

# Subtelomere-binding protein Tbf1 and telomere-binding protein Rap1 collaborate to inhibit localization of the Mre11 complex to DNA ends in budding yeast

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**ABSTRACT** Chromosome ends, known as telomeres, have to be distinguished from DNA double-strand breaks that activate DNA damage checkpoints. In budding yeast, the Mre11-Rad50-Xrs2 (MRX) complex associates with DNA ends and promotes checkpoint activation. Rap1 binds to double-stranded telomeric regions and recruits Rif1 and Rif2 to telomeres. Rap1 collaborates with Rif1 and Rif2 and inhibits MRX localization to DNA ends. This Rap1-Rif1-Rif2 function becomes attenuated at shortened telomeres. Here we show that Rap1 acts together with the subtelomere-binding protein Tbf1 and inhibits MRX localization to DNA ends. The placement of a subtelomeric sequence or TTAGGG repeats together with a short telomeric TG repeat sequence inhibits MRX accumulation at nearby DNA ends in a Tbf1-dependent manner. Moreover, tethering of both Tbf1 and Rap1 proteins decreases MRX and Tel1 accumulation at nearby DNA ends. This Tbf1- and Rap1-dependent pathway operates independently of Rif1 or Rif2 function. Depletion of Tbf1 protein stimulates checkpoint activation in cells containing short telomeres but not in cells containing normal-length telomeres. These data support a model in which Tbf1 and Rap1 collaborate to maintain genomic stability of short telomeres.

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## INTRODUCTION

Double-strand breaks (DSBs) are induced by exogenous DNA-damaging agents and carcinogens or by endogenous byproducts including reactive oxygen species. The repair of DSBs is crucial for maintaining genome stability (Pierce *et al.*, 2001; O'Driscoll and Jeggo, 2006). Linear eukaryotic chromosomes have specialized structures at their ends called telomeres (Vega *et al.*, 2003; Smogorzewska and de Lange, 2004; Louis and Vershinin, 2005). Telomeres prevent the recognition of chromosome ends as DSBs,

but dysfunctional or short telomeres behave like DSB lesions and initiate the DNA damage response (Longhese, 2008; Palm and de Lange, 2008). The cellular responses to DSBs consist of activation of checkpoint signaling and DNA repair processes (Zhou and Elledge, 2000; Harrison and Haber, 2006).

Checkpoint signals are initiated through two large protein kinases—ataxia-telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) (Zhou and Elledge, 2000; Abraham, 2001). ATM and ATR are highly conserved among eukaryotes. In budding yeast, ATM and ATR correspond to Tel1 and Mec1, respectively (Harrison and Haber, 2006). Several lines of evidence have established that the Mre11-Rad50-Nbs1 (Xrs2 in budding yeast) complex is the primary sensor that recruits ATR/Mec1 and ATM/Tel1 to DSBs (Nakada *et al.*, 2003a, 2004; Falck *et al.*, 2005; You *et al.*, 2005). In budding yeast, the Mre11-Rad50-Xrs2 (MRX) complex recruits Tel1 to DNA ends through its interaction with Xrs2 (Nakada *et al.*, 2003a) and modulates Tel1 catalytic activity at DNA ends (Fukunaga *et al.*, 2011). MRX collaborates with Sae2 and initiates the generation of single-stranded DNA (ssDNA) at DSB ends (Krogh and Symington, 2004). The heterotrimeric replication protein A (RPA) binds ssDNA with

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Abbreviations used: ATM, ataxia-telangiectasia mutated; ChIP, chromatin immunoprecipitation; DSB, double-strand break; 5-FOA, 5-fluoroorotic acid; MMS, methyl methanesulfonate; MRX, Mre11-Rad50-Xrs2; RPA, replication protein A; STR, subtelomeric repeat element.

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high affinity and plays a pivotal role in homologous recombination repair of DSBs (Wold, 1997; Krogh and Symington, 2004). Mec1 accumulates at DNA ends through its interaction with the RPA-ssDNA complex (Zou and Elledge, 2003; Nakada *et al.*, 2005). Mec1 and Tel1 subsequently phosphorylate the downstream kinase, Rad53, and contribute to full activation of the kinase (Schwartz *et al.*, 2002; Sweeney *et al.*, 2005). Activation of the Mec1/Tel1-Rad53 pathway leads to transient cell-cycle arrest and transcriptional activation of genes involved in DNA repair (Harrison and Haber, 2006).

