild-type and a *tel1Δ* strain. However, in the *ddc1Δ* mutant, Rad9 phosphorylation was undetectable, while that of Mrc1 was significantly reduced (Supplemental Fig. 3B). In the *ddc1Δ tel1Δ* double mutant, phosphorylation of both Rad9 and Mrc1 was below the detection limit (Supplemental Fig. 3B), but significant Rad53 phosphorylation

was still observed (Fig. 3C). The residual S-phase DNA damage checkpoint activity remaining in the *tel1Δ ddc1Δ* double mutant was abrogated in the triple *tel1Δ ddc1Δ dna2* mutants, as indicated by a complete lack of Rad53 phosphorylation upon 4-NQO treatment of S-phase cells (Fig. 3C).

Mec1 kinase activation is required for growth

During these studies, we noticed a very low viability (<1% plating efficiency) of the *tel1Δ ddc1Δ dna2* triple mutants (Fig. 4A; Supplemental Fig. 4). Mutants defective for both *MEC1* and *TEL1* show poor viability and display a highly increased rate of genome instability compared with the single mutants (Myung and Kolodner 2002; Vernon et al. 2008). We determined that the poor viability of our triple mutants was a direct consequence of a defect in Mec1 kinase activation. First, reduced viability was

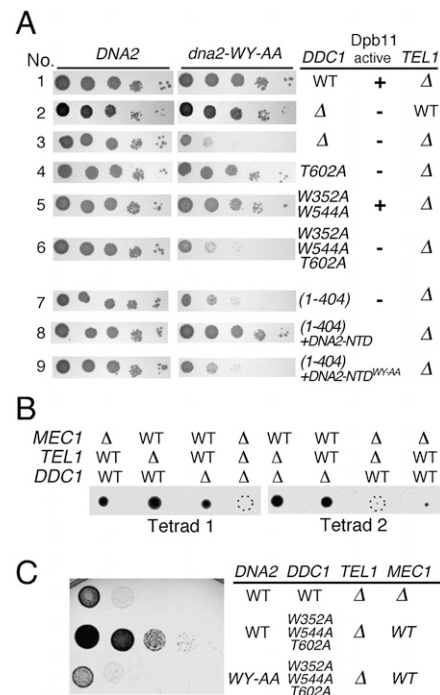


Figure 4. Cell viability and S-phase progression require minimal checkpoint kinase activity. (A) Tenfold serial dilutions of cells containing the indicated *DDC1* and/or *TEL1* mutation and either wild-type *DNA2* (left) or *dna2-WY-AA* (right) were plated on 5-fluoroorotic acid medium, which reveals the phenotype of mutant *DNA2* by eviction of a wild-type *DNA2*-complementing plasmid (see the Supplemental Material). All strains are also *sml1Δ*. Complete plates are in Supplemental Figure 4. (B) Meiotic tetrads from a cross between PY305 (*tel1Δ*, *sml1Δ*) and PY310 (*ddc1Δ mec1Δ sml1Δ*) were dissected on YEPD plates, which were incubated for 5 d at room temperature. The relevant genotypes are indicated on top, and *mec1Δ tel1Δ sml1Δ* mutants are circled. (C) Comparison of growth rate between a *mec1Δ tel1Δ sml1Δ* triple deletion strain (colony 3 from tetrad 2) and strain PY286 with the indicated *ddc1* allele and either wild-type *DNA2* or *dna2-WY-AA* (see row 6 in A). See the Supplemental Material for further information and Supplemental Figure 4C for full plates with additional mutants.

observed only in the triple mutant *ddc1Δ tel1Δ dna2-WY-AA*, and restoring any of the three mutations to wild type restored robust growth (Fig. 4A, rows 1–3). Second, instead of the *ddc1Δ* mutant, we used individual *DDC1* point mutants that eliminate specific checkpoint functions. Stimulation of Mec1 by 9-1-1 is eliminated in a *ddc1-WW352,544AA* mutant, and participation of Dpb11 in checkpoint activation is eliminated in a *ddc1-T602A* mutant (Navadgi-Patil and Burgers 2009). Neither the *ddc1-WW352,544AA* mutant (Fig. 4A, row 5) nor the *ddc1-T602A* mutant (Fig. 4A, row 4) caused poor growth in a *tel1Δ dna2-WY-AA* background; however, the triple *ddc1-WWT352,544,602AAA* mutant (Fig. 4A, row 6), which eliminated stimulation by both Ddc1 and Dpb11, resulted in poor growth.

