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Published in:
Genetics

DOI:
10.1534/genetics.118.300809

Publication date:
2018

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (APA):
A Critical Role for Dna2 at Unwound Telomeres

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ABSTRACT Dna2 is a nuclease and helicase that functions redundantly with other proteins in Okazaki fragment processing, double-strand break resection, and checkpoint kinase activation. Dna2 is an essential enzyme, required for yeast and mammalian cell viability. Here, we report that numerous mutations affecting the DNA damage checkpoint suppress dna2Δ lethality in Saccharomyces cerevisiae. dna2Δ cells are also suppressed by deletion of helicases Pif1 and MPH1, and by deletion of POL32, a subunit of DNA polymerase δ. All dna2Δ cells are temperature sensitive, have telomere length defects, and low levels of telomeric 3′ single-stranded DNA (ssDNA). Interestingly, Rfa1, a subunit of the major ssDNA binding protein RPA, and the telomere-specific ssDNA binding protein Cdc13, often colocalize in dna2Δ cells. This suggests that telomeric defects often occur in dna2Δ cells. There are several plausible explanations for why the most critical function of Dna2 is at telomeres. Telomeres modulate the DNA damage response at chromosome ends, inhibiting resection, ligation, and cell-cycle arrest. We suggest that Dna2 nuclease activity contributes to modulating the DNA damage response at telomeres by removing telomeric C-rich ssDNA and thus preventing checkpoint activation.

KEYWORDS Dna2; telomere; yeast

The conserved nuclease/helicase Dna2 affects 5′ processing of Okazaki fragments during lagging strand replication (Budd and Campbell 1997), resection of double-strand breaks (DSBs)/uncapped telomeres (Ngo et al. 2014), activation of DNA damage checkpoint pathways (Kumar and Burgers 2013), resolution of G quadruplexes (Lin et al. 2013), and mitochondrial function (Budd et al. 2006; Duxin et al. 2009). Increased expression of DNA2 is found in a broad spectrum of cancers, including leukemia, melanoma, breast, ovarian, prostate, pancreatic, and colon cancers (Peng et al. 2012; Dominguez-Valentin et al. 2013; Strauss et al. 2014; Jia et al. 2017; Kumar et al. 2017; Wellcome Sanger Institute). Dna2 is an important enzyme because its loss is lethal in human cell lines, mice, Caenorhabditis elegans, budding yeast, and fission yeast (Budd et al. 1995; Kang et al. 2000; Lin et al. 2013). The amount of Dna2 in cells also seems to be important as dna2Δ/DNA2 heterozygous mice show increased levels of aneuploidy-associated cancers and cells from these mice contain high numbers of anaphase bridges and dysfunctional telomeres (Lin et al. 2013).

In budding yeast Dna2 functions redundantly with other proteins in its various roles and intriguingly, unlike Dna2, most of these proteins are not essential. For example, Rad27, Rnh201, and Exo1 are all nonessential and are also involved in processing of 5′ ends of Okazaki fragments (Bae et al. 2001; Kao and Bambara 2003). Exo1, Sgs1, Sae2, Mre11, Rad50, and Xrs2 are all nonessential and are involved in DSB resection (Mimitou and Symington 2008; Zhu et al. 2008; Shim et al. 2010). Ddc1 (nonessential) and Dpb11 (essential) are involved in Mec1 (essential) checkpoint kinase activation (Puddu et al. 2008; Navadgi-Patil and Burgers 2009a,b; Kumar and Burgers 2013). Given that Dna2 often functions redundantly with non-essential proteins, it is unclear what specific function or functions of Dna2 is/are so critical for cell viability.

Several genetic and biochemical experiments have suggested that the most critical function of Dna2 is in processing long flaps at a small subset of 5′ ends of Okazaki fragments (Budd et al. 2011; Balakrishnan and Bambara 2013). Dna2 is unique in that, unlike the other 5′ nucleases (Rad27, Exo1, Rnh201), it can cleave RPA-coated single-stranded DNA (ssDNA) (Stewart et al.
2008; Cejka et al. 2010; Levikova et al. 2013; Levikova and Cejka 2015; Myler et al. 2016). RPA, the major eukaryotic ssDNA binding protein, binds ssDNA of 20 bases or more (Sugiyama et al. 1997; Rossi and Bambara 2006; Balakrishnan and Bambara 2013). Furthermore, RPA-coated ssDNA is potentially lethal because it stimulates DNA damage checkpoint responses (Lee et al. 1998; Zou and Elledge 2003).