Telomeres contain a double-stranded DNA region of tandem repeats (e.g., vertebrates and most higher eukaryotes, T<sub>2</sub>AG<sub>3</sub>; budding yeast, TG<sub>1-3</sub>) and a 3' protruding ssDNA region of the G-rich strand (Louis and Vershinin, 2005; Palm and de Lange, 2008). 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 forms a complex with Stn1 and Ten1 and acts as a telomere cap to protect telomeres from degradation (Garvik *et al.*, 1995; Nugent *et al.*, 1996; Grandin *et al.*, 2001; Pennock *et al.*, 2001; Petreaca *et al.*, 2007; Xu *et al.*, 2009). Cdc13-mediated telomere capping inhibits RPA recruitment and subsequent Mec1 accumulation (Rouse and Jackson, 2002; Hirano and Sugimoto, 2007) but does not affect accumulation of Tel1 or the MRX complex at DNA ends (Hirano and Sugimoto, 2007). Several lines of evidence have established 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). Short telomeric TG repeat acts as a seed for the addition of telomere sequence at DNA ends (Diede and Gottschling, 1999). MRX or Tel1 associates efficiently with DNA breaks adjacent to a short (81 base pair) telomeric TG repeat, whereas little or no Mre11 or Tel1 binding is detected at DNA ends next to long telomere tracts (Negrini *et al.*, 2007; Hirano *et al.*, 2009). 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; Lustig *et al.*, 1990; Hardy *et al.*, 1992; Wotton and Shore, 1997). Rif1 and Rif2 inhibit Tel1 association, but not MRX association, with DNA ends (Hirano *et al.*, 2009). Rap1 inhibits MRX localization to DNA ends, but inhibition is not effective in the absence of Rif1 and Rif2 (Negrini *et al.*, 2007; Hirano *et al.*, 2009). If Tel1 is absent, however, Rap1 does not require Rif1 or Rif2 function to repress MRX localization (Hirano *et al.*, 2009). Thus Rap1 collaborates with Rif1 and Rif2 and inhibits MRX accumulation at telomeres. Rif2 competes with Tel1 for binding to the C-terminus of Xrs2 (Hirano *et al.*, 2009), but the functions of Rap1 or Rif1 remain to be determined.

Telomeres are connected to blocks of homologous sequences termed subtelomeres. Subtelomeric elements include the X core, the subtelomeric repeat element (STR), and the Y' repeat (Louis and Vershinin, 2005). All telomeres contain the X core element, most contain STR, and two-thirds of chromosome ends contain the Y' repeat (Louis, 1995). The STR sequence consists of multiple copies of the vertebrate telomeric motif, TTAGGG (Louis, 1995). In budding yeast, TTAGGG is recognized by Tbf1 protein, which contains a Myb domain at its C-terminus (Brigati *et al.*, 1993; Koering *et al.*, 2000; Lavoie *et al.*, 2010). Y' elements also contain the Tbf1-binding TTAGGG sequence (Louis, 1995). Overall, most telomere-subtelomere junctions contain multiple Tbf1-binding sites. Previous studies showed that targeting of Tbf1 to telomeres causes reduction in telomere length (Brevet *et al.*, 2003; Berthiau *et al.*, 2006). It is not precisely understood, however, how Tbf1 controls telomere length. Because Tbf1 binds to subtelomeric regions immediately adjacent

to telomeres, it is possible that Tbf1 works together with Rap1 or its partners Rif1 and Rif2.

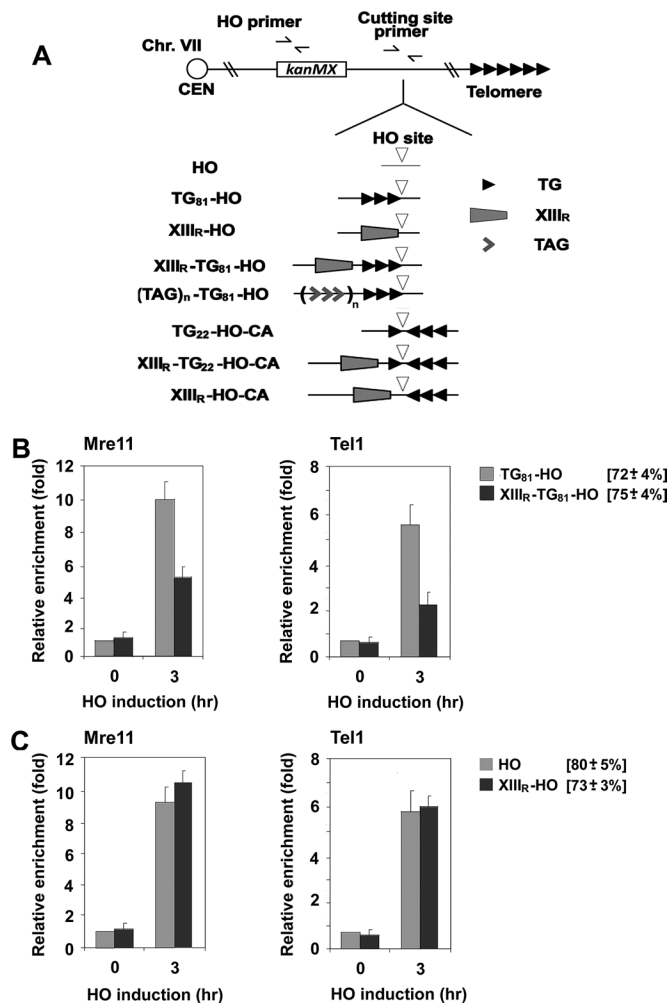
In this study, we investigated how the subtelomere-binding protein Tbf1 contributes to telomere homeostasis. We show that Tbf1 and Rap1 act together and inhibit MRX localization to DNA ends independently of Rif1 or Rif2. The presence of TTAGGG repeats reduces MRX and Tel1 accumulation at nearby DNA ends mimicking short telomeres. Moreover, tethering of both Tbf1 and Rap1 proteins decreases MRX and Tel1 accumulation at adjacent DNA ends. In both cases, inhibition of MRX accumulation occurs independently of Rif1 and Rif2. We also show that Tbf1 and Rap1 suppress checkpoint activation at short telomeres. Depletion of Tbf1 protein increases checkpoint activation in cells containing short telomeres but not in those containing normal-length telomeres. Moreover, Tbf1 is required to repress checkpoint activation at DNA ends where a subtelomeric sequence is placed together with a short TG repeat. These data support a model in which Tbf1 and Rap1 collaborate to protect DNA ends at short telomeres.

