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Rif1 Supports the Function of the CST Complex in Yeast Telomere Capping

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Abstract

Telomere integrity in budding yeast depends on the CST (Cdc13-Stn1-Ten1) and shelterin-like (Rap1-Rif1-Rif2) complexes, which are thought to act independently from each other. Here we show that a specific functional interaction indeed exists among components of the two complexes. In particular, unlike *RIF2* deletion, the lack of Rif1 is lethal for *stn1ΔC* cells and causes a dramatic reduction in viability of *cdc13-1* and *cdc13-5* mutants. This synthetic interaction between Rif1 and the CST complex occurs independently of *rif1Δ*-induced alterations in telomere length. Both *cdc13-1 rif1Δ* and *cdc13-5 rif1Δ* cells display very high amounts of telomeric single-stranded DNA and DNA damage checkpoint activation, indicating that severe defects in telomere integrity cause their loss of viability. In agreement with this hypothesis, both DNA damage checkpoint activation and lethality in *cdc13 rif1Δ* cells are partially counteracted by the lack of the Exo1 nuclease, which is involved in telomeric single-stranded DNA generation. The functional interaction between Rif1 and the CST complex is specific, because *RIF1* deletion does not enhance checkpoint activation in case of CST-independent telomere capping deficiencies, such as those caused by the absence of Yku or telomerase. Thus, these data highlight a novel role for Rif1 in assisting the essential telomere protection function of the CST complex.

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Introduction

Telomeres, the specialized nucleoprotein complexes at the ends of eukaryotic chromosomes, are essential for genome integrity. They protects chromosome ends from fusions, DNA degradation and recognition as DNA double-strand breaks (DSBs) that would otherwise lead to chromosome instability and cell death (reviewed in [1]). Telomeric DNA in the budding yeast *Saccharomyces cerevisiae*, as well as in nearly all other eukaryotes examined to date, comprise short TG-rich repeated sequences ending in a short single-stranded 3′ overhang (G tail) that corresponds to the strand bearing the TG-rich repeats. The addition of telomeric repeats depends on the action of telomerase, a specialized reverse transcriptase that extends the TG-rich strand of chromosome ends. Recruitment/activation of this enzyme requires the Cdc13 protein that binds to the telomeric TG-rich single-stranded DNA (ssDNA) [2–6]. The direct interaction between Cdc13 and the Est1 regulatory subunit of telomerase is essential for telomerase recruitment, and it is disrupted by the *cdc13-2* mutation that leads to gradual telomere erosion and accompanying senescence [2,4,7].

The average length of *S. cerevisiae* telomeric 3′ overhangs is 12–14 nucleotides, although it can increase to ~50 nucleotides during the late S/G2 phase of the cell cycle [8–10]. While single-stranded telomeric G-tails can arise after removal of the last RNA primer during lagging-strand replication, the blunt ends of the leading-strand telomere must be converted into 3′ overhangs by resection of the 5′ strand. This 5′ to 3′ nucleolytic degradation involves several proteins, such as the MRX complex, the nucleases Exo1 and Dna2 and the helicase Sgs1 [10,11]. Cyclin-dependent kinase activity (Cdk1 in *S. cerevisiae*) is also required for generation of the extended single-stranded overhangs in late S phase [12,13]. As Cdk1 activity is low in G1, telomere resection can occur only during S/G2 [8], coinciding with the time frame in which G-tails are lengthened and can serve to recruit telomerase.

Keeping the G tail in check is crucial to ensure telomere stability, and studies in budding yeast have shown that Cdc13 prevents inappropriate generation of ssDNA at telomeric ends [2,14,15]. This essential capping function depends on Cdc13 interaction with the Stn1 and Ten1 proteins to form the so-called CST (Cdc13-Stn1-Ten1) complex. This complex binds to telomeric ssDNA repeats and exhibits structural similarities with the heterotrimeric ssDNA binding complex Replication protein A (RPA) [16], suggesting that CST is a telomere-specific version of RPA. Loss of Cdc13 function through either the *cdc13-1* temperature sensitive allele or the *cdc13-td* conditional degron allele results in telomere C-strand degradation, leading to activation of the DNA damage checkpoint [13,14,17,18]. Similarly, temperature sensitive mutations in either *STN1* or *TEN1* genes cause telomere degradation and checkpoint-mediated cell cycle arrest [19–21]. Interestingly, Stn1 interacts with Pol12 [22], a subunit of the DNA polymerase α (polα)-primase complex with putative regulatory functions, while Cdc13 interacts with the polα catalytic subunit of the same complex [7], suggesting that CST function might be tightly coupled to the priming of telomeric

Author Summary

Protection of chromosome ends is crucial for maintaining chromosome stability and genome integrity, and its failure leads to genome rearrangements that may facilitate carcinogenesis. This protection is achieved by the packaging of chromosome ends into protective structures called telomeres that prevent DNA repair/recombination activities. Telomeric DNA is bound and stabilized by two protein complexes named CST and shelterin, which are present in a wide range of multicellular organisms. Whether structural and functional connections exist between these two capping complexes is an important issue in telomere biology. Here, we investigate this topic by analyzing the consequences of disabling the two *Saccharomyces cerevisiae* shelterin-like components, Rif1 and Rif2, in different hypomorphic mutants defective in CST components. We demonstrate that Rif1 plays a previously unanticipated role in assisting the essential telomere protection function of the CST complex, indicating a tight coupling between CST and Rif1. As CST complexes have been recently identified also in other organisms, including humans, which all rely on shelterin for telomere protection, this functional link between CST and shelterin might be an evolutionarily conserved common feature to ensure telomere integrity.

C strand synthesis. In any case, it is so far unknown whether the excess of telomeric ssDNA in *cst* mutants arises because the CST complex prevents the access of nuclease/helicase activities to telomeric ends and/or because it promotes polα-primase-dependent C strand synthesis.

In addition to the capping function, a role for the CST complex in repressing telomerase activity has been unveiled by the identification of *cdc13*, *stn1* and *ten1* alleles with increased telomere length [2,21,23,24]. The repressing effect of Cdc13 appears to operate through an interaction between this protein and the C-terminal domain of Stn1 [25,26], which has been proposed to negatively regulate telomerase by competing with Est1 for binding to Cdc13 [4,24].

A second pathway involved in maintaining the identity of *S. cerevisiae* telomeres relies on a complex formed by the Rap1, Rif1 and Rif2 proteins. Although only Rap1 is the only shelterin subunit conserved in budding yeast, the Rap1-Rif1-Rif2 complex functionally recapitulates the shelterin complex acting at mammalian telomeres (reviewed in [27]). Rap1 is known to recruit its interacting partners Rif1 and Rif2 to telomeric double-stranded DNA via its C-terminal domain [28–30]. This complex negatively regulates telomere length, as the lack of either Rif1 or Rif2 causes telomere lengthening, which is dramatically increased when both proteins are absent [30]. The finding that telomere length in *rif1*Δ *rif2*Δ double mutant is similar to that observed in *RAP1* C-terminus deletion mutants [30] suggests that Rap1-dependent telomerase inhibition is predominantly mediated by the Rif proteins. However, Rif proteins have been shown to regulate telomere length even when the Rap1 C-terminus is absent [31], suggesting that they can be brought to telomeres independently of Rap1.

In addition to negatively regulate telomere length, Rap1 and Rif2 inhibit both nucleolytic processing and non homologous end joining (NHEJ) at telomeres [32–34]. Telomeric ssDNA generation in both *rif2*Δ and *rap1*Δ*C* cells requires the MRX complex [33], and the finding that MRX association at telomeres is enhanced in *rif2*Δ and *rap1*Δ*C* cells [33,35] suggests that Rap1 and Rif2 likely prevent MRX action by inhibiting MRX recruitment onto telomeric ends. Interestingly, the checkpoint response is not elicited after inactivation of Rap1 or Rif2, suggesting that either the accumulated telomeric ssDNA is insufficient for triggering checkpoint activation or this ssDNA is still covered by Cdc13, which can inhibit the association of the checkpoint kinase Mec1 to telomeres [36]. Notably, Rif1 is not involved in preventing telomeric fusions by NHEJ [32] and its lack causes only a slight increase in ssDNA generation at a de novo telomere [33]. These findings, together with the observation that Rif1 prevents telomerase action independently of Rif2, indicate that Rif1 and Rif2 play different functions at telomeres.

As both CST and the shelterin-like complex contribute to telomere protection, we asked whether and how these two capping complexes are functionally connected. We found that the viability of cells with defective CST complex requires Rif1, but not Rif2. In fact, *RIF1* deletion increases the temperature sensitivity of *cdc13-1* cells and impairs viability of *cdc13-5* cells at any temperature. Furthermore, the *rif1*Δ and *stn1*Δ*C* alleles are synthetically lethal. By contrast, the lack of Rif2 has no effects in the presence of the same *cdc13* and *stn1* alleles. We also show that *cdc13-1 rif1*Δ and *cdc13-5 rif1*Δ cells accumulate telomeric ssDNA that causes hyperactivation of the DNA damage checkpoint, indicating that loss of Rif1 exacerbates telomere integrity defects in *cdc13* mutants. By contrast, deletion of *RIF1* does not enhance either cell lethality or checkpoint activation in *yku70*Δ or *est2*Δ telomere capping mutants. Thus, Rif1 is required for cell viability specifically when CST activity is reduced, highlighting a functional link between Rif1 and CST.