Two reported null suppressors of dna2Δ lethality, rad9Δ and pif1Δ, delete proteins that interact with RPA-coated ssDNA (Budd et al. 2006, 2011). Rad9 is important for the checkpoint pathway stimulated by RPA-coated ssDNA (Lydall and Weinert 1995). Pif1, a 5′ to 3′ helicase, increases the length of 5′ ssDNA flaps on Okazaki fragments, creating substrates for RPA binding and therefore checkpoint activation and Dna2 cleavage (Pike et al. 2009; Levikova and Cejka 2015). These genetic and biochemical data supported a model in which Dna2 is critical for cleaving RPA-coated long flaps from a subset of Okazaki fragments (Budd et al. 2011). However, more recently it was reported that other checkpoint mutations (dde1Δ or mec1Δ) also affecting the response to RPA-coated ssDNA did not suppress dna2Δ (Kumar and Burgers 2013). It was suggested that specific interactions between Rad9 and Dna2 were important for the viability of dna2Δ rad9Δ cells, rather than the response to RPA-coated ssDNA per se (Kumar and Burgers 2013).

In budding yeast, checkpoint mutations such as rad9Δ and ddc1Δ exacerbate fitness defects caused by general DNA replication defects (e.g., defects in DNA ligase, Pol α, Pol ε, or Pol δ) (Weinert et al. 1994; Dubarry et al. 2015), but suppress defects caused by mutations affecting telomere function (e.g., defects in Cdc13, Stn1, Yku70) (Addinall et al. 2008; Holstein et al. 2017). The opposing effects of checkpoint mutations in general DNA replication or telomere-defective contexts is most likely explained by damage to noncoding telomeric DNA being comparatively benign in comparison to damage to coding DNA in the middle of chromosomes. By this logic, the suppression of dna2Δ by rad9Δ implies that dna2Δ might cause telomere-specific rather than general chromosome replication defects. Furthermore, Dna2 localizes to human and yeast telomeres (Choe et al. 2002; Chai et al. 2013; Lin et al. 2013), and pif1Δ, which suppresses dna2Δ, affects a helicase that is active at telomeres and affects telomere length (Dewar and Lydall 2010; Budd and Campbell 2013; Lin et al. 2013; Phillips et al. 2015). Thus, several lines of evidence suggest that Dna2 might play critical function(s) at telomeres.

To further explore whether Dna2 is important at telomeres, we set out to clarify the effects of checkpoint pathways on fitness of dna2Δ mutants. We find that deletion of numerous DNA damage checkpoint mutations, all affecting responses to RPA-coated ssDNA, as well as deletions of Pif1 and Mph1 helicases, and Pol32, a subunit of Pol δ, suppress dna2Δ to a similar extent. These findings, along with a number of other telomere phenotypes lead us to suggest that the most critical function of Dna2 for cell viability is at telomeres. There are three possible substrates for Dna2 activity at telomeres: unwound telomeres, long flaps on terminal telomeric Okazaki fragments, and G4 quadruplexes formed on the G-rich ssDNA. We propose that the critical function of Dna2 is removing RPA-coated, 5′ C-rich, ssDNA at telomeres.

**Materials and Methods**

**Yeast culture and passage**

All yeast strains were in W303 background and RAD5+ and ade2-1, except strains used for microscopy, which were ADE2. Strains and plasmids details are in Supplemental Material, Tables S1 and S2 in File S1, respectively. Strains and plasmids are available upon request. Media were prepared as described previously and standard genetic techniques were used to manipulate yeast strains (Sherman et al. 1986). YEPD (1 liter: 10 g yeast extract, 20 g bactopeptone, 50 ml 40% dextrose, 15 ml 0.5% adenine, 935 ml H2O) medium was generally used. Dissected spores were germinated for 10–11 days at 20°, 7 days at 23°, or 3–4 days at 30°. Colonies from spores on germination plates were initially, instead of patched onto YEPD medium plates and grown for 3 days. Next, these were streaked for single colonies and incubated for 3 days at 23°. Thereafter, 5–10 colonies of each strain were pooled by toothpick and streaked for single colonies every 3 days.

**Yeast spot test assays**

A total of 5–10 colonies were pooled, inoculated into 2 ml YEPD medium and grown to saturation on a wheel at 23°. Saturated cultures were fivefold serially diluted in sterile water (40:160 μl) in 96-well plates. Cultures were transferred onto rectangular YEPD medium agar plates with a rectangular pin tool, and incubated at the indicated temperatures for 3 days before photography, unless stated otherwise.

**In-gel assay/Southern blots**

In-gel assays were performed as previously described (Dewar and Lydall 2012), with minor modifications. Infrared 5′ IRDye 800 probes were used (AC probe: M3157, CCCACCCACACA CCCACACCC; TG probe: M4462, GGGTTGGGTGTTG TGGTTGG; Integrated DNA Technologies). No RNase was used during nucleic acid purification. Samples were run on a 1% agarose gel in 0.5× TBE (50 V for 3 hr), and the probe was detected on a LI-COR (Odyssey) imaging system. ssDNA was quantified using ImageJ. The gel was then placed back in an electrophoresis tank, run for 2 hours, and processed for Southern blotting. Then, gel was stained using SYBR Safe, and DNA was detected using a Syngene’s G:BOX imaging system. DNA was then transferred to a positively charged nylon membrane. The membrane was hybridized with a 1 kbp Y′ and TG probe, as previously described (Holstein et al. 2014). Loading controls were generated by foreshortening the full-sized SYBR Safe-stained gel images with Adobe Illustrator CS6.

