Cell Cycle 10:10, 1690-1698; May 15, 2011; © 2011 Landes Bioscience

Inviability of a *DNA2* deletion mutant is due to the DNA damage checkpoint

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Key words: yeast, *RAD27, RAD9, RAD53*, Okazaki fragment processing, DNA replication, exo1

Abbreviations: OF, Okazaki fragment; OFP, Okazaki fragment processing; DSB, double-strand break

Dna2 is a dual polarity exo/endonuclease, and 5' to 3' DNA helicase involved in Okazaki Fragment Processing (OFP) and Double-Strand Break (DSB) Repair. In yeast, *DNA2* is an essential gene, as expected for a DNA replication protein. Suppression of the lethality of *dna2*Δ mutants has been found to occur by two mechanisms: overexpression of *RAD27*^{scFEN1}, encoding a 5' to 3' exo/endo nuclease that processes Okazaki fragments (OFs) for ligation, or deletion of *PIF1*, a 5' to 3' helicase involved in mitochondrial recombination, telomerase inhibition and OFP. Mapping of a novel, spontaneously arising suppressor of *dna2*Δ now reveals that mutation of *rad9* and double mutation of *rad9 mrc1* can also suppress the lethality of *dna2*Δ mutants. Interaction of *dna2*Δ and DNA damage checkpoint mutations provides insight as to why *dna2*Δ is lethal but *rad27*Δ is not, even though evidence shows that Rad27^{ScFEN1} processes most of the Okazaki fragments, while Dna2 processes only a subset.

Introduction

Chromosomal DNA is subjected to constant damage not only from exogenous agents, such as ionizing radiation, ultraviolet radiation and base-damaging drugs, but also from damage arising from faulty DNA replication and spontaneous base loss. Unrepaired or misrepaired damage can lead to cell lethality or genome instability, such as translocations or aneuploidy. The DNA damage checkpoint involves a cascade of protein kinases that arrests or slows down the cell cycle, allowing time to repair the damage. Additional functions of the checkpoint include stabilizing the DNA replication fork and stimulating DNA repair. Yeast (*Saccharomyces cerevisiae*) Mec1 and Tel1 are two phosphoinositol kinase-related protein kinases that initiate the DNA damage response in response to single-strand and double-strand DNA breaks.[1,2] Two downstream kinases, Rad53 and Chk1, are activated by Mec1/Tel1 kinases after DNA damage, although Mec1 is the primary upstream kinase.[3] Activated Rad53 then positively regulates an additional kinase, Dun1.[4] Dun1 regulates levels of ribonucleotide reductase and arrests cells having DNA damage in late anaphase.[4] Additional activities of Rad53 after DNA damage include stimulation of DNA repair by phosphorylation of Rad55, inhibition of firing of late origins, induction of transcription of DNA repair genes and phosphorylation of the nuclease Exo1 and its apparent inhibition.[5-7] Exo1 is a 5' to 3' nuclease involved in mismatch base repair, double-strand break (DSB) repair and 5' to 3' degradation of uncapped telomeres.[8-12]

Rad9 and Mrc1 are "mediators" that transduce the signal between Mec1/Tel1 and the Rad53, Chk1 and Dun1 kinases.[13-15] *RAD9* is, in fact, the original defining gene of the DNA damage checkpoint response.[16] Rad9 is a scaffold protein that binds the forkhead domain of Rad53 after phosphorylation by Mec1.[17] The Rad9/Rad53 interaction results in Rad53 kinase activation through Rad53 dimerization and subsequent transphosphorylation.[14,18] Activated Rad53 delays the G_2/M transition by inhibiting the degradation of cohesin and inhibiting spindle elongation.[19,20] Activated Rad53 stabilizes stalled replication forks.[21-23] *rad9*Δ mutants are sensitive to IR, UV and prematurely enter mitosis with damaged DNA.[24] Mrc1 is a non-essential DNA replication protein that binds to Mcm2, the replicative helicase, Cdc45, a subunit of the replicative helicase and DNA polymerase ε (pol ε), the leading strand polymerase.[25,26] Mrc1 couples DNA unwinding with replicative synthesis, and in *mrc1*Δ mutants, replication fork rate is reduced.[13,27,28] After DNA damage Mrc1 is phosphorylated by Mec1, primarily in the N terminus, allowing Mrc1 to activate Rad53 by an undefined mechanism.[29] The mechanism of activation of Rad53 by Mrc1 presumably differs from Rad9 activation of Rad53, since Mrc1 travels with the replication fork.[30] However, to test whether Mrc1 phosphorylation by Mec1 is required for Rad53 activation, Osborn and Elledge mutated all the potential Mec1/Tel1 phosphorylation sites in Mrc1, creating the mutant *mrc1-AQ*. Activation of Rad53 by HU treatment is abolished in the *rad9*Δ *mrc1-AQ* mutant, and the *rad9*Δ *mrc1-AQ* mutant is sensitive to low doses of HU. Unlike the *mrc1*Δ mutant,

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Submitted: 03/28/11; Accepted: 03/29/11
DOI: 10.4161/cc.10.10.15643