Results

Rif1, but not Rif2, is required for cell viability when Cdc13 or Stn1 activities are reduced

Yeast cells harbouring the *cdc13-1* temperature-sensitive allele of the gene encoding the essential telomeric protein Cdc13 are viable at permissive temperature (20–25°C), but die at restrictive temperature (26–37°C), likely due to accumulation of ssDNA at telomeres caused by the loss of Cdc13 capping functions [14]. As also the shelterin-like complex contributes to the maintenance of telomere integrity, we investigated its possible functional connections with Cdc13 by disabling either Rif1 or Rif2 in *cdc13-1* cells. Deletion of *RIF2* did not affect *cdc13-1* cell viability in YEPD medium at any tested temperature (Figure 1A). By contrast, *cdc13-1 rif1*Δ cells showed a maximum permissive temperature for growth of 20°C and were unable to grow at 25°C, where *cdc13-1* single mutant cells could grow at almost wild type rate (Figure 1A). The enhanced temperature-sensitivity of *cdc13-1 rif1*Δ cells was due to the lack of *RIF1*, because the presence of wild type *RIF1* on a centromeric plasmid allowed *cdc13-1 rif1*Δ cells to grow at 25°C (Figure 1B). The synthetic effect of the *cdc13-1 rif1*Δ combination was not uncovered during a previous genome wide search for gene deletions enhancing the temperature-sensitivity of *cdc13-1* cells [37], likely because that screening was done at 20°C, a temperature at which *cdc13-1 rif1*Δ double mutants do not show severe growth defects (Figure 1A). Our data above indicate that Rif1, but not Rif2, is required to support cell viability when Cdc13 protective function is partially compromised.

If the lack of Rif1 in *cdc13-1* cells increased the temperature-sensitivity by exacerbating the telomere end protection defects of these cells, Rif1 overexpression might suppress the temperature sensitivity caused by the *cdc13-1* allele. Indeed, high copy number plasmids carrying wild type *RIF1*, which had no effect on wild type cell viability, improved the ability of *cdc13-1* cells to form colonies on synthetic selective medium at the semi-permissive temperature of 26–27°C (Figure 1C).

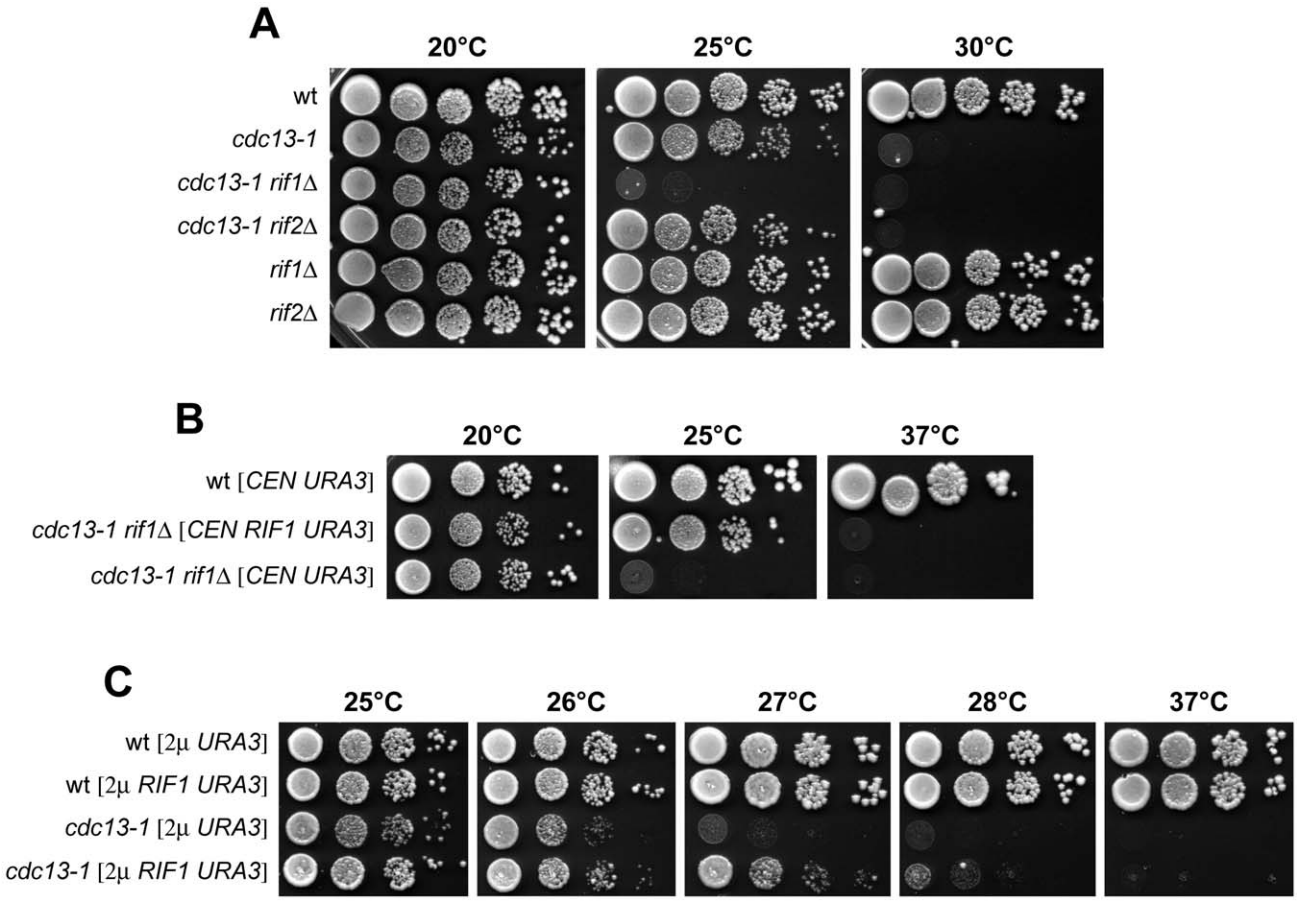

Figure 1. Synthetic effects between the *rif1*Δ and *cdc13-1* mutations. (A) Strains with the indicated genotypes were grown overnight in YEPD at 20°C. Serial 10-fold dilutions were spotted onto YEPD plates and incubated at the indicated temperatures for 2–4 days. (B–C) Strains containing the indicated plasmids were grown overnight at 20°C in synthetic liquid medium lacking uracil. Serial 10-fold dilutions were spotted onto plates lacking uracil that were incubated at the indicated temperatures for 3–5 days.
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The function of Cdc13 in telomere protection is mediated by its direct interactions with Stn1 and Ten1, leading to formation of the CST complex (reviewed in [38]). In addition to the capping function, the CST complex is implicated in repression of telomerase action [2,21,23,24]. This CST-dependent negative regulation of telomerase can be separated from CST capping function, as yeast cells either carrying the *cdc13-5* allele or lacking the Stn1 C-terminus (residues 282–494) (*stn1*Δ*C*) display extensive telomere elongation but no or minimal growth defects [24–26]. We evaluated the specificity of the genetic interaction between *rif1*Δ and *cdc13-1* by analysing the consequences of deleting *RIF1* and *RIF2* in *cdc13-5* or *stn1*Δ*C* cells. Deletion of *RIF1* turned out to reduce cell viability of *cdc13-5* mutant cells at any temperatures, while deletion of *RIF2* did not (Figure 2A). Furthermore, meiotic tetrad dissection of *stn1*Δ*C/STN1 rif1*Δ*/RIF1* diploid cells did not allow the recovery of viable *stn1*Δ*C rif1*Δ double mutant spores (Figure 2B), indicating that *rif1*Δ and *stn1*Δ*C* were synthetic lethal. By contrast, viable *rif2*Δ *stn1*Δ*C* spores were found with the expected frequency after tetrad dissection of *stn1*Δ*C/STN1 rif2*Δ*/RIF2* diploid cells (Figure 2C). The observed synthetic phenotypes suggest that both *stn1*Δ*C* and *cdc13-5* cells have capping deficiencies and that the lack of Rif1 enhances their protection defects. Consistent with this hypothesis, *cdc13-5* and *stn1*Δ*C* mutants were shown to accumulate telomeric ssDNA, although the amount of this ssDNA was not enough to invoke a DNA damage response [24,25]. We conclude that Rif1, but not Rif2, is required to support cell viability when a partial inactivation of CST capping function occurs.

A Cdc13 specific function that is not shared by the other subunits of the CST complex is its requirement for recruitment/activation of telomerase at chromosome ends [2–6]. Cdc13-mediated telomerase recruitment is disrupted by the *cdc13-2* mutation, which leads to progressive telomere shortening and senescence phenotype [4]. We therefore asked whether *RIF1* deletion influences viability and/or senescence progression of *cdc13-2* cells. Viable *cdc13-2 rif1*Δ spores were recovered after tetrad dissection of *cdc13-2/CDC13 rif1*Δ*/RIF1* diploid cells (data not shown), indicating that the lack of Rif1 does not affect the overall viability of *cdc13-2* cells. When spores from the dissection plate were streaked on YEPD plates for 4 successive times, the decline in growth of *cdc13-2* and *cdc13-2 rif1*Δ spores occurred with similar kinetics (Figure 2D), indicating that *RIF1* deletion did not accelerate the senescence phenotype of *cdc13-2* cells specifically defective in telomerase recruitment. Taken together, these genetic interactions indicate that Rif1, but not Rif2, has a role in assisting the essential function of the CST complex in telomere protection.

