Cell cycle–dependent spatial segregation of telomerase from sites of DNA damage

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Telomerase can generate a novel telomere at DNA double-strand breaks (DSBs), an event called de novo telomere addition. How this activity is suppressed remains unclear. Combining single-molecule imaging and deep sequencing, we show that the budding yeast telomerase RNA (TLC1 RNA) is spatially segregated to the nucleolus and excluded from sites of DNA repair in a cell cycle–dependent manner. Although TLC1 RNA accumulates in the nucleoplasm in G1/S, Pif1 activity promotes TLC1 RNA localization in the nucleolus in G2/M. In the presence of DSBs, TLC1 RNA remains nuclear in most G2/M cells but accumulates in the nucleoplasm and colocalizes with DSBs in rad52Δ cells, leading to de novo telomere additions. Nucleoplasmic accumulation of TLC1 RNA depends on Cdc13 localization at DSBs and on the SUMO ligase Siz1, which is required for de novo telomere addition in rad52Δ cells. This study reveals novel roles for Pif1, Rad52, and Siz1–dependent sumoylation in the spatial exclusion of telomerase from sites of DNA repair.

Introduction

DNA double-strand breaks (DSBs) are one of the most cytotoxic forms of DNA damage, and their repair is critical for maintenance of genome integrity and cell survival. Classically, two pathways of DSB repair have been defined: nonhomologous end joining (NHEJ) and homologous recombination (HR). NHEJ, which occurs preferentially in G1, directly rejoins the DNA ends and often results in loss of genetic information at the break site (Moore and Haber, 1996; Takata et al., 1998). HR, which occurs during S and G2 phase, requires an homologous template for repair and generally preserves genetic information at the break site (Moore and Haber, 1996; Pâques and Haber, 1999). The choice of DSB repair by the HR or NHEJ pathway is dictated in part by the presence or absence of 5′-to-3′ resection, which generates 3′ single-stranded DNA (ssDNA) tails at the DSB ends and commits DSB repair to HR.

In addition to HR and NHEJ, DSBs can be repaired by the action of telomerase at the break site, a phenomenon referred to as telomere healing or de novo telomere addition, which often leads to gross chromosomal rearrangements (GCRs; Kramer and Gottschling, 1993; Pennaneach et al., 2006). Telomere healing has been particularly well studied in the budding yeast *Saccharomyces cerevisiae*. In yeast, telomerase is composed of an RNA moiety called TLC1, which contains the template sequence and acts as scaffold on which the catalytic reverse transcription Est2 and associated proteins (Est1, Est3, and yKu70/80) bind (Singer and Gottschling, 1994; Counter et al., 1997; Lingner et al., 1997). Recruitment of telomerase to telomeres during S phase depends on the interaction between Est1 and the telomeric DNA single-strand binding protein Cdc13 (Pennock et al., 2001; Bianchi et al., 2004; Bianchi and Shore, 2008).

Telomere healing events in yeast are suppressed by numerous mechanisms. One includes the 5′–3′ DNA helicase Pif1 that acts as a potent inhibitor of telomere addition at telomeres and DSBs (Schulz and Zakian, 1994; Zhou et al., 2000). Pif1-deficient cells have overelongated telomeres and a rate of telomere healing that is dramatically increased compared with WT cells (Myung et al., 2001). Pif1 is phosphorylated upon DNA damage in a Mec1-dependent manner, and a phosphorylation-defective mutant of Pif1 (pif1-4A) displays an increased propensity to add telomeric repeats on an HO endonuclease–induced DSB (Makovets and Blackburn, 2009). In addition to Pif1, the Mec1 kinase phosphorylates Cdc13 on its S306 residue, a phosphorylation event suggested to suppress Cdc13 recruitment to DSB ends that have little or no telomere-like sequences (Zhang and Durocher, 2010). However, recent evidence shows that these mechanisms are not sufficient to repress all Cdc13 and telomerase recruitment events at DSBs because Cdc13 and telomerase subunits can be detected at a DSB by chromatin immunoprecipitation, even if no telomere is...
During the cell cycle, TLC1 RNA nuclear distribution varies, indicating its function in telomere elongation (Teixeira et al., 2002; Gallardo et al., 2008). Because the telomerase RNA is the limiting component of the telomerase holoenzyme in yeast (Mozdzy and Cech, 2006), its dynamics should reflect the dynamics of the active telomerase complex in vivo better than other telomerase components. However, a systematic analysis of the distribution of TLC1 RNA in each phase of the cell cycle has not yet been conducted. To do so, quantification of telomerase RNA in the nucleus of yeast cells at different phases of the cell cycle was performed using FISH on the endogenous TLC1 RNA. Budding index was used to determine the cell cycle phase of each cell. Beside TLC1 RNA, the yeast nucleolus was detected using a probe against the ITS1 ribosomal RNA spacer precursor, and the nucleoplasm was labeled using DAPI. We detected between 3 and 10 nuclear TLC1 RNA foci per cell, with each focus corresponding to a single TLC1 RNA molecule, as recently reported (Bajon et al., 2015). Although cells in G1 and S phase displayed a nucleoplasmic accumulation of TLC1 RNA foci, as previously shown (Gallardo et al., 2008), a predominantly nucleolar accumulation of TLC1 RNA foci was observed in G2/M cells, as shown by colocalization with the ITS1 ribosomal RNA precursor (Fig. 1, A and B). Accumulation of TLC1 RNA in the nucleolus in G2/M was further validated by cell synchronization with nocodazole (see Fig. 2 C).

To identify factors required for this intranuclear trafficking of TLC1 RNA, we focused on factors known to regulate telomere length. One of these factors is the Pif1 5’–3’ helicase, which removes telomerase from telomeres in late S/G2 and acts as a negative regulator of telomerase activity (Schulz and Zakian, 1994; Zhou et al., 2000). Quantification of TLC1 RNA localization during the cell cycle in a pif1Δ strain shows that deletion of PFI1 inhibits the nucleolar accumulation of TLC1 RNA in G2/M, as TLC1 RNA accumulated in the nucleoplasm in all the phases of the cell cycle (Figs. 1 C and S1 A). This result was validated using the pif1Δ-m2 mutant, an allele with reduced nuclear activity of Pif1 (Schulz and Zakian, 1994). This mutant displays a phenotype midway between WT and pif1Δ strains, with TLC1 RNA present in both nucleoplasm and nucleolus in G2/M (Fig. 1 D and S1B). Altogether, these results suggest that Pif1 promotes the trafficking of TLC1 RNA from the nucleolus to the nucleolus in G2/M.

