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Rif1 and Rif2 inhibit localization of Tel1 to DNA ends

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Summary

Chromosome ends, known as telomeres, have to be distinguished from DNA double-strand breaks (DSBs) that activate the DNA damage checkpoint. In budding yeast, the ATM homolog Tel1 associates preferentially with short telomeres and promotes telomere addition. Here we show that the telomeric proteins Rif1 and Rif2 attenuate Tel1 recruitment to DNA ends through distinct mechanisms. Both Rif1 and Rif2 inhibit the localization of Tel1, but not the Mre11-Rad50-Xrs2 (MRX) complex, to adjacent DNA ends. Rif1 function is weaker at short telomeric repeats compared with Rif2 function, and is partly dependent on Rif2. Rif2 competes with Tel1 for binding to the C-terminus of Xrs2. Once Tel1 is delocalized, MRX does not associate efficiently with Rap1-covered DNA ends. These results reveal a mechanism by which telomeric DNA sequences mask DNA ends from Tel1 recognition for the regulation of telomere length.

Introduction

Telomeres are nucleoprotein complexes at the ends of linear eukaryotic chromosomes, which are distinguished from DNA double-strand breaks (DSBs). To maintain genomic integrity, all organisms respond to DSBs by promptly launching the DNA damage response. This response involves the recruitment of DNA repair factors to sites of DNA damage and the activation of signal transduction pathways, often termed DNA damage checkpoint pathways (Zhou and Elledge, 2000). Telomeres are protected from checkpoint activation (de Lange, 2005; Longhese, 2008). Paradoxically, checkpoint proteins are essential for telomere length maintenance.

Checkpoint signals are initiated through two large protein kinases, ataxia-telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) (Abraham, 2001; Zhou and Elledge, 2000). ATM and ATR are highly conserved among eukaryotes. In budding yeast, ATM and ATR correspond to Tel1 and Mec1, respectively. Several lines of evidence indicate that the Mre11-Rad50-Nbs1 (Xrs2 in budding yeast) complex is the primary sensor that recruits ATR/Mec1 and ATM/Tel1 to DSBs (Falck et al., 2005; Nakada et al., 2004; Nakada et al., 2003a; You et al., 2005). In budding yeast, the Mre11-Rad50-Xrs2 (MRX) complex collaborates with exonucleases in the generation of single-stranded DNA (ssDNA) at DSB ends (Krogh and Symington, 2004). Tel1 interacts with the C-terminus of Xrs2 to localize to DNA ends (Falck et al., 2005; Nakada et al., 2003a; You et al., 2005). Once ssDNA is generated, replication protein A (RPA) recognizes ssDNA (Krogh and Symington, 2004; Wold, 1997), and recruits Mec1 to DNA ends (Nakada et al., 2005; Zou and Elledge, 2003). Mec1 and Tel1 activate the

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downstream Rad53 kinase (Nakada et al., 2003b; Sanchez et al., 1996; Schwartz et al., 2002; Sun et al., 1996; Sweeney et al., 2005; Usui et al., 2001), thereby leading to transient cell-cycle arrest and transcriptional activation of genes involved in DNA repair.

Telomeres contain a double-stranded DNA region of tandem repeats (e.g. human, T_2AG_3; budding yeast, $TG_{1\text{-}3}$) and a 3′ protruding ssDNA region of the G-rich strand (Smogorzewska and de Lange, 2004; Vega et al., 2003). Single-stranded tails on telomeres are bound by sequence-specific ssDNA binding proteins, such as Cdc13 in budding yeast (Lin and Zakian, 1996; Nugent et al., 1996). Cdc13 acts as a telomere cap to protect telomeres from degradation (Garvik et al., 1995; Nugent et al., 1996; Pennock et al., 2001), thereby inhibiting RPA recruitment and subsequent Mec1 accumulation (Hirano and Sugimoto, 2007; Rouse and Jackson, 2002). However, Cdc13-mediated capping does not affect accumulation of Tel1 or MRX complex at DNA ends (Hirano and Sugimoto, 2007). Recent evidence indicates that Tel1 associates preferentially with short telomeres and promotes telomere addition (Bianchi and Shore, 2007; Chang et al., 2007; Hector et al., 2007; Sabourin et al., 2007; Viscardi et al., 2007). However, it has not been determined how Tel1 is inhibited from localizing to telomeres of normal length. Double-stranded telomeric DNA repeats are bound by the sequence-specific binding protein Rap1, which recruits Rif1 and Rif2 proteins via its C-terminal domain (Conrad et al., 1990; Hardy et al., 1992; Lustig et al., 1990; Wotton and Shore, 1997). The Rap1-Rif1-Rif2 complex creates a negative feedback loop that regulates telomere length (Levy and Blackburn, 2004; Marcand et al., 1997; Wotton and Shore, 1997), but the molecular details of this feedback mechanism are not fully understood.

In this study, we defined the functions of Rif1 and Rif2 using a system that tethers telomeric proteins adjacent to non-telomeric DNA ends. We show that Rif1 and Rif2 proteins inhibit Tel1 association, but not MRX association, with non-telomeric DNA ends. Rif2 interacts with the Xrs2 C-terminus to inhibit MRX-Tel1 interaction, whereas Rif1 appears to act through a different mechanism. We also show that the MRX complex does not efficiently associate with Rap1-bound DNA ends in the absence of Tel1. These data provide a model in which Rif1 and Rif2 cooperate with Rap1 in regulation of MRX and Tel1 localization at telomeres.

Results

Decreased MRX and Tel1 accumulation at DNA ends containing a longer TG sequence

The 81 bp TG_{81} sequence acts as a seed for the addition of telomere sequence at DNA ends (Diede and Gottschling, 1999, 2001). Although the TG_{81} sequence decreases Mec1 localization, it does not affect Tel1 localization to the nearby DNA end (Hirano and Sugimoto, 2007). Indeed, Tel1 associates effectively with short telomeres (Bianchi and Shore, 2007; Chang et al., 2007; Hector et al., 2007; Sabourin et al., 2007; Viscardi et al., 2007). To understand the mechanism by which longer telomeric sequence prevents Tel1 from localizing to DNA ends, we placed two copies of TG_{81} (the TG_{162} sequence) in tandem adjacent to the HO cleavage site (Fig. 1A). We first compared telomere addition at TG_{81} and TG_{162} ends (Fig. 1B). Cells transformed with the GAL-HO plasmid were grown in sucrose to prevent activation of the GAL promoter and arrested with nocodazole at G2/M. After arrest, cells were incubated with galactose to induce *HO* expression. DNA samples were collected at various times and examined by Southern blot analysis. DSBs were generated within one hour after *HO* expression, as shown by the appearance of cleaved fragments (CUT) and disappearance of intact DNA fragments (PRE). As found previously (Hirano and Sugimoto, 2007), the CUT fragments were elongated if the DNA end contained the TG_{81} sequence. However, no telomere addition was detected at TG_{162} ends over the course of the experiment. This observation is consistent with the recent finding that 125 bp or shorter telomeres are elongated more extensively *in vivo* (Chang et al., 2007).

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Tel1 associates with short telomeres in an MRX-dependent manner and promotes telomere addition at DNA ends (Bianchi and Shore, 2007; Chang et al., 2007; Hector et al., 2007; Sabourin et al., 2007). We next compared Mre11 and Tel1 binding to TG_{81} and TG_{162} ends (Fig. 1C and 1D). As found previously (Hirano and Sugimoto, 2007), Mre11 and Tel1 associated efficiently with TG_{81} ends. However, Mre11 and Tel1 association was greatly reduced at TG_{162} ends. MRX is required for efficient ssDNA accumulation at DNA ends (Lee et al., 1998; White and Haber, 1990). Cdc13 proteins bind to telomeric ssDNA for end protection and telomere addition (Lin and Zakian, 1996; Nugent et al., 1996; Pennock et al., 2001). We expected that Cdc13 accumulation would also be decreased at TG_{162} ends compared with TG_{81} ends. However, Cdc13 associated similarly with TG_{81} and TG_{162} ends (Fig. 1E), suggesting that high accumulation of the MRX complex is not required for creation of ssDNA tracts at TG_{162} ends. Cdc13 binds to normal length telomeres as efficiently as short telomeres (Bianchi and Shore, 2007; Chang et al., 2007; Sabourin et al., 2007). These results suggest that TG_{162} ends are converted to DNA ends that behave similarly to a normal length telomere.