The CST complex functionally and physically interacts with the polα-primase complex [7,21,22,25], which is essential for telomeric C-strand synthesis during telomere elongation. Thus, we analyzed the genetic interactions between *rif1*Δ and temperature

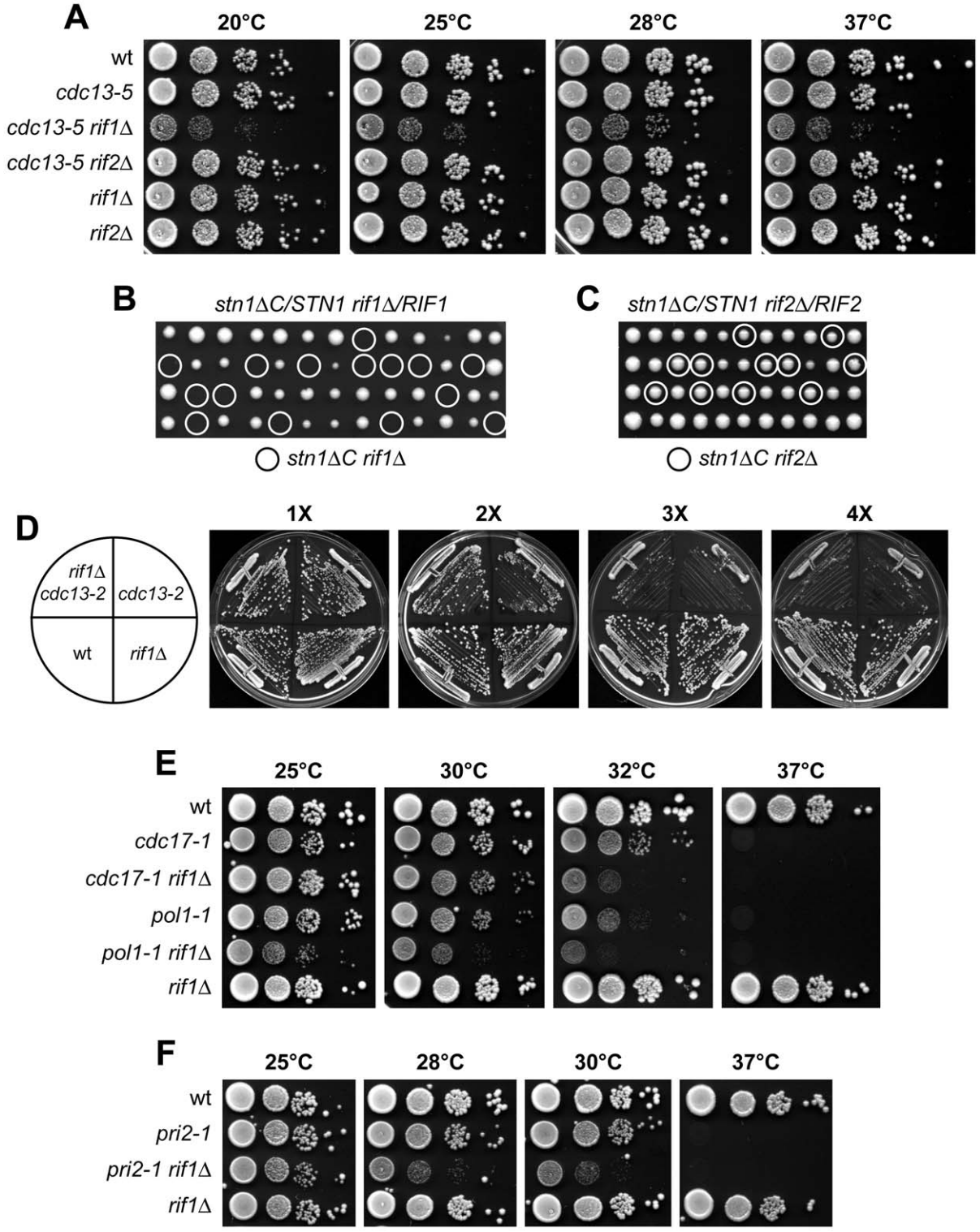

Figure 2. *RIF1* deletion affects viability of both *cdc13-5* and *stn1ΔC* cells. (A) Strains with the indicated genotypes were grown overnight in YEPD at 25°C. Serial 10-fold dilutions were spotted onto YEPD plates and incubated at the indicated temperatures for 2–4 days. (B–C) Viability of spores derived from diploids heterozygous for the indicated mutations. Spores with the indicated double mutant genotypes are circled. (D) Meiotic tetrads from a *CDC13/cdc13-2 RIF1/rif1Δ* diploid strain were dissected on YEPD plates. After ~25 generations on the dissection plate, spore clones from 20 tetrads were subjected to genotyping and concomitantly to four successive streak-outs (1X to 4X), corresponding to ~25, ~50, ~75 and ~100 generations of growth, respectively. All tetratype tetrads behaved as the one shown in this panel. (E–F) Cell cultures were grown overnight in YEPD at 25°C. Serial 10-fold dilutions were spotted onto YEPD plates that were incubated at the indicated temperatures for 2–3 days.
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sensitive alleles affecting DNA primase (*pri2-1)* [39] or polα (*cdc17-1* and *pol1-1*) [40,41]. Both *cdc17-1 rif1*Δ and *pol1-1 rif1*Δ cells were viable, but their temperature-sensitivity was greatly enhanced compared to *cdc17-1* and *pol1-1* single mutants (Figure 2E). Similarly, the maximal permissive temperature of the *pri2-1 rif1*Δ double mutant was reduced relative to that of *pri2-1* single mutant cells (Figure 2F). Moreover both *pol1-1 rif1*Δ and *pri2-1 rif1*Δ cells showed growth defects even at the permissive temperature of 25°C (Figure 2E and 2F). Thus, Rif1, like CST, functionally interacts with the polα-primase complex.

The lack of Rif1 enhances the DNA damage checkpoint response in *cdc13* mutant cells

The synthetic effects of combining *rif1*Δ with *cdc13* and *stn1* mutations suggest that Rif1 might normally assist the Cdc13 and Stn1 proteins in carrying out their essential telomere protection functions. It is known that *cdc13-1* cells undergo checkpoint-dependent metaphase arrest when incubated at the restrictive temperature [14]. Failure to turn on the checkpoint allows *cdc13-1* cells to form colonies at 28°C [42,43], indicating that checkpoint activation can partially account for the loss of viability of *cdc13-1* cells. We then asked whether the enhanced temperature sensitivity of *cdc13-1 rif1*Δ cells compared to *cdc13-1* cells might be due to upregulation of the DNA damage checkpoint response. Deletion of the checkpoint gene *RAD9*, which partially suppressed the temperature sensitivity of *cdc13-1* mutant cells, slightly improved the ability of *cdc13-1 rif1*Δ cells to grow at 23–25°C (Figure 3A), indicating that the synthetic interaction between Rif1 and Cdc13 can be partially alleviated by checkpoint inactivation. Furthermore, when wild type, *rif1*Δ, *cdc13-1* and *cdc13-1 rif1*Δ cell cultures were arrested in G1 with α-factor at 20°C (permissive temperature) and then released from G1 arrest at 25°C (non-permissive temperature for *cdc13-1 rif1*Δ cells), they all replicated DNA and budded with similar kinetics after release (Figure 3B and 3C). However, most *cdc13-1 rif1*Δ cells then arrested in metaphase as large budded cells with a single nucleus, while wild type, *cdc13-1* and *rif1*Δ cells divided nuclei after 75–90 minutes (Figure 3D).

To assess whether the cell cycle arrest of *cdc13-1 rif1*Δ cells was due to DNA damage checkpoint activation, we examined the Rad53 checkpoint kinase, whose phosphorylation is necessary for checkpoint activation and can be detected as changes in Rad53 electrophoretic mobility. Rad53 was phosphorylated in *cdc13-1 rif1*Δ cells that were released from G1 arrest at 25°C, whereas no Rad53 phosphorylation was seen in any of the other similarly treated cell cultures (Figure 3E).

RIF1 deletion caused a checkpoint-mediated G2/M cell cycle arrest also in *cdc13-5* cells. In fact, exponentially growing *cdc13-5 rif1*Δ cell cultures at 25°C contained a higher percentage of large budded cells with a single nucleus than *rif1*Δ or *cdc13-5* cell cultures under the same conditions (Figure 3F). Furthermore, Rad53 phosphorylation was detected in these *cdc13-5 rif1*Δ cells, but not in the *rif1*Δ and *cdc13-5* cell cultures (Figure 3G). Thus, the lack of Rif1 results in DNA damage checkpoint activation in both *cdc13-1* and *cdc13-5* cells under conditions that do not activate the checkpoint when Rif1 is present.

The synthetic interaction between Rif1 and CST is independent of *rif1*Δ-induced telomere overelongation

The lack of Rif1 is known to cause telomere overelongation [29]. Thus, we examined telomere length in *cdc13-1 rif1*Δ double mutant cells. The length of duplex telomeric DNA was examined after transferring at 25°C cell cultures exponentially growing at 20°C, followed by Southern blot analysis with a TG-rich probe of XhoI-digested genomic DNA prepared at different times after shift at 25°C (Figure 4A). As expected [29], *rif1Δ* mutant cells had longer telomeres than wild type and *cdc13-1* cells (Figure 4A). Telomeres in *cdc13-1 rif1*Δ double mutant cells either at 20°C or after incubation at 25°C were longer than those of wild type and *cdc13-1* cells, but undistinguishable from those of *rif1Δ* cells (Figure 4A). Not only *RIF1* deletion, but also the *cdc13-5* mutation is known to cause telomere overelongation [24] (Figure 4B). Interestingly, when telomere length was analyzed in *cdc13-5 rif1Δ* double mutant cells grown at 25°C, telomeres were longer in *cdc13-5 rif1*Δ double mutant cells than in *cdc13-5* and *rif1*Δ single mutants (Figure 4B), indicating that the *cdc13-5* mutation exacerbates the telomere overelongation defect caused by the lack of Rif1.