Regulation of TLC1 RNA nuclear trafficking by Rad52 after DNA damage

Given that TLC1 RNA molecules accumulate preferentially in the nucleolus during G2/M, we hypothesized that the nucleolar localization of TLC1 RNA could reduce the capacity of telomerase to interfere with HR to repair DSBs in G2/M (Diede and Gottschling, 1999). Indeed, in the yeast S. cerevisiae, HR is mostly excluded from the nucleolus, and DSBs in ribosomal DNA (rDNA) are repaired by HR in the nucleoplasm, suggesting that HR is exclusively nucleoplasmic (Lisby et al., 2003; Torres-Rosell et al., 2007). Furthermore, Pif1, which promotes the trafficking of TLC1 RNA to the nucleolus, inhibits de novo telomere addition by telomerase at DSBS (Schulz and Zakian, 1994; Phillips et al., 2015). To test this hypothesis, the distribution of TLC1 RNA molecules was determined by FISH after induction of DNA damage using bleomycin, a radiomimetic agent that preferentially generates DSBS (Chen and Stubbe, 2005). Yeast cells were exposed to 5 µg/ml bleomycin for 180 min, conditions that cause 90% of the cells to have at least one DSB per cell (Fig. S2 A). After treatment with bleomycin, FISH was

### Results

**TLC1 RNA nuclear distribution varies during the cell cycle**

Previous studies used FISH to show that TLC1 RNA accumulates in the nucleolus in G1 and S phase, which is related to its function in telomere elongation (Teixeira et al., 2002; Gallardo et al., 2008). Because the telomerase RNA is the limiting component of the telomerase holoenzyme in yeast (Mozdzy and Cech, 2006), its dynamics should reflect the dynamics of the active telomerase complex in vivo better than other telomerase components. However, a systematic analysis of the distribution of TLC1 RNA in each phase of the cell cycle has not yet been conducted. To do so, quantification of telomerase RNA in the nucleus of yeast cells at different phases of the cell cycle was performed using FISH on the endogenous TLC1 RNA. Budding index was used to determine the cell cycle phase of each cell. Beside TLC1 RNA, the yeast nucleolus was detected using a probe against the ITS1 ribosomal RNA spacer precursor, and the nucleoplasm was labeled using DAPI. We detected between 3 and 10 nuclear TLC1 RNA foci per cell, with each focus corresponding to a single TLC1 RNA molecule, as recently reported (Bajon et al., 2015). Although cells in G1 and S phase displayed a nucleoplasmic accumulation of TLC1 RNA foci, as previously shown (Gallardo et al., 2008), a predominantly nucleolar accumulation of TLC1 RNA foci was observed in G2/M cells, as shown by colocalization with the ITS1 ribosomal RNA precursor (Fig. 1, A and B). Accumulation of TLC1 RNA in the nucleolus in G2/M was further validated by cell synchronization with nocodazole (see Fig. 2 C).

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performed to detect both TLC1 and ITS1 RNA. TLC1 RNA foci remained predominantly nucleolar in both bleomycin treated and untreated G2/M cells (Fig. 2 A). However, quantification of the phenotypes revealed a small but significant accumulation of G2/M cells with TLC1 RNA in the nucleoplasm in bleomycin-treated cells (Fig. 2 B), suggesting that DNA damage affect the nucleolar distribution of this RNA. To confirm the link between TLC1 RNA accumulation in the nucleolus and repair of DSB in the nucleoplasm, HR was inhibited by deletion of RAD52. Surprisingly, the percentage of G2/M cells with nucleolar TLC1 RNA foci decreased from 70% in WT cells to 30% in rad52Δ cells treated with bleomycin, and most cells accumulated TLC1

Figure 1. Intranuclear trafficking of TLC1 RNA during the cell cycle depends on Pif1. (A) Monitoring TLC1 RNA localization during the cell cycle by FISH. The nucleolus was stained with a probe against the ITS1 spacer of the rRNA precursor (labeled with Cy5). Arrowheads mark the position of the TLC1 RNA in selected cells. DAPI was used to stain DNA. Bar, 1 µm. (B) Quantification of TLC1 RNA localization during the cell cycle; n = 900 cells. (C) Quantification of TLC1 RNA localization during the cell cycle in pif1Δ cells; n = 900 cells. (D) Quantification of TLC1 RNA localization in G2/M in WT, pif1Δ, and pif1-m2 cells; n = 200–900 cells. Error bars represent ±SD. *, P < 0.05; **, P < 0.01; ****, P < 0.001 (two-tailed t test).
Figure 2. **TLC1 RNA accumulates in the nucleoplasm in G2/M in rad52Δ cells after DNA damage.** (A) FISH on TLC1 RNA in WT or rad52Δ cells treated or not with bleomycin (blm). Arrowheads indicate position of TLC1 RNA foci in the nucleus. Bar, 1 µm. (B) Quantification of TLC1 RNA distribution in the nucleus in WT or rad52Δ cells after treatment with bleomycin (blm); n = 200 cells. (C) TLC1 RNA can relocalize to the nucleoplasm from the nucleolus in rad52Δ cells treated with bleomycin. Quantification of TLC1 RNA distribution in the nucleus in WT or rad52Δ cells synchronized with nocodazole (Noc), followed by treatment with bleomycin (blm); n = 200–300 cells. (D) FISH on TLC1 RNA in rad52Δ cells synchronized with nocodazole (Noc) and treated or not with bleomycin (blm). Arrowheads indicate position of TLC1 RNA foci in cells. Bars, 1 µm. Error bars represent ±SD. **, P < 0.01; ***, P < 0.005 (two-tailed t-test).
RNA foci predominantly in the nucleoplasm or in both compartments (Fig. 2, A and B).

To determine if the nucleoplasmic accumulation of \( TLC1 \) RNA in the presence of DSBs is caused by its retention in the nucleoplasm or by its relocalization from the nucleolus to nucleoplasm, yeast cells were synchronized in G2 with nocodazole (Fig. S2 B), followed by treatment with bleomycin. Although nocodazole-treated cells displayed \( TLC1 \) RNA foci in the nucleolus, treatment with bleomycin still resulted in the redistribution of \( TLC1 \) RNA foci toward the nucleoplasm in \( rad52 \) \( \Delta \) cells, suggesting a dynamic trafficking of this RNA between these two compartments (Fig. 2, C and D). Altogether, these results suggest that in cells containing DSBs, \( TLC1 \) RNA can relocalize from the nucleolus to the nucleoplasm and that its nucleolar retention depends on \( RAD52 \).