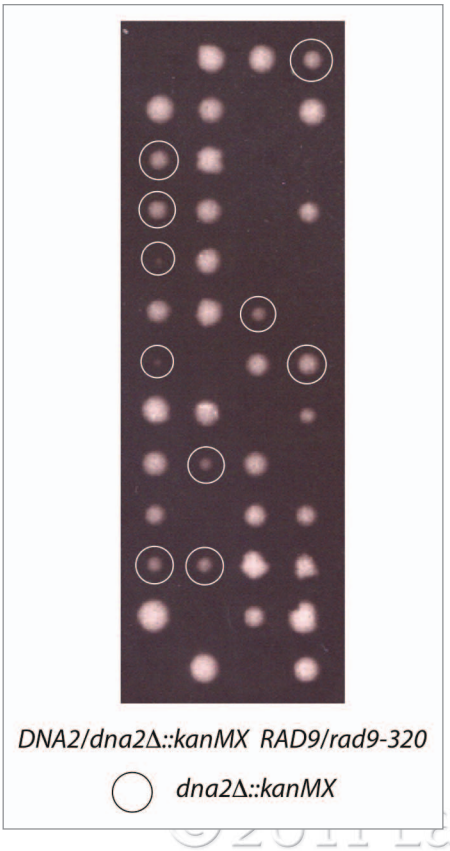

Figure 1. Mutation of *RAD9* suppresses the lethality of *dna2*Δ. Tetrads from a dissection of MB221 *DNA2/dna2*Δ*::kanMX RAD9/rad9-320* illustrate the presence of a mutation suppressing the lethality of *dna2*Δ. The *dna2*Δ colonies are circled.

the *mrc1-AQ* mutant does not exhibit a slow DNA replication phenotype and is specifically checkpoint-defective.[30]

Dna2 is a 5' to 3' helicase and 5' to 3' and 3' to 5' exo/endonuclease with single-strand annealing and strand exchange activity.[31-36] *dna2*-1 mutants are defective in DNA replication and accumulate short DNA fragments at the restrictive temperature,[37,38] and *dna2*Δ mutants are inviable. Rad27^{scFEN1} (Flap Endonuclease) is a 5' to 3' exo/endonuclease that processes the majority of the Okazaki fragments.[39-41] Dna2 assists Rad27^{scFEN1} in RNA primer removal through a coordinated Rad27^{scFEN1}, Dna2, RPA interaction at a subset of Okazaki fragments.[42-46] *rad27*Δ mutants are viable but exhibit a very high frequency of mutation at repeated DNA sequences resulting from delayed OFP.[47] The *dna2-1* mutants do not show an increased frequency of these mutations, and therefore, the high mutational rate of *rad27*Δ mutants suggests Rad27^{scFEN1} processes the majority of the Okazaki fragments. This raises an obvious question: since OFP is essential, why are *dna2*Δ mutants inviable and *rad27*Δ mutants viable?

Comparison of the genetic interaction network of *DNA2* and *RAD27* provided a clue to the answer.[48-51] *dna2* mutants show synthetic lethality with approximately 46 other genes, and two-dimensional clustering of these revealed that *DNA2* and *RAD27* share the greatest number of interactions, consistent with function in the same pathway. This overlapping set of mutants affect DNA replication, DNA repair, histone modification and the cellular stress response. However, we also noticed that there are differences. In particular, *dna2* mutants are not synthetically lethal with DNA damage checkpoint mutants *mec1, rad53, rad9, rad24, rad17, ddc1, mec2,* while *rad27* mutants are synthetically lethal with these checkpoint genes. The only DNA damage checkpoint mutants that are synthetically lethal with *dna2* are *mrc1*Δ*, tof1*Δ and *csm3*Δ; however, these are also part of the replication progression complex and important for DNA synthesis.[25] Therefore, the synthetic lethality presumably results from the role of *MRC1, TOF1* and *CSM3* in DNA replication.

Previously, all suppressors of *dna2*Δ affected components of the OFP system. In this work, we report the surprising observation of a spontaneously arising suppressor of *dna2*Δ mutants that, instead, disrupts the DNA damage checkpoint through inactivation of the Rad9 "mediator." We propose that the DNA repair functions of Dna2 contribute to its essential function, and in the absence of this repair function, the cell cycle inhibition by the checkpoint leads to permanent arrest rather than to survival, as it does in *rad27*Δ cells, which are repair proficient.

Results

Identification of an extragenic suppressor of the lethality of *dna2*Δ. *dna2*Δ*::kanMX* strains are inviable; however, the inviability is suppressed by a deletion of *PIF1*. When a *DNA2/dna2*Δ*::kanMX PIF1/pif1*Δ*::HIS3* diploid is sporulated and dissected, the *dna2*Δ*::kanMX* spores always segregate with *pif1*Δ*::HIS3.* During one dissection of a *DNA2/dna2*Δ*::kanMX PIF1/pif1*Δ*::HIS3* diploid, however, we noticed a *kanMX* (kanomycin resistant) *his3* (did not grow on histidine minus plates) colony, suggesting that it was *dna2*Δ *PIF1*. This indicated that the *dna2*Δ mutant could be suppressed by a mutation other than *pif1*Δ*::HIS3*. To check if the suppressor (*supX*) represented a genetic suppressor or a nonspecific suppressor (e.g., suppression resulting from overproduction of heat shock proteins), the viable *dna2*Δ *PIF1 supX* strain was crossed to a BY4741-derived strain and sporulated. Thirteen tetrads were dissected (**Fig. 1**). If the *dna2*Δ strain is suppressed by one suppressor, the expected number of *dna2*Δ strains recovered would be 13. In keeping with this, 28 *DNA2* spores and 10 *dna2*Δ*::KanMX* spores were recovered. The recovery of *dna2*Δ strains at about 20 to 25% of dissected tetrads suggests that one mutation was segregating in the cross that is suppressing the lethality of *dna2*Δ. The *dna2*Δ spores in this cross were temperature-sensitive and ionizing radiation (IR)-sensitive (not shown). The IR sensitivity of the *dna2*Δ *supX* strain is significantly different from the IR resistance of a *dna2*Δ *pif1*Δ strain.[52] Therefore, *supx* was not likely *pif1*Δ.