Effect of *rif1Δ* or *rif2Δ* mutation on MRX and Tel1 accumulation at TG_{162} ends

Rif1 and Rif2, which interact with the C-terminus of Rap1, are known to act synergistically as negative regulators of telomere elongation (Hardy et al., 1992; Wotton and Shore, 1997). However, increased telomere elongation at TG_{81} ends was detected in *rif2Δ* cells, whereas telomere addition at TG_{81} ends was not observed in *rif1Δ* cells (data not shown; Diede and Gottschling, 1999; Frank et al., 2006). We therefore tested the effects of the *rif1Δ* or *rif2Δ* mutations on telomere synthesis on TG_{162} ends (Fig. 2A). Telomere synthesis was restored at TG_{162} ends in *rif1Δ* or *rif2Δ* cells, although the *rif1Δ* mutation exhibited a weak effect. In addition, telomere addition in *rif1Δ rif2Δ* double mutants was significantly greater than in either of the single mutants.

We then examined the effect of *rif1Δ* or *rif2Δ* mutation on Mre11 and Tel1 localization to TG_{162} ends (Fig. 2B and 2C). Consistent with a model in which MRX and Tel1 act in the same pathway and control telomere length (Ritchie and Petes, 2000), *rif1Δ* or *rif2Δ* mutations increased association of Mre11 and Tel1 proteins with TG_{162} ends. Again, the *rif2Δ* mutation conferred stronger effects than the *rif1Δ* mutation. The *rif1Δ rif2Δ* double mutation exhibited greater effects on Mre11 association than either of the singe mutations, whereas it did not apparently enhance Tel1 association compared with the *rif2Δ* single mutation. Tel1 accumulation might be therefore saturated at TG_{162} ends in the absence of Rif2. Consistent with inhibitory roles of Rif1 and Rif2 in Tel1 binding, deletion of *TEL1* significantly decreased telomere addition in *rif2Δ* single or *rif1Δ rif2Δ* double mutants (Fig. 2D). Thus, Rif1 and Rif2 are involved in attenuation of Tel1 and MRX localization to TG_{162} ends.

Since Rif1 and Rif2 play inhibitory roles at TG_{82} and TG_{162} ends, Rif1 and Rif2 should associate with these ends. We therefore addressed whether Rif1 or Rif2 binds to the TG_{81} and TG_{162} sequences before or after DSB induction (Fig. 2E and 2F). As a control, we analyzed the localization of Rif1 or Rif2 proteins to HO-induced DNA ends containing no TG sequence (HO ends). Rif1 associated with the TG_{162} sequence more efficiently than the TG_{81} sequence. Association of Rif1 with the TG_{162} repeat was detected before *HO* expression and increased three-fold after *HO* expression. Rif1 association is in part dependent on Rif2, because Rif1 association was decreased in the absence of Rif2. No apparent Rif2 binding was detected at the TG_{162} sequence before *HO* expression (Supp. Fig. 1S), and unlike Rif1, Rif2 was only modestly enriched at the TG sequence following *HO* expression. Rif2 association was also TG-length dependent; Rif2 bound more abundantly to TG_{162} than to TG_{81}. However, these results do not exclude the possibility that Rif2 binds to the TG tracts before *HO* expression, as previous studies have shown that detection of Rif2 association with telomeres by ChIP assay is lower compared with that of Rif1 association (Sabourin et al., 2007; Smith et al., 2003).

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Rap1 binds double-stranded TG repeats and recruits Rif1 and Rif2 to telomeric DNA regions (Conrad et al., 1990; Hardy et al., 1992; Lustig et al., 1990; Wotton and Shore, 1997). We found that Rap1 associated with the TG_{81} and TG_{162} repeats before *HO* expression and that Rap1 association at the TG_{162} increased two-fold after *HO* expression (Fig. 2G). Not all proteins bind to the binding sites more efficiently after DSB induction nearby. For example, association of LacI proteins with the lacO sequence was not affected after *HO* expression (Supp. Fig. 2S). No significant association of Rap1, Rif1 or Rif2 proteins was detected at HO ends (Fig. 2E, 2F and 2G). Together, these results support the idea that Rif1 and Rif2 promote the inhibition of MRX and Tel1 binding at telomeric DNA ends.

Inhibition of Tel1 localization to DNA ends by tethering of Rif1 or Rif2 nearby

The above results indicated that Rif1 and Rif2 are required to decrease MRX and Tel1 association at DNA ends containing TG sequences. However, it is not clear whether Rif1 or Rif2 directly inhibits localization of the MRX complex or Tel1. Since Rif1 and Rif2 proteins localize to double-stranded TG sequences in a Rap1-dependent manner, they could stimulate functions of Rap1 proteins and *vice versa*. To further explore Rif1 and Rif2 function, we set up a system to tether Rif1 and Rfi2 proteins adjacent to DSBs independent of Rap1 (Fig. 3A). We constructed TetR-Rif1 and TetR-Rif2 fusion genes, both of which were found to be fully functional. TetR-Rif1 and TetR-Rif2 restored wild-type telomere length to *rif1Δ* and *rif2Δ* mutants, respectively (data not shown). To target these chimeric proteins, we placed different numbers of the TetR-binding sequence (TetO) adjacent to the HO cleavage site. We observed that TetR fusion protein binding is proportional to number of the TetO sequences (Supp. Fig. 3S). To exclude the possibility that Rap1 is recruited by interacting with tethered Rif1 or Rif2, we introduced a C-terminal truncation *rap1* (*rap1-ΔC*) mutation into the strain. We first examined whether expression of TetR-Rif1 or TetR-Rif2 fusion proteins inhibits Mre11 and Tel1 binding to DNA ends containing the 8×TetO repeat ($TetO_8$ ends) (Fig. 3B and Fig. 3C). Both TetR-Rif1 and TetR-Rif2 fusion proteins decreased Tel1 binding to $TetO_8$ ends (Fig. 3B). Curiously, however, neither TetR-Rif1 nor TetR-Rif2 fusion proteins interfered with Mre11 binding (Fig. 3C). No inhibition of Tel1 binding was detected if cells contained no TetO repeat near the HO cleavage site (Supp. Fig. 4S) or expressed the TetR protein by itself (Supp. Fig. 5S). Tel1 localizes to DSBs by interacting with the C-terminus of Xrs2 (Nakada et al., 2003a). It is therefore possible that Rif1 or Rif2 disrupts Tel1 localization by inhibiting MRX complex formation. However, neither TetR-Rif1 nor TetR-Rif2 decreased Xrs2 association with $TetO_8$ ends (Fig. 3D), suggesting against this possibility. Furthermore, this Rif1/Rif2-dependent inhibition appears to be specific to Tel1, as no inhibition of Mec1 was observed (Supp. Fig. 6S). Together, these results indicate that tethering of Rif1 and Rif2 near DSB ends inhibited Tel1 accumulation but not MRX accumulation. We further addressed whether there was a dosage effect of TetR-Rif1 or TetR-Rif2 by examining Tel1 and Mre11 binding at 4×TetO ($TetO_4$) or 2×TetO ($TetO_2$) ends (Fig. 3E and 3F). Both TetR-Rif1 and TetR-Rif2 decreased Tel1 binding at $TetO_4$ ends as efficiently as at $TetO_8$ ends, whereas neither of them inhibited Tel1 binding at $TetO_2$ ends. No attenuation of Mre11 binding by TetR-Rif1 or TetR-Rif2 was detected at $TetO_2$ or $TetO_4$ ends (Supp. Fig. 7S). These results suggest that Rif1 and Rif2, once recruited to DNA ends, can operate independently of Rap1, and support the view that Rif1 and Rif2 act as dosage-dependent inhibitors of telomere lengthening (Levy and Blackburn, 2004). Our results, however, do not exclude the possibility that Rap1-recruited Rif1 and Rif2 proteins can directly decrease MRX binding at DNA ends.

As shown above, Rif1 recruitment to TG ends partially depends on Rif2 function (see Fig. 2E). Therefore, inhibition of Tel1 binding by Rif1 might require Rif2 function, and *vice versa*. To uncover Rif1- or Rif2-specific functions, we investigated the effect of TetR-Rif1 or TetR-Rif2 at $TetO_4$ or $TetO_8$ ends in *rif1Δ rif2Δ* double mutants (Fig. 3G and 3H). Inhibition of Tel1 binding by TetR-Rif1 was attenuated in *rif1Δ rif2Δ* mutant cells. Attenuation of the inhibition

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was more noticeable at $TetO_4$ ends than at $TetO_8$ ends. Introduction of the *rif1Δ* mutation did not alter TetR-Rif1-dependent inhibition, whereas the *rif2Δ* mutation exhibited the same effect as the *rif1Δ rif2Δ* mutation (Supp. Fig. 8S). In contrast, TetR-Rif2-dependent inhibition was not affected by the *rif1Δ rif2Δ* mutation. Thus, inhibition by TetR-Rif1 becomes dependent on Rif2 at DNA ends containing a short TetO array, although TetR-Rif2 exerts its function independently of Rif1. These results also suggest that Rif1 is a weaker inhibitor of Tel1 binding than Rif2.