**Yeast live-cell imaging**

Cells were grown shaking in liquid synthetic complete medium supplemented with 100 μg/ml adenine at 25°, to OD600 = 0.2–0.3, and processed for fluorescence microscopy as described previously (Silva et al. 2012). Rfa1 was tagged with...
cyan fluorescent protein (clone W7) (Heim and Tsien 1996) and Cdc13 with yellow fluorescent protein (clone 10C) (Ormö et al. 1996; Khadaroo et al. 2009). Fluorophores were visualized with oil immersion on a widefield microscope (AxioImager Z1; Carl Zeiss, Thornwood, NY) equipped with a 100× objective lens (Plan Apochromat, numerical aperture 1.4; Carl Zeiss), a cooled charge-coupled device camera (Orca-ER; Hamamatsu Photonics), DIC, and an illumination source (HXP120C; Carl Zeiss). Eleven optical sections with 0.4 μm spacing through the cell were imaged. Images were acquired and analyzed using Volocity software (PerkinElmer). Images were pseudocolored according to the approximate emission wavelength of the fluorophores.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. Table S1 in File S1 lists all strains.

Results

dna2Δ lethality is suppressed by checkpoint inactivation

To clarify the effect of DNA damage checkpoint gene deletions in dna2Δ cells, heterozygous dna2Δ checkpointΔ diploid strains were sporulated, tetrads were dissected, and viable genotypes determined. We examined the effects of RAD9, DDC1, and MEC1, affecting a checkpoint mediator protein, a component of the 9-1-1 checkpoint sliding clamp, and the central checkpoint kinase (homolog of human ATR), respectively, and all previously studied in the context of dna2Δ (Budd et al. 2011; Kumar and Burgers 2013). We also examined RAD17, encoding a partner of Ddc1 in the checkpoint sliding clamp; CHK1, encoding a downstream checkpoint kinase; RAD53, a parallel downstream kinase; and TEL1, encoding the homolog of human ATM. As a positive control for suppression, we also examined the effects of PIF1, encoding a 5' to 3' helicase, because pif1Δ (like rad9Δ) suppresses dna2Δ (Budd et al. 2006).

dna2Δ rad9Δ and dna2Δ pif1Δ strains are temperature sensitive (Budd et al. 2006, 2011) and therefore spores were germinated at 20, 23, and 30° to allow comparison of dna2Δ suppression frequencies at different temperatures. Interestingly, the effects of rad9Δ, ddc1Δ, rad17Δ, chk1Δ, and mec1Δ were very similar, as they each permitted dna2Δ strains to form colonies at 20 and 23° but not at 30° (Figure 1, Figure S1a in File S1, and Table 1). In comparison, pif1Δ suppressed dna2Δ with higher efficiency and at higher temperatures, and pif1Δ dna2Δ colonies on germination plates were larger than those permitted by checkpoint gene deletions (Figure 1, Figure S1a).
contain telomere defects. 

(Figure S1a in File S1, and Table 1). tel1Δ and rad53Δ did not suppress dna2Δ, presumably because they have different roles in the DNA damage response. We conclude that rad9Δ, ddc1Δ, rad17Δ, chk1Δ, and mec1Δ, but not rad53Δ and tel1Δ checkpoint mutations, suppress inviability caused by dna2Δ. These data suggest that dna2Δ causes lethal Rad9, Rad17, Ddc1, Chk1, and Mec1 mediated cell-cycle arrest. Given that checkpoint mutations suppress dna2Δ and telomere defects (cdc13Δ, yku70Δ, and stn1-13) (Addinall et al. 2008; Holstein et al. 2017) but enhance DNA replication defects (Weinert et al. 1994; Dubarry et al. 2015), the pattern of dna2Δ genetic interactions strongly suggests that dna2Δ cells contain telomere defects.