To identify the *supX* gene, DNA was prepared from a segregant (MB220) of the cross of BY4741(WT) x *dna2*Δ, and the genome was sequenced and compared to strain BY4741 (see Materials and Methods). The complete list of mutations in MB220 can be found in **Supplemental Table 1**. Three genes were found to have C-terminal deletions. Among these, the only logical candidate for a suppressor of *dna2*Δ is *RAD9*. No mutations were found in the *PIF1* gene.

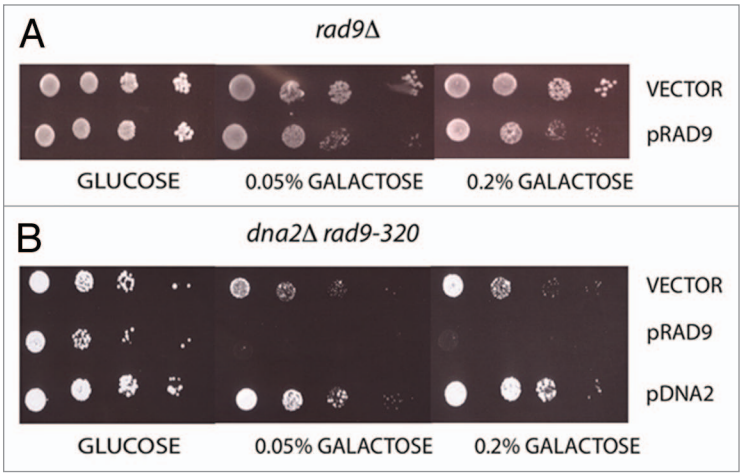

Figure 2. Expression of *RAD9* in a *dna2*Δ *rad9-320* strain results in lethality. (A) Contol showing that *RAD9* expression in *DNA2 rad9*Δ is not lethal. Strain MB228-*DNA2::9MYC rad9*Δ*::HIS3* was transformed with the plasmids pSEY-18GAL and BG1805-Rad9. Cells carrying the plasmids were incubated on medium lacking uracil containing 2% glucose, 0.05% galactose 2% raffinose or 0.2% galactose 2% raffinose. (B) Expression of *RAD9* in *dna2*Δ *rad9-320* is lethal. Strain MB220 *dna2*Δ *rad9-320* was trainsformed with the plasmids pSEY18GAL, BG1805-Rad9 and pSEY18GAL-DNA2 and incubated with 2% glucose, 0.05% galactose 2% raffinose, 0.2% galactose 2% raffinose.

RAD9 encodes a 1,309 amino acid protein, and the suppressor mutation consisted of a stop codon at amino acid 320. Rad9 possesses at least four domains required for full activity. Amino acids 1–231 are required to activate Chk1; residues 390–458 and residues 593–620 are phosphorylated by Mec1 and contain the Rad53 interaction domain; residues 754–947 contain a Tudor domain, which binds methylated histones, and residues 1,027–1,310 contain two BRCT repeats that bind phosphorylated histones.[14,53-55] The BRCT repeats are required for Rad9 dimerization, a necessary step in Rad53 activation. The 1–320 amino acid fragment in the *rad9* suppressor thus possesses the region required for activation of Chk1 but is missing the Rad53 interaction domain, the Tudor domain and the BRCT domain.[14] Since the chromatin binding region, dimerization region and Mec1 phosphorylation sites are missing, the mutant is therefore presumably completely defective in activation of Rad53 and Chk1. The *rad9-320* mutation partially accounts for the IR sensitivity of the strain, although the *dna2*Δ *rad9-3*20 strain is significantly more sensitive to irradiation than a *rad9*Δ strain (Budd M and Campbell JL, unpublished data). We designate the suppressive allele (*supX* above) as *rad9-320*, indicative of the site of the mutation.

***RAD9* reverses the suppression of *dna2*Δ by *rad9-320*.** To confirm that the *rad9-320* mutation was suppressing *dna2*Δ, we asked whether expression of *RAD9* could restore lethality to *dna2*Δ *rad9-320*. *RAD9* cloned into a yeast plasmid under control of the *GAL1* promoter was introduced into *dna2*Δ *rad9-320*. Since overexpression of *RAD9* can in itself cause slow growth, we first determined induction conditions that were not lethal in a *rad9*Δ *DNA2* strain (**Fig. 2A**). On 2% glucose plates, the plasmid had no effect on the growth of the strain. 0.05% galactose is the lowest concentration of galactose that results in detectable induction of the *GAL1* promoter, with a level induction of 1/100 that of full induction of 2%.[56] Concentrations of 0.05% and 0.2% galactose resulted in minor inhibition of growth of *rad9*Δ *DNA2*. This level of expression was sufficient to completely suppress the IR sensitivity of the strain, however (not shown). We then used these induction conditions in the *dna2*Δ *rad9-320* strain carrying the *GAL1-RAD9* plasmid. On glucose containing plates, the *RAD9* plasmid resulted in no detectable growth inhibition. On 0.05% and 0.2% galactose, the *RAD9* plasmid, but not control plasmids, completely blocked growth of the *dna2*Δ *rad9-320* strain. These results show that expression of *RAD9* causes inviability of the strain *dna2*Δ *rad9-320*. We conclude that the DNA damage checkpoint is deleterious rather than beneficial in the *dna2*Δ mutant.