We carried out several other control studies to investigate the possibility of growth defects in the *DNA2* mutants that would be unrelated to its checkpoint function. The essentiality of *DNA2* stems from its function during Okazaki fragment maturation (for review, see Burgers 2009). Both the flap endonuclease FEN1 and the nuclease function of Dna2 process 5' flaps generated during the process of Okazaki fragment maturation. Growth defects of conditional *dna2* mutants are suppressed by overexpression of *RAD27*, which encodes FEN1 (Budd and Campbell 1997). However, *RAD27* overexpression did not suppress the growth defect of our checkpoint-defective mutants, indicating that the growth phenotype of the *dna2* mutants is checkpoint-specific (Supplemental Fig. 5A). Furthermore, our hypothesis predicts that any action that restores some checkpoint activity should also restore robust growth. Above, we showed that a chimeric *ddc1-dna2* construct partially suppressed the checkpoint defects of *ddc1(1-404^{W352A})* lacking its C-terminal tail (Fig. 2). This chimeric gene restored partial G1 checkpoint activity (Fig. 2C) and also partial S-phase DNA damage checkpoint activity (Supplemental Fig. 5B). The chimeric gene also suppressed the growth defect of the *ddc1Δ tel1Δ dna2-WY-AA* triple mutant, but suppression was lost when the critical aromatic amino acids in the Dna2 tail of the chimera were mutated (Fig. 2C, cf. rows 7 and 8,9). Finally, the *dna2* mutants showed no growth defect in a *mec1Δ sml1Δ ddc1Δ* strain that was wild type for *TEL1* (Supplemental Fig. 5C). From these data, we conclude that Mec1 needs to exert its kinase function, mediated by one of its three activator proteins (Ddc1, Dpb11, or Dna2), for robust growth in a *tel1Δ* mutant. Consistent with this conclusion, we found that a *mec1Δ tel1Δ* double mutant showed a growth defect comparable with that of the activation-defective *MEC1 tel1Δ* strain (all strains were also *sml1Δ*). A tetrad analysis of a diploid strain that was heterozygous *MEC1/mec1Δ* and *TEL1/tel1Δ* but homozygous *sml1Δ/sml1Δ* showed poor or no spore viability of *mec1Δ tel1Δ* double mutants (Fig. 4B), and those double mutants that did grow were as defective for growth as the activation-defective *MEC1 tel1Δ* strain (Fig. 4C).

The poor growth that we observed in the multiple checkpoint-defective mutants is in part caused by their poor progression through S phase. A dramatic deterioration

in S-phase progression was observed when *tel1Δ ddc1Δ* double mutants lost the Mec1 stimulatory function of Dna2 (Fig. 5A, panel 4). The response of these mutants to hydroxyurea also showed strong differences. *Tel1Δ ddc1Δ* double mutants progressed slowly but synchronously through S phase in the presence of hydroxyurea, indicative of a functional checkpoint (Fig. 5B, panel 3). However, mutants that, in addition, have lost the Mec1 stimulatory function of Dna2 terminally accumulate in S phase (Fig. 5B, panel 4). The mutant *dna2* allele alone showed no cell cycle defect with or without hydroxyurea. This cell cycle analysis suggests that cells fail to properly complete DNA replication in checkpoint-defective mutants. Unreplicated chromosomes fail to migrate through agarose gels in a pulsed-field gel electrophoresis (PFGE) experiment (Hennessy et al. 1991). The migration defect of a *ddc1Δ tel1Δ* double mutant is strongly increased when this mutant also carries the *dna2-WY-AA* allele (Fig. 5C).

