RESEARCH COMMUNICATION

Multiple pathways inhibit NHEJ at telomeres

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The nonhomologous end-joining (NHEJ) repair pathway is inhibited at telomeres, preventing chromosome fusion. In budding yeast *Saccharomyces cerevisiae*, the Rap1 protein directly binds the telomere sequences and is required for NHEJ inhibition. Here we show that the Rap1 C-terminal domain establishes two parallel inhibitory pathways through the proteins Rif2 and Sir4. In addition, the central domain of Rap1 inhibits NHEJ independently of Rif2 and Sir4. Thus, Rap1 establishes several independent pathways to prevent telomere fusions. We discuss a possible mechanism that would explain Rif2 multifunctionality at telomeres and the recent evolutionary origin of Rif2 from an origin recognition complex (ORC) subunit.

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Nonhomologous end-joining (NHEJ) inhibition at telomeres prevents fusions between two telomeres and between a telomere and a double-stranded broken end (DuBois et al. 2002; Chan and Blackburn 2003; Mieczkowski et al. 2003; Riha et al. 2006). In fission yeast *Schizosaccharomyces pombe*, the Taz1 protein binds telomeric DNA and recruits the Rap1 protein. Both Taz1 and Rap1 are required for NHEJ inhibition (Ferreira and Cooper 2001; Miller et al. 2005). In mammals, TRF1 and TRF2 are the two orthologs of Taz1, and RAP1 is recruited to the telomeres by TRF2 (Li et al. 2000). In vivo, TRF2 is essential for NHEJ inhibition (van Steensel et al. 1998; Celli and de Lange 2005; Celli et al. 2006). In vitro, it has been found that NHEJ inhibition by telomeric repeats requires TRF2 and RAP1 (Bae and Baumann 2007). In *Saccharomyces cerevisiae*, there is no ortholog of Taz1/TRF1/TRF2. Rap1 directly binds the telomere sequences, and its loss causes telomere fusions dependent on all the factors known to be required for NHEJ (Pardo and Marcand 2005; Pardo et al. 2006).

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Rap1 is a modular protein with three conserved domains: a BRCT domain of unknown function in the N-terminal region, a centrally located DNA-binding domain with two Myb-like folds, and an independent C-terminal domain called RCT (Rap1 C-terminal) (Fig. 1A; Li et al. 2000; Dreesen et al. 2007). In budding yeast, Rap1 also binds to ~5% of the promoters and is required for viability. The BRCT and RCT domains are dispensable (Kyrion et al. 1992; Moretti et al. 1994). The RCT is required for the negative feedback loop that represses telomere elongation by telomerase (Kyrion et al. 1992; Marcand et al. 1997) and for the establishment of a silent chromatin near telomeres (Kyrion et al. 1993; Buck and Shore 1995). It interacts with the proteins Rif1, Rif2, Sir3, and Sir4. Rif1 and Rif2 are required for the inhibition of telomere elongation (Wotton and Shore 1997; Levy and Blackburn 2004; Teixeira et al. 2004). Sir3 and Sir4 establish transcriptional silencing, which also requires Sir2, a conserved histone deacetylase. In this work, we ask which Rap1 domains and interacting factors are involved in NHEJ inhibition at telomeres.