***rad9*Δ suppresses the *dna2*Δ lethality, and *rad9*Δ *mrc1-AQ* suppresses even more efficiently.** The *dna2*Δ *rad9-320* strain was viable but grew more slowly than wild type. We reasoned that this might be due to continued induction of the S-phase checkpoint by *MRC1,* and that mutation of the checkpoint function of *MRC1* might further enhance the growth *of dna2*Δ *rad9-320*. We first tested the combined effect of *rad9*Δ *mrc1-AQ* double mutation on the temperature sensitivity of *dna2-1* strains. The suppressive effect of *rad9*Δ, *mrc1-AQ* and *rad9 mrc1-AQ* mutants on *dna2-1* growth at 23°C, 28°C and 34°C is shown in **Figure 3**. *dna2-1* strains cease growth at 28°C; *dna2-1 rad9*Δ and *dna2-1 mrc1-A*Q cease growth at 34°C. Thus, although *rad9*Δ suppresses *dna2*Δ lethality, it does not suppress *dna2-1* temperature sensitivity. However, *dna2-1 rad9*Δ *mrc1-AQ* is capable of growth at 34°C. Thus, the combined *rad9*Δ *mrc1-AQ* mutation is a strong suppressor of the temperature-sensitive phenotype of *dna2-1* strains, similar to the *pif1*Δ suppression of *dna2-1* strains.[52] We then tested the ability of *mrc1-AQ* combined with *rad9*Δ as an additional suppressor of the *dna2*Δ lethality. Five independent WT/*dna2*Δ::*natR rad9*Δ::*kanMX mrc1-AQ*::*HIS3* diploids were sporulated and dissected with an average of 12 to 13 tetrads per diploid. The data from one of the crosses is illustrated in **Figure 4**. The results of the 62 total diploids scored were as follows: (the number following the genotype indicates the number of spores identified): WT, 22; *rad9*Δ, 32; *mrc1-AQ*, 29; *rad9*Δ *mrc1-AQ*, 27; *dna2*Δ *rad9*Δ. 5; *dna2*Δ *mrc1-AQ*, 1; *dna2*Δ *rad9*Δ *mrc1-AQ*, 17; *dna2*Δ, 0. Assuming 100% viability, about 31 spores from each genotype should be recovered (62 tetrads x 4 spores ÷ 8 genotypes = 31). From this data, we first point out that the five viable *dna2*Δ *rad9*Δ spores serve as an independent control, showing that complete deletion of *RAD9* suppresses *dna2*Δ inviability, consistent with *rad9-320* likely functioning as a Rad9-null in suppression. Second, 23 *dna2*Δ strains were recovered in combination with either or both of the checkpoint mutants, *rad*9 and/or *mrc1-AQ,* and none were recovered when *dna2*Δ was not combined with a checkpoint mutant. All the *dna2*Δ *rad9*Δ, *dna2*Δ *mrc1-AQ* and *dna2*Δ *rad9*Δ *mrc1-AQ* strains could form colonies upon restreaking at 23°C but not at 36°C. *mrc1-AQ* alone barely

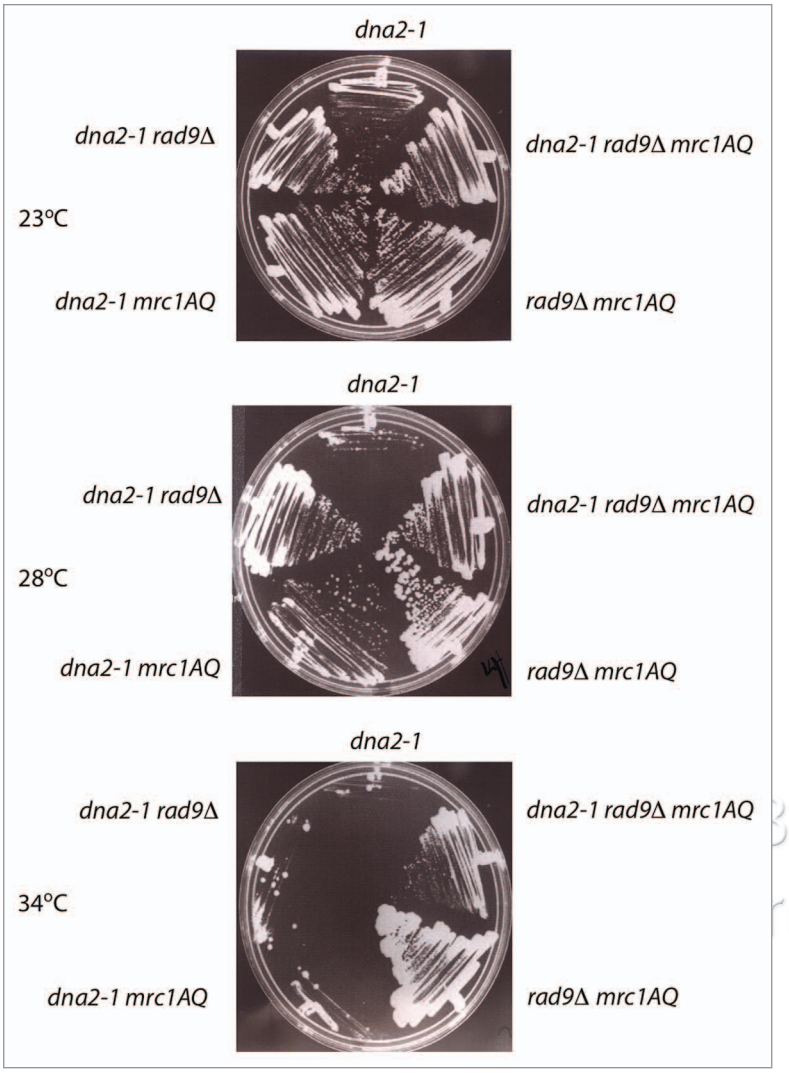

Figure 3. *rad9Δ mrc1AQ* double mutation suppresses the temperature-sensitive growth of *dna2-1* mutants. Mutants *dna2-1, dna2-1 rad9Δ, dna2-1 mrc1AQ, dna2-1 rad9Δ mrc1AQ, rad9Δ mrc1AQ* were incubated on plates at 23°C, 28°C and 34°C.

suppressed *dna2*Δ, and only one suppressor was recovered. *rad9*Δ is a more efficient suppressor than *mrc1-AQ,* and *rad*9Δ *mrc1-AQ* provided more efficient suppression, since they were recovered at higher frequency, although the *dna2*Δ *rad*9Δ *mrc1-AQ* colonies were not necessarily larger than the *dna2*Δ *rad9*Δ colonies (**Fig. 4,** compare squares and circles).