Discussion

The three Mec1 activators described in this study show a remarkable similarity of structure and mechanism of action. The activity of all three is localized to a region of the protein that is predicted to be disordered (Fig. 1B; Navadgi-Patil et al. 2011). The activity is anchored by the presence of two aromatic amino acids; however,

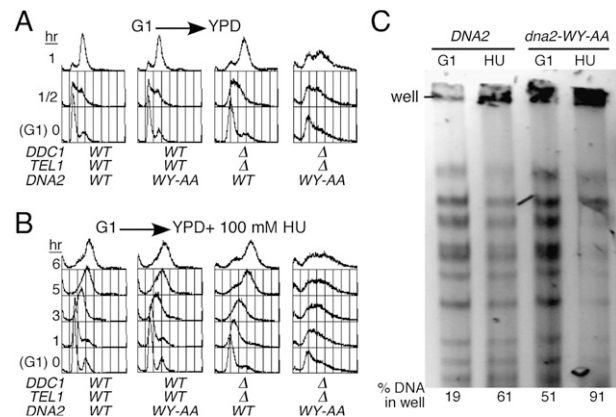


Figure 5. Checkpoint-defective mutant shows poor progression through S phase. FACS scans for strain PY288 (panels 1,2) and PY286 (panels 3,4). The relevant genotypes are indicated at the bottom of the profiles. Strains were synchronized in G1 with α factor and, at $t = 0$, released into either fresh YPD (A) or YPD with 100 mM hydroxyurea (B). (C) Strain PY286 with either *DNA2* or *dna2-WY-AA* was synchronized in G1 with α factor and released in YPD with or without 100 mM hydroxyurea for 4 h. Chromosomes were analyzed using PFGE (see the Supplemental Material). Values at the bottom represent the percentage of DNA remaining in the wells. Under normal growth conditions, only Chromosome XII fails to migrate into the gel (Carle and Olson 1985). Incompletely replicated chromosomes such as those that exist when cells are treated with hydroxyurea also tend to remain in the wells (Hennessy et al. 1991). The high percentage of DNA remaining in the well in the *dna2-WY-AA* mutant indicates that chromosomes fail to complete replication.

surprisingly, in *S. cerevisiae*, these aromatics can be separated by as much as 191 amino acids in Ddc1 and as little as one in Dna2. In other organisms, a similar apparent lack of constraint exists. In the case of 9-1-1, the separation of the aromatics is reduced to 67 amino acids in human and 45 amino acids in *S. pombe* Rad9, the ortholog of Ddc1. This large variation in spacing makes predicting a region that will function as a Mec1/ATR-activating domain currently very difficult. Although all Dna2s, including vertebrate species, have an unstructured NTD containing several aromatic amino acids, predicting which ones will be involved in activation has not been possible, and experimental verification is necessary. It is quite evident, however, that there are definite constraints on the type of unstructured domain that can function as a Mec1/ATR activator. Other proteins with long unstructured regions, containing aromatic amino acids (e.g., Pif1 and Rrm3) (Fig. 1B), do not activate Mec1. In addition, our genetic analysis strongly suggests that the number of activators is limited to the three discovered so far: 9-1-1, Dpb11/TopBP1, and now Dna2. Elimination of the activation function of all three proteins leads to an essentially complete elimination of the DNA damage and replication checkpoints, provided a secondary pathway involving Tel1, the ortholog of human ATM, is also eliminated (Figs. 3, 6). *Mec1 tel1* mutants also show telomere dysfunction (Myung and Kolodner 2002). The senescence phenotype of telomere dysfunction is generally expressed after prolonged cultivation of mutants (Lundblad and Szostak 1989). However, the immediate growth defects that we observed in our checkpoint-defective mutants suggest that some Mec1/Tel1 kinase activity is essential for normal mitotic cell growth (Figs. 4B, 5A).