Results and Discussion

Rap1 C-terminal domain is required for NHEJ inhibition at telomeres

In *S. cerevisiae*, the TG₁₋₃ telomere repeats display a variability that to some degree disrupts the palindrome formed by a telomere–telomere fusion, allowing the detection of a fraction of the fusions directly by PCR (Fig. 1B; Mieczkowski et al. 2003; Pardo and Marcand 2005). Initially, we looked for fusions in a *rap1-ΔC* mutant lacking the RCT (Δ670–807), but failed to detect them (Fig. 1C; data not shown). In *rap1-ΔC* cells, telomeres are elongated over several kilobases (Kyrion et al. 1992), and fusions between long telomeres may not be prone to amplification. In *tel1* and *tel1 rap1-ΔC* cells, telomeres are short (Craven and Petes 1999), at a steady mean length of ~160 and ~200 base pairs (bp), respectively, in the strains used here. On its own, the absence of Tel1 does not influence the frequency of fusions caused by full-length Rap1 loss (Pardo and Marcand 2005) and only moderately changes the frequency of rearrangements between two double-strand breaks and between a double-strand break and a telomere (DuBois et al. 2002; Chan and Blackburn 2003; Pennaneach and Kolodner 2004; Lee and Lee 2007), suggesting that we could take advantage of the *tel1-Δ* mutant to facilitate the detection of fusions by PCR. As shown in Figure 1C, telomere fusions are amplified by PCR in the *tel1 rap1-ΔC* double mutant. Telomere length distribution being heterogeneous, telomere fusions are detected as a smearing PCR signal. The mean length of the PCR products suggests fusions with ~350 bp of telomeric repeats, a length consistent with fusions between telomeres close to the mean telomere length in this strain. Fusions remain undetected in wild-type cells, in a *tel1* single mutant, and in *tel1 rap1-ΔC* cells lacking the NHEJ factor Lif1. This result suggests that the Rap1 C-terminal domain is required for NHEJ inhibition at telomeres, at least in a context where the telomeres remain short.

Figure 2. Amplification of telomere-telomere fusions in *TEL1* cells. For each strain, PCRs from two independent samples are displayed. DNA was diluted with DNA from the wild-type strain.

silent chromatin. In *a/α* cells, NHEJ is repressed globally (Astrom et al. 1999). Thus, in *tel1 rif2 sir4 a/α* cells, the frequency of fusions is reduced, as expected.

The requirement of Rif2 and Sir4 for NHEJ inhibition is apparent in *tel1-Δ* strains in which telomeres are shorter than in wild-type cells. We then asked whether this requirement is detectable in *TEL1* cells. As shown in Figure 2, fusions are amplified after a limited number of cycles in *rif2 sir4* cells but not in *rif2* and *sir4* single mutant cells nor in *rif2 sir4 a/α* cells. A very low frequency of telomere-telomere fusions in *rif2* cells is consistent with a previous report showing that fusions between telomeres and a double-strand break remain rare in *rif2* cells (DuBois et al. 2002).

NHEJ inhibition by Rap1 in the absence of Rif2 and Sir4

To compare the frequency of fusions between cells lacking Rif2 and Sir4 and cells losing Rap1, we used a previously described conditional allele of *RAP1*, a double shut-off degon called *rap1-Δ* (Pardo and Marcand 2005). This allele causes Rap1 levels to drop in cells exiting exponential growth. As shown in Figure 2, fusions are much more frequently amplified in *rap1-Δ* cells than in *rif2 sir4* cells, suggesting that NHEJ remains partially inhibited in cells lacking Rif2 and Sir4. However, telomeres are longer in *rif2 sir4* cells than in *rap1-Δ* cells and, remarkably, the mean length of the amplified

fusions in *rif2 sir4* cells changes with the number of PCR cycles from ~600 bp at 26 cycles to ~540 at 30 cycles. The preferential amplification of shorter products during the PCR makes the comparison between two strains with different telomere lengths less convincing.

To address this difficulty, the frequencies of fusions in cells lacking Rif2 and Sir4 and in cells losing Rap1 were compared in a *tel1-Δ* context. As shown in Figure 3, the highest level of fusions is observed in *tel1 rif2 sir4 rap1-Δ* cells in which telomeres are also the longest (~240 bp). PCR performed on serial dilutions of their genomic DNA allows an estimation of the relative fusion frequencies between the tested *tel1* strains. Fusions are at least 30 times more frequent in *tel1 rif2 sir4 rap1-Δ* cells than in *tel1 rif2 sir4* cells or in *tel1 rif2 sir4 rap1-Δ* cells complemented with a wild-type copy of *RAP1* [*tel1 rif2 sir4 rap1-Δ* + *RAP1*]. This indicates that Rap1 still inhibits NHEJ in the absence of Rif2 and Sir4. *tel1 rif2 sir4 rap1-Δ* cells display a significantly higher level of fusions than *tel1 rap1-Δ* cells (Fig. 3, 20 cycles), indicating that the *rap1-Δ* allele is not a complete loss-of-function mutant for the Rif2 and Sir4 pathways. Fusions are at least 10 times more frequent in *tel1 rif2 sir4* cells than in *tel1 rif2* cells or in *tel1 sir4* cells, confirming the synergistic interaction between Rif2 and Sir4 to inhibit NHEJ.