Discussion

We have identified *rad9*Δ as a novel suppressor of the lethality of *dna2*Δ using full genome sequencing of a spontaneous suppressor. Suppression of the complete absence of Dna2 is surprising, because *rad9*Δ suppresses the growth defect of only a subset of *dna2* point mutants and does not suppress the inability of *dna2-1* mutants to grow at 34°C. The *rad9-320* mutation identified in this work gives rise to a stop codon at amino acid 320, which is expected to create a functionally null allele. Even if the fragment survives nonsense-mediated decay, the only functions associated, to date, with the N-terminal domain remaining are Chk1 activation and binding to the replication initiator protein Dbp11.[53] Indeed, in independent experiments, we verified that *rad9*Δ suppressed *dna2*Δ (**Fig. 4**), and that expression of *RAD9* reversed the suppression by *rad9-320* (**Fig. 2**). None of the additional mutations identified by DNA sequencing of the original suppressor strain appears to be a candidate suppressor mutation. DNA sequencing did not detect any mutations in the *PIF1* gene, which we had previously identified as a *dna2*Δ suppressor. Interestingly, the *rad9*Δ *mrc1AQ* double mutation resulted in more efficient suppression than either mutation alone. We conclude that inefficiently processed DNA intermediates arising due to the absence of Dna2 do not activate either the Rad53 or Chk1 cell cycle checkpoint functions in the *dna2*Δ *rad9*Δ *mrc1AQ* mutants. In this way, terminal arrest is avoided, allowing further division in the complete absence of Dna2.

Previously, specific endogenous suppressors of *dna2* lethality were attributed to increasing the efficiency of OFP. This may be mediated by overexpression of Rad27^{ScFEN1}, which is directly involved in OFP, and of BLM, WRN, Exo1 and Mph1, which provide auxiliary functions and stimulate the nuclease activities of Dna2 and Rad27^{ScFEN1}.[57-61] In *Schizosaccharomyces pombe,* overexpression of FEN1, pol δ or DNA ligase suppress a *dna2* helicase mutant.[62] A related phenomenon involves suppressing the production of irreparable toxic structures such as long 5' flaps by Pif1 helicase. *pif1* mutation allows *dna2-1* strains to grow at 37°C.[52,57] Pif1 is a 5' to 3' helicase that unwinds forked DNA substrates, RNA/DNA duplexes and removes telomerase bound to telomeres.[63,64] A *dna2*Δ *pif1*Δ strain is viable but temperature sensitive; however, a *dna2*Δ *pif1*Δ *pol32*Δ strain does grow at 37°C.[52] Thus, Pol32 is an additional suppressor of *dna2* mutants. Pol32 is a stimulatory subunit of pol δ.[65] The suppression of *dna2*Δ by *pif1*Δ and further suppression by *pol32*Δ suggests that long flaps created during OPF by pol δ, Pol32 and Pif1 are potentially lethal substrates unless they are processed by Dna2 (**Fig. 5**). Additional suppressors of *dna2* mutants are not involved in OFP. E1A overexpression was one of the first identified suppressors of Dna2.[66] E1A is encoded by the human adenovirus, activates viral transcription and can promote cell cycle progression. *TOR* overexpression also suppresses some *dna2* helicase mutants.[67] The mechanisms involved are not understood.

Our current work identifies a new compensatory pathway that we propose involves desensitizing *dna2* mutants to replication stress encountered in its absence. In addition to our newly documented suppression of the lethality of deletion of *DNA2* by deletion of *RAD9*, *rad9*Δ and *mec1* mutants have been observed to suppress the growth defects of a subset of *dna2* point mutants and the synthetic lethality of a *dna2-2 ctf4*Δ mutant.[67,68] *rad9* and *mrc1* mutants desensitize other DNA replication mutants to replication damage as well. *cdc9 rad9 mrc1* mutants progress

further into DNA synthesis than *cdc9 rad*9, which progress further than *cdc9* mutants at 36°C.[69] The lethality of *orc2-1* and *orc1-4* mutants at the restrictive temperature is suppressed by *rad9*Δ mutation.[70] This is of general interest, because desensitizing cells to DNA damage during DNA replication is a hallmark of cancer cells and is the cause of genome instability. The most widespread desensitizing mutation is p53, a common mutation in human cancer.

We propose that cell cycle checkpoint activation in the absence of Dna2 probably results from persistant 5' flaps created by combined polδ/Pif1 DNA strand displacement synthesis during Okazaki fragment processing (see **Fig. 5** for details). Supporting this mode of checkpoint activation, Rad53 is highly phosphorylated in *dna2-1* strains, and phosphorylation is Mec1-dependent, while Rad53 remains largely unphosphorylated in *dna2-1 pif1*Δ strains.[52] Furthermore, we calculate that this mechanism could easily generate sufficient single-stranded DNA to activate the DNA damage response. It has been estimated that at least 10^4 bp of single-stranded DNA is required to activate Rad53 after an HO-induced DSB, based on the rate of 5' to 3' resection and the time of appearance of phosphorylated Rad53.[71,72] Yeast contains about 2.3 x 10^7 nt, and therefore, about 2 x 10^5 Okazaki fragments, which are about 125 nt in length, are synthesized during S phase.[73] If polδ/Pif1 strand displacement causes about 1% of the flaps to become 30 nt or longer, then about 2 x 10^3 flaps greater than 30 nt long or 1.2 x 10^5 nt of single-stranded DNA, is expected to be generated during S phase in a *dna2*Δ mutant, more than is necessary to activate the checkpoint. After checkpoint activation, cells normally repair the DNA damage, deactivate the checkpoint and resume cell division. *dna2-1* strains repair the DNA damage (long RPA bound 5' flaps) inefficiently since Rad53 is constitutively activated and *dna2-1* strains divide very slowly, a 4 hour generation time at 23°C. *dna2*Δ strains would be unable to repair the unprocessed Okazaki fragments during the G_2 division delay; thus the Rad9/Mrc1-dependent G_2 division delay contributes to cell death rather than recovery.