The finding that telomeres in *cdc13-1 rif1*Δ double mutant cells at 25°C were longer than those of *cdc13-1* cells, but undistinguishable from those of *cdc13-1 rif1Δ* cells grown at 20°C (Figure 4A) suggests that the growth defects of *cdc13-1 rif1Δ* cells at 25°C are not due to *rif1Δ*-induced telomere overelongation. Telomere lengthening in *rif1Δ* mutant cells is telomerase-dependent [44] and requires the action of the checkpoint kinase Tel1 that facilitates telomerase recruitment [45,46]. To provide additional evidences that loss of viability in *cdc13 rif1Δ* mutants occurs independently of *rif1Δ*-induced alterations in telomere length, we asked whether *RIF1* deletion was still deleterious in *cdc13-1*, *cdc13-5* and *stn1ΔC* cells in a context where telomeres cannot be elongated due to the lack of Tel1 [45]. We found that *TEL1* deletion did not alleviate the growth defects of *cdc13-1 rif1*Δ cells (Figure 5A). Rather, *cdc13-1 tel1Δ* and *cdc13-1 rif1Δ tel1Δ* cells showed an enhanced temperature sensitivity compared to *cdc13-1* and *cdc13-1 rif1Δ* cells, respectively, presumably due to the combined effects of loss of a telomere elongation mechanism and inability to protect telomeres from shortening activities. Furthermore, the growth defects of *cdc13-5 rif1*Δ double mutant cells were similar to those of *cdc13-5 rif1*Δ *tel1*Δ triple mutant cells (Figure 5B). Finally, viable *stn1ΔC rif1*Δ *tel1Δ* mutant spores could not be recovered after meiotic tetrad dissection of *stn1ΔC/STN1 rif1*Δ*/RIF1 tel1Δ/TEL1* diploid cells (data not shown), indicating that *stn1ΔC* and *rif1*Δ were synthetic lethal even in the absence of Tel1.

As telomere lengthening is dramatically increased when both Rif1 and Rif2 are absent [30], we also investigated whether the absence of Rif2 exacerbates *cdc13-1 rif1*Δ growth defects. As shown in Figure 5C, *cdc13-1 rif1*Δ *rif2*Δ cells formed colonies at the maximum temperature of 20°C and behaved similarly to *cdc13-1 rif1*Δ cells. We therefore conclude that the synthetic interaction between *rif1Δ* and *cdc13* alleles is not due to *rif1*Δ-induced alterations in telomere length, but it is a direct consequence of Rif1 loss.

The lack of Rif1 causes generation of telomeric ssDNA in *cdc13* cells

It is known that *cdc13-1* cells at 37°C accumulate telomeric ssDNA that triggers checkpoint-mediated cell cycle arrest [14]. Thus, we investigated whether *cdc13-1 rif1*Δ and *cdc13-5 rif1*Δ cells contained aberrant levels of single-stranded TG sequences at their telomeres that could be responsible for loss of viability in *cdc13-1 rif1*Δ and *cdc13-5 rif1*Δ cells at 25°C. The integrity of chromosome ends was analyzed by an in-gel hybridization procedure [9], probing for the presence of single-stranded TG sequences. Both *cdc13-1* and *rif1*Δ single mutants either grown at 20°C (Figure 6A, lanes 2 and 4) or incubated at 25°C for 3 hours (Figure 6A, lanes 6 and 8) showed only a very slight increase in single-stranded TG sequences compared to wild type (Figure 6A, lanes 1 and 5). By contrast, *cdc13-1 rif1*Δ double mutant cells contained higher

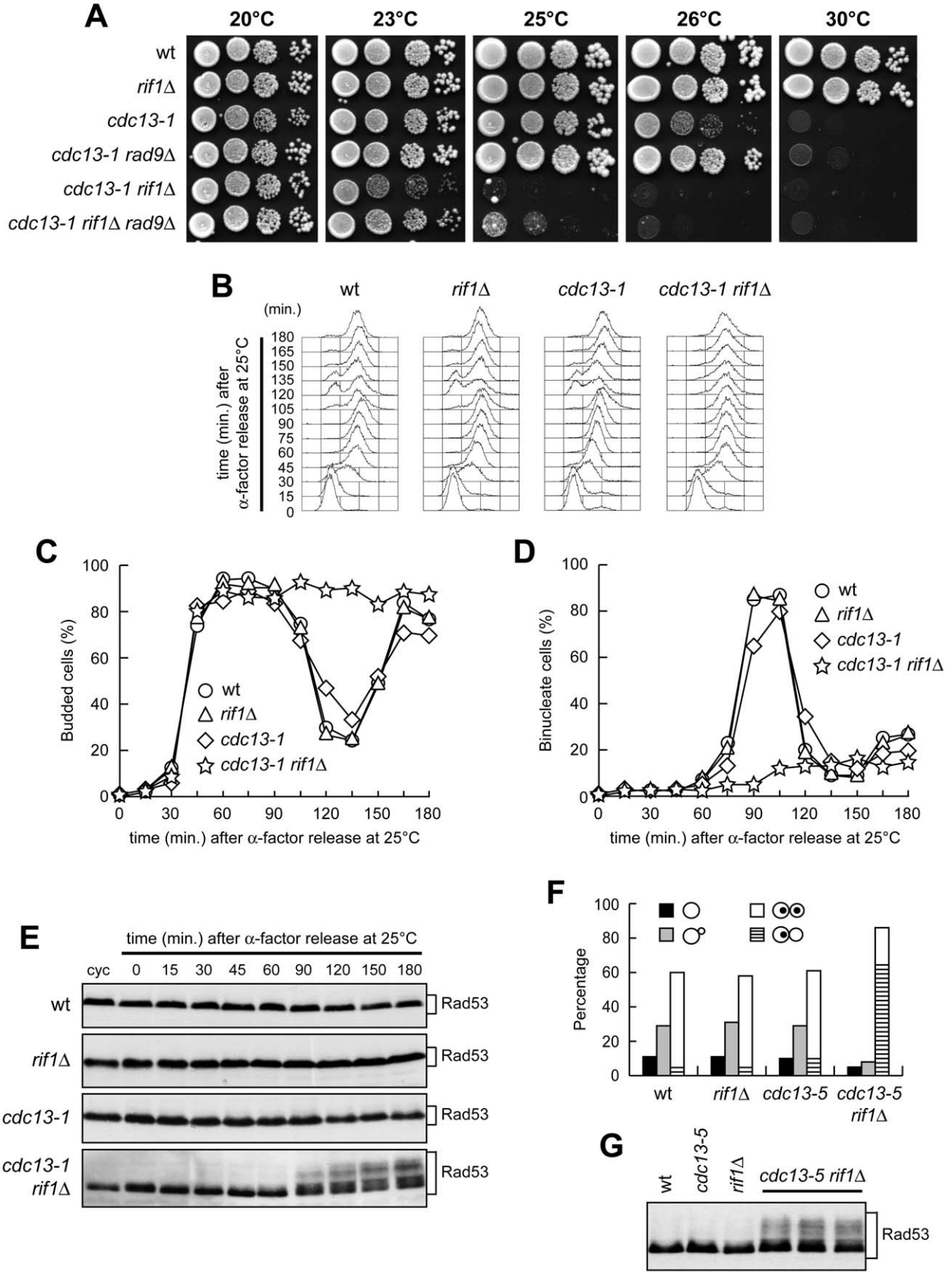

Figure 3. Metaphase arrest and checkpoint activation in *cdc13 rif1*Δ cells. (A) Strains with the indicated genotypes were grown overnight in YEPD at 20°C. Serial 10-fold dilutions were spotted onto YEPD plates and incubated at the indicated temperatures for 2–4 days. (B–E) Cell cultures exponentially growing at 20°C in YEPD were arrested in G1 with α-factor and then released from G1 arrest in YEPD at 25°C (time zero). Samples were

taken at the indicated times after release from α-factor for FACS analysis of DNA content (B), for determining the kinetics of bud emergence (C) and nuclear division (D), and for western blot analysis of Rad53 using anti-Rad53 antibodies (E). cyc, cycling cells. (F–G) Cell cultures were grown exponentially in YEPD at 25°C. (F) The frequency of cells with no, small or large buds was determined by analyzing a total of 200 cells for each strain. The percentage of large budded cells with one or two nuclei was evaluated by fluorescence microscopy. (G) Rad53 in cell cultures exponentially growing at 25°C was visualized as in panel E. Three independent *cdc13-5 rif1*Δ strains were analyzed.
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amounts of telomeric ssDNA than *cdc13-1* and *rif1*Δ cells already at 20°C (Figure 6A, lane 3) and the amount of this ssDNA increased dramatically when *cdc13-1 rif1*Δ cells were incubated at 25°C for 3 hours (Figure 6A, lane 7). A similar telomere deprotection defect was observed also for *cdc13-5 rif1*Δ cells grown at 25°C (Figure 6A, lane 11), which displayed an increased amount of telomeric ssDNA compared to similarly treated wild type and *cdc13-5* cells (Figure 6A, lanes 9 and 10).

Because the length of single-stranded G overhangs increases during S phase [8], the strong telomeric ssDNA signals observed in *cdc13-1 rif1*Δ cell cultures at 25°C (Figure 6A) might be due to an enrichment of S/G2 cells. We ruled out this possibility by monitoring the levels of single-stranded TG sequences in *cdc13-1 rif1*Δ cell cultures that were arrested in G2 with nocodazole at 20°C and then transferred to 25°C in the presence of nocodazole for 3 hours (Figure 6B). Similarly to what we observed in exponentially growing cell cultures, G2-arrested *cdc13-1 rif1*Δ cells at 20°C displayed increased amounts of ssDNA compared to each single mutant under the same conditions, and incubation at 25°C led to further increase of this ssDNA (Figure 6B). Taken together, these findings indicate that the lack of Rif1 causes a severe defect in telomere protection when Cdc13 activity is partially compromised.

