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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 (Saccharomyces 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

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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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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 metazoans [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 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...
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 nucleolar 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 activity (~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-(1–404WA)] lacks its unstructured C-terminal tail and carries an additional W352A mutation in the PCNA-like domain is defective 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 Mrcl mediator [Alcasabas et al. 2001; Osborn and Elledge 2003]. Rad9 is the putative ortholog of human 53BP1 and primarily transduces the checkpoint in response to DNA damage, while Mrcl 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, Mrcl, 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 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 Mrcl 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 Mrcl and Rad53 phosphorylation [Fig. 3B]. Similar results were obtained when we introduced the DNA2 mutations into the single dna2Δ strain; a minor reduction in Mrcl and Rad53 phosphorylation was observed in the double mutants [Fig. 3B].

Next, we examined the phenotype of DNA2 mutations in the dna2ΔΔ tel1Δ double mutant. In a wild-type DNA2 background, the dna2ΔΔ tel1Δ double mutant still showed considerable Mrcl phosphorylation in response to hydroxyurea treatment, although less robust than either the dna2Δ 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 Mrcl 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 a factor, then allowed to proceed into S phase in fresh growth medium and treated with 4-NQO. Robust phosphorylation of both Rad9 and Mrcl was observed in a wild-type and a tel1Δ strain. However, in the dna2ΔΔ mutant, Rad9 phosphorylation was undetectable, while that of Mrcl was significantly reduced [Supplemental Fig. 3B]. In the dna2ΔΔ tel1Δ double mutant, phosphorylation of both Rad9 and Mrcl 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 dna2Δ double mutant was abrogated in the triple tel1Δ dna2Δ dna2Δ 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 dna2Δ dna2Δ 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
observed only in the triple mutant \textit{ddc1Δ tel1Δ dna2Δ-WY-}
\textit{AA}, and restoring any of the three mutations to wild type
restored robust growth (Fig. 4A, rows 1–3). Second, instead of the
\textit{ddc1Δ} mutant, we used individual \textit{DDC1}
point mutants that eliminate specific checkpoint functions.
Stimulation of Mec1 by 9-1-1 is eliminated in a
\textit{ddc1Δ-WWT352,544AA} mutant, and participation of Dpb11
in checkpoint activation is eliminated in a
\textit{ddc1Δ-T602AA} mutant (Navadgi-Patil and Burgers 2009).
Neither the \textit{ddc1Δ-WWT352,544AA} mutant (Fig. 4A, row 5) nor the
\textit{ddc1Δ-T602AA} mutant (Fig. 4A, row 4) caused poor growth
in a \textit{tel1Δ dna2Δ-WY-AA} background; however, the triple
\textit{ddc1Δ-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 in-
vestigate the possibility of growth defects in the \textit{DNA2}
mutants that would be unrelated to its checkpoint func-
tion. The essentiality of \textit{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 \textit{dna2} mutants are sup-
pressed by overexpression of \textit{RAD27}, which encodes
FEN1 (Budd and Campbell 1997). However, \textit{RAD27} over-
expression did not suppress the growth defect of our
checkpoint-defective mutants, indicating that the growth
phenotype of the \textit{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 \textit{ddc1Δ–dna2} construct partially
suppressed the checkpoint defects of \textit{ddc1Δ(1–404W352A)}
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 (Sup-
plemental Fig. 5B). The chimeric gene also suppressed the
growth defect of the \textit{ddc1Δ tel1Δ dna2Δ-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 \textit{dna2}
mutants showed no growth defect in a \textit{mec1Δ sml1Δ}
\textit{ddc1Δ} strain that was wild type for \textit{TEL1} (Supple-
mental 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 \textit{tel1Δ} mutant. Consistent with this conclu-
sion, we found that a \textit{mec1Δ tel1Δ} double mutant showed
a growth defect comparable with that of the activation-
defective \textit{MEC1 tel1Δ} strain [all strains were also \textit{sml1Δ}].
A tetrad analysis of a diploid strain that was heterozygous
\textit{MEC1/mec1Δ} and \textit{TEL1/tel1Δ} but homozygous \textit{sml1Δ/}
\textit{sml1Δ} showed poor or no spore viability of \textit{mec1Δ tel1Δ}
double mutants [Fig. 4B], and those double mutants that
did grow were as defective for growth as the activation-
defective \textit{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 \textit{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. \textit{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]. How-
ever, mutants that, in addition, have lost the Mec1
stimulatory function of DNA2 terminally accumulate in
S phase [Fig. 5B, panel 4]. The mutant \textit{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 mu-
tants. Unrelicated chromosomes fail to migrate through
agarose gels in a pulsed-field gel electrophoresis (PFGE)
experiment [Hennessy et al. 1991]. The migration defect
of a \textit{ddc1Δ tel1Δ} double mutant is strongly increased
when this mutant also carries the \textit{dna2Δ-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,
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].

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 proteins 9-1-1 and Dpb11.

**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 a factor [5 μg/mL] and released into S phase with or without hydroxyurea (100 or 200 mM) or 4-NQO (2 μg/mL); G2/M arrest was accomplished with nocodazole (20 μg/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-μL phosphorylation assay consisted of 25 mM Hepes-NaOH (pH 7.8), 5 mM MgCl₂, 100 μM unlabeled ATP, 0.5 μCi [γ-32P]ATP, 1 mM DTT, 100 mM NaCl, 100 μg/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 μL of 5X 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).

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Dna2 activates Mec1 during S phase


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

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