In addition to failing to induce cell cycle arrest, another mechanism is also probably involved in the suppression of the *dna2*Δ lethality by *rad*9 and *rad*9 *mrc1AQ*. Dna2 has recently been shown to be involved in resection of DSBs in yeast.[9,74-76] Although the DSB break repair function of Dna2 is missing, the viability of *dna2*Δ *rad9-320* suggests that in the absence, but not in the presence, of Rad9, lethal endogenous DSBs are channeled into an alternative repair pathway. The Exo1-dependent DSB repair pathway is a good candidate, given previously documented genetic interactions and overlaps of function between Dna2 and Exo1.[52] Exo1 is a 5' to 3' nuclease with homology to $Rad27^{scFEN1}$ and is involved in mismatch base repair, resection at the ends of DSBs and decapped telomeres and can function in OFP when either $Rad27^{scFEN1}$ or Dna2 is defective.[9,10,77-79] Overproduction of Exo1 partially suppresses the growth defect of *dna2-1* cells at 30°C and suppresses the growth defect of *rad*27Δ mutants at 37°C.[50,78] *dna2-1*, *dna2-2*, *dna2*Δ *pif1*Δ and *rad27*Δ mutants are synthetically lethal with *exo1*Δ mutants.[50,52,78] In processing of DSBs, either Exo1 or Dna2 can provide the resection function.[9,74-76] Several recent lines of evidence suggest that activation

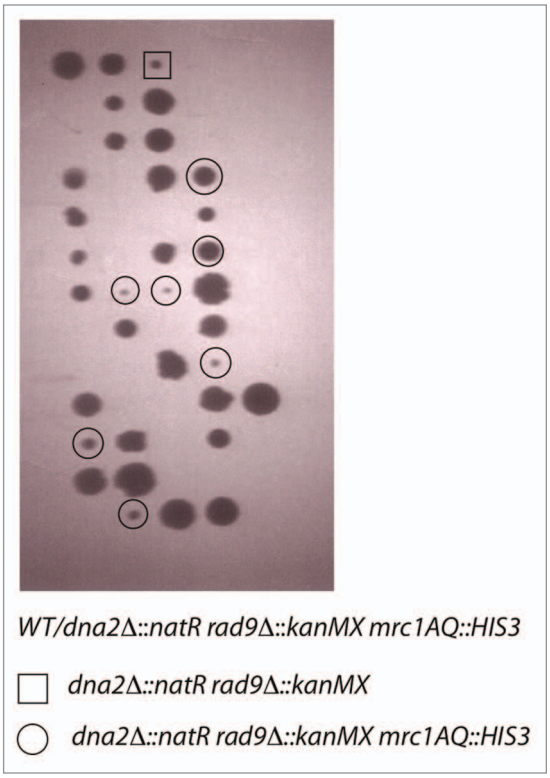

Figure 4. *rad9*Δ *mrc1AQ* double mutation suppresses the lethality of *dna2*Δ better than *rad9*Δ alone. Tetrads from a dissection of MB227 *DNA2/dna2Δ::natR MRC1/mrc1AQ::HIS3 RAD9/rad9Δ::kanMX*. The *dna2*Δ *rad9*Δ colonies are enclosed by a square, and *dna2*Δ *rad9*Δ *mrc1AQ* colonies are enclosed by a circle.

of *RAD9* and *RAD53* inhibit processes dependent on Exo1 nucleolytic functions. Cdc13 is an essential telomere capping protein. After Cdc13 inactivation, extensive 5' to 3' degradation occurs at the telomere end, continuing as far as 15 kb from the end.[80,81] The degradation is mainly Exo1-dependent.[10] In the absence of Rad9, 5' to 3' degradation proceeds significantly further and faster, suggesting Rad9/Rad53 inhibits Exo1.[82,83] Other evidence that Exo1 is inhibited by a Rad53-dependent step is the observation that in *rad53* mutants, replication forks stalled by MMS or HU exhibit long regions of single-stranded DNA on the nascent lagging strand, and the appearance of single-stranded DNA is dependent on Exo1. In *exo1*Δ *rad53* mutants, there is no single-stranded DNA, and *exo1*Δ suppresses the MMS sensitivity of a *rad53* mutant.[84] In *RAD53* cells, stalled forks remain fully duplex and do not collapse, which could be explained if Exo1 is inhibited.[85] In the *rad*9Δ and *rad*9 *mrc1* mutants, Rad53 is deactivated, and the putative Rad53-dependent inhibition of Exo1 is relieved. In addition, Rad9 and the H3K79 histone methyltransferase Dot1 inhibit 5' to 3' degradation at DSBs and telomeres.[86] A *rad9*Δ mutant would relieve much of the inhibition of Exo1, and *rad*9Δ *mrc1AQ* may relieve the remainder of the inhibition, allowing Exo1 to function in OFP/DSB repair as a backup repair pathway in the absence of Dna2 (**Fig. 5**).