The lack of Exo1 counteracts DNA damage checkpoint activation and telomeric ssDNA accumulation in *cdc13 rif1*Δ cells

If telomeric ssDNA accumulation contributes to checkpoint activation in *cdc13-1 rif1*Δ and *cdc13-5 rif1*Δ cells, then mutations reducing ssDNA generation should alleviate the arrest and relieve the lethality caused by the lack of Rif1 in *cdc13-1* and *cdc13-5* background. Because the Exo1 nuclease contributes to generate telomeric ssDNA in *cdc13-1* cells [47], we examined the effect of deleting *EXO1* in *cdc13 rif1*Δ cells. When G2-arrested cell cultures at 20°C were transferred to 25°C for 3 hours, *cdc13-1 rif1*Δ *exo1*Δ triple mutant cells contained significantly lower amounts of telomeric ssDNA than *cdc13-1 rif1*Δ cells (Figure 6B). A similar behaviour of the triple mutant was detectable even when G2-arrested cultures where kept at 20°C, although the quantity of telomeric ssDNA accumulated by *cdc13-1 rif1*Δ cells at this

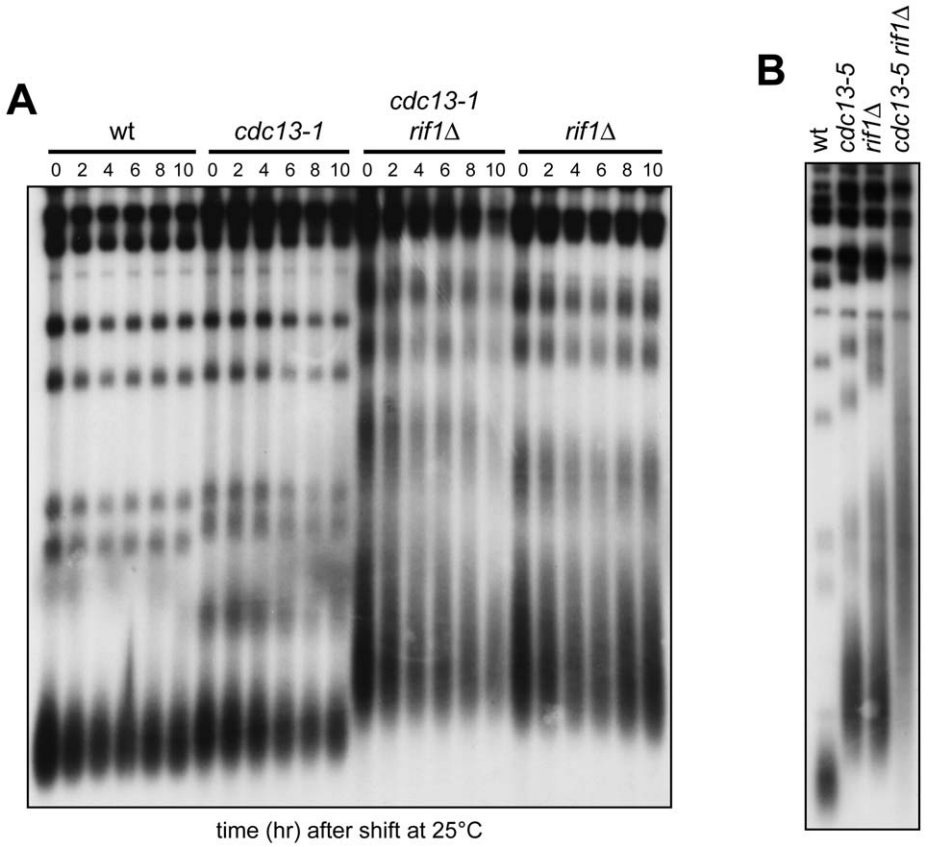

Figure 4. Native telomere length in *cdc13 rif1*Δ cells. (A) Cells with the indicated genotypes exponentially growing in YEPD at 20°C were shifted at 25°C at time 0. Cells were collected at the indicated time points after shift and XhoI-cut genomic DNA was subjected to Southern blot analysis using a radiolabeled poly(GT) telomere-specific probe. (B) XhoI-cut genomic DNA extracted from cells with the indicated genotypes exponentially growing at 25°C was subjected to Southern blot analysis as in panel A.
doi:10.1371/journal.pgen.1002024.g004

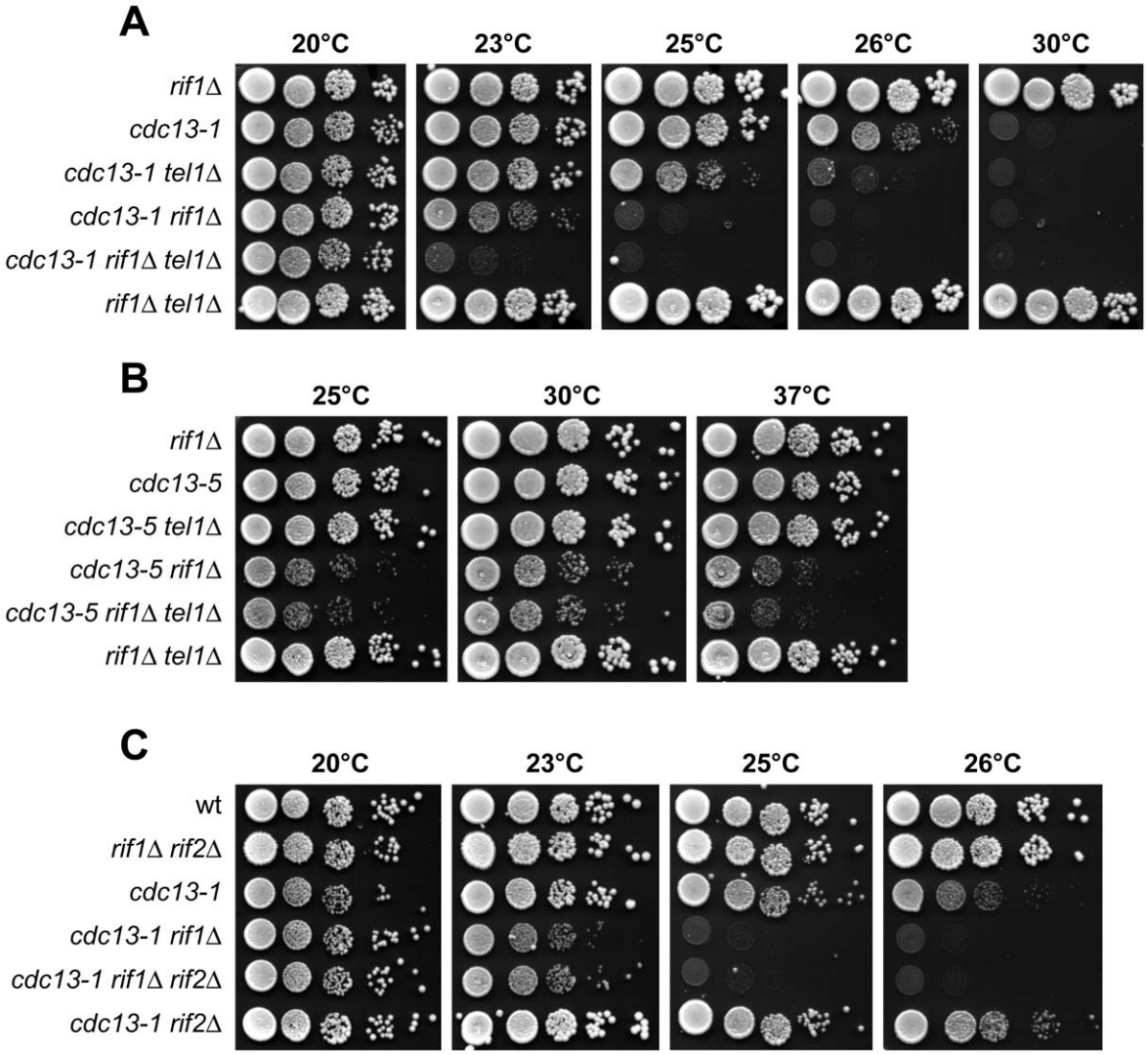

Figure 5. Effect of deleting *TEL1* or *RIF2* on growth of *cdc13 rif1Δ* cells. Cells with the indicated genotypes were grown overnight in YEPD at 20°C (A and C) or 25°C (B). Serial 10-fold dilutions were spotted onto YEPD plates that were then incubated at the indicated temperatures for 2–4 days.
doi:10.1371/journal.pgen.1002024.g005

temperature was lower than at 25°C (Figure 6B). Furthermore, *EXO1* deletion partially suppressed both the temperature-sensitivity of *cdc13-1 rif1*Δ cells (Figure 7A) and the loss of viability of *cdc13-5 rif1*Δ cells (Figure 7B), further supporting the hypothesis that reduced viability in these strains was due to defects in telomere protection.

Exo1-mediated suppression of the *cdc13 rif1*Δ growth defects correlated with alleviation of checkpoint-mediated cell cycle arrest. In fact, when cell cultures exponentially growing at 20°C were incubated at 25°C for 3 hours, the amount of both metaphase-arrested cells and Rad53 phosphorylation was reproducibly lower in *cdc13-1 rif1*Δ *exo1*Δ cells than in *cdc13-1 rif1*Δ cells (Figure 7C and 7D). Similar results were obtained also with *cdc13-5 rif1*Δ *exo1*Δ cells growing at 25°C, which accumulated less metaphase-arrested cells and phosphorylated Rad53 than similarly treated *cdc13-5 rif1*Δ cells (Figure 7E and 7F). Thus, both cell lethality and checkpoint-mediated cell cycle arrest in *cdc13 rif1*Δ cells appear to be caused, at least partially, by Exo1-dependent telomere DNA degradation.