\( TLC1 \) RNA colocalizes with DSB sites in the nucleoplasm

To understand the significance of the nucleoplasmic accumulation of \( TLC1 \) RNA in DNA damaged cells devoid of Rad52, the colocalization between \( TLC1 \) RNA and a cytological marker of DSBs was measured. Rfa1, the largest subunit of the heterotrimeric complex RPA, was used as a marker, because it is recruited to DSBs and binds ssDNA generated by resection (Alani et al., 1992; Krogh and Symington, 2004). For this reason, Rfa1 is frequently used as a specific cytological marker for DNA end processing in vivo (Gasiorek et al., 1998; Raderschall et al., 1999; Barlow et al., 2008). Rfa1 was fused to GFP and, similarly to ionizing radiation–induced DSBs, a single Rfa1-GFP focus was observed in cells treated with bleomycin, in both WT and \( rad52 \) \( \Delta \) cells (Fig. 3 A). To validate this assay, colocalization between \( TLC1 \) RNA and Rfa1-GFP foci in G2/M was measured in \( pif1-m2 \) cells treated with bleomycin. Because Pif1 plays a key role in removing telomerase from DSBs and inhibits de novo telomere addition (Myung et al., 2001), we expect an increased colocalization between \( TLC1 \) RNA and Rfa1-GFP foci in \( pif1-m2 \) compared with WT cells. Indeed, we observed a colocalization between a Rfa1-GFP focus and a \( TLC1 \) RNA focus in 29% of \( pif1-m2 \) cells treated with bleomycin, whereas such colocalization was observed in 16% of WT cells (Fig. S3 A). FISH was performed on Rfa1-GFP \( rad52 \) \( \Delta \) cells to detect endogenous \( TLC1 \) RNA after bleomycin treatment. In 31% of the cells, one of the \( TLC1 \) RNA foci colocalized with an Rfa1-GFP focus (Fig. 3 A and Fig. S3 B). This percentage is higher than in WT cells (Fig. S3 A) or compared to the colocalization of \( TLC1 \) RNA foci with a random nuclear focus (corresponding to the \( MDN1 \) transcription site), which colocalize only in 9% of the cells (Fig. S3 C).

It is possible that \( TLC1 \) RNA nucleoplasmic foci that are not colocalized with a Rfa1-GFP focus could be associated with other DNA damage sites (Fig. S3 D). To test this possibility, another cytological marker of DSB, \( \gamma-H2A \), was combined with FISH on \( TLC1 \) RNA. In contrast to Rfa1-GFP, \( \gamma-H2A \) form several foci in mammalian and yeast cells after DNA damage (Rogakou et al., 1999; Mazumder et al., 2013). In yeast, \( \gamma-H2A \) accumulation is a direct readout of Mec1 activity at a DSB (Ira et al., 2004). Simultaneous FISH against \( TLC1 \) RNA and immunofluorescence (IF) with an antibody specific to \( \gamma-H2A \)
was performed in cells treated or not with bleomycin. In these conditions, yeast cells exposed to 5 μg/ml bleomycin during 180 min contained between two and four γ-H2A foci per cell, whereas untreated cells did not contain any foci (Fig. 3 B). As observed with Rfa1-GFP, most TLC1 RNA foci were in the nucleoplasm in rad52Δ cells treated with bleomycin, but only one TLC1 RNA focus colocalized with one of the γ-H2A foci in 45% of the cells (Fig. 3 B). Altogether, these results show that during DNA damage and in the absence of Rad52, most TLC1 RNA molecules are excluded from the nucleolus and accumulate in the nucleoplasm in G2/M, and a fraction of these molecules accumulate at DSBs.

TLC1 RNA nucleoplasmic accumulation after DNA damage depends on Cdc13 and the MRX complex and is regulated by the DNA damage response pathway

The observation that TLC1 RNA molecules colocalize with only a fraction of the DSBs raises questions concerning the link between TLC1 RNA trafficking and the DNA damage response. To confirm that TLC1 RNA accumulation in the nucleoplasm depends on the DNA damage response, factors upstream of Rad52, like the MRX complex (Mre11 and Xrs2), the ATM-like kinase Tel1 and the ATR-like kinase Mec1 were deleted in rad52Δ background cells to test their ability to suppress the nucleoplasmic accumulation of TLC1 RNA (Fig. 4 A). None of these deletions had any effect on the nucleolar distribution of TLC1 RNA in G2/M cells in the absence of DSBs. After induction of DSBs, deletion of MRE11 or XRS2 completely suppressed the nucleoplasmic accumulation of TLC1 RNA in rad52Δ cells (Fig. 4 B). This result is consistent with a function of MRX complex in DSBs processing and de novo telomere addition (Frank et al., 2006). However, deletion of TEL1 partially suppressed the nucleoplasmic accumulation of TLC1 RNA in rad52Δ cells, as this RNA accumulated in both nucleolus and nucleoplasm (Fig. 4 B). Given the role of Tel1 in DNA resection, it is possible that Tel1 might regulate TLC1 RNA nucleoplasmic accumulation by positively influencing the function of the MRX complex in the processing of DSBs, as it was shown to do at telomeres (Martina et al., 2012). On the other hand, deletion of MEC1 has no significant effect on TLC1 RNA trafficking (Fig. 4 B). Although Mec1 inhibits telomerase activity at DSBs by decreasing Cdc13 binding and by phosphorylation of the telomerase inhibitor Pif1 (Makovets and Blackburn, 2009; Zhang and Durocher, 2010), this activity is not required for TLC1 RNA trafficking, because this RNA still accumulated in the nucleolus. Altogether, these results are consistent with a model in which DSB processing and resection is required for TLC1 RNA relocation from the nucleolus to nucleoplasm in the absence of RAD52.

This model predicts that resected DSBs may retain telomerase in the nucleoplasm, and this retention should be mediated by a ssDNA-binding protein. Indeed, the single-strand telomeric binding protein Cdc13 was previously shown to be essential for the recruitment of telomerase at DNA breaks (Bianchi et al., 2004). This was revealed by using the cdc13-2 mutant, which disrupts the interaction between Cdc13 and the telomerase subunit Est1 (Nugent et al., 1996). We therefore tested the effect of the cdc13-2 mutation on the distribution of TLC1 RNA after DNA damage. In a WT RAD52 background, induction of DSBs in the cdc13-2 strain reduced the accumulation of TLC1 RNA in the nucleoplasm compared with a WT strain (Fig. S4 A). This effect is even more striking in a cdc13-2 rad52Δ strain, as TLC1 RNA did not accumulate in the nucleoplasm after exposure to bleomycin, showing that the cdc13-2 mutation completely suppressed the rad52Δ phenotype (Fig. 4, B and C). This result suggests that the Cdc13-Est1 interaction is essential for nucleoplasmic accumulation of TLC1 RNA after DNA damage.