The question remains why *rad27*Δ mutants are viable and *dna2*Δ mutants are inviable. We have previously proposed

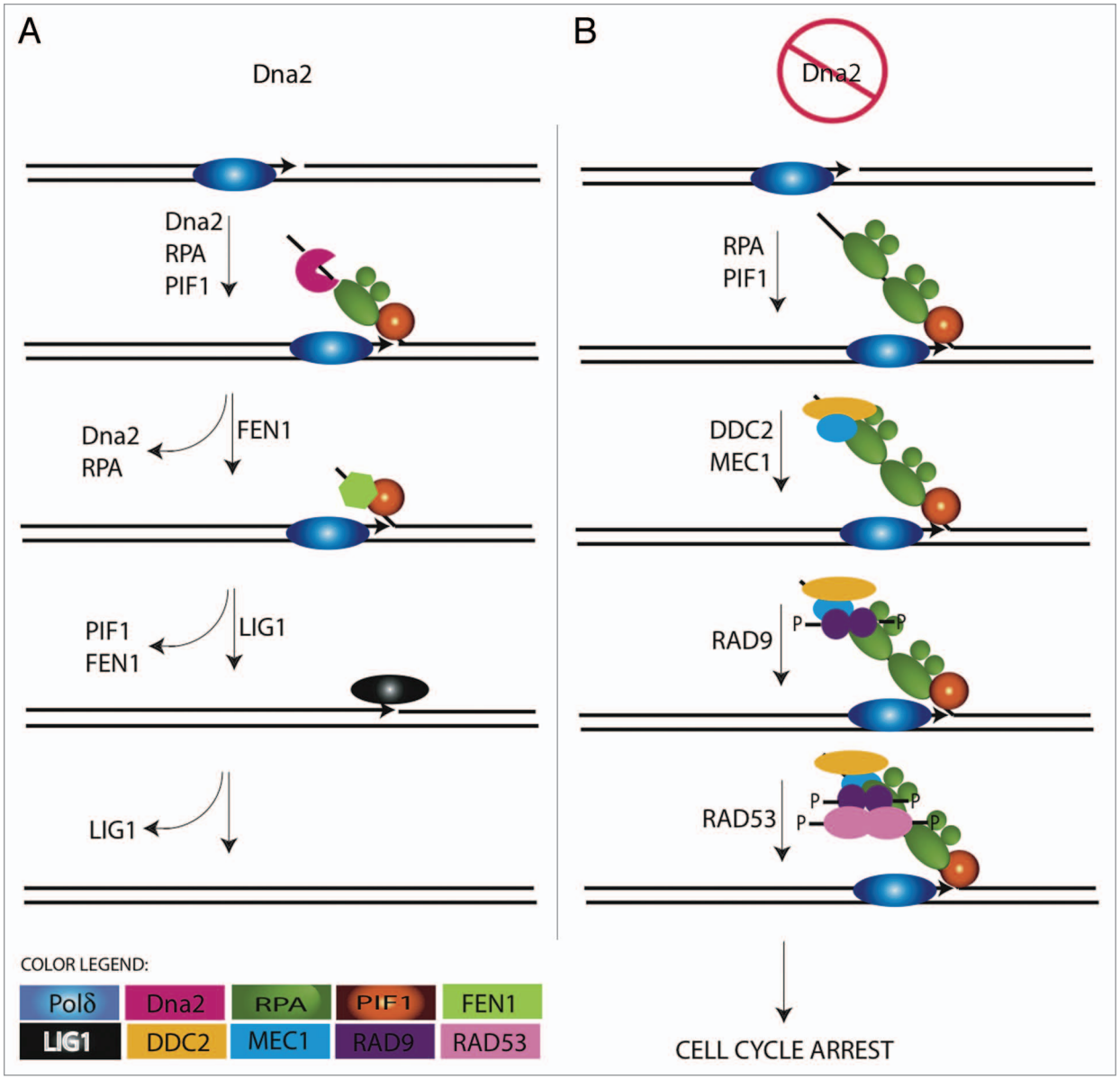

Figure 5. Checkpoint activation due to replication errors in the absence of Dna2. In vitro, DNA strand displacement synthesis by pol δ in the presence of Rad27^{scFEN1} protein on a primer adjacent to an upstream fragment results in the appearance of cleaved flaps. Although most of the cleaved flaps are short, 8–12 nt, a small subset are about 30 nt.[45,89] Pol δ strand displacement is stimulated by Pif1, and a significant increase in the 30 bp cleaved product is observed in the presence of Pif1, although the 30 nt cleaved product is still only 1–2% of the total cleaved products.[90,91] The 30 nt flap synthesized by Polδ/Pif1 becomes refractory to cleavage by Rad27^{scFEN1} in the presence of RPA, which may result in even further lengthening of the flap.[91,92] As shown in (A), in normal cells containing Dna2, the RPA bound flap is bound by Dna2, which cleaves the long flap, releasing RPA and allowing Rad27^{scFEN1} to further cleave the flap, resulting in a ligatable nick.[43-45,91,93] In the absence of Dna2, (B), the RPA-bound flap, however, persists and can bind Mec1/Ddc2 kinase. The 5' flap-bound Mec1/Ddc2 then recruits and phosphorylates Rad9, which binds methylated histone H3-K79 with its Tudor domain. Mec1/Ddc2 may also phosphorylate histone H2A, providing an additional site for Rad9 binding to chromatin. Chromatin bound Rad9 then recruits Rad53, resulting in dimerization, autophosphorylation and Rad53 kinase activation, allowing Rad53 to activate the DNA damage response and cell cycle arrest. (Similarly, the MCM helicase and pol ε may target Mrc1 to Mec1-bound 5' flaps). After checkpoint activation, cells normally repair the DNA damage, deactivate the checkpoint and resume cell division. *dna2*Δ strains would be unable to repair the unprocessed Okazaki fragments during the G_2 division delay; thus the Rad9/Mrc1-dependent G_2 division delay contributes to cell death rather than recovery.