Effect of MRX-Tel1 interaction on MRX accumulation at TG ends

Tethered Rif1/Rif2 proteins decreased Tel1 binding but not MRX binding to DSB ends. In contrast, Rif1/Rif2 proteins are required for delocalization of both Tel1 and MRX from TG ends. One explanation could be that localization of MRX to TG ends is dependent on Tel1. We therefore compared Mre11 accumulation at HO ends and TG_{81} ends in the absence of Tel1 (Fig. 4A and 4B). Mre11 associated with HO-ends in *tel1Δ* mutants as efficiently as in wild-type cells. Although detectable, Mre11 association with TG_{81} ends was significantly decreased in *tel1Δ* mutants. It is possible that Tel1-dependent phosphorylation regulates association of MRX with TG_{81} ends. However, Mre11 association was not lost in a strain harboring a kinase-deficient *tel1* (*tel1-KN*) mutation (Fig. 4C). As kinase activity is dispensable for Tel1 localization to short telomeres (Hector et al., 2007), the *tel1-KN* mutation did not affect Tel1 localization to TG_{81} ends (Fig. 4D). We also addressed whether Tel1 is required for efficient MRX localization to actual telomeric ends. Similarly to Tel1, the MRX complex binds preferentially to shortened telomeres (Viscardi et al., 2007). Telomere shortening occurs equally in cells carrying a *tel1Δ* or *tel1-KN* mutation (Supp. Fig. 9S)(Greenwell et al., 1995; Mallory and Petes, 2000). We therefore compared the association of Mre11 with telomeres VI-R and XV-L in *tel1Δ* and *tel1-KN* mutants (Fig. 4E and 4F). Mre11 association was increased in both *tel1Δ* and *tel1-KN* mutants compared with wild-type cells. However, MRX association was more pronounced in *tel1-KN* mutants than in *tel1Δ* mutants. It seems unlikely that the *tel1Δ* or *tel1-KN* mutation increases MRX binding at DNA ends containing longer TG tracts, because introduction of the *tel1Δ* mutation did not increase Mre11 localization to TG_{162} ends (Supp. Fig. 10S). These results indicate that Tel1 binding promotes MRX accumulation at telomeric DNA ends, and explain why Rif1 and Rif2 are required for inhibition of MRX association with TG ends.

Rap1-dependent inhibition of MRX accumulation at DNA ends in *tel1Δ* mutants

Since Rap1 directly binds telomeric DNA sequences, it is possible that Rap1 decreases localization of MRX to DNA ends in the absence of Tel1. We investigated whether the TetR-Rap1 fusion protein decreases Mre11 association with a DSB near the TetO sequence (Fig. 5). Rap1 contributes to the formation of heterochromatin by interacting with Sir2, Sir3 and Sir4 proteins (Grunstein, 1997). We introduced a *sir2Δ* mutation in $TetO_8$ cells, because expression of TetR-Rap1 partially decreased efficiency of HO cleavage at the $TetO_8$-HO cassette in a *SIR2*-dependent manner (data not shown). The C-terminus of Rap1 interacts with and recruits Rif1 and Rif2 (Conrad et al., 1990;Hardy et al., 1992;Lustig et al., 1990;Wotton and Shore, 1997). As shown above, tethered Rif1 or Rif2 protein decreases Tel1 localization to adjacent DNA ends. TetR-Rap1 might recruit Rif1 or Rif2 protein and thereby decrease MRX association with the DNA end. As expected, TetR-Rap1 expression partially reduced Mre11 association with $TetO_8$ ends in wild-type cells, whereas no reduction was observed in *rif1Δ rif2Δ* mutants (Fig. 5A). Likewise, TetR-Rap1 expression diminished Tel1 binding in wild-type cells but not in *rif1Δ rif2Δ* mutants (Supp. Fig. 11S). We then examined the effect of TetR-Rap1 expression on MRX association in *tel1Δ* mutants (Fig. 5B). Mre11 association was further decreased in *tel1Δ* mutants compared with wild-type cells. The observed TetR-Rap1-mediated effect in *tel1Δ* mutants did not require Rif1 or Rif2 function; Mre11 association was decreased similarly in *tel1Δ* single and *tel1Δ rif1Δ rif2Δ* triple mutants (Fig. 5B). Thus, MRX associates

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less efficiently with Rap1-bound DNA ends in *tel1Δ* mutants. As discussed above, Rif1 and Rif2 attenuate Tel1 association with adjacent DNA ends. Together, these results support the idea that once Rif1 and Rif2 delocalize Tel1, Rap1 decreases MRX binding at telomeric DNA ends.

Rif2 binds to the C-terminus of Xrs2 and inhibits the Tel1-Xrs2 interaction

To further understand the mechanism by which Rif1/Rif2 proteins inhibit Tel1 localization to DNA ends, we attempted to determine biochemical activities of Rif1 and Rif2 proteins. Tel1 interacts with the C-terminus of Xrs2 to localize to DNA ends (Nakada et al., 2003a). We therefore examined whether Rif1 or Rif2 interacts with the Xrs2 C-terminus by pull-down assay (Fig. 6A). A GST fusion of the Xrs2 C-terminus (GST-Xrs2C) or GST alone was captured on glutathione beads and incubated with extracts from cells expressing HA-tagged Rif1 or Rif2 proteins. Bound proteins were then analyzed by immunoblotting with anti-HA antibodies. Rif2-HA was precipitated by GST-Xrs2C but not GST alone. In contrast, no specific interaction between Rif1 and the Xrs2 C-terminus was detected. Furthermore, Rif1 was not precipitated with full-length Xrs2 proteins (data not shown). Thus, Rif2 interacts with the C-terminus of Xrs2, whereas Rif1 does not.

It is possible that Rif2 interacts directly with the Xrs2 C-terminus and inhibits the Tel1-Xrs2 interaction. To test this hypothesis, we first set up an *in vitro* assay to detect the Tel1-Xrs2 interaction (Fig. 6B). FLAG-tagged Tel1 protein was incubated with GST-Xrs2C or GST alone and precipitated by glutathione beads. Bound proteins were then analyzed by immunoblotting with anti-FLAG antibodies. Consistent with a model in which Tel1 interacts with the C-terminus of Xrs2, Tel1 protein was specifically pulled-down by the C-terminus of Xrs2. We then asked whether Rif2 directly inhibits the Tel1-Xrs2 interaction *in vitro* (Fig. 6B). We purified His-FLAG-tagged Rif2 proteins to near homogeneity from *E. coli* by tandem affinity column chromatography (Supp. Fig. 12S). Inclusion of Rif2 decreased Tel1-Xrs2 interaction, whereas control BSA had no effect. Moreover, higher concentrations of Rif2 increased the Rif2-Xrs2 interaction. We also found that Rif2 interacts with GST-Xrs2C but not GST alone (Fig. 6C). Thus, Rif2 competes with Tel1 for binding to the Xrs2 C-terminus *in vitro*.

We mapped the region of Rif2 involved in Xrs2 binding (Fig. 6D). Purified N-terminal (amino acids 1-170), central (amino acids 91-250) or C-terminal (amino acids 171-395) fragments of Rif2 protein were incubated with GST-Xrs2C or GST and interaction was analyzed by pull-down assay as above. The N-terminal fragment was found to bind to the Xrs2 C-terminus. However, no interaction with Xrs2 was observed for the central or C-terminal fragment. We also found that tethering of the Rif2 N-terminus inhibited Tel1 localization to DNA ends, whereas the central or C-terminal region did not (Fig. 6E). These results indicate that the N-terminus of Rif2 plays a key role in the interaction with the Xrs2 C-terminus. Taken together, our results support a model in which Rif2 binds to the Xrs2 C-terminus and blocks the recruitment of Tel1 to the MRX complex at DNA ends.

Discussion

As natural DNA ends, telomeres must escape recognition by checkpoint proteins that activate the DNA damage response pathway. In budding yeast, Cdc13-mediated telomere capping inhibits Mec1 localization to the telomeric DNA end, but does not affect Tel1 accumulation. Recent evidence supports a model in which Tel1 preferentially associates with short telomeres and promotes telomere synthesis. However, it has not been determined how Tel1 accumulation at extended telomeres is inhibited. Rap1 binds double-stranded TG repeats and recruits Rif1 and Rif2 to telomeres via its C-terminal domain (Conrad et al., 1990; Hardy et al., 1992; Lustig et al., 1990; Wotton and Shore, 1997). In this study, we show that binding of multiple Rif1 and Rif2 proteins decreases localization of Tel1 to adjacent DNA ends through two different

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mechanisms. First, Rif1 and Rif2 inhibit recruitment of Tel1 to MRX at DNA ends. Second, in the absence of Tel1, Rap1 attenuates MRX association with DNA ends. Together, these results indicate that Rif1 and Rif2 co-operate with Rap1 and decrease Tel1 accumulation at extended telomeres (Fig. 7).