Cdc13 clusters increase after DNA damage and accumulate at DSB sites

The aforementioned results show that in the absence of Rad52, Cdc13 plays a key role in the nucleoplasmic accumulation of TLC1 RNA after induction of DSBs. Recent work has shown that Cdc13 can be detected at a DSB by chromatin immunoprecipitation (Oza et al., 2009; Chung et al., 2010; McGee et al., 2010; Ribaud et al., 2012). We therefore examined whether TLC1 RNA nucleoplasmic accumulation is caused by the accumulation of Cdc13 at DSBs in WT and rad52Δ cells. Endogenous Cdc13 was tagged with 13 myc epitopes, and IF was performed to detect the distribution of myc-tagged Cdc13. In WT cells treated with bleomycin, Cdc13-13Myc accumulated as dim subnuclear foci in 20% of G2/M cells (Fig. 5, A and B). Surprisingly, deletion of RAD52 increased the number of cells with Cdc13 foci to more than 85% (Fig. 5 B). These results were confirmed using a Cdc13-GFP fusion protein in living yeast cells, which revealed the presence of a Cdc13 focus in over 50% of rad52Δ cells after bleomycin treatment (Fig. S4 B). The Cdc13 foci formed in rad52Δ cells were two- to threefold bigger and brighter than those observed in WT cells (Fig. 5 A and Fig. S4, C and D), likely reflecting an increased number of Cdc13 proteins associated with DSBs. To distinguish the larger Cdc13 foci observed in rad52Δ from the dimmer Cdc13 foci, we renamed the larger foci Cdc13 clusters. Notably, these clusters were only formed in rad52Δ cells in G2/M after DNA damage, with 65% of the cells containing one cluster and fewer cells containing two clusters (Fig. 5 D), suggesting that Rad52 inhibits Cdc13 accumulation at DSBs. However, we still detected Cdc13 foci in the same percentage of cells in both rad52Δ and WT cells (Fig. 5 C).

To determine if Cdc13 accumulates at DSBs after bleomycin addition, the DSB marker Rfa1 was used to investigate the colocalization between Cdc13-GFP and Rfa1-mCherry in G2/M. Cdc13-GFP clusters colocalized with a Rfa1-mCherry focus in 69% of the cells (Fig. 5 E), indicating that Cdc13 accumulates at DSBs. To test if Cdc13 foci/clusters are also formed in the absence of another mediator of HR, such as Rad51, Cdc13 foci/cluster formation was measured in rad51Δ cells after bleomycin treatment. Indeed, a previous study had shown that Cdc13 recruitment to an irreparable HO-induced DSB is highly reduced in rad51Δ cells (Oza et al., 2009). Strikingly, both Cdc13 foci and clusters disappeared in rad51Δ cells (Fig. 5, B and C), whereas TLC1 RNA accumulated in the nucleolus in those cells (Fig. 5 F), reflecting that Rad51 positively influences Cdc13 accumulation and TLC1 RNA nucleoplasmic localization at DSBs. To determine the epistatic relationship between RAD51 and RAD52 in TLC1 RNA trafficking, TLC1 FISH was performed in a double-mutant rad51Δ rad52Δ strain. Interestingly, deletion of RAD51 suppresses the nucleoplasmic accumulation of TLC1 RNA observed in rad52Δ cells, suggesting that Rad51 is epistatic to RAD52 in this pathway (Fig. 5 F). Altogether, these results show that nucleoplasmic accumulation of TLC1 RNA observed in rad52Δ cells is linked, in part, to an increased accumulation of Cdc13 at DSBs. In the absence of Rad52,
sected DSBs may be more accessible to Cdc13 binding, which may accumulate on the ssDNA and recruit telomerase.

**Temporal analysis of Cdc13 foci formation and TLC1 RNA localization at DNA damage sites**

Although the aforementioned data show that Cdc13 and TLC1 RNA can accumulate at DSBs after induction of DNA damage, it is not clear if their association with DSBs is an early or late event during DNA repair. Indeed, a late recruitment of Cdc13 and TLC1 RNA would probably suggest an indirect effect caused by the absence of DNA repair at DSBs and the accumulation of longer strands of ssDNA. To answer this question, we measured the kinetics of accumulation of Cdc13 and TLC1 RNA at DSBs in rad52Δ cells. Yeast cells were treated with bleomycin, and culture samples were taken at different time points. Yeast cells were fixed with formaldehyde and processed for IF and/or FISH. Immunofluorescence on Cdc13-myc showed that 50% of the cells already displayed a Cdc13 focus/cluster 30 min after induction of DNA damage (Fig. 6 A). The kinetics of Cdc13 foci/cluster formation was similar to the kinetics of accumulation of γ-H2A foci (Fig. 6 A), suggesting that Cdc13 accumulated rapidly at DSB sites in the absence of Rad52. A refined analysis of Cdc13 accumulation at DSBs was performed by separately quantifying cells with Cdc13 foci or clusters over time. This analysis revealed that the number of cells containing Cdc13 foci reached a peak at 30 min and stayed stable for the remainder of the treatment (Fig. 6 B). Surprisingly, Cdc13 clusters also appeared in over 30% of the cells after 30 min of bleomycin treatment (Fig. 6 B). However, the number of cells containing a Cdc13 cluster still increased over time. These results show that Cdc13’s association with DSBs is an early process after DNA damage in the absence of Rad52.