**DNA2 deletion causes temperature sensitivity**

On germination plates dna2Δ checkpointΔ colonies were often small and heterogeneous in size in comparison with dna2Δ pif1Δ colonies, implying that mutating checkpoint genes did not suppress the dna2Δ growth defects as efficiently as removing the Pif1 helicase (Figure 1). One explanation for this difference in colony size was that checkpoint mutations permitted only a limited number of cell divisions, but that ultimately the dna2Δ checkpointΔ double-mutant clones would senesce and cease growth. To test this hypothesis, dna2Δ checkpointΔ double mutants were passaged further. Interestingly, the opposite to senescence was observed, and dna2Δ checkpointΔ mutants in fact became fitter and more homogeneous in colony size with passage and grew indefinitely (Figure 2A and Figure S2a in File S1). This suggests that dna2Δ checkpointΔ double mutants originally grow quite poorly and that some type of adaptation to the absence of Dna2 occurs in dna2Δ checkpointΔ mutants. We considered that additional suppressor mutations had arisen in dna2Δ checkpointΔ mutants, but backcross experiments did not support this hypothesis (Figure S1b in File S1). It was also clear that even different strains of the same genotype became similarly fit when passaged at 23°, which is inconsistent with different suppressor mutations arising. However, all strains remained temperature sensitive for growth at higher temperatures, and growth at high temperature was more heterogeneous than growth at low temperature (Figure 2B). Overall, passage of dna2Δ checkpointΔ strains shows that they adapt to the absence of Dna2 but remain temperature sensitive for growth, presumably because ongoing cellular defects are more penetrant at higher temperature. Consistent with a previous study (Budd et al. 2006), dna2Δ pif1Δ strains, the least temperature-sensitive genotype, formed smaller colonies at 36° than at 30°, showing that even these cells also have a temperature-sensitive molecular defect (Figure 2B). We noted a similarity between yku70Δ and dna2Δ strains as each genotype exhibits a temperature-sensitive phenotype and is suppressed by checkpoint mutations (Maringele and Lydall 2002). In the case of yku70Δ mutants, high levels of 3′ ssDNA are generated at telomeres at high temperature (Maringele and Lydall 2002).

**dna2Δ cells have abnormal telomere length with limited ssDNA**

We next tested whether Dna2 affects the structure of telomeric DNA. We first tested for increased levels of 3′ ssDNA at telomeres in dna2Δ cells because this is seen in yku70Δ cells (Maringele and Lydall 2002). Furthermore, in fission yeast, Dna2 was shown to be involved in the generation of G-rich ssDNA at telomeres (Tomita et al. 2004). Importantly, it was reported that dna2Δ rad9Δ cells have anomalously low levels of telomeric 3′ G-rich ssDNA (Budd and Campbell 2013). Consistent with what was reported for rad9Δ dna2Δ, chk1Δ dna2Δ, mec1Δ dna2Δ, rad17Δ dna2Δ, ddc1Δ dna2Δ, and pif1Δ dna2Δ cells all showed low levels of 3′ G-rich ssDNA at telomeres in comparison with DNA2 strains (Figure 3, A and B and Figures S3 and S4 in File S1). We conclude that all

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20, 23, and 30° are the temperatures at which spores were germinated. The leftmost column shows the gene deleted in each dna2Δ+ diploid. Viable dna2Δ xyzΔ is the number of spores that germinated and formed visible colonies. Expected dna2Δ xyzΔ is the expected number of viable dna2Δ xyzΔ strains if xyzΔ completely suppressed the dna2Δ invisible phenotype, based on the total number of tetrads dissected. For example, 25% of dna2Δ rad9Δ+ spores should be dna2Δ rad9Δ, and 12.5% of mec1Δ+/ sml1Δ+ dna2Δ+ should be mec1Δ sml1Δ dna2Δ.

**Table 1 dna2Δ suppression efficiency**

We next tested whether Dna2 affects the structure of telomeric DNA. We first tested for increased levels of 3′ ssDNA at telomeres in dna2Δ cells because this is seen in yku70Δ cells (Maringele and Lydall 2002). Furthermore, in fission yeast, Dna2 was shown to be involved in the generation of G-rich ssDNA at telomeres (Tomita et al. 2004). Importantly, it was reported that dna2Δ rad9Δ cells have anomalously low levels of telomeric 3′ G-rich ssDNA (Budd and Campbell 2013). Consistent with what was reported for rad9Δ dna2Δ, chk1Δ dna2Δ, mec1Δ dna2Δ, rad17Δ dna2Δ, ddc1Δ dna2Δ, and pif1Δ dna2Δ cells all showed low levels of 3′ G-rich ssDNA at telomeres in comparison with DNA2 strains (Figure 3, A and B and Figures S3 and S4 in File S1). We conclude that all
DNA2 Δ mutants have low levels of telomeric 3' ssDNA. Interestingly, the DNA2 Δ ssDNA phenotype is opposite to that observed in other telomere-defective strains (cdc13-1 and yku70Δ mutants), which contain high levels of 3' telomeric ssDNA (Maringele and Lydall 2002). We also checked for 5' C-rich ssDNA and saw no evidence for increased levels of telomeric C-rich ssDNA (Figure S5 in File S1).

To search for other telomeric DNA phenotypes in DNA2 Δ strains, we examined telomere length by Southern blotting. Interestingly, the telomeres of chk1Δ DNA2 Δ, mec1Δ DNA2 Δ, rad17Δ DNA2 Δ, and ddc1Δ DNA2 Δ cells were long, and in fact longer and more diffuse than pif1Δ strains, known to have very long telomeres (Schulz and Zakian 1994) (Figure 3C and Figures S4 and S6 in File S1). In contrast, and as reported before, rad9Δ DNA2 Δ telomeres were slightly shorter than the wild-type length (Budd and Campbell 2013). Rad9 is unique among checkpoint proteins because it binds chromatin and inhibits nuclease activity at telomeres and DSBs (Bonetti et al. 2015; Ngo and Lydall 2015). Perhaps, therefore, the comparatively short telomere length in rad9Δ DNA2 Δ mutants reflects this chromatin-binding function of Rad9 at telomeres.