Tel1 localizes to DNA ends by interacting with the C-terminus of Xrs2 (Nakada et al., 2003a; Sabourin et al., 2007). It is possible that Rif1 and Rif2 decrease Tel1 localization to DNA ends by interfering with the Tel1-Xrs2 interaction. Supporting this hypothesis, Rif2 directly binds to the C-terminus of Xrs2 and inhibits the Tel1-Xrs2 interaction *in vitro* (see Fig. 6). Even though Rif1 and Xrs2 interact with each other, the Rif2-Xrs2 interaction may be weak compared with the Tel1-Xrs2 interaction. For example, MRX recruits Tel1, but not Rif2, to DSBs (see Fig. 2). Moreover, TetR-Rif2 does not recruit MRX to a region adjacent to the $TetO_8$ sequence (see Fig. 3). These observations support a model in which Rif2 inhibits the Tel1-Xrs2 interaction by covering the Tel1 binding site on Xrs2 if multiple Rif2 proteins are placed nearby. It has not been determined whether Rif2 and Tel1 interact with the same sequence on the Xrs2 C-terminus; it therefore remains possible that Rif2 recognizes a different sequence from the Tel1 binding site and stimulates dissociation of Tel1 from MRX at telomeric ends. Interaction of Rif1 with Xrs2 has not been detected; thus, it remains unclear how Rif1 inhibits Tel1 localization at DNA ends.

Rif1 and Rif2 appear to have different functions, consistent with the fact that they are unrelated. First, Rif2 is a more potent inhibitor of Tel1 localization than Rif1, as shown by the observation that TetR-Rif2 fusion decreases Tel1 localization at TetO ends more effectively than the TetR-Rif1 fusion (see Fig. 3G and 3H). Initial telomere addition occurs at TG_{162} ends more quickly in *rif2Δ* mutants than *rif1Δ* mutants (see Fig. 2A). However, like native telomeres, TG_{162} ends become longer in *rif1Δ* mutants than in *rif2Δ* mutants (data not shown; Wotton and Shore, 1997). Thus, Rif1 plays a more important role at longer TG repeats, suggesting that Rif1 acts in a more dosage-dependent manner than Rif2. Secondly, Rif1 function is partially dependent on Rif2; inhibition of Tel1 localization by TetR-Rif1 is decreased in the absence of Rif2 (see Fig. 3G and 3H). These different properties of Rif1 and Rif2 could explain why decreased binding of Rif2 alone leads to Tel1 enrichment at short telomeres (Sabourin et al., 2007). Several lines of evidence indicate that Rif1 association with telomeres fluctuates during cell-cycle progression, peaking at G2/M (Sabourin et al., 2007; Smith et al., 2003). Increased Rif1 binding at the TG_{162} sequence after DSB induction might result from telomere formation at the TG ends in G2/M-arrested cells (see Fig. 2E).

Rif1 homologs have been identified and characterized in fission yeast and human (Kanoh and Ishikawa, 2001; Silverman et al., 2004; Xu and Blackburn, 2004). Although human Rif1 is not involved in telomere length control, fission yeast Rif1 localizes to telomeres and negatively regulates telomere length, suggesting that Rif1 also controls Tel1 localization at telomeres in fission yeast. There are no recognizable Rif2 homologs in other eukaryotes. Even though the N-terminal half of Rif2 can interact with the Xrs2 C-terminus (see Fig. 6D), it does not share meaningful similarities with other proteins. The C-termini of Nbs1 and Xrs2 are involved in binding to ATM and Tel1, respectively (Falck et al., 2005; Nakada et al., 2003a; You et al., 2005). However, the Nbs1/Xrs2 proteins are not highly conserved compared with Mre11 and Rad50 proteins. It is possible that Rif2 has evolved by adapting to the diversification of Nbs1/Xrs2 proteins.

Rif1 and Rif2 inhibit Tel1 localization, but not MRX localization, to DNA ends independent of Rap1 function (see Fig. 3). In the absence of Tel1, however, Rap1 decreases MRX accumulation at DNA ends independent of Rif1 or Rif2 (see Fig. 5). It is therefore possible that once Rif1 and Rif2 delocalize Tel1 from Rap1-covered telomeric DNA ends, Rap1 could promote the dissociation of MRX from the ends. Since Rif2 interacts with the C-terminus of

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Xrs2, it might transiently tether MRX to telomeric DNA ends; however, Rap1 could subsequently remove. Supporting this model, Rif2 overexpression has been shown to inhibit MRX association with telomeres (Viscardi et al., 2007). Thus, the Rap1-Rif1-Rif2 complex appears to act as a highly organized machinery to remove MRX and Tel1 from extended telomeres. At present, it is not known why MRX requires Tel1 protein to associate efficiently with telomeric DNA ends. We speculate that MRX might associate more stably with histone-bound DNA than Rap1-covered DNA.

Rap1 can recruit Rif1 and Rif2 not only to telomeric DNA ends but also to intra-chromosomal regions (see Fig. 2E and 2F). On natural chromosomes, however, extensive Rap1 binding is observed mostly at telomeric DNA regions (Smith et al., 2003). Telomeres might possess specific chromatin structures that promote Rap1 binding, since Rap1 binding at TG repeats increases slightly after DSB induction (see Fig. 2G). Telomeres are extended to 4 kb, but not significantly longer, in cells expressing C-terminal truncated Rap1 proteins or in cells carrying *rif1Δ rif2Δ* double mutation (Kyrion et al., 1992; Wotton and Shore, 1997). It is thus possible that extensive binding of Rap1 on longer telomeres blocks MRX association before recruiting Tel1 to the DNA end. In this situation, Rif1 or Rif2 would not be required for inhibition of MRX localization. Consistent with this view, binding of multiple Rap1 proteins has been shown to decrease MRX accumulation at DNA ends independently of the Rap1 C-terminus (Negrini et al., 2007). In budding yeast, MRX plays an essential role in non-homologous end-joining (NHEJ) as well as homologous recombination repair of DSBs (D'Amours and Jackson, 2002; Krogh and Symington, 2004). In agreement with an inhibitory role of MRX localization, Rap1 prevents telomere fusions by NHEJ (Pardo and Marcand, 2005). Rap1 homologs have been identified as telomeric proteins across species (Smogorzewska and de Lange, 2004; Vega et al., 2003). Human RAP1 is also involved in protecting telomeres from NHEJ (Bae and Baumann, 2007). The conservation of Rap1/RAP1 suggests that Rap1/RAP1 proteins universally protect telomeres from DSB repair machinery.

In summary, we have shown that Rif1 and Rif2 cooperate with Rap1 to shelter telomeric DNA ends from Tel1 recognition. In mammals, the telomeric DNA binding protein TRF2 is involved in inhibition of ATM activation at telomeres (Karlseder et al., 1999). TRF2 interacts with the Mre11-Rad50-Nbs1 complex (Zhu et al., 2000) and recruits other telomere binding proteins to telomeres (Chen et al., 2008). It will be interesting to see whether telomere binding proteins play a similar role in inhibition of ATM localization in mammalian cells.

Experimental Procedures

Strains and plasmids

Cells containing the TG_{162}-HO, $tetO_2$-HO, $tetO_4$-HO or $tetO_8$-HO cassette were generated by the plasmid pTG_{162}-HO, $ptetO_2$-HO, $ptetO_4$-HO or $ptetO_8$-HO, respectively. Cells carrying the TG_{81}-HO cassette were described previously (Hirano and Sugimoto, 2007). The *rap1-ΔC* strain was constructed by transformation with the PCR fragment amplified from pDL106 as described (Levy and Blackburn, 2004). All the strains used in this study are isogenic to KSC1516 (Nakada et al., 2003a). Details of the strain and plasmid construction are described in Supplemental Information.

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation was performed using anti-HA (16B2) or anti-myc (9E10) antibodies as previously described (Hirano and Sugimoto, 2007). Quantification of immunoprecipitated DNAs was achieved by using a real-time PCR detection system (Bio-Rad). Relative enrichment was determined by normalizing signals from a region near the DSB (HO), telomere VI-R or telomere XV-L to control signals from a region in *SMC2*. The signals

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were then normalized to input signals for each primer set. Enrichment is defined as accumulation of proteins at the DSB or the telomere relative to that at the *SMC2* locus. Sequence of the PCR primers is described in Supplementary Information.