The lack of Rif1 does not enhance the checkpoint response to CST-independent capping deficiencies or to an irreparable DSB

The lack of Rif1 might increase the lethality of cells with reduced CST activity just because it causes a telomere deprotection defect that exacerbates the inherent telomere capping defects of *cdc13* or *stn1* mutants. If this hypothesis were correct, *RIF1* deletion should affect viability also of other non-CST mutants defective in end protection. Alternatively, Rif1-CST functional interaction might be specific, thus reflecting a functional connection between Rif1 and CST. To distinguish between these two possibilities, we analyzed the effects of deleting *RIF1* in Yku70 lacking cells, which display Exo1-dependent accumulation of telomeric ssDNA, as well as checkpoint-mediated cell cycle arrest at elevated temperatures (37°C) [47,51]. Loss of Yku in *est2Δ* cells, which lack the telomerase catalytic subunit, leads to synthetic lethality, presumably due to the combined effects of telomere shortening and capping defects [48–50,52]. As expected [53], *yku70*Δ cells were viable at 25°C and 30°C, but they were unable

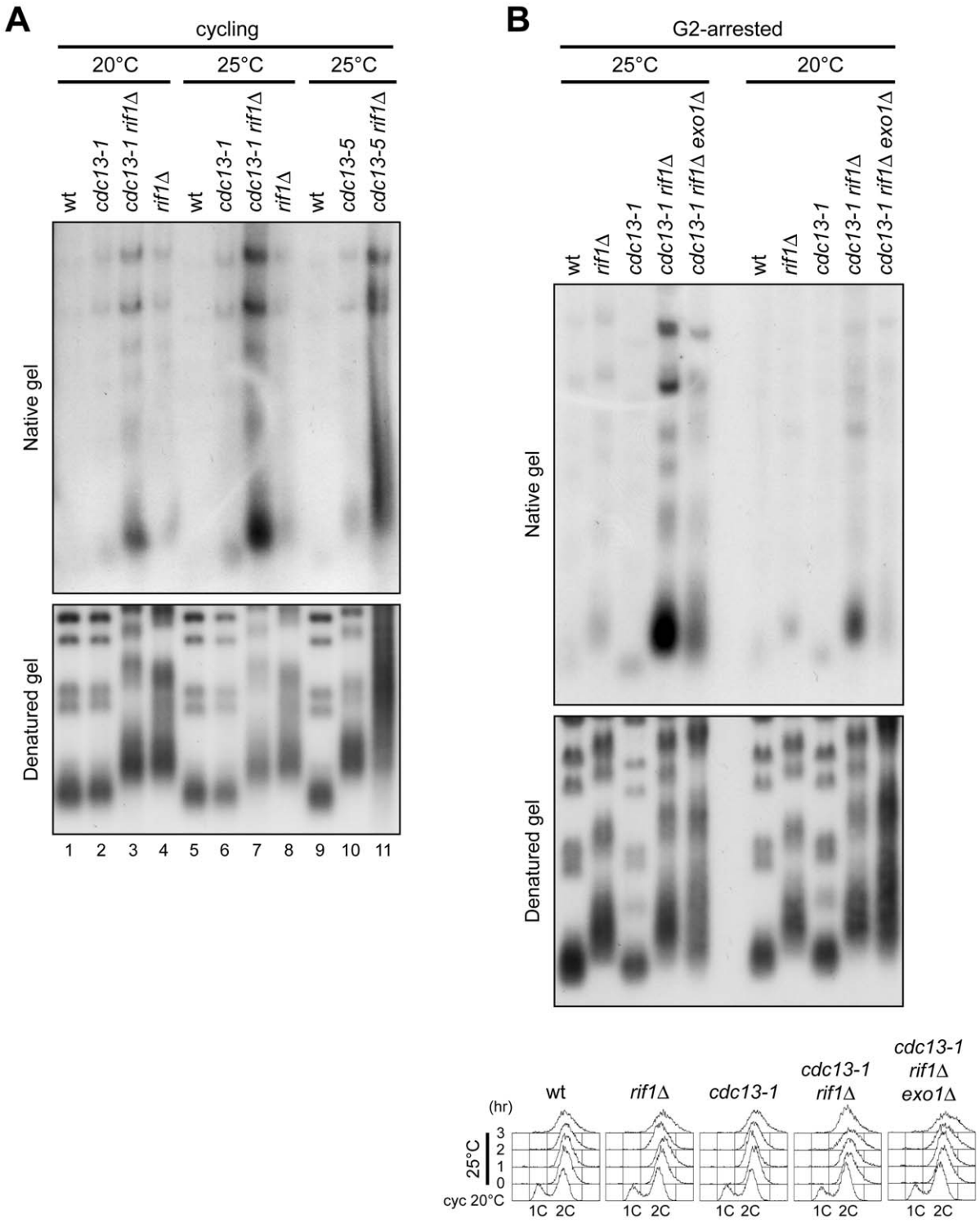

Figure 6. *RIF1* deletion enhances ssDNA formation at native telomeres of *cdc13* cells. (A) Wild type and otherwise isogenic *cdc13-1*, *cdc13-1 rif1*Δ and *rif1*Δ cell cultures exponentially growing at 20°C (lanes 1–4) were incubated at 25°C for 3 hours (lanes 5–8). Wild type and otherwise isogenic *cdc13-5* and *cdc13-5 rif1*Δ cell cultures were grown exponentially at 25°C (lanes 9–11). Genomic DNA was digested with XhoI and single-stranded telomere overhangs were visualized by in-gel hybridization (native gel) using an end-labelled C-rich oligonucleotide. The same DNA samples were separated on a 0.8% agarose gel, denatured and hybridized with the end-labeled C-rich oligonucleotide for loading and telomere length control (denatured gel). (B) Cell cultures were arrested in G2 with nocodazole at 20°C (right) and then transferred at 25°C in the presence of nocodazole for 3 hours (left), followed by analysis of single-stranded telomere overhangs (top) as in panel A, and FACS analysis of DNA content (bottom).

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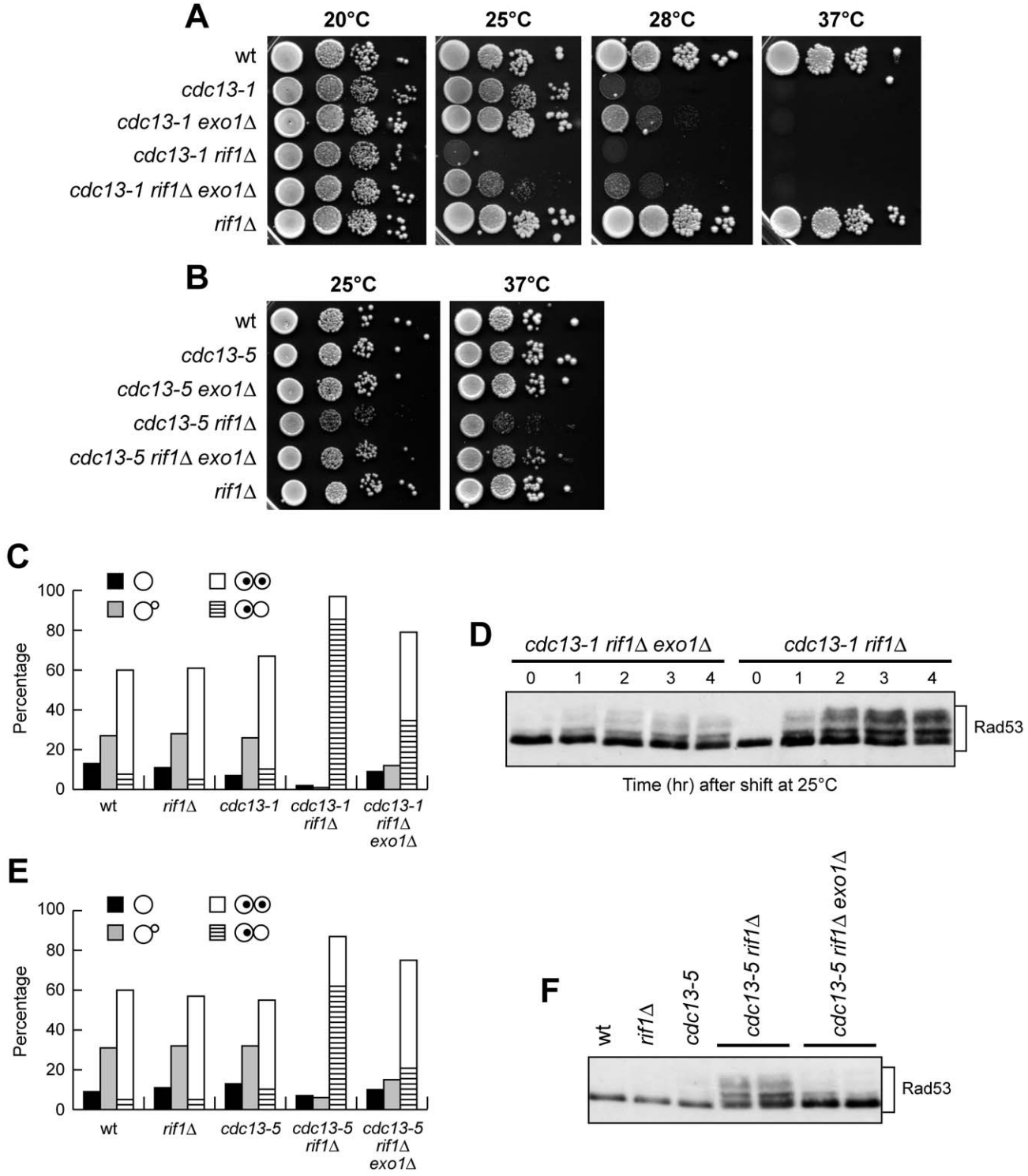

Figure 7. *EXO1* deletion partially suppresses cell lethality and checkpoint activation in *cdc13 rif1*Δ cells. (A–B) Serial 10-fold dilutions of cell cultures grown overnight in YEPD at 20°C (A) and 25°C (B) were spotted onto YEPD plates and incubated at the indicated temperatures for 2–4 days. (C–D) Cell cultures exponentially growing in YEPD at 20°C were shifted to 25°C. (C) The frequencies of cells with no, small or large buds and of large budded cells with one or two nuclei were determined after 3 hours at 25°C as in Figure 3F, by analyzing a total of 200 cells for each strain. (D) Western blot analysis with anti-Rad53 antibodies of total protein extracts prepared at the indicated times. This experiment was repeated three times with similar results. (E–F) Cultures of cells with the indicated genotypes, exponentially growing at 25°C in YEPD, were analyzed as in panels C (E) and D (F), respectively. Two independent *cdc13-5 rif1*Δ and *cdc13-5 rif1*Δ *exo1*Δ strains were analyzed for Rad53 phosphorylation.
doi:10.1371/journal.pgen.1002024.g007

to form colonies at 37°C (Figure 8A). Similarly, *yku70Δ rif1Δ* double mutant cells grew well at 25°C and 30°C (Figure 8A) and did not show Rad53 phosphorylation when grown at 25°C (Figure 8B, time 0). Furthermore, similar amounts of phosphorylated Rad53 were detected in both *yku70Δ* and *yku70Δ rif1Δ* cell cultures that were kept at 37°C for 4 hours (Figure 8B), indicating that loss of Rif1 does not enhance the telomere protection defects already present in *yku70Δ* cells. Consistent with a previous