**Figure 4. Genetic requirements for nucleoplasmic accumulation of TLC1 RNA after DNA damage in rad52Δ cells.** [A] TLC1 RNA does not accumulate in the nucleoplasm in G2/M in the absence of DNA damage in rad52Δ, mre11Δ rad52Δ, xrs2Δ rad52Δ, tel1Δ rad52Δ, sml1Δ rad52Δ, sml1Δ mec1Δ rad52Δ and cdc13-2 rad52Δ cells. WT, rad52Δ, and sml1Δrad52Δ were used as control strains; n = 200–300 cells. [B] TLC1 RNA nucleoplasmic accumulation after induction of DNA damage requires Mre11 and Xrs2 of the MRX complex and Cdc13 and is regulated by Tel1. p-values are included in Table S2. Error bars represent ±SD; n = 150–300 cells. [C] FISH on TLC1 RNA in cdc13-2 rad52Δ cells treated or not with bleomycin. Arrowheads indicate the position of TLC1 RNA foci in cells. Bar, 1 µm. ***, P < 0.005 (two-tailed t test).
Figure 5. Cdc13 accumulates at sites of DNA break in rad52Δ, but not in rad51Δ cells. (A) Cdc13 foci appear in WT and rad52Δ cells after DNA damage. Immunofluorescence (IF) analysis of Cdc13-myc was conducted in WT and rad52Δ cells, with or without bleomycin (blm) treatment. Arrowheads mark the position of Cdc13 foci. Bar, 1 µm. (B) Rad52 and Rad51 have opposite effect on Cdc13 foci formation. Quantification of Cdc13 foci/cluster formation in WT, rad52Δ, and rad51Δ strains, with or without bleomycin (blm) treatment; n = 200 cells. (C) Analysis of Cdc13 clusters and foci formation in DNA-damaged cells; n = 200 cells. (D) Quantification of the number of Cdc13 cluster per G2/M-damaged cells; n = 200 cells.
tion of DNA damage. Unlike γ-H2A foci, which accumulated rapidly after DNA damage (Fig. 6 A), the nucleoplasmic accumulation of TLC1 RNA molecules started later, between 30 and 60 min after bleomycin treatment, and reached a plateau after 120 min (Fig. 6 C). We do not think that the difference in the kinetics of TLC1 RNA versus γ-H2A is a result of the lower sensitivity of the FISH assay versus IF, because single TLC1 RNA molecules are detected as single foci with this assay (Bajon et al., 2015). Interestingly, 30 min after induction of DNA damage, TLC1 RNA remained in the nucleolus even if 50% of the cells contain Cdc13 foci/clusters (Fig. 6, A and C), indicating that the association of Cdc13 with the DNA break sites is not the limiting step for the nucleoplasmic accumulation of TLC1 RNA.

These results show that nucleoplasmic accumulation of TLC1 RNA proceeds more slowly than the formation of Cdc13 foci/clusters. However, it is possible that a small number of TLC1 RNA molecules may associate rapidly with a DSB, whereas the majority of TLC1 RNA molecules still remain in the nucleolus.

To test this possibility, colocalization of TLC1 RNA with Rfa1-GFP was measured in rad52Δ cells during a time-course treatment with bleomycin. Surprisingly, early colocalization of a single TLC1 RNA focus with the DNA break site was detected even in rad52Δ cells (Fig. 6, D and E). This indicates that the association of Cdc13 with the DNA break sites is not the limiting step for the nucleoplasmic accumulation of TLC1 RNA.
RNA focus with the Rfa1-GFP focus was detected in 27% of the cells 30 min after initiation of DNA damage (Fig. 6, D and E). At this time point, most $TLC1$ RNA molecules remained outside of the DAPI-stained nucleoplasm (presumably in the nucleolus) in these cells. The colocalization between $TLC1$ RNA and Rfa1-GFP increased to nearly 40% of the cells after 60 min of bleomycin treatment and remained stable afterward (Fig. 6 E).

These results could be explained by a two-step process in the accumulation of $TLC1$ RNA in the nucleoplasm after DNA damage. One early step, which leads to the accumulation of a small number of $TLC1$ RNA molecules at a resected DSB (Fig. 6 E), and a late step, which corresponds to the global redistribution of bulk $TLC1$ RNA molecules from the nucleolus to the nucleoplasm, but not directly at DSBs (Fig. 6 C). This late redistribution may be triggered by the accumulation of unrepaired DSBs in the absence of $RAD52$. Once in the nucleoplasm, the $TLC1$ RNA molecules might be associated with Cdc13 at telomeres, which would explain why in $cdc13-2$ mutant, $TLC1$ RNA remains in the nucleolus even in the presence of DNA damage (Figs. 4 and S4). This raises questions regarding how unrepaired DSBs could trigger this relocalization of telomerase molecules.

The E3 SUMO ligase Siz1 regulates the nucleoplasmic accumulation of $TLC1$ RNA after DNA damage

The observation that $TLC1$ RNA molecules are excluded from the nucleolus and accumulate in the nucleoplasm in the presence of unrepaired DSBs suggests a specific regulation of this trafficking during DNA damage. To identify regulators of the intranuclear trafficking of $TLC1$ RNA, we focused on factors involved in sumoylation, a posttranslational modification that regulates DNA damage repair. Indeed, mutation or depletion of sumoylation enzymes in yeast and human cells notably results in defects in DNA repair, including recombination abnormalities and impaired DSB repair (Maeda et al., 2004; Branzei et al., 2006). Furthermore, sumoylation is known to regulate Rad52 and DSBs nuclear distribution in yeast (Torres-Rosell et al., 2007). In budding yeast, three mitotic SUMO E3 ligases have been identified, including two homologous Siz proteins (Siz1 and Siz2) and the Mms21 subunit of the essential Smc5/6 complex (Johnson and Gupta, 2001; Zhao and Blobel, 2005). Several factors involved in telomere maintenance are known to be sumoylated, including Cdc13, which is a substrate of both Siz1 and Siz2 (Hang et al., 2011). Interestingly, Cdc13 sumoylation increases after DNA damage, suggesting that Cdc13 activity may be regulated by SUMO during DNA damage (Hang et al., 2011).

To determine if sumoylation regulates the spatial distribution of telomerase after DNA damage, $TLC1$ RNA localization was determined in a single mutant of two SUMO E3 ligases, $siz1\Delta$ or $siz2\Delta$, in $rad52\Delta$ background with + or without − exposure to bleomycin [blm]; $n = 200–300$ cells. Error bars represent ±SD. ***, $P < 0.005$ (two-tailed t test). (B) Cdc13 clusters are still formed in the $siz1\Delta$ $rad52\Delta$ strain after DNA damage. Immunofluorescence (IF) analysis of Cdc13-myc was conducted in $rad52\Delta$ and $siz1\Delta$ $rad52\Delta$ cells after bleomycin [blm] treatment. Bar, 1 μm; $n = 200$ cells.
that these SUMO E3 ligases do not regulate the cell cycle–dependent trafficking of TLC1 RNA. Interestingly, during bleomycin-induced DNA damage, the deletion of SIZ1, but not SIZ2, strongly decrease the nucleoplasmic accumulation of TLC1 RNA in a rad52Δ strain (Fig. 7 A). These results show that Siz1, but not Siz2, controls the spatial distribution of TLC1 RNA after DNA damage.