Pull-down assay

FLAG-Tel1 proteins were purified from budding yeast cells. GST, GST-Xrs2C, His-Rif2-FLAG or His-Rif2 fragments were purified from *E. coli*. Details for purification were described in Supplementary information. The interaction of Rif1 or Rif2 with the Xrs2 C-terminus was examined using extracts from cells expressing Rif1-HA or Rif2-HA. Purified GST or GST-Xrs2C was coupled to CNBr-activated Sepharose 4B. Extracts were prepared in the lysis buffer by glass-bead beating as described in Supplementary information, and incubated with GST- or GST-Xrs2C-coupled Sepharose beads. Since Rif2-HA is expressed two-fold more than Rif1-HA, extracts from Rif2-HA expressing cells were mixed with extracts from *rif2Δ* cells. After incubation for 120 min at 4°C, Sepharose beads were washed three times and bound proteins were analyzed by immunoblotting. Interaction of purified Tel1 and Rif2 with Xrs2 was examined as follows. Purified GST or GST-Xrs2C was bound to glutathione sepharose beads and then incubated with purified FLAG-Tel1 or purified Rif2 proteins in the binding buffer (20 mM Tris-HCl at pH 8.0, 100 mM NaCl, 0.01% Triton X-100) at 4°C for 1 hr. Bound proteins were released from the beads by addition of glutathione (reduced form) at a final concentration of 10 mM, and characterized by immunoblotting analysis.

Other methods

Immunoblotting analysis was performed using anti-FLAG, anti-T7 or anti-HA antibodies as described (Hirano and Sugimoto, 2007). Southern blotting was performed as previously described (Hirano and Sugimoto, 2007).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank C. Newlon and H. Araki for critical reading and discussion; Y. Tanaka for construction of pGAL-FLAG-Tel1; D. Nakada for construction of pGST-XRS2C; H. Araki, E. Blackburn, A. Matsuura, D. Shore, A. Straight and V. Zakian for materials. This work was supported by National Institutes of Health grant GM073876.

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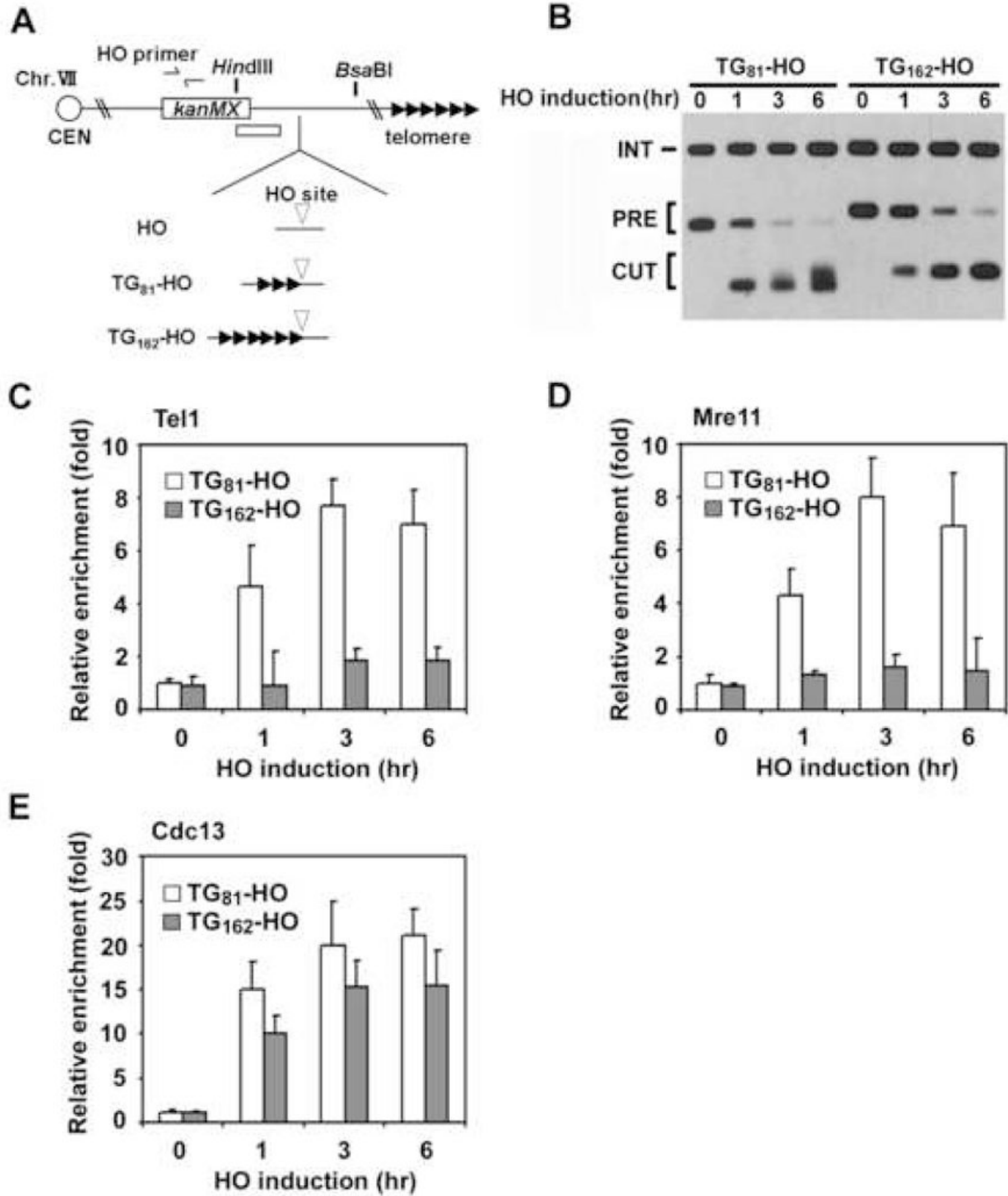

Fig. 1. Effect of the 162 bp TG repeats on HO-induced DNA ends

(A) Schematic of the HO cleavage site and the TG repeats at the *ADH4* locus on chromosome VII-L. In HO cells, the *ADH4* locus was replaced with the cassette containing the *KanMX* gene and an HO cleavage site (inverted triangle). 81 bp or 162 bp of TG repeat sequence (three or six repetitive arrowheads) was placed centromere-proximal to the HO site in TG_{81}-HO cells or TG_{162}-HO cells, respectively. Centromere is shown as a circle on the left (CEN). The white bar indicates a probe used to monitor telomere synthesis by Southern blot. The HO primer pair was designed to amplify a region 1 kb away from the HO site.

(B) Effect of the 162 bp TG repeats on telomere addition. TG_{81}-HO and TG_{162}-HO cells were transformed with the GAL-HO plasmid. Transformed cells were grown in sucrose and

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synchronized at G2/M with nocodazole. After arrest, galactose was added to the culture to induce *HO* expression. Aliquots of cells were collected at the indicated times after *HO* expression. Genomic DNA was digested with *Hin*dIII and *Bsa*BI and then analyzed by Southern blot using the probe shown in A. The band labeled PRE indicates the 1 kb or 1.1 kb *Hin*dIII-*Bsa*BI fragment containing the HO site. After cleavage with HO, this band is converted into a new band (0.6 or 0.7 kb, marked CUT). Telomere addition retards migration of the CUT fragment. The probe also detects a 1.8 kb *Hin*dIII fragment from the *SMC2* locus on chromosome VI. This band is marked INT and serves as a loading control.
(C-E). Association of Tel1, Mre11 and Cdc13 with TG_{81} or TG_{162} ends. TG_{81}-HO and TG_{162}-HO cells expressing Tel1-HA (C), Mre11-myc (D) or Cdc13-myc (E) were transformed with the GAL-HO plasmid, and cultured as in B. Aliquots of cells were collected at the indicated times after *HO* expression and subjected to ChIP assay. Co-precipitated DNA was analyzed by real-time PCR using the HO primer pair shown in A and the SMC2 primer pair for the control *SMC2* locus on chromosome VI. Relative enrichment was determined from three independent experiments, and bars represent averages with standard deviations indicated by lines above.