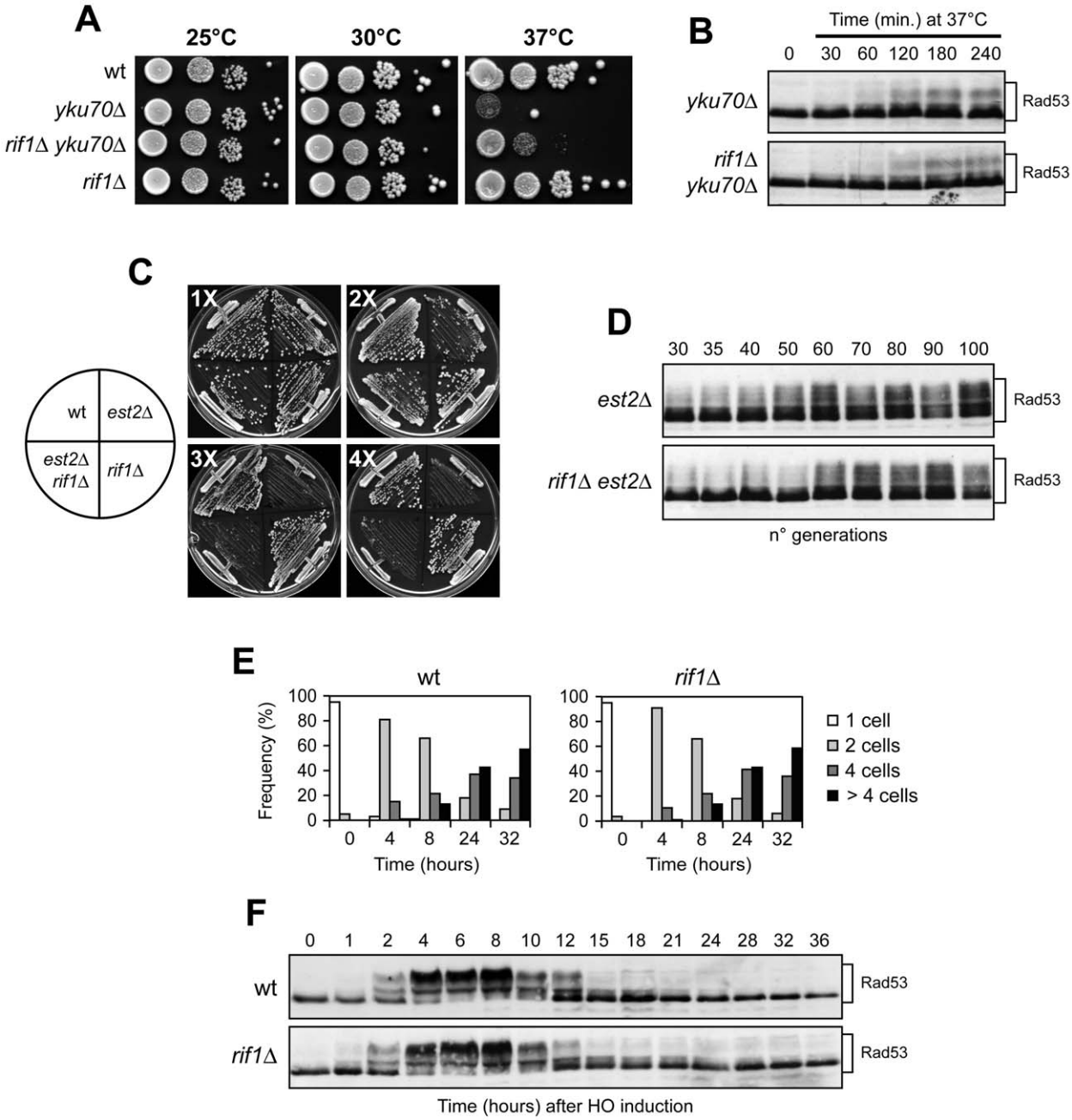

Figure 8. *RIF1* deletion does not influence the checkpoint response to CST-independent telomere capping deficiencies or to an irreparable DSB. (A) Cell cultures with the indicated genotypes were grown overnight in YEPD at 25°C. Serial 10-fold dilutions were spotted onto YEPD plates and incubated at the indicated temperatures for 2–3 days. (B) Cell cultures exponentially growing in YEPD at 25°C were shifted to 37°C at time 0. Rad53 was visualized at the indicated time points as in Figure 3E. (C) Meiotic tetrads from a *EST2/est2Δ RIF1/rif1Δ* diploid strain were dissected on YEPD plates. After ~25 generations, spore clones from 20 tetrads were subjected to genotyping and to four successive streak-outs (1X to 4X), corresponding to ~25, ~50, ~75 and ~100 generations of growth, respectively. All tetratype tetrads behaved as the one shown in this panel. (D) After ~25 generations of growth on the dissection plates, *est2Δ* and *est2Δ rif1Δ* spore clones were propagated in liquid YEPD medium. At the indicated generations, protein extracts were subjected to western blot analysis with anti-Rad53 antibodies. (E–F) Checkpoint response to an irreparable DSB. (E) YEP+raf G1-arrested cell cultures of wild type JKM139 and its isogenic *rif1Δ* derivative strain were spotted on galactose-containing plates that were incubated at 28°C (time zero). At the indicated time points, 200 cells for each strain were analyzed to determine the frequency of single cells and of cells forming microcolonies of 2, 4 or more than 4 cells. (F) Galactose was added at time zero to cell cultures of the strains in panel E exponentially growing in YEP+raf. Protein extracts were subjected to western blot analysis with anti-Rad53 antibodies.
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observation [54], *RIF1* deletion partially suppressed the temperature sensitivity (Figure 8A) and the telomere length defect (data not shown) caused by the lack of Yku70, suggesting that the elongated state of the telomeres could be the reason why *yku70*Δ *rif1*Δ cells can proliferate at 37°C.

Checkpoint activation can also be induced during telomere erosion caused by insufficient telomerase activity [55,56]. Thus, we asked whether *RIF1* deletion accelerated senescence progression and/or upregulated checkpoint activation in cells lacking the telomerase catalytic subunit Est2. Meiotic tetrads were dissected from a diploid strain heterozygous for the *est2*Δ and *rif1*Δ alleles, which are recessive and therefore do not affect telomere length in the diploid. After 2 days of incubation at 25°C (approximately 25 generations), spore clones from the dissection plate were both streaked for 4 successive times (Figure 8C) and propagated in YEPD liquid medium to prepare protein extracts for Rad53 phosphorylation analysis at different time points (Figure 8D). Similar to what was previously observed [44], *RIF1* deletion did not accelerate senescence progression in *est2*Δ cells, as *est2*Δ *rif1*Δ clones showed a decline in growth similar to that of *est2*Δ clones (Figure 8C). Furthermore, *est2*Δ and *est2*Δ *rif1*Δ cell cultures showed similar patterns of Rad53 phosphorylation with increasing number of generations (Figure 8D). Thus, the lack of Rif1 does not enhance either DNA damage checkpoint activation or senescence progression during telomere erosion caused by the lack of telomerase.

Finally, because the telomerase machinery is known to be recruited to an unrepaired DSB [57], we ruled out the possibility of a general role for Rif1 in inhibiting checkpoint activation by examining activation/deactivation of the checkpoint induced by an unrepaired DSB. To this end, we used JKM139 derivative strains, where a single DSB can be generated at the *MAT* locus by expressing the site-specific HO endonuclease gene from a galactose-inducible promoter [58]. This DSB cannot be repaired by homologous recombination, because the homologous donor sequences *HML* or *HMR* are deleted. As shown in Figure 8E, when G1-arrested cell cultures were spotted on galactose containing plates, both wild type and *rif1*Δ JKM139 derivative cells overrode the checkpoint-mediated cell cycle arrest within 24–32 hours, producing microcolonies with 4 or more cells. Moreover, when galactose was added to exponentially growing cell cultures of the same strains, Rad53 phosphorylation became detectable as electrophoretic mobility shift in both wild type and *rif1*Δ cell cultures about 2 hours after HO induction, and it decreased in both cell cultures after 12–15 hours (Figure 8F), when most cells resumed cell cycle progression (data not shown). Thus, Rif1 does not affect the checkpoint response to an irreparable DSB. Altogether these data indicate that Rif1 supports specifically CST functions in telomere protection.

Discussion

Both shelterin and CST complexes are present in a wide range of unicellular and multicellular organisms, where they protect the integrity of chromosomes ends (reviewed in [38]). Thus, the understanding of their structural and functional connections is an important issue in telomere regulation. We have approached this topic by analysing the consequences of disabling the shelterin-like *S. cerevisiae* proteins Rif1 or Rif2 in different hypomorphic mutants defective in CST components. We provide evidence that Rif1, but not Rif2, is essential for cell viability when the CST complex is partially compromised. In fact, *RIF1* deletion exacerbates the temperature sensitivity of *cdc13-1* mutant cells that are primarily defective in Cdc13 telomere capping functions. Furthermore, cells carrying the *cdc13-5* or the *stn1*Δ*C* mutation, neither of which causes per se DNA damage checkpoint activation and growth defects [24,26], grow very poorly or are unable to form colonies, respectively, when combined with the *rif1*Δ allele. By contrast, *RIF1* deletion does not affect either viability or senescence progression of *cdc13-2* cells, which are specifically defective in telomerase recruitment. This Cdc13 function is not shared by the other CST subunits, suggesting that Rif1 is specifically required to support the essential capping functions of the CST complex.