For each activator, we determined how elimination of its function by mutation affected phosphorylation of the mediator proteins Mrc1 and Rad9 and of the effector

kinase Rad53. Our analysis suggests that the hydroxyurea-induced replication checkpoint is predominantly transduced through Mrc1 phosphorylation (Fig. 3B; Supplemental Fig. 3A). The Mrc1 phosphorylation signal is significantly affected by mutation of *TEL1* or *DNA2* but less so by mutation of *DDC1*. On the other hand, the S-phase DNA damage checkpoint is transduced through both Mrc1 and Rad9 phosphorylation. Phosphorylation of Rad9 in response to S-phase damage is attenuated most strongly by mutation of *DDC1*, and that of Mrc1 is attenuated most strongly by mutation of both *DDC1* and *TEL1* (Supplemental Fig. 3B). However, complete elimination of Rad53 phosphorylation in response to S-phase damage was only accomplished by mutation of all three genes: *TEL1*, *DDC1*, and *DNA2*. A tentative conclusion from these studies is that during S phase, 9-1-1 signals primarily through the Rad9 mediator, whereas both 9-1-1 and Dna2 signal through Mrc1. Since these studies were carried out with a *ddc1Δ* strain and Dpb11's checkpoint function depend on its recruitment by Ddc1 (Navadgi-Patil and Burgers 2009), the individual checkpoint contributions of Ddc1 and Dpb11 could not be separated in this analysis.

Our data show an important and well-defined role for Dna2 in checkpoint initiation during S phase. Previous studies have shown a connection between Dna2 and the replication checkpoint. The growth defects of a *tel1Δ mec1-21* strain, containing a hypomorphic allele of *MEC1*, was partially suppressed by overexpression of *DNA2* (Vernon et al. 2008). In light of the demonstrated Mec1 stimulation function of Dna2, we propose that the *mec1-21* allele is defective for kinase activation, and the increased abundance of Dna2 partially suppressed this defect.

The essential function of Dna2 is thought to stem from its role in Okazaki fragment maturation. During maturation, DNA polymerase δ carries out strand displacement synthesis that is coupled to concomitant cutting of the emerging flap by FEN1 nuclease and ligation by DNA ligase I (for review, see Burgers 2009). However, occasional excessive strand displacement synthesis generates long flaps that require cutting by Dna2. The essentiality of *DNA2* is suppressed by mutations/conditions that disfavor strand displacement synthesis and therefore disfavor the generation of long flaps (Budd et al. 2006; Stith et al. 2008). Overexpression of *RAD27*, the gene encoding FEN1, suppresses the temperature sensitivity of several conditional mutants of *DNA2*, including that of an N-terminal truncation mutant [*dna2-(Δ1-405)*] (Budd and Campbell 1997; Bae et al. 2001), indicating that this extensive truncation mutant showed Okazaki fragment maturation defects. However, both the *dna2-WY-AA* and *dna2-(Δ1-257)* mutants showed only a detectable phenotype when combined with mutations in *DDC1* and *TEL1*, and this growth phenotype was not suppressed by *RAD27* overexpression (Supplemental Fig. 5A), suggesting that these latter mutants are not defective for Okazaki fragment maturation but for checkpoint function. *DNA2* essentiality is also partially suppressed by deletion of *RAD9* (Formosa and Nittis 1999; Budd et al. 2011),