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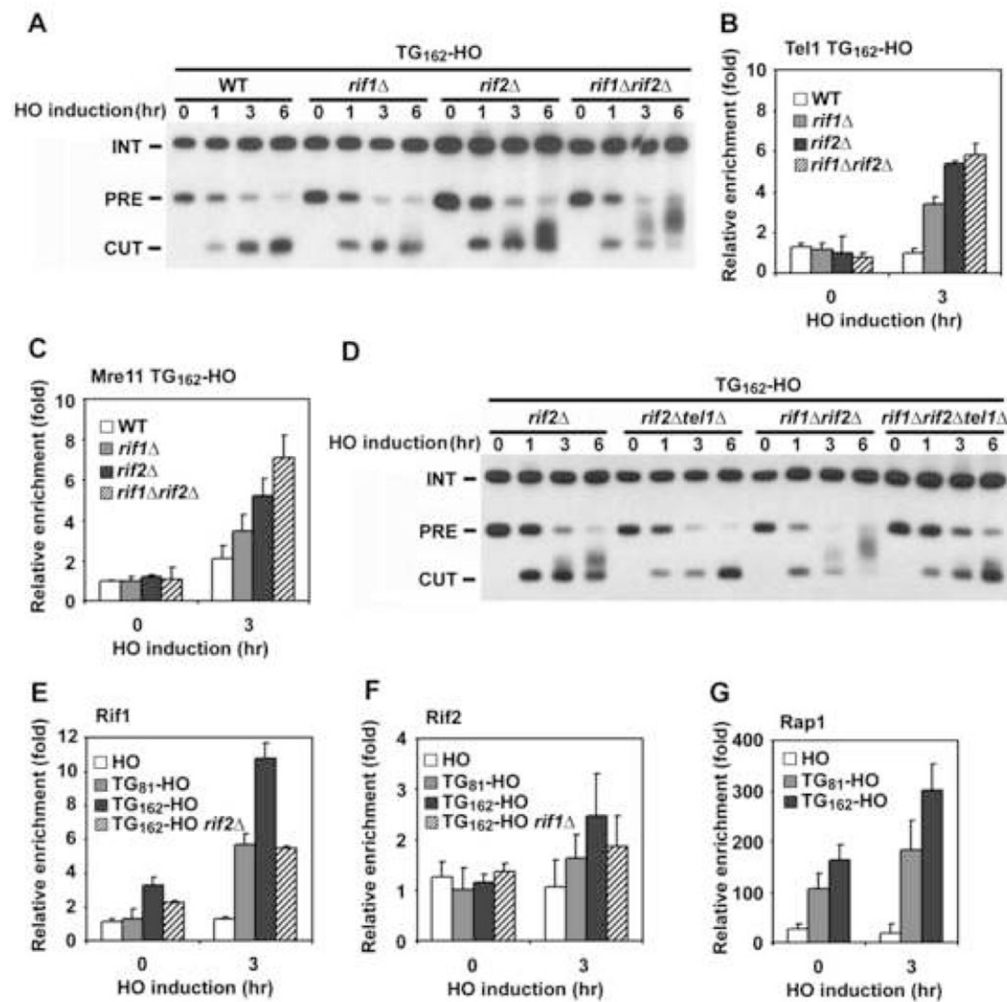

Fig. 2. Effect of *rif1Δ* or *rif2Δ* mutation on association of Tel1 and Mre11 with TG_{162} ends

(A) Effect of *rif1Δ* or *rif2Δ* mutation on telomere addition at TG_{162} ends. TG_{162}-HO cells were analyzed by Southern blot as in Fig. 1B.

(B, C) Effect of *rif1Δ* or *rif2Δ* mutation on association of Tel1 and Mre11 with TG_{162} ends. TG_{162}-HO cells expressing Tel1-HA (B) or Mre11-myc (C) were analyzed by ChIP assay as in Fig. 1C.

(D) Effect of *tel1Δ* mutation on telomere addition at TG_{162} ends in *rif2Δ* or *rif1Δ rif2Δ* mutants. TG_{162}-HO cells were analyzed by Southern blot as in A.

(E) Association of Rif1 with TG_{81} or TG_{162} ends. HO, TG_{81}-HO, TG_{162}-HO and TG_{162}-HO *rif2Δ* cells expressing Rif1-HA were analyzed by ChIP assay as in B.

(F) Association of Rif2 with TG_{81} or TG_{162} ends. HO, TG_{81}-HO, TG_{162}-HO and TG_{162}-HO *rif1Δ* cells expressing Rif2-HA were analyzed by ChIP assay as in B.

(G) Association of Rap1 with TG_{81} or TG_{162} ends. HO, TG_{81}-HO, TG_{162}-HO and TG_{162}-HO cells expressing Rap1-HA were analyzed by ChIP assay as in B.

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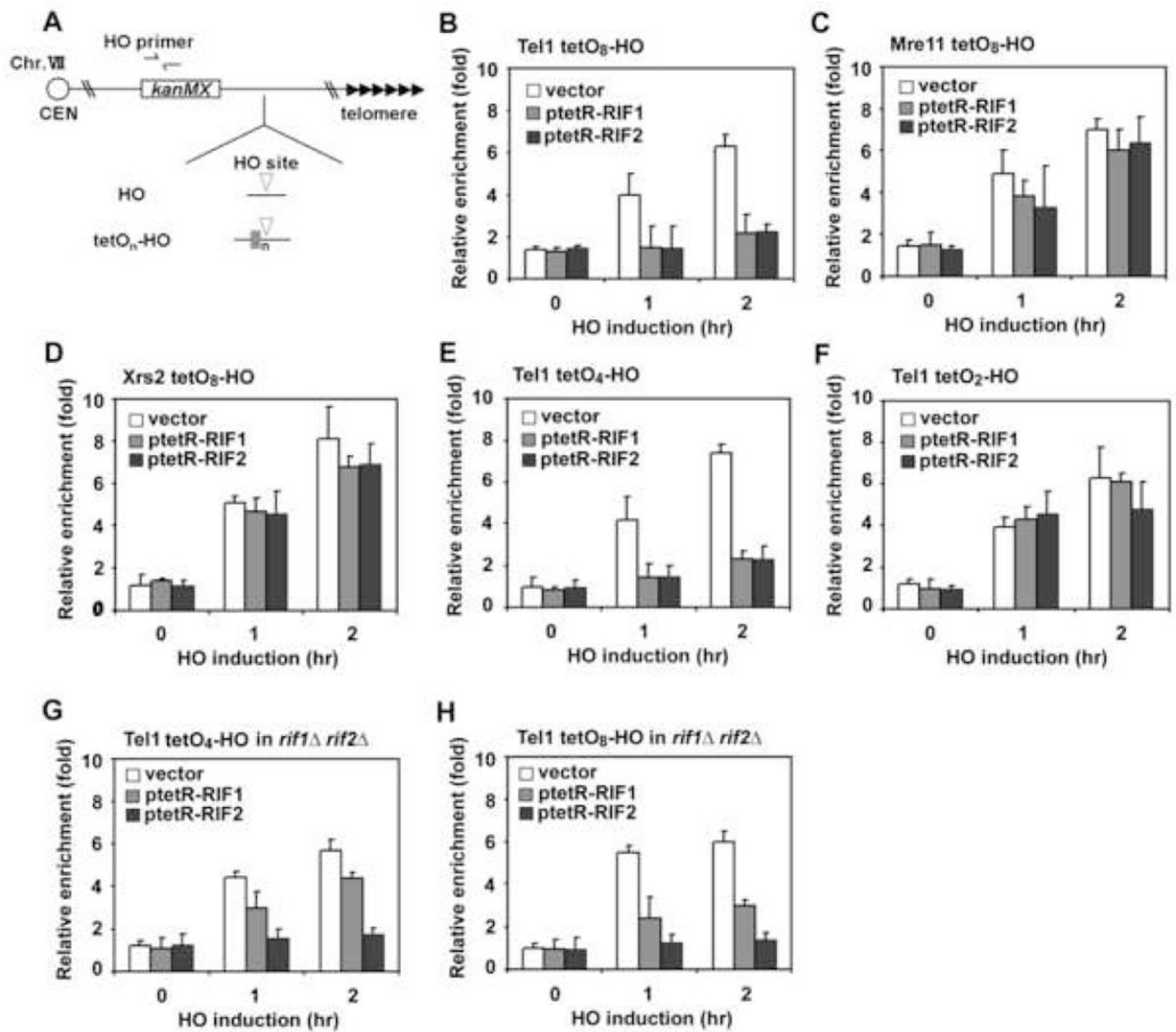

Fig. 3. Effect of Rif1 or Rif2 tethering on association of Tel1 and Mre11 with nearby DSBs

(A) Schematic of the HO cleavage site and the adjacent TetO arrays at the *ADH4* locus on chromosome VII-L. The *ADH4* locus was replaced with the cassettes containing the *KanMX* gene, an HO cleavage site (inverted triangle) and different copies (n) of the TetO sequence (a grey box). The *RAP1* gene was replaced with a *rap1-ΔC* mutation. The HO primer pair was designed to amplify a region 1 kb away from the HO site (see Fig. 1A).

(B, C) Effect of TetR-Rif1 or TetR-Rif2 expression on association of Tel1 and Mre11 with $TetO_8$ ends. $TetO_8$-HO cells expressing Tel1-HA (B) or Mre11-myc (C) were transformed with ptetR-RIF1, ptetR-RIF2 or the control vector, together with the GAL-HO plasmid, and analyzed by ChIP assay as in Fig. 1C.

(D) Effect of TetR-Rif1 or TetR-Rif2 expression on association of Xrs2 with $TetO_8$ ends. $TetO_8$-HO cells expressing Xrs2-myc were analyzed as in B.

(E, F) Effect of TetR-Rif1 or TetR-Rif2 expression on association of Tel1 with $TetO_2$ ends or $TetO_4$ ends. $TetO_4$-HO (E) or $TetO_2$-HO cells (F) expressing Tel1-HA were analyzed by ChIP assay as in B.