Since *tel1 rif2 sir4 rap1-Δ* cells do not display more fusions than *tel1 rif2 sir4* cells (Fig. 1), the loss of the Rap1 C-terminal domain does not explain the increased level of fusions caused by full-length Rap1 loss in a *tel1 rif2 sir4* mutant context. Hence, we used a *rap1-ΔN* allele encoding a protein truncated for its nonessential N-terminal part ($\Delta 2-228$) to determine whether the Rap1

Figure 3. NHEJ inhibition by Rap1 in the absence of Rif2 and Sir4. For each strain, PCRs from two independent samples are displayed. The amount of amplified product is indicated in nanograms below each visible smear. DNA were diluted with DNA from the wild-type strain.

BRCT domain could be involved in NHEJ inhibition. As shown in Figure 3, fusions in *tel1 rif2 sir4 rap1-ΔN* cells remain ~30 times less frequent than *tel1 rif2 sir4 rap1-Δ* cells. This indicates that NHEJ inhibition in the absence of Rif2 and Sir4 does not entail the Rap1 N-terminal domain. Together, these results show that the part of Rap1 essential for viability, including the DNA-binding domain, establishes NHEJ inhibition through a pathway independent of Rif2 and Sir4.

NHEJ inhibition in cis at double-strand breaks

We asked whether NHEJ inhibition could also be established at another position in the genome. To test this, two I-SceI sites were inserted in opposite orientation on each side of the *URA3* gene on chromosome V as displayed in Figure 4A. The sequence encoding the nuclease was inserted at a different locus and placed under the control of a galactose-inducible promoter. Cells are haploid and cannot survive the loss of the broken chromosome. Simple rejoining by NHEJ recreates the I-SceI sites. Upon continuous expression of the endonuclease on galactose, only ~0.4% of the cells form a colony. These cells survive by mutating the cleavage sites. In

most of the survivors, the *URA3* gene is lost and the two distal ATAA 3' overhangs are fused to form an –ATAT– joint (data not shown). This event requires Lif1 and the DNA polymerase Pol4 (data not shown).

First, we asked whether a telomeric sequence adjacent to the break could inhibit NHEJ. Two-hundred-fifty-six base pairs of telomeric repeats were inserted in the vicinity of one of the I-SceI sites. The distance between the 5' end generated by I-SceI and the edge of the telomeric repeats is 12 bp. The TG strand runs 5' to 3' toward the end. As shown in Figure 4B, the presence of the telomeric repeats reduced survival on galactose to the frequency observed in cells lacking Lif1. Thus, an internal telomeric sequence near a break inhibits a rearrangement by NHEJ. In cells lacking Rif2 or Sir4 alone, the frequency of survival is slightly increased by about fourfold. In cells lacking both Rif2 and Sir4, the frequency of survival is increased by ~300-fold compared with the single mutants. This indicates that Rif2 and Sir4 synergistically inhibit NHEJ in this assay. In *rif2 sir4 a/a* cells, NHEJ is globally repressed in *trans* and survival is reduced to a low frequency. The loss of Rif1 does not influence survival on galactose. Survival in a *rif2 sir4* double mutant remains about sevenfold lower than in the absence of the telomeric sequence, suggesting that rearrangements by NHEJ are still partially inhibited in this context.

Next, we asked whether NHEJ inhibition could be established independently of the telomeric repeats. Five *UAS_{Gal4}*-binding sites were inserted in the vicinity of one of the I-SceI sites replacing the internal telomeric sequence. The distance between the 5' end generated by I-SceI and the edge of the first Gal4-binding site is 23 bp. In cells expressing the Gal4 DNA-binding domain alone (Gbd) and the native Gal4 protein, the presence of the *UAS_{Gal4}* sites does not influence survival on galactose (Fig. 4C). To target the Rap1 C-terminal domain, we used a chimera fusing the Gal4 DNA-binding domain and the Rap1 C-terminal domain (Gbd–Rap1Cter; amino acids 653–827). This hybrid establishes transcriptional silencing and participates in the negative feedback loop on telomere elongation when targeted to the internal edge of a telomere (Buck and Shore 1995; Marcand et al. 1997).