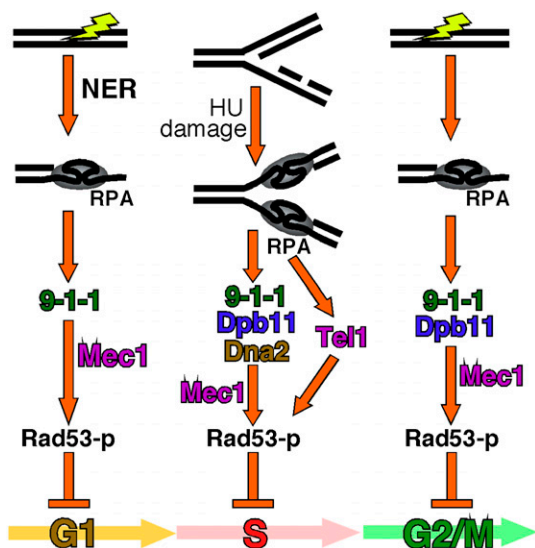


Figure 6. Model for Mec1 activation during the cell cycle (see the text for details).

suggesting that lethality of *dna2Δ* was the result of unrecoverable checkpoint arrest due to the generation of long ssDNA flaps. Surprisingly, however, we found that *dna2Δ* lethality was not suppressed in *ddc1Δ* mutants that are defective for the G1 and G2/M DNA damage checkpoints or in the *tel1Δ ddc1Δ sml1Δ* mutants that lost checkpoint activation in all phases of the cell cycle because of the inability to stimulate Mec1 kinase activity (Supplemental Fig. 4). *Dna2Δ* lethality was also not suppressed in a *mec1Δ ddc1Δ sml1Δ* mutant (Supplemental Fig. 5C). Therefore, we conclude that the suppression of *dna2Δ* lethality is *RAD9*-specific, and the connection to the DNA damage checkpoint may be more indirect.

Interestingly, both Dna2 and 9-1-1 are lagging strand-specific factors. Dna2 engages 5' flaps that are generated during the process of Okazaki fragment maturation, and 9-1-1 specifically loads onto 5' double-stranded to single-stranded junctions, which occur naturally during Okazaki fragment priming (Ellison and Stillman 2003; Majka et al. 2006a). However, the possibility exists that checkpoint signaling on the leading strand could be possible after restart of a stalled replication fork by priming on the leading strand, which would provide the necessary 5' junction for 9-1-1 or Dna2 loading. Further studies to determine the role of 9-1-1 and Dna2 in replication restart and checkpoint activation may shed more light on checkpoint signaling initiated at the leading versus the lagging strand.

Materials and methods

All yeast strains and plasmids used in this study are listed in the Supplemental Tables. Cells were synchronized in G1 phase with α factor (5 μ g/mL) and released into S phase with or without hydroxyurea (100 or 200 mM) or 4-NQO (2 μ g/mL); G2/M arrest was accomplished with nocodazole (20 μ g/mL). Details are given in the Supplemental Material. Cell extracts were subjected to Western analysis with antibodies to Rad53, Mrc1, and Rad9 as described in the Supplemental Material.

Phosphorylation assay

The 20- μ L phosphorylation assay consisted of 25 mM Hepes-NaOH (pH 7.8), 5 mM MgCl₂, 100 μ M unlabeled ATP, 0.5 μ Ci [γ -³²P] ATP, 1 mM DTT, 100 mM NaCl, 100 μ g/mL BSA, and 100 nM Rad53-kd with/without 2.5 nM deca-primed single-stranded BlueScript DNA (3 kb), 150nM RPA, and 10nM Mec1/Ddc2. Reactions were initiated by adding the indicated amounts of (mutant) Dna2 and incubated at 30°C. Reactions were terminated after 10 min by the addition of 5 μ L of 5 \times SDS-PAGE loading dye. Ten microliters of the samples was loaded onto 12% SDS-PAGE gel, dried, and exposed to a phosphor screen (GE Healthcare).

Acknowledgments

We thank Bonnie Yoder and Carrie Stith for strain construction and protein purification, Roberto Galletto for a gift of Pif1 and Rrm3 DNA helicases, Stephen Elledge for a gift of Mrc1 antibodies, Lynn White and Helen Piwnica-Worms for help with FACS analysis, and John Majors for critical discussions. This work was supported in part by Grants GM032431 and GM083970 from the National Institutes of Health.

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