In summary, all DNA2 Δ mutants analyzed have abnormal telomere lengths and low levels of 3' G-rich ssDNA.

Long telomeres are present in telomerase-deficient, recombination (RAD52)-dependent survivors (Wellinger and Zakian 2012). Recombination is also important to rescue stalled replication forks in telomeric sequences because the terminal location of telomeric DNA means that stalled forks cannot be rescued by forks arriving in the opposite direction, as in elsewhere in the genome. Because the telomeres in DNA2 Δ strains were often long, we wondered if recombination contributed to the viability of DNA2 Δ strains. Interestingly, Rad52 did seem to contribute to the viability of rad9Δ DNA2 Δ and ddc1Δ DNA2 Δ strains (Figure S7 in File S1). This strongly suggests that recombination-dependent mechanisms help DNA2 Δ cells maintain viability.

**DNA2 nuclease is critical in checkpoint-defective cells**

DNA2 is a nuclease as well as a helicase, and directly activates the central checkpoint kinase Mec1 (Kumar and Burgers 2013). Any of these functions might be important at telomeres or elsewhere. To test which biochemical activity is
most important to cell fitness, we transformed nuclease-, helicase-, or checkpoint-defective alleles of DNA2 into rad9Δ dna2Δ or ddc1Δ dna2Δ cells, and measured growth at high temperature. It was clear that helicase dead and checkpoint-defective alleles rescued the dna2Δ defect and permitted growth at high temperatures (Figure 4B and Figure S8 in File S1). In contrast, the nuclease-defective allele of DNA2 did not rescue the dna2Δ growth defect. We conclude that the most critical function of Dna2 in checkpoint-defective yeast cells is its nuclease function.

**dna2Δ mutants contain RPA-bound telomeres**

dna2Δ cells are temperature sensitive, have telomere length phenotypes, and stimulate checkpoint pathways. However, paradoxically, dna2Δ cells have reduced levels of telomeric ssDNA when measured by in-gel assay. We reasoned that one plausible function for Dna2 nuclease activity was removal of ssDNA present in vivo that was not detectable in vitro. That is, unwound terminal telomeric DNA formed Y-shaped structures in vivo, with spliced arms of G-rich and C-rich ssDNA. The 5′ C-rich and 3′ G-rich ssDNA should bind RPA and CST (Cdc13, Stn1, and Ten1) (Nugent et al. 1996), respectively, with the RPA-coated 5′ ssDNA stimulating DNA damage checkpoint pathways. The ssDNA present on the arms of Y-shaped telomeres in vivo might not be detected by in-gel assays because complementary ssDNA strands would reanneal during DNA purification. Finally, telomere unwinding might be catalyzed by helicases (for example, Pif1) and high temperature, explaining the effects of pif1Δ and temperature on fitness of dna2Δ cells.

Most eukaryotic cells contain 3′ ssDNA overhangs on the G-rich strand of telomeric DNA, and this ssDNA is bound by proteins such as Pot1 and CST. If unwound telomeres occur in dna2Δ cells, then CST should still bind the 3′ strand, but in addition, RPA could bind the C-rich 5′ strand and stimulate the checkpoint. Presumably, in such a case, both RPA and CST complexes would colocalize at telomeres and stop the stimulation of the checkpoint pathway. To explore RPA and CST localization, the two largest subunits of each complex, Cdc13 and Rfa1, were tagged with yellow and cyan fluorescent proteins, respectively, and their localization in dna2Δ cells was examined by live-cell microscopy.

We examined Cdc13 and Rfa1 foci in ddc1Δ dna2Δ, pif1Δ dna2Δ cells and wild-type, ddc1Δ, pif1Δ controls. Because
some of these cells grew poorly and may have altered cell-cycle distributions, we counted foci in budded cells (S/G2/M) as this is when Rfa1 foci are more likely to be present (Figure 5). We observed broadly similar fractions of cells with Cdc13 foci in all cultures at the level of 30–70%, but checkpoint-defective strains ddc1Δ and ddc1Δ dna2Δ had somewhat higher levels (closer to 70%) (Figure 5A). In G1 cells the number of Cdc13 foci was smaller (<20%), but ddc1Δ dna2Δ cells tended to have consistently slightly higher levels (on average 15%) (Figure S9a in File S1). We conclude that DNA2 deletion has no strong effect on Cdc13 foci formation.