that the synthetic lethality of *rad27*Δ with recombinational repair mutants and the viability of *dna2* mutants in combination with the same recombinational repair mutants suggests that DSBs occur more frequently in *rad27*Δ, and that, therefore, Rad27^{ScFEN1} processes most of the Okazaki fragments. The remaining OFs are inefficiently processed by Dna2, Exo1 and RnaseH. The synthetic lethalities also suggest that Rad27^{ScFEN1} is not required for DSB repair but is dedicated to DNA replication, thus a *rad27*Δ mutant requires the use of the G_2 division delay to repair damage, allowing its survival (albeit with high frequency

Table 1. Strains

Y238	*MATa pep4-3 trp4-580 ura3-52 leu2-3,112*
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*
BY4741	*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*
MB220	*MATa dna2Δ::kanMX rad9-320 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*
MB221	*4742/MB220*
MB222	*MATa dna2Δ::natR his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 trp1Δ (pSEY18-DNA2)*
580-10D	*MATa trp1-1 ura3-1 his3-3-11,15 leu2-3,112 ade2-1 can1-100*
MB223	*MATa rad9Δ::kanMX mrc1AQ::HIS3 trp1-1 ura3-1 his3-3-11,15 leu2-3,112 ade2-1 can1-100*
MB224	*MATa dna2-1 rad9Δ::kanMX trp1-1 ura3-1 his3-3-11,15 leu2-3,112 ade2-1 can1-100*
MB225	*MATa dna2-1 mrc1AQ trp1-1 ura3-1 his3-3-11,15 leu2-3,112 ade2-1 can1-100*
MB226	*MATα dna2-1rad9Δ::kanMX mrc1AQ trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100*
MB227	*MATa/MATα DNA2/dna2Δ::natR MRC1/ mrc1AQ::HIS3 RAD9/ rad9Δ::kanMX his3-11,15/his3Δ1 leu2-3,112/leu2Δ0 ura3-1/ura2Δ0 trp1-1/trp1Δ ADE2/ade2-1 CAN/can1-100 MET15/met15Δ0*
Y438	*MATa rad9Δ::HIS3 trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100*
MB228	*MATa DNA2::9MYC::LEU2 rad9Δ::HIS3 trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100*

of mutation). Hence, *rad27 rad9* is lethal.[48,51,87] With *dna2*Δ, instead, even though there are fewer OFP failures and fewer replication-induced DSBs, G_2 division delay is lethal because Dna2 is required for efficient repair. Potential backup repair pathways are suppressed in the presence of *RAD9,* and its deletion results in *dna2* survival.

Materials and Methods

Strains and plasmids. The strains are listed in **Table 1**. The plasmid BG1805-GAL1-Rad9 (2 μ ori, *URA3*) was obtained in the strain Y258 from Open Biosystems. The plasmid BG1805-Rad9 was purified from Y258, transformed into *Eschericia coli* and then purified. Standard genetic techniques were used for tetrad analysis and genetic analysis. The chromosomal DNA from MB220 was isolated using the Qiagen Genomic-tip P/20. The *kanMX* disruption cassette confers resistance to G418/geneticin, and *natR* cassette confers resistance to Nourseothricin.

DNA sequencing and mutation detection. Libraries were prepared from genomic DNA isolated from BJ4741 and its derivative strain MB220 carrying the *dna2*Δ suppressor, following the standard Illumina protocol. The libraries were sequenced on the Illumina Genome Analyzer II using a single read sequencing protocol producing 15.4 and 20.2 million 64 nt reads passing quality filtering, respectively. The sequence output corresponds to 80.2x coverage of the *S. cerevisiae* genome for BJ4741 and 105.2x coverage for MB220. To identify polymorphisms in MB220, each strain was compared to the reference *S. cerevisiae* genome (version S288C, downloaded from the Saccharomyces Genome Database, www.yeastgenome.org) and to each other using the VAAL software package, which can detect both single nucleotide polymorphisms (SNPs) and deletion/insertion events (indels).[88] To identify genes affected by the mutations, genome annotations in GFF format (downloaded from SGD ftp://ftp.yeastgenome.org/yeast/data_download/chromosomal_feature/saccharomyces_cerevisiae.gff) were loaded into a MySQL database, and the output of the TruePoly module of VAAL containing the differences between MB220 and BJ4741 was compared against it. For each polymorphism, we recorded MB220, BJ4741 and reference genome sequences flanking the mutation, overlapping gene(s), gene description, associated GO terms and other available functional annotations, nature of the mutation (i.e., stop codon, nonsynonymous substitution, synonymous substitution) and amino acid change produced by it. Our analysis[88] identified 476 SNPs and 21 small indels. 347 of those either did not fall within genes (115) or resulted in nucleotide substitutions that did not affect amino acid sequence (232). One hundred and forty-five mutations produced a single amino acid change, two deleted one and six amino acids (without frame change), and three produced stop codons. The complete list of identified polymorphisms can be found in **Supplemental Table 1** (MB220_vs._BJ4741_polymorphisms_all). Mutations producing stop codons, their genomic coordinates and affected genes are: polymorphism 19, chr4 902499, *RAD9* (DNA damage-dependent checkpoint protein); polymorphism 432, chr13 74415, YML099W-A (dubious open reading frame unlikely to encode a protein); polymorphism 471, chr15 286230, *TAT2* (high-affinity tryptophan and tyrosine permease).

Acknowledgments

This work was supported by Public Health Service, NIGMS 078666 and Army Research Office (ARO) 09-1-0041.

Note

Supplemental materials can be found at:
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