(G, H) Effect of TetR-Rif1 or TetR-Rif2 expression on association of Tel1 in the absence of Rif1 and Rif2. $TetO_4$-HO *rif1Δ rif2Δ* (G) or $TetO_8$-HO *rif1Δ rif2Δ* cells (H) expressing Tel1-HA were analyzed as in B.

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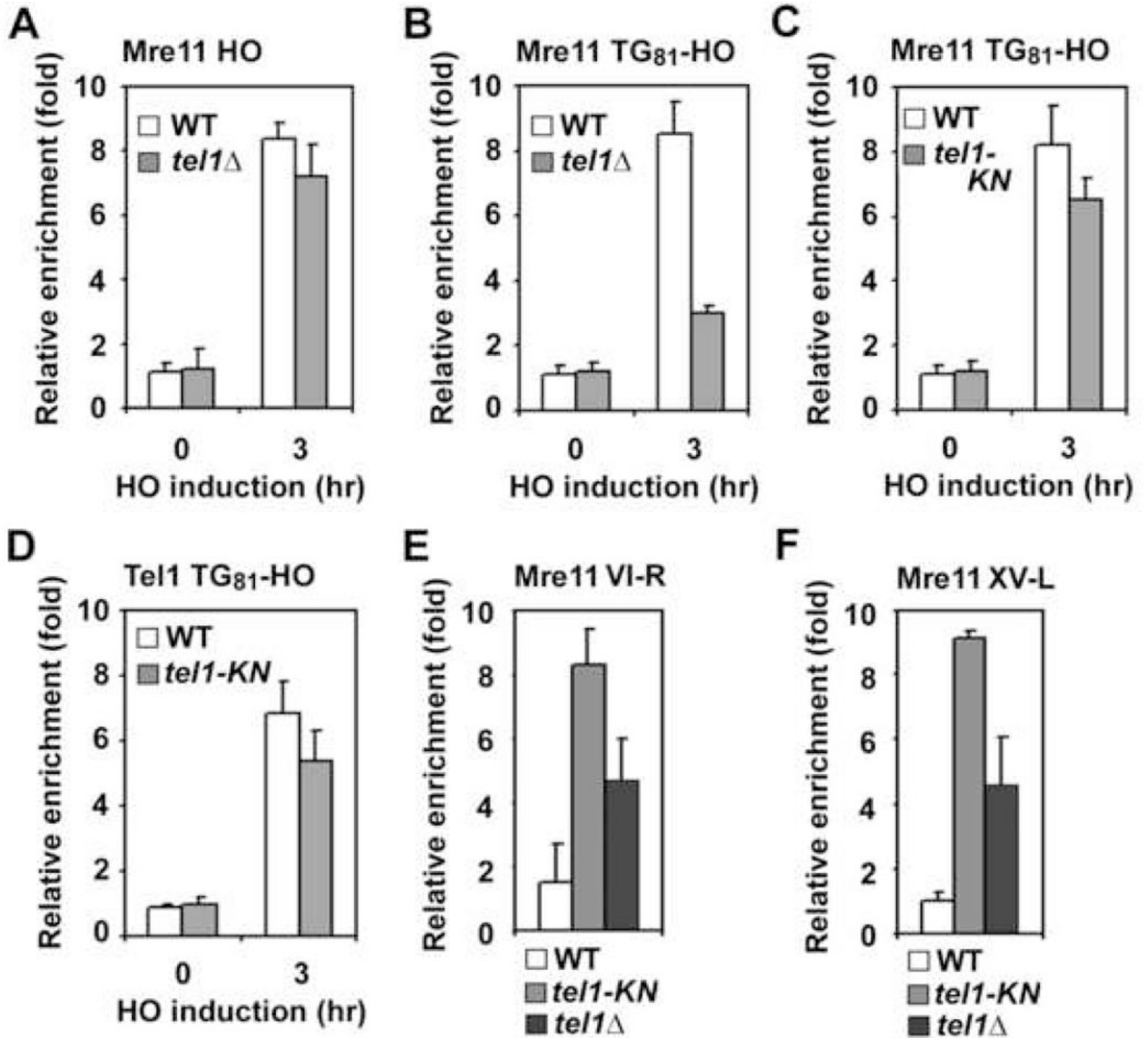

Fig. 4. Effect of Tel1-MRX interaction on Mre11 association with telomeric DNA ends

(A, B) Effect of *tel1Δ* mutation on Mre11 association with HO-induced DSBs or TG_{81} ends. HO (A) or TG_{81}-HO (B) cells expressing Mre11-myc were analyzed by ChIP assay as in Fig. 1C. Strains used carried the wild-type *TEL1* gene or *tel1Δ* mutation.

(C, D) Effect of *tel1-KN* mutation on Mre11 association with TG_{81} ends. TG_{81}-HO cells expressing Mre11-myc and wild-type Tel1-HA or mutant Tel1-KN-HA proteins were analyzed by ChIP assay as in A to monitor Mre11 association (C) or Tel1 association (D).

(E, F) Effect of *tel1-KN* or *tel1Δ* mutation on Mre11 association with the telomere VI-R or XV-L. Wild-type, *tel1-KN* or *tel1Δ* cells expressing Mre11-myc were synchronized at G2/M with nocodazole and subjected to ChIP assay as in A. Mre11 association with the telomere VI-R (E) or XV-L (F) were monitored.

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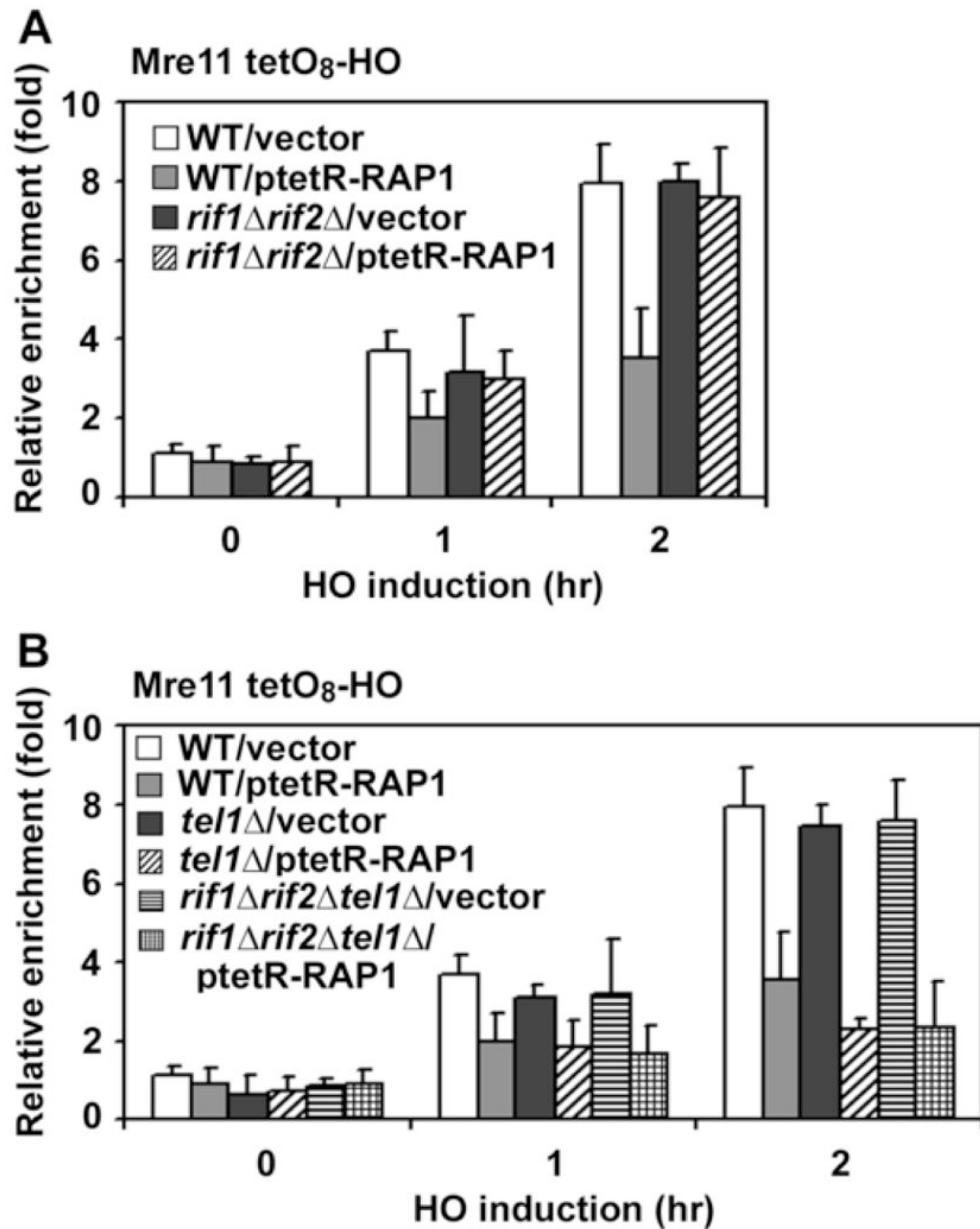

Fig. 5. Inhibitory effect of TetR-Rap1 expression on Mre11 accumulation at $TetO_8$ ends

(A) Effect of TetR-Rap1 expression on Mre11 association in *rif1Δ rif2Δ* cells. $TetO_8$-HO or $TetO_8$-HO *rif1Δ rif2Δ* cells expressing Mre11-myc were transformed with ptetR-RAP1 or the control vector, together with the GAL-HO plasmid, and analyzed as in Fig. 1C to monitor Mre11 association. The strains used here contained a *sir2Δ* mutation to suppress inefficient HO cleavage.