In the NHEJ assay described here, Gbd–Rap1Cter reduces survival on galactose by ~50-fold in cells with *UAS_{Gal4}* sites adjacent to the break, and does not influence survival in cells without *UAS_{Gal4}* sites (Fig. 4C). Thus, targeting the Rap1 C-terminal domain is sufficient to inhibit a rearrangement by NHEJ at an ectopic position. This inhibition is not complete compared with a *lif1-Δ* mutant. Remarkably, inhibition is strengthened in cells lacking Tel1. In cells lacking Rif2, Gbd–Rap1Cter loses its ability to reduce the frequency of rearrangements. In cells lacking only Rif1 or Sir4, Gbd–Rap1Cter retains its repressive effect. In *sir4 a/a* cells, Gbd–Rap1Cter does not influence the residual frequency of survival. The requirement for Rif2 but not Sir4 in this assay indicates that NHEJ inhibition by Gbd–Rap1Cter acts through the Rif2 pathway established by Rap1.

Possible mechanisms of inhibition

We observed that in budding yeast Rap1 establishes several parallel pathways to inhibit NHEJ at telomeres. The coexistence of independent inhibitory pathways ensures

Figure 4. NHEJ inhibition at double-strand breaks. (A) Schematic representation of the NHEJ assay. (B) NHEJ inhibition by internal telomeric repeats. (C) NHEJ inhibition by ectopic targeting of Rap1 C-terminal domain. The mean relative survival on galactose versus glucose were observed in three experiments.

Figure 5. Alignment of the Rif2 and Orc4 sequences. [A] The first domain (domain I) of the AAA⁺ module. The positions of the observed secondary structures of *Pyrobaculum aerophilum* Cdc6 (Liu et al. 2000) are shown at the bottom, together with those of AAA⁺ motifs. [WA] Walker A; [WB] Walker B; [S1] sensor 1. Identities are shown white on a black background, and similarities are shaded gray [dark gray for hydrophobic residues, light gray for other similarities]. Positions of the Walker A lysine, Walker B aspartate, and *S. cerevisiae* Orc4 Arg 267 are indicated by red arrowheads. [B] The second domain (domain II) of the AAA⁺ module. Only the sequences of Rif2 and Orc4 are aligned, as the alignment cannot be extended in an accurate way to Cdc6. However, secondary structure prediction made on the basis of this alignment using PHD (Rost et al. 2004) indicated an all- α fold, with four main helices, reminiscent of the domain II structure observed in Cdc6. Note that in Cdc6, the domain II is discontinuous, with a short N-terminal fragment located upstream of domain I. Such an N-terminal extension might be found in the Rif2 and Orc4 sequences.

that fusions do not occur unless the telomeres become extremely short (Chan and Blackburn 2003; Mieczkowski et al. 2003). This buffering sees to it that fusions never form when telomeres deviate slightly from the range of lengths found in wild-type cells. In humans, short tracts of telomeric DNA are also sufficient to inhibit NHEJ in vitro (Bae and Baumann 2007).

NHEJ inhibition by an ectopic targeting of the Rap1 C-terminal domain near a double-strand break reveals that the inhibitory pathway that requires Rif2 can act without telomeric DNA. This suggests that Rif2 acts on protein complexes and not through a telomere-specific DNA structure. It also reveals that NHEJ inhibition by Rif2 is a standard *cis* effect in which the recruitment of a factor locally inhibits a molecular function. Rif2 inhibits not only NHEJ but also telomerase-mediated telomere elongation (Wotton and Shore 1997; Teixeira et al. 2004) and a form of homologous recombination between telomeres (Teng et al. 2000). A unique common requirement for these three inhibited processes is the Mre11-Rad50-Xrs2 complex. Thus, the simplest hypothesis is that the Rif2 pathway inhibits locally the Mre11-Rad50-Xrs2 complex. This single molecular activity for Rif2 would explain its multifunctionality. Wild-type-length telomeres are refractory to Mre11-Rad50-Xrs2 binding (Negrini et al. 2007; Viscardi et al. 2007), suggesting that Rif2 may inhibit the recruitment of the complex.