We also searched for Rfa1 foci and observed that, on average, 30% of budded and 10% of unbudded control cells contained Rfa1 foci (Figure 5B and Figure S9b in File S1). In contrast, ddc1Δ dna2Δ and pif1Δ dna2Δ cultures contained a much higher fraction of budded cells with Rfa1 foci. Generally, >80% of ddc1Δ dna2Δ and pif1Δ dna2Δ cells, and ~40% of pif1Δ cells contained at least one Rfa1 focus (Figure 5B), suggesting that high levels of DNA damage and ssDNA are present in these strains. In G1 cells, the number of Rfa1 foci was smaller (up to 80%), and cells hardly ever contained more than one Rfa1 focus (Figure S9b in File S1).

If the Rfa1 foci observed in dna2Δ cells were primarily at telomeres, rather than at DSBs or long flaps on Okazaki fragments elsewhere in the genome, then Rfa1 foci in dna2Δ cells should preferentially localize at telomeres. Assuming Cdc13 foci are at telomeres (Khadaroo et al. 2009), then >60% of these telomeric foci in ddc1Δ dna2Δ budded cells colocalized with Rfa1 (Figure 5C). In contrast, <10% of Cdc13 foci contained Rfa1 in wild-type or ddc1Δ budded cells, suggesting low Rfa1 at telomeres in wild-type and ddc1Δ strains. This suggests that RPA-bound ssDNA occurs at high frequency near telomeres in ddc1Δ dna2Δ cells. pif1Δ dna2Δ cells contained nearly as many Rfa1 foci and Cdc13 foci as ddc1Δ dna2Δ cells, but less Cdc13 foci contained Rfa1 (~30%). We conclude that pif1Δ dna2Δ cells have less RPA-bound ssDNA at telomeres than ddc1Δ dna2Δ cells. Interestingly, pif1Δ single mutants also contained more Rfa1 foci than wild-type cells, and more colocalization of Rfa1 and Cdc13 (~5%) (Figure 5, B–D). This suggests that pif1Δ cells, which contain long telomeres, show comparatively high levels of RPA binding at telomeres, possibly due to the difficulty of replicating through long stretches of telomeric DNA.

Overall, of all the genotypes examined, ddc1Δ dna2Δ mutants had the highest fraction of Cdc13 foci that contain Rfa1, Rfa1 foci that contain Cdc13, and Cdc13-Rfa1 foci (Figure 5, C and D and Figure S9f in File S1). These data are consistent with a model in which both G-rich and C-rich ssDNA are found at high levels at telomeres in ddc1Δ dna2Δ cells. Interestingly, pif1Δ dna2Δ cells also contained increased levels of CST/RPA-bound ssDNA, suggesting that Pif-independent helicases may unwind telomeric C-rich and G-rich ssDNA in the absence of Pif1, to generate substrates for RPA binding.

**dna2Δ lethality is suppressed by mph1Δ and pol32Δ, but not sgs1Δ**

To search for additional activities that might unwind telomeric DNA, like Pif1, we examined genes affecting likely candidates. Sgs1 was a candidate since it functions with Dna2 in resection of DSBs and uncapped telomeres (Cejka et al. 2010; Ngo et al. 2014), but its deletion did not suppress dna2Δ (Figure S10a in File S1), as has been reported by others (Hoopes et al. 2002; Weitao et al. 2003; Budd et al. 2005). On this basis Sgs1 does not seem to contribute to telomere unwinding, or if it does, it also has other functions that are essential in dna2Δ strains.
We examined Mph1, because like Pif1, Mph1 stimulates Dna2 activity in vitro (Kang et al. 2009). Interestingly, mph1Δ suppressed dna2Δ. The effect of mph1Δ was similar to checkpoint mutations, but not as strong as pif1Δ (Figure S10, a–c in File S1). Therefore loss of Mph1, a 3’ to 5’ helicase, like loss of Pif1, a 5’ to 3’ helicase, suppresses the inviability of dna2Δ cells. Given the polarity of the Mph1 helicase, it would most likely engage with the 3’ G-rich overhanging strand to unwind telomeric DNA, and compete with CST for this substrate. To test this hypothesis, mph1Δ was combined with cdc13-1 and the temperature-sensitive phenotype was scored. Interestingly, mph1Δ mildly suppresses the temperature-dependent growth defects of cdc13-1 mutants (Figure S10d in File S1). This suggests that Mph1 and CST compete to bind the same G-rich strand at telomeres, and is consistent with the idea that Mph1 engages with the 3’ telomeric overhang to unwind telomeric double-stranded DNA.

Finally, we tested Pol32, a DNA Pol δ subunit, which helps displace 5’ ends of Okazaki fragments. It had been reported that pol32Δ suppresses some alleles of DNA2, and weakly suppresses dna2Δ (Budd et al. 2006; Stith et al. 2008). Interestingly, we confirmed that pol32Δ suppressed dna2Δ. In contrast to checkpoint mutations, pol32Δ suppressed dna2Δ at high temperature (30°C and 23°C) but not at 20°C (Figure S10, a–c in File S1). This temperature-dependent suppression may be explained by the fact that pol32Δ mutants are cold sensitive (Gerik et al. 1998).