(B) Effect of TetR-Rap1 expression on Mre11 association in *tel1Δ* or *tel1Δ rif1Δ rif2Δ* cells. $TetO_8$-HO, $TetO_8$-HO *tel1Δ* or $TetO_8$-HO *tel1Δ rif1Δ rif2Δ* cells expressing Mre11-myc were examined as in A.

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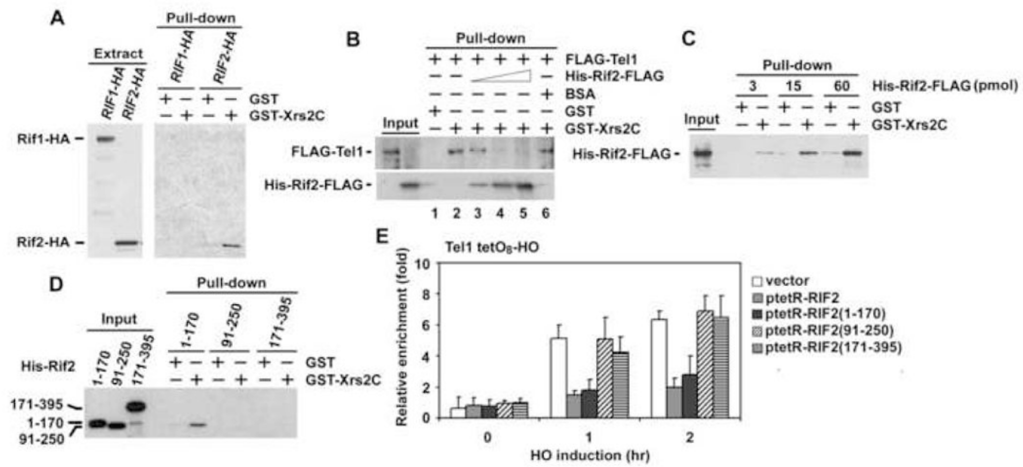

Fig. 6. Interaction of Rif2 with Xrs2 C-terminus

(A) Interaction of Rif1 and Rif2 with Xrs2 C-terminus. GST or GST-Xrs2C was immobilized on CNBr-activated Sepharose, and incubated with yeast extracts (2 mg of protein) containing Rif1-HA or Rif2-HA. One fifth of the eluted proteins were resolved on a SDS-gel for immunoblotting analysis with anti-HA antibodies (Pull-down), whereas cell extracts (7 μg of protein) were analyzed as control.

(B) Effect of Rif2 on Tel1 interaction with the C-terminus of Xrs2. FLAG-Tel1 or His-Rif2-FLAG was incubated with GST or GST-Xrs2C and glutathione beads. Proteins bound to glutathione beads were subjected to immunoblotting analysis with anti-FLAG antibodies. 0.1 pmol of FLAG-Tel1 and 3 pmol of GST or GST-Xrs2C were used for the binding assay. His-Rif2-FLAG was added in the reaction at the dose of 3, 15 or 60 pmol (lanes 3-5). 3 μg of BSA, which is equivalent to 60 pmol of His-Rif2-FLAG, was added in the reaction as a control (lane 6).

(C) Interaction of Rif2 with the C-terminus of Xrs2. The same amounts of His-Rif2-FLAG as those used in B were incubated with 3 pmol of GST or GST-Xrs2C, and pulled down with glutathione beads. Bound proteins were analyzed by immunoblotting with anti-FLAG antibodies.

(D) Interaction of the Rif2 N-terminus with the Xrs2 C-terminus. 3 pmol of His-Rif2 fragments were incubated with 3 pmol of GST or GST-Xrs2C and then precipitated by glutathione beads. All the His-tagged proteins contain a T7 epitope at the N-terminus. Bound proteins were analyzed by immunoblotting with anti-T7 antibodies.

(E) Association of Tel1 with $TetO_8$ ends in cells expressing TetR-Rif2 fusion proteins. $TetO_8$-HO cells expressing Tel1-HA were transformed with ptetR-RIF2, ptetR-RIF2(1-170), ptetR-RIF2(91-250), ptetR-RIF2(171-395) or the control vector, together with the GAL-HO plasmid, and analyzed by ChIP assay as in Fig. 1C.

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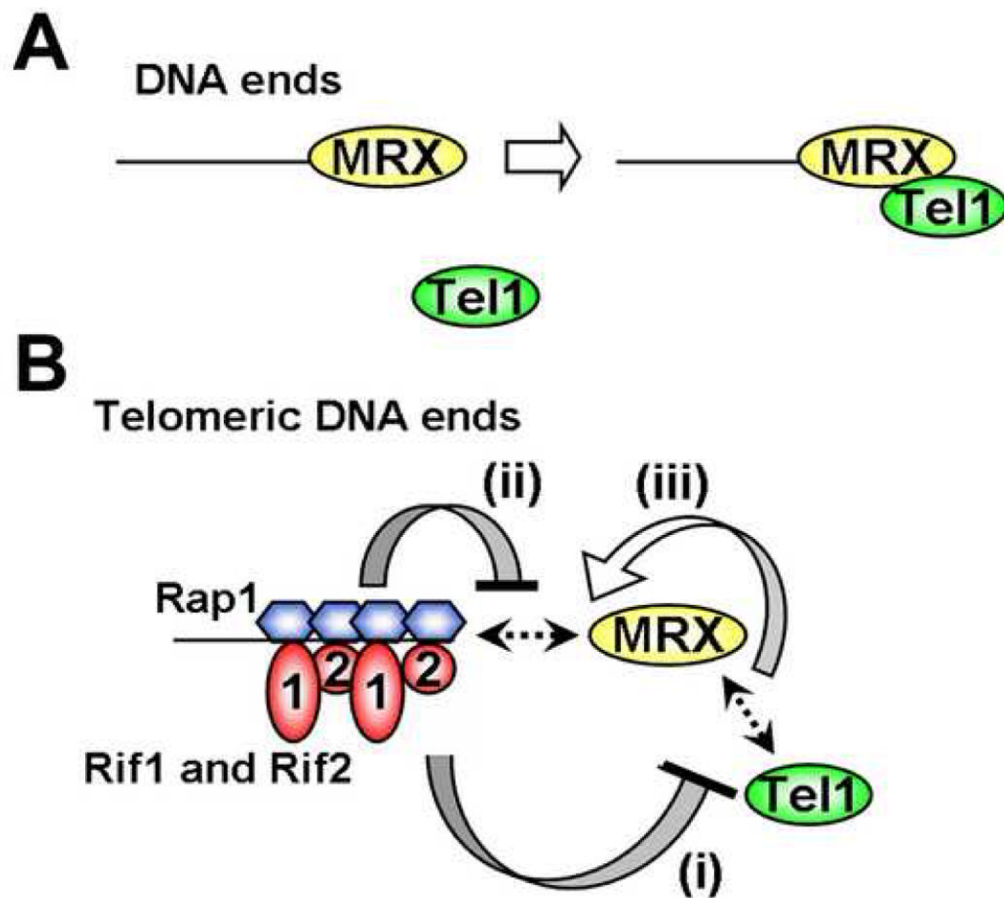

Fig. 7. Model for localization of MRX and Tel1 to telomeric DNA ends

(A) MRX-dependent localization of Tel1 to DNA ends. MRX recognizes DNA ends and then recruits Tel1 to the DNA ends. MRX associates with DNA ends independently of Tel1.

(B) Inhibitory network for MRX and Tel1 localization at telomeric DNA ends.

(i) Rif1 and Rif2 by themselves inhibit MRX-dependent Tel1 localization to DNA ends. Rif2 competes with Tel1 for binding to the C-terminus of Xrs2. It is not known how Rif1 inhibits Tel1 localization to DNA ends.

(ii) MRX does not efficiently localize to Rap1-bound DNA ends in the absence of Tel1.

(iii) MRX-Tel1 interaction could stabilize MRX association with Rap1-bound DNA ends. If Rif1 and Rif2 disrupt Tel1-MRX interaction at DNA ends, MRX could no longer associate efficiently with Rap1-bound DNA ends.