One trivial explanation could be that Siz1 indirectly affects the trafficking of TLC1 RNA by modulating the processing of DSBs. To determine if Siz1 plays a role in the resection of DSBs, the kinetic of resection of a single HO cut site was measured in rad52Δ and siz1Δ rad52Δ strains. No difference in the kinetic of DSB resection was observed in the absence of Siz1 (Fig. S5, A and B). Also, after DNA damage, we found that deletion of SIZ1 did not affect the formation of Rfa1-GFP foci, a marker of DSB resection (Fig. S5 C). To identify where Siz1 acts in the DNA repair pathway, serial dilutions of siz1Δ, siz2Δ, rad52Δ, and double-mutant siz1Δ rad52Δ strains were spotted on plates containing different concentrations of bleomycin. Unlike SIZ2, deletion of SIZ1 increased the sensitivity of yeasts to bleomycin, although only at a higher concentration compared with a RAD52 deletion (Fig. S5 D). Interestingly, the siz1Δ rad52Δ strain showed the same sensitivity to bleomycin as a rad52Δ strain, showing that RAD52 is epistatic to SIZ1. These results suggest that Siz1 acts downstream of Rad52 in the DNA repair pathway.

Because Siz1 is known to promote Cdc13 sumoylation after DNA damage (Hang et al., 2011), it may regulate Cdc13 clustering at DSBs and subsequent TLC1 RNA trafficking during DNA damage. To test this hypothesis, IF on Cdc13-myc was performed in siz1Δ rad52Δ and siz2Δ rad52Δ cells after bleomycin treatment. Cdc13 clusters and foci still formed in the siz1Δ rad52Δ strain as in rad52Δ cells (Fig. 7 B). Altogether, these results show that Siz1 is not involved in DSBs processing or Cdc13 accumulation at resected DSB but acts downstream of this process.

Siz1 promotes de novo telomere addition in the absence of RAD52

One key question is whether this cell cycle–dependent TLC1 RNA trafficking plays a role in de novo telomere addition at spontaneous DSBs. To answer this question, we used the GCR assay developed by the Kolodner laboratory (Chen and Kolodner, 1999). In this assay, two counterselectable markers (URA3 and CAN1) are inserted near the left telomere of chromosome V. Cells that undergo a GCR event (i.e., telomere addition, translocation) that leads to simultaneous loss of CAN1 and URA3 can be selected by growth on medium containing 5-FOA and canavanine. Afterward, the number of GCR events corresponding to telomere healing was quantified. The GCR assay was first performed in rad52Δ, pipf1-m2, and pipf1-m2 rad52Δ strains. As previously reported (Myung et al., 2001), deletion of RAD52 or the pipf1-m2 mutation increase telomere healing rates (Fig. 8 A).
Combining the pif1-m2 mutation with a rad52 deletion results in a 12-fold increase in de novo telomere addition compared with the pif1-m2 strain, as previously shown (Myung et al., 2001).

Deletion of SIZ1 decreases the accumulation of TLC1 RNA in the nucleoplasm of rad52Δ cells after DNA damage (Fig. 6 A), which should reduce de novo telomere addition and GCR rates observed in a rad52Δ strain. In fact, an 11-fold reduction in telomere healing rates was observed in a siz1Δ rad52Δ strain compared with the rad52Δ strain (Fig. 8 A). Because Pif1 is required to promote TLC1 RNA trafficking to the nucleolus, our model predicts that inhibition of Pif1 should suppress the nucleolar accumulation of TLC1 RNA observed in the siz1Δ rad52Δ strain and increase GCR rates. Indeed, the pif1-m2 mutation partially suppresses the nucleolar accumulation of TLC1 in the rad52Δ siz1Δ mutant, as TLC1 RNA molecules accumulate in both nucleolus and nucleolus in the triple mutant pif1-m2 siz1Δ rad52Δ, with or without bleomycin treatment (Fig. 8 B). Deletion of SIZ1 in the pif1-m2 strain reduces GCR rates by sixfold, close to the twofold reduction previously reported for this double mutant (see Fig. 8 A). This could be because Pif1 is sumoylated during DNA damage (Hang et al., 2011), and the absence of Siz1 may affect the activity of Pif1 at sites of DNA damage. Strikingly, the triple mutant pif1-m2 siz1Δ rad52Δ restores the telomere healing rate to levels similar to the pif1-m2 rad52Δ strain (Fig. 8 A), which is consistent with our model that mutation in Pif1 bypasses the need for Siz1 activity for de novo telomere addition in rad52Δ cells.

Identification of telomere healing events in bleomycin treated cells

Although the GCR assay shows that Pif1, Rad52, and Siz1 regulate the frequency of telomere healing events, it remains unclear if de novo telomere addition really occurs in bleomycin-treated cells. To identify de novo telomere events in cells treated with bleomycin, WT, pif1-m2, rad52Δ, and rad52Δ siz1Δ cells were synchronized in G2/M with nocodazole prior treatment with bleomycin for 3 h. Genomic DNA was extracted and submitted to paired-end Illumina sequencing. Analysis of Illumina paired reads identified 73 reads containing de novo telomere events (Fig. 9 A and Table S3). 96% of these reads were identified in bleomycin-treated cells, and only three reads were identified from untreated cells, suggesting that these reads are not sequencing artifacts. Although this analysis most likely underestimates the occurrence of de novo telomere addition (see Materials and methods for details), more telomere healing events were identified per million reads in pif1-m2, rad52Δ, and rad52Δ siz1Δ cells treated with bleomycin compared with WT treated cells.

Telomere healing events were observed on most chromosomes (Table S3). Surprisingly, 14% of the telomere healing events identified occurred in the rDNA locus on chromosome 12, which is close to the percentage of the yeast genome occupied by this locus (~11%). Analysis of the sequences upstream de novo telomere addition events revealed the absence of nucleotide insertions or deletions, which is a feature of nonhomologous end joining and microhomology-mediated end joining repair (Sfeir and Symington, 2015). However, a TG-rich bias in the 10 nt upstream of the telomere addition sites was observed (Fig. 9 B), which was also previously reported for telomerase-dependent de novo telomere events (Putnam et al., 2004). Finally, the majority of these reads were found in the pif1-m2 strain, suggesting that these events are mediated by telomerase activity.

Discussion

In this study, we used single-molecule imaging to study TLC1 RNA trafficking during the cell cycle and after induction of
DNA damage. Our results demonstrate that TLC1 RNA, the limiting component of yeast telomerase, is engaged in an intranuclear trafficking during the cell cycle. In G1/S, TLC1 RNA molecules are present in the nucleoplasm, whereas in G2/M, they accumulate predominantly in the nucleolus. We also show that this trafficking depends on the helicase Pif1 and is linked to the presence of DNA damage. In addition to Pif1, the key recombination protein Rad52 strongly controls this trafficking in the presence of DNA damage, as it specifically suppresses the accumulation of TLC1 RNA molecules in the nucleoplasm and at DSBs generated by bleomycin by antagonizing the accumulation of Cdc13 at DNA breaks (Fig. 10).