Cell lethality caused by the absence of Rif1 in both *cdc13-1* and *cdc13-5* cells appears to be due to severe telomere integrity defects. In fact, telomeres in both *cdc13-1 rif1*Δ and *cdc13-5 rif1*Δ double mutant cells display an excess of ssDNA that leads to DNA damage checkpoint activation. Deleting the nuclease *EXO1* gene partially restores viability of *cdc13-1 rif1*Δ and *cdc13-5 rif1*Δ cells and reduces the level of telomeric ssDNA in *cdc13-1 rif1*Δ cells, indicating that cell lethality in *cdc13 rif1*Δ cells is partially due to Exo1-dependent telomere DNA degradation and subsequent activation of the DNA damage checkpoint.

Although Rif1 and Rif2 interact both with the C-terminus of Rap1 and with each other [29,30], our finding that only Rif1 is required for cell viability when Cdc13 or Stn1 capping activities are reduced indicates that Rif1 has a unique role in supporting CST capping function that is not shared by Rif2. Earlier studies are consistent with the idea that Rif1 and Rif2 regulate telomere metabolism by different mechanisms [30,31,35]. Furthermore, while the content of Rif2 is lower at shortened than at wild type telomeres, the level of Rif1 is similar at both, suggesting that these two proteins are distributed differently along a telomere [59]. Finally, inhibition of telomeric fusions requires Rif2, but not Rif1 [32].

Noteworthy, although *RIF1* deletion is known to cause telomere overelongation [29], the synthetic interaction between Rif1 and CST occurs independently of *rif1*Δ-induced alterations in telomere length. In fact, the lack of Tel1, which counteracts *rif1Δ*-induced telomere overelongation [45], does not alleviate the growth defects of *cdc13 rif1Δ* cells. Furthermore, deletion of *RIF2*, which enhances telomere elongation induced by the lack of Rif1 [30], does not exacerbate the synthetic phenotypes of *cdc13 rif1Δ* double mutant cells. Thus, loss of viability in *cdc13 rif1*Δ cells is not due to telomere overelongation caused by *RIF1* deletion, but it is a direct consequence of Rif1 loss.

By analyzing the effects of combining *RIF1* deletion with mutations that cause telomere deprotection without affecting CST functions, we found that the functional interaction between Rif1 and the CST complex is highly specific. In fact, the lack of Rif1 does not enhance the DNA damage checkpoint response in telomerase lacking cells, which are known to experience gradual telomere erosion leading to activation of the DNA damage checkpoint [55,56]. Furthermore, *RIF1* deletion does not upregulate DNA damage checkpoint activation in *yku70*Δ cells, which display Exo1-dependent accumulation of ssDNA and checkpoint-mediated cell cycle arrest at 37°C [47–51]. This is consistent with previous observations that comparable signals for G strand overhangs can be detected on telomeres derived from *yku70*Δ and *yku70*Δ *rif1*Δ cells [54], indicating that *RIF1* deletion does not exacerbate the end protection defect due to the absence of Yku. By contrast, the lack of Rif1 partially suppresses both temperature-sensitivity and telomere shortening in *yku70*Δ cells (Figure 8A) [54], possibly because the restored telomere length helps to compensate for *yku70*Δ capping defects. Notably, although *RIF1* deletion leads to telomere overelongation in *cdc13-1* and *cdc13-5* mutants, this elongated telomere state does not help to increase viability in *cdc13-1 rif1*Δ and *cdc13-5 rif1*Δ cells.

The simplest interpretation of the specific genetic interactions we found between Rif1 and CST is that a functional connection exists

between Rif1 and the CST complex, such that Rif1 plays a previously unanticipated role in assisting the CST complex in carrying out its essential telomere protection function. Indeed, this functional interaction is unexpected in light of Rif1 and CST localization along a telomere. In fact, while CST is present at the very ends of chromosomes, Rif1 is thought to be distributed centromere proximal on the duplex telomeric DNA [59]. However, as yeast telomeres have been proposed to fold back onto the subtelomeric regions to form a ~3-kb region of core heterochromatin [60,61], this higher-order structure could place Rif1 and CST in close proximity, thus explaining their functional interaction.

The function of Rif1 in sustaining CST activity cannot be simply attributable to the Rif1-mediated suppression of ssDNA formation at telomeres, as *rif1*Δ cells show only a very slight increase in ssDNA at both native (Figure 6) and HO-induced telomeres [33] compared to wild type. Furthermore, although deletion of Rif2 leads to increased amounts of telomeric ssDNA [33], *cdc13-1 rif2*Δ, *cdc13-5 rif2*Δ and *stn1*Δ*C rif2*Δ double mutants are viable and do not display growth defects. Finally, other mutants defective in telomere capping or telomere elongation (*yku70*Δ and *est2*Δ) are perfectly viable in the absence of Rif1.

One possibility is that Rif1 physically interacts, directly or indirectly, with the CST complex. Indeed, human Stn1 was found to copurify with the shelterin subunit TPP1 [62], suggesting the existence of CST-shelterin complexes in mammals. Unfortunately, we were so far unable to coimmunoprecipitate Rif1 with Cdc13 or Stn1, and further analyses will be required to determine whether Rif1 and the CST complex undergo stable or transient association during the cell cycle.

Indeed, not only 5′-3′ resection, but also incomplete synthesis of Okazaki fragments is expected to increase the size of the G tail during telomere replication. The yeast CST complex genetically and physically interacts with the polα-primase complex [7,22,25] and the human CST-like complex increases polα-primase processivity [63,64]. Furthermore, the lack of CST function in G1 and throughout most of S phase does not lead to an increase of telomeric ssDNA [13], suggesting that the essential function of CST is restricted to telomere replication in late S phase. Altogether, these observations suggest that CST may control overhang length not only by blocking the access of nucleases, but also by activating polα-primase-dependent C-strand synthesis that can compensate G tail lengthening activities. Based on the finding that Rif1 regulates telomerase action and functionally interacts with the polα-primase complex (Figure 2), it is tempting to propose that Rif1 favours CST ability to replenish the exposed ssDNA at telomeres through activation/recruitment of polα-primase, thus coupling telomerase-dependent elongation to the conventional DNA replication process.

The recent discoveries that human TPP1 interacts physically with Stn1 [62] and that CST-like complexes exist also in *S. pombe*, plants and mammals [65–68] raise the question of whether functional connections between the two capping complexes exist also in other organisms. As telomere protection is critical for preserving genetic stability and counteracting cancer development, to address this question will be an important future challenge.

Materials and Methods

Strains and plasmids

Strain genotypes are listed in supplementary Table S1. Unless otherwise stated, the yeast strains used during this study were derivatives of W303 (*ho MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3 can1-100*). All gene disruptions were carried out by PCR-based methods. The *cdc13-1* mutant was kindly provided by D. Lydall (University of Newcastle, UK). The *cdc13-2* mutant was kindly provided by V. Lundblad (Salk Institute, La Jolla, USA). The *stn1*Δ*C* and *cdc13-5* alleles carried a stop codon following amino acids 282 and 694 respectively [24,25], and were generated by PCR-based methods. Wild type and *cdc13-1* strains carrying either the 2 μ vector or 2 μ *RIF1* plasmid were constructed by transforming wild type and *cdc13-1* strains with plasmids YEplac195 (2 μ *URA3*) and pML435 (2 μ *RIF1 URA3*), respectively. The strains used for monitoring checkpoint activation in response to an irreparable DSB were derivatives of strain JKM139 (*MATa ho hml*Δ *hmr*Δ *ade1 lys5 leu2-3,112 trp1::hisG ura3-52 ade3::GAL-HO*), kindly provided by J. Haber (Brandeis University, Waltham, MA, USA) [58]. To induce *HO* expression in JKM139 and its derivative strains, cells were grown in raffinose-containing yeast extract peptone (YEP) and then transferred to raffinose- and galactose-containing YEP.

Cells were grown in YEP medium (1% yeast extract, 2% bactopeptone, 50 mg/l adenine) supplemented with 2% glucose (YEPD) or 2% raffinose (YEP+raf) or 2% raffinose and 2% galactose (YEP+raf+gal). Synthetic complete medium lacking uracil supplemented with 2% glucose was used to maintain the selective pressure for the 2 μ *URA3* plasmids.

Southern blot analysis of telomeres and in-gel hybridization

Genomic DNA was digested with *Xho*I. The resulting DNA fragments were separated by electrophoresis on 0.8% agarose gel and transferred to a GeneScreen nylon membrane (New England Nuclear, Boston), followed by hybridization with a ^{32}P-labelled poly(GT) probe and exposure to X-ray sensitive films. Standard hybridization conditions were used. Visualization of single-stranded overhangs at native telomeres was done by in-gel hybridization [9], using a single-stranded 22-mer CA oligonuleotide probe. The same DNA samples were separated on a 0.8% agarose gel, denatured and hybridized with an end-labeled C-rich oligonucleotide for loading control.

Other techniques

For western blot analysis, protein extracts were prepared by TCA precipitation. Rad53 was detected using anti-Rad53 polyclonal antibodies kindly provided by J. Diffley (Clare Hall, London, UK). Secondary antibodies were purchased from Amersham and proteins were visualized by an enhanced chemiluminescence system according to the manufacturer. Flow cytometric DNA analysis was determined on a Becton-Dickinson FACScan on cells stained with propidium iodide.

Supporting Information

Table S1 Yeast strains used in this study.
(DOC)

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Author Contributions

Conceived and designed the experiments: SA MPL. Performed the experiments: SA DB. Analyzed the data: SA DB GL MPL. Wrote the paper: GL MPL.

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