**Discussion**

We report that loss of proteins affecting numerous aspects of the DNA damage response permit budding yeast cells to divide indefinitely in the absence of the essential protein Dna2. Loss of DNA damage checkpoint proteins (Rad9, Ddc1, Rad17, Chk1, and Mec1) or Pif1, a 5’ to 3’ helicase, Mph1, a 3’ to 5’ helicase, or Pol32, a DNA polymerase δ subunit, suppress the inviability of dna2Δ cells. The suppression of dna2Δ by checkpoint mutations makes dna2Δ mutants more similar to telomere-defective

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**Figure 5** dna2Δ mutants accumulate CST and RPA, the ssDNA binding complexes. (A–D) Percentages of Cdc13 foci, Rfa1 foci, or colocalized Cdc13-Rfa1 foci in dna2Δ and control strains are shown. (A) Percentage of budded (S/G2/M) cells with either Cdc13 foci only or Cdc13-Rfa1 foci. (B) Percentage of budded cells with either Rfa1 foci only or Cdc13-Rfa1 foci. (C) Percentage of budded cells with Cdc13 foci that colocalize with Rfa1 foci. (D) Percentage of budded cells with colocalizing Cdc13-Rfa1 foci. Error bars indicate 95% confidence intervals (n = 213–437, from two independent cultures of each strain). * indicates statistical significance (P < 0.05) determined using Fisher’s exact test. Strains are as follows: wild type (WT) (DLY12342, DLY12343, ddc1Δ (DLY12282, DLY12280, DLY12283), ddc1Δ dna2Δ (DLY12281, DLY12341, DLY12284, DLY12279), pif1Δ (DLY12346, DLY12347), and pif1Δ dna2Δ (DLY12344, DLY12345). (E) An example of live-cell images is shown. Cdc13-Rfa1 colocalized foci are indicated by green arrows, Cdc13 foci by yellow arrows, and Rfa1 foci by blue arrows. Bar, 3 μm. Strain details are in Table S1 in File S1.
strains than general DNA replication-defective strains (Dubarry et al. 2015). Consistent with this, dna2Δ strains show telomere length phenotypes and a high degree of colocalization of Cdc13, a telomeric G-rich ssDNA binding protein, and Rfa1, a more general ssDNA binding protein in vivo. dna2Δ mutants are also temperature sensitive and have low levels of telomeric G-rich ssDNA. The nuclease function of Dna2, but not helicase and checkpoint functions, is critical to confer the viability of dna2Δ checkpoint strains at high temperature.

The low levels of telomeric 3' ssDNA that we detect at telomeres of dna2Δ mutants by in vitro in-gel assay is the opposite phenotype to the high levels of 3' ssDNA found at telomeres in other telomere-defective strains suppressed by checkpoint gene mutations (for example, cdc13-1 and yku70Δ mutants) (Maringele and Lydall 2002; Ngo et al. 2014). Our explanation is that high levels of RPA-coated C-rich ssDNA and comparatively normal levels of CST-coated G-rich ssDNA are present at unwound telomeres of dna2Δ cells in vivo. This is detected as colocalization by live-cell imaging, but when DNA is extracted, it renatures during purification and ssDNA is not detected.

There are at least three plausible scenarios for why Dna2 might have its most critical functions at or near telomeres (Figure 6A). One model that best fits all our data is that Dna2 nuclease activity removes potentially harmful, RPA-coated 5' C-rich ssDNA at the termini of telomeres (Figure 6A, scenario I). In this model, helicases like Pif1 or Mph1 unwind the telomeric termini. The G-rich strand is bound by CST and the 5' C-rich strand is bound by RPA, a substrate for Dna2 cleavage. Scenario II: Processing of long flaps of Okazaki fragments near telomeres. DNA polymerase δ displacement activity, stimulated by helicase(s), generates long flaps on an Okazaki fragment near telomere. Long C-rich flap, bound by RPA, are subjected to Dna2 cleavage. Scenario III: G-quadruplex unwinding and processing. G-quadruplexes formed on telomeric G-rich ssDNA are unwound or processed by Dna2. All proteins were drawn to scale. (B) Lagging and leading strand replication at telomeres. Short red arrows indicate Okazaki fragments on the lagging strand. The long red arrow indicates replicated leading strand. The brown circle indicates the flap formed on an internal Okazaki fragment. The green circle indicates no flap on the terminal telomeric Okazaki fragment. The blue circle indicates no flap on the leading strand template.