However, the effect of a RAD52 deletion on the accumulation of Cdc13 at DSBs may not be simply caused by the accumulation of longer resected DNA in this mutant, because the deletion of RAD51, which also produces long ssDNA at DSBs (Sugawara et al., 1995; Zhu et al., 2008), completely abolishes the formation of Cdc13 foci. Interestingly, deletion of RAD51 suppresses the effect of a RAD52 deletion on TLC1 RNA trafficking. This result supports a previous observation that Rad51 is required for the recruitment of Cdc13 and Est2 to a nonrepairable DSB (Oza et al., 2009). Why Rad52 and Rad51 have opposite effects on the formation of Cdc13 foci is still unclear.

Using Illumina paired-end sequencing, we identified several reads containing de novo telomere addition events in the genome of yeasts treated with bleomycin. De novo telomere addition occurred downstream of TG-rich sequences less than 10 nt. A recent characterization of an endogenous hotspot of de novo telomere addition in yeast revealed a bipartite structure, with a TG-rich core sequence where telomere addition occurs, and an upstream proximal Stim sequence that binds Cdc13 and stimulates telomere addition (Obodo et al., 2016). Interestingly, de novo telomere addition is increased at this bipartite hotspot in the absence of Rad52. Hence, because of their short TG-rich core sequence, it is possible that several of the sites of de novo telomere addition identified in the DNA-sequencing study may contain this bipartite structure. Another surprising result is that 14% of the reads containing de novo telomeres were from the rDNA locus, suggesting the presence of telomerase activity in the nucleolus. Because DSBs in rDNA in yeast are processed in the nucleolus but repaired by HR in the nucleoplasm (Torres-Rosell et al., 2007), the unusual dynamics of these DSBs between the nucleolus and the nucleoplasm may explain in part their accessibility to telomerase activity in the nucleoplasm.

In addition, we demonstrate that the E3 SUMO ligase Siz1 regulates the spatial distribution of TLC1 RNA after DNA damage without affecting DSB processing or Cdc13 accumulation at DSBs. DNA damage is known to trigger a wave of sumoylation leading to simultaneous multisite modifications of several DNA repair proteins of the HR pathway in yeast (Psakhye and Jentsch, 2012). However, these sumoylation events depend on the Siz2 SUMO ligase and not on Siz1, suggesting that the effect of Siz1 on TLC1 RNA localization is independent of the main role of SUMO in the modulation of the HR pathway. Although the effect of a SIZ1 deletion on TLC1 RNA trafficking might be explained by the Siz1-dependent sumoylation of Cdc13 (Hang et al., 2011), this sumoylation event was shown to inhibit telomerase binding to Cdc13. Therefore, other targets of Siz1 may be involved in this process. Sumoylation by Siz1 is required for de novo telomere addition in the absence of Rad52 and, to a lesser extent, in a pif1-m2 strain. This requirement for Siz1 activity is completely suppressed in a double pif1-m2 rad52 Δ strain (Fig. 10).

In line with the observation that DNA repair is excluded from the nucleolus (Dion et al., 2013), our study reveals how the spatial segregation of telomerase and HR activities restricts telomerase access to DSBs. This process is not specific to budding yeast telomerase, because the catalytic subunit of human telomerase (hTERT) was also shown to accumulate in the nucleolus after ionizing radiation–induced DSBs (Wong et al., 2002), indicating that nucleolar localization of telomerase after DNA damage may be a conserved process during evolution.

Materials and methods

Constructs and strains

Table S1 lists strain genotypes. Yeast strains were generated by disrupting the RAD52, RAD51, SIZ2, SIZ1, MRE11, XRS2, TEL1, or MEC1 genes in W303 strain by a one-step PCR disruption method using KAN marker (Wach et al., 1994). The mecl Δ strains were kept viable by deletion of the SML1 gene (Zhao et al., 1998). In this strain, the KAN marker was removed by transforming cells with pSH47 expressed Cre recombinase. The cdc13-2 strain transformed with the plasmid pVL438 CDC13 (Ycp33 CEN, URA3, and CDC13: Chandra et al., 2001) was obtained from R. Wellinger’s laboratory (Université de Sherbrooke, Sherbrooke, Canada). Deletion of RAD52 was followed by the elimination of the pVL438 CDC13 plasmid using 5-FOA. Strains carrying Rfa1-GFP, Rfa1-mCherry, Cdc13-GFP, and Cdc13-13Myc allele at the RFA1 and CDC13 chromosomal loci were generated by a PCR one-step tagging method using plasmids pFA6A-GFP-KANMX4, pFA6A-
mCherry-Kan, or pFA6A-13Myc-KANMX4 (Knop et al., 1999). Verification of these strains was performed by PCR, microscopy, and/or Western blot. For Cdc13-GFP and Cdc13-13Myc strains, senescence experiments were conducted by repeatedly streaking strains. For all these strains, the RAD52 gene disruptions were obtained by using TRP1, KAN, or Hygro markers. RAD51 deletion was also obtained by using TRP1 marker.

Cell cycle–dependent localization of TLC1 RNA and induction of DSBs Asynchronously growing WT yeast cells (W303) were grown in YEPD (yeast extract peptone-dextrose) until OD600 0.3 to 0.4, fixed with paraformaldehyde, and processed for FISH analysis to detect TLC1 RNA and ITS1 pre-rRNA. ITS1 FISH probe was provided by D. Zenklusen. (Université de Montréal, Montréal, Quebec, Canada). The cell cycle stage of the cells was estimated by measuring the bud-to-mother size ratio (bud-ding index) as G1 (cells without bud), S phase (cells with small to mid-size bud), and G2/M (cells with large bud or sharing the nucleus between mother and daughter). This method is highly reproducible for scoring G2/M cells, because nearly identical percentages of TLC1 RNA distribution were measured in nocodazole-treated cells and nontreated cells (see Fig. 2, B and C). For induction of DSBs, haploid WT and rad52Δ strains were grown in YEPD medium (at OD600 0.2) and exposed to 5 µg/ml bleomycin (BLE011.10; Bioshop) for 180 min. In these conditions, 90% of the cells have at least one DSB after 180 min (Fig. S2). The presence and number of DNA damage per cell after bleomycin treatment were quantified using the number of γ-H2A foci, which correlates with the number of DSBs per cell (Sedelnikova et al., 2002). For nocodazole treatment, WT and rad52Δ cells were grown in YEPD medium to OD600 ∼0.2 before addition of 15 µg/ml nocodazole for 90 min, and synchronization of cells in G2/M was monitored by microscopy. In these conditions, more than 90% of cells are in G2/M. 5 µg/ml bleomycin was then added, and cells were incubated for another 180 min.