Sir4 inhibits NHEJ through an unknown mechanism. Interestingly, Sir4 interacts at telomeres with the Yku80 subunit of KU (Ribes-Zamora et al. 2007). This interaction might be important for NHEJ inhibition. The central domain of Rap1 inhibits NHEJ independently of Rif2 and Sir4. Rap1 interaction with DNA might directly out-compete the stable recruitment of the NHEJ machinery or act through a DNA secondary structure similar to the one proposed for telomeres in higher eukaryotes (Griffith et al. 1999; Amiard et al. 2007).

Evolutionary origin of Rif2

Rap1 is required for NHEJ inhibition in fission yeast (Miller et al. 2005) and in vitro with human cell extracts (Bae and Baumann 2007), suggesting that the pathways observed in budding yeast might be conserved in evolution. An ortholog of Sir4 in higher eukaryotes remains to be identified. Remarkably, *RIF2* is syntenic to *ORC4*, indicating that the two genes diverged from a common ancestor after the last whole-genome duplication of the *S. cerevisiae* lineage (Scannell et al. 2007). Despite this synteny information, the similarity between the Rif2 and Orc4 sequences can be detected only using very sensitive methods of sequence analysis (Fig. 5; Supplemental Fig. 2). Like Orc4, Rif2 possesses two domains typical of AAA⁺ modules but is distinct from Orc4, since an additional C-terminal domain, adopting a winged-helix fold in Cdc6, is absent. The Walker A and Walker B motifs are highly degenerated in Rif2: The Walker A lysine is

substituted by a histidine, while the Walker B aspartate, which is located at the end of a β -strand, is substituted by a glutamic acid. Arg 267 of *S. cerevisiae* Orc4, essential for origin recognition complex (ORC) ATPase activity [Bowers et al. 2004], is not conserved in Rif2. This suggests that, although it shares the same overall AAA⁺ fold as Orc4, Rif2 probably lacks ATPase activity.

The recent origin of Rif2 suggests two scenarios. Rif2 may have established a new pathway at telomeres; for instance, to compensate for the weakening of another pathway. Alternatively, the duplication of the gene may have allowed a separation of functions and a specialization of Rif2 at telomeres. If the latter is true, ORC may play the role of Rif2 at telomeres in yeast species that diverged from *S. cerevisiae* prior to the whole-genome duplication and, by extension, in other eukaryotes. Interestingly, ORC is recruited to telomeres by TRF2 in human cells (Deng et al. 2007). Whether ORC directly inhibits NHEJ, elongation by telomerase, and homologous recombination in some species remains to be tested.

Materials and methods

Strains and plasmids

The strains used in this study are listed in Supplemental Table 1. Silencing-defective strains are deleted of *HML α* or *HMRa* to remain NHEJ-proficient unless mentioned. Details of strain construction are provided with the Supplemental Material.

Amplifications of the telomere–telomere fusions

Cells were grown to saturation in rich medium for 4 d (Fig. 1) or 3 d (Figs. 2, 3). Telomere–telomere fusions were amplified by PCR from ~10 ng of genomic DNA (see Supplemental Material). The mean length of the fusions was obtained by subtracting the size of the amplified X and Y sequences (460 bp) from the mean length of the amplified products.

Telomere length

Telomere length was measured by PCR after end-labeling with terminal transferase (Supplemental Material; Teixeira et al. 2004). The mean telo-

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mere length was obtained by subtracting the size of the end primer (26 bp) and the size of the amplified X or Y' sequences (340 or 120 bp, respectively) from the mean length of the amplified products.

I-SceI assay

Cells were spread at appropriate dilutions on glucose- and galactose-containing plates (Supplemental Material). Colonies were counted after incubation for 4 d at 30°C.

Alignment of the Rif2 and Orc4 sequences

A similarity between Rif2 and *Kluyveromyces lactis* Orc4 could be revealed by PSI-BLAST searches just below the threshold value when no compositional adjustment was preferred. This similarity was supported at the secondary structure level using Hydrophobic Cluster Analysis (HCA) (Supplemental Material; Supplemental Fig. 2).

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