Figure 6 Three plausible roles for Dna2 in removing unwound RPA-coated ssDNA at telomeres. (A) Three scenarios for Dna2 activity. Scenario I: 5' RPA-coated ssDNA cleavage at telomeric termini. Telomere ends are unwound by helicases, for example, Pif1 or Mph1. The 3' G-rich strand is bound by CST and the 5' C-rich strand is bound by RPA, a substrate for Dna2 cleavage. Scenario II: Processing of long flaps of Okazaki fragments near telomeres. DNA polymerase δ displacement activity, stimulated by helicase(s), generates long flaps on an Okazaki fragment near telomere. Long C-rich flap, bound by RPA, are subjected to Dna2 cleavage. Scenario III: G-quadruplex unwinding and processing. G-quadruplexes formed on telomeric G-rich ssDNA are unwound or processed by Dna2. All proteins were drawn to scale. (B) Lagging and leading strand replication at telomeres. Short red arrows indicate Okazaki fragments on the lagging strand. The long red arrow indicates replicated leading strand. The brown circle indicates the flap formed on an internal Okazaki fragment. The green circle indicates no flap on the terminal telomeric Okazaki fragment. The blue circle indicates no flap on the leading strand template.
experiments in yeast suggest that Dna2 is critical for removing protect telomeric DNA from being unwound by helicases. Our damage response. Telomeric structures like t-loops, and pro-need to be protected from the harmful aspects of the DNA essential gene in budding and human cells. Interestingly, Dna2 affects telomere phenotypes in all these organisms (Choe et al. 2012; Ngo et al. 2014), but our evidence is that telomeres are particularly reliant on Dna2.

If Dna2 acts at the very termini of telomeres (Figure 6A, scenario I), either the lagging strand, the leading strand, or both might be targets for Dna2 (Figure 6B). It is well-established that the leading and lagging strands of telomeres are pro-cessed by different mechanisms (Parenteau and Wellinger 1999; Wu et al. 2012; Bonetti et al. 2013; Soudet et al. 2014). After lagging strand replication is complete, the very terminus cannot be fully replicated because of the end replication problem. Irrespective of whether the most terminal Okazaki fragment is created by passage of the replication fork or CST recruitment of Pol α, it is unusual as unlike >99% of the other Okazaki fragments, it will not contain a flap at its 5’ end (Figure 6B). Perhaps the absence of a flap and/or a polymerase facilitates helicase engagement. The leading strand telomere end, which is thought to be blunt after the replication fork has passed, may also be susceptible to helicase activities.

We and others (Budd and Campbell 2013) have shown that dna2Δ rad9Δ cells have a short telomere phenotype. All other dna2Δ strains, including other checkpoint-defective strains, have long telomeres. Hence it is not telomere length per se that determines the survival of dna2Δ cells. Rad9, like its human ortholog 53BP1, binds chromatin and inhibits resection at telomere-defective ddc1Δ-1 cells and at DSBs (Iwabuchi et al. 2003; Lazzaro et al. 2008; Bunting et al. 2010; Ngo and Lydall 2015). Perhaps Rad9 binding to chromatin also inhibits helicase activity, telomere unwinding, and nuclease activity. Presumably unwound telomeres are also more susceptible to nucleases (other than Dna2). Consistent with this, the 9-1-1 complex recruits Dna2 and Exo1 nuclease to uncapped telomeres (Ngo and Lydall 2015), and ddc1Δ dna2Δ and rad17Δ dna2Δ mutants, defective in 9-1-1, have long telomeres.

Telomeres in all organisms are difficult to replicate and need to be protected from the harmful aspects of the DNA damage response. Telomeric structures like t-loops, and proteins like CST, shelterin, and the Ku heterodimer may help protect telomeric DNA from being unwound by helicases. Our experiments in yeast suggest that Dna2 is critical for removing RPA-coated C-rich ssDNA at unwound telomeres. DNA2 is an essential gene in budding and fission yeasts, C. elegans, mice, and human cells. Interestingly, C. elegans dna2Δ mutants show temperature-dependent delayed lethality (Lee et al. 2003), suggesting that temperature-dependent telomere unwinding in C. elegans creates substrates for Dna2 nuclease activity at high temperatures.

Dna2 localizes at telomeres in yeast, humans, and mice, and Dna2 affects telomere phenotypes in all these organisms (Choe et al. 2002; Lin et al. 2013). Dna2, checkpoint proteins, Pif1 and Mph1 helicases, and Pol32 are all conserved between human and yeast cells, and affect telomere-related human diseases such as cancer, suggesting our observations may be relevant to human disease (Paeschke et al. 2013; Byrd and Raney 2015; Ceccaldi et al. 2016). It will be interesting to see if telomere-specific functions for Dna2 are conserved across eukaryotes.

**Acknowledgments**

We are particularly grateful to Lata Balakrishnan, Peter Burgers, Judy Campbell, Laura Marinegele, and Duncan Smith for advice and input. This work was funded by European Union Marie Curie International Training Network (ITN) network CodeAge (FP7-PEOPLE-2011-ITN) and the Biotechnol-ogy and Biological Sciences Research Council (BBSRC) (BB/M002314/1). The Danish Agency for Science, Technology and Innovation (DFF) and the Villum Foundation supported the work performed by M.L.

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Communicating editor: D. Bishop