**FISH and IF**

The yeast fixation protocol and fluorescent in situ hybridization to detect endogenous TLC1 RNA have been described previously (Ptingsten et al., 2012). Five Cy3-labeled 50-nt probes were used to detect the endogenous TLC1 RNA, and one Cy5-labeled 50-nt probe was used to detect the ITS1 pre-rRNA. For colocalization experiments with endogenous Rfa1-GFP, yeast was cultured at room temperature, and fixation was performed in 3% formaldehyde, and processed for FISH analysis to detect TLC1 RNA, ITS1 pre-rRNA (nucleolus) or DAPI (nucleoplasm), with reproducible results obtained with two or three different individuals. For each yeast strain, a total of 200 G2/M cells were randomly scored in two biologically independent experiments. The linescanning application of Fiji or Meta Morph software was also used to quantify TLC1 RNA, ITS1 RNA, and DAPI fluorescence distribution in the nucleus of yeast cells. Numbers are expressed as percentage of cells with TLC1 RNA located in the nucleoplasm or nucleolus or evenly distributed between both compartments. Simultaneous FISH on TLC1 and MDN1 RNA was performed to determine the percentage of colocalization of TLC1 RNA foci with a random nuclear focus, represented by the MDN1 transcription site. The MDN1 FISH probes were provided by D. Zenklusen.

**Live-cell imaging**

Cells expressing fusion protein Rfa1-GFP, Cdc13-GFP, or Cdc13-GFP and Rfa1-mCherry in rad52Δ genetic background were incubated overnight at 25°C in 2 ml SC-TRP and then diluted to OD600 ∼0.2 and grown for one cell cycle. Cdc13-GFP foci and colocalization between Cdc13-GFP and Rfa1-mCherry were induced by 5 µg/ml bleomycin for 3 h. Cells were collected by centrifugation at 2,000 g, mounted on standard glass slides, and covered with a coverslip appropriate to the optics of the microscope. All the images were acquired using an Axio Imager 2 epifluorescence upright microscope (ZEISS) equipped with a 100x DIC H (1.4 NA) objective and an Evolve fx EM-CCD camera or microscope AXIO OBSERVER Z1 confocal spinning disk (ZEISS) equipped with the same objective and camera. For each field of cells, 11 fluorescent images at each of the relevant wavelengths were obtained at 0.4-µm intervals along the Z axis. Inspection of all focal planes of each cell was performed to quantify colocalization. Image acquisition times for DIC, bright-field, GFP, and mCherry were 40, 40, 900, and 60 ms, respectively.

**Quantitative analysis of Cdc13 foci and clusters**

Determination of Cdc13 focus and cluster diameter and maximal fluorescence intensity was calculated as described previously (Gallardo et al., 2011).

**DSB resection assay**

Yeast was grown to early–mid log phase in YEP-raffinose medium at 30°C. HO endonuclease was induced by the addiction of 3% galactose. Samples were harvested before galactose addition and every 30 min after induction for 4 h. Genomic DNA was extracted and digested by PciI restriction enzyme. DNA fragments were separated on a 1% agarose gel and transferred on a neutral nylon membrane. Southern blot was performed with a probe generated by PCR amplification of a sequence close to the HO cut site. DNA loaded at each time point was normalized using a probe against the AGXI gene. Intensities of bands on the Southern blot were quantified with Image Lab. Resection was measured as the ratio of the signal intensity for each time point relative to the signal intensity of the initial HO cleavage time point.

**GCR assay and characterization of telomere healing events**

All GCR rates were determined by fluctuation tests. Mutation rates were calculated using the median method (Putnam and Kolodner, 2010). Re-
paired-end sequencing

Cultures were started from an OD600 of 0.2. When the OD600 reached 0.4, 15 µg/ml nocodazole was added to the culture. Cultures were incubated for 3 h until >80% of the cells were synchronized in G2/M. Synchronization was assessed by microscopy. If needed, 5 µg/ml bleomycin was added and cultures were incubated another 3 h before harvesting the cells. Genomic DNA was extracted, and 1 µg was sent for paired-end Illumina sequencing on an Illumina HiSeq2000. Each condition was done in duplicate. Genome coverage for each sample ranged from 25 to 210× (2,792,066 to 14,219,231 sequences).

Datasets were analyzed using a Galaxy (Afgan et al., 2016) modified version of a previously published workflow (Zampini et al., 2015). Sequences were filtered for a minimum length of 40 nt, an average quality of at least 20, and no more than 50 nt under a quality of 20. They were then aligned using the Burrows-Wheeler aligner against the sacCer3 reference genome (NCBI Assembly accession no. GCA_000146045.2) from which telomere sequences were removed. Read pairs for which both reads were successfully mapped do not contain de novo telomere additions and were discarded. The first 25 nt of the remaining pairs were aligned using Bowtie, and sequences which matched downstream of a very high number of reads with pseudo-palindromes, possibly because rad52Δ cells have longer resated DNA at DSBs. Lastly, because telomere sequences shorter than 12 nt were not analyzed, short de novo telomere additions were ignored. Fragmentation of the DNA near a site of telomere addition during library preparation therefore means that some occurrences were missed.

Statistical analysis

A two-tailed Student’s t test or analysis of variance was used to calculate the statistical significance. A Mann–Whitney t test was used for the GCR assay. Statistical significance was defined as P < 0.05 (*). Calculations were performed using GraphPad.

Online supplemental material

Fig. S1 presents TLC1 RNA localization in pif1Δ and pif1-m2 strains. Fig. S2 describes quantification of γ-H2A foci and cell cycle synchronization. Fig. S3 shows the analysis of TLC1 RNA colocalization with DSBs. Fig. S4 presents a quantitative analysis of Cdc13 foci. Fig. S5 shows that Siz1 is not involved in DSBs processing but acts downstream of Rad52 in DNA repair. Table S1 shows all yeast strains genotypes. Table S2 lists p-values of all the strains tested in Fig. 4 B. Table S3 lists all the de novo telomere addition identified by DNA sequencing.

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Author contributions: F. Ouenzar, M. Lalonde, and F. Gallardo performed FISH and IF experiments, analyzed microscopy data, and prepared strains. G. Morin helped to perform the FISH experiments shown in Fig 4. M. Lalonde prepared strains and performed GCR assays and analysis of Illumina sequencing. H. Laprade performed OCR assays and DNA resection. S. Tremblay-Belzile helped with bioinformatics analysis. F. Ouenzar, M. Lalonde, and P. Chartrand designed the experiments and wrote the paper.