## RESULTS

### Effect of a subtelomeric sequence on localization of MRX and Tel1 to DNA ends

Rap1 collaborates with Rif1 and Rif2 to inhibit MRX localization to DNA ends (Hirano *et al.*, 2009). Subtelomere-binding proteins Reb1 and Tbf1 have been shown to negatively control telomere elongation (Berthiau *et al.*, 2006). Tbf1 binding, but not Reb1 binding, shortens telomere length in the presence of Tel1 (Berthiau *et al.*, 2006). We thus tested a hypothesis that Tbf1 stimulates the Rap1 function that inhibits MRX localization to DNA ends.

Tbf1 has been identified as a protein that binds to the vertebrate telomeric TTAGGG repeat sequence (Brigati *et al.*, 1993; Koering *et al.*, 2000), and recent chip-on-chip studies showed that Tbf1 has a well-defined specificity for the TAGGG(C/T) motif (Lavoie *et al.*, 2010). The sequence information for telomere-subtelomere junctions is available for 22 chromosome ends in the *Saccharomyces* Genomic Database. Of these, 21 sequences contain one to eight copies of the TAGGG(C/T) sequence with random spacing. The XIII-R subtelomere region contains six TAGGG(C/T) motifs within 40 base pairs of the telomeric TG sequence. We placed the 40-base pair XIII-R subtelomeric sequence (XIII<sub>R</sub>) near the TG<sub>81</sub>-HO sequence or just the HO cleavage site generating the XIII<sub>R</sub>-TG<sub>81</sub>-HO or XIII<sub>R</sub>-HO cassette, respectively (Figure 1A). We then examined whether the subtelomere sequence XIII<sub>R</sub> decreases MRX accumulation near TG<sub>81</sub> or nontelomeric HO ends (Figure 1, B and C). TG<sub>81</sub> ends have been shown to behave similarly to a short telomere; the 81-base pair TG<sub>81</sub> sequence acts as a seed for the addition of telomere sequence (Diede and Gottschling, 1999) and allows MRX or Tel1 to accumulate at nearby DNA ends (Hirano *et al.*, 2009). Cells transformed with the GAL-HO plasmid were grown in sucrose to prevent activation of the GAL1 promoter and arrested with nocodazole at G2/M. After arrest, cells were incubated with galactose to induce HO expression. Aliquots of cells were collected at the indicated times after HO expression and subjected to chromatin immunoprecipitation (ChIP) assay to monitor Mre11 accumulation at HO-induced DNA ends. The introduction of XIII<sub>R</sub> decreased Mre11 binding at adjacent TG<sub>81</sub> ends (XIII<sub>R</sub>-TG<sub>81</sub> ends; Figure 1B). Tel1 localizes to telomeric or nontelomeric DNA ends in an Xrs2-dependent manner (Nakada *et al.*, 2003a; Sabourin *et al.*, 2007). Similar inhibition was observed for Tel1 binding (Figure 1B). The introduction of XIII<sub>R</sub>, however, did not affect Mre11 or Tel1 binding at nontelomeric DNA ends (XIII<sub>R</sub>-HO ends; Figure 1C). These results raised the possibility that subtelomere-binding Tbf1



**FIGURE 1:** Effect of a subteleromic sequence on localization of MRX and Tel1 to DNA ends with telomeric sequences. (A) Schematic of the HO cleavage site, the 81-base pair telomeric TG repeat, the XIII-R subteleromic region, the 22-base pair telomeric TG repeat, and the TTAGGG repeats at the *ADH4* locus on chromosome VII-L. In HO cells, the *ADH4* locus was replaced with a DNA fragment containing the KanMX gene and an HO cleavage site (inverted triangle). The 81-base pair TG<sub>81</sub> sequence (three repetitive arrowheads) or the XIII-R subteleromic (XIII<sub>R</sub>) sequence (trapezoid) was placed centromere-proximal to the HO cleavage site, generating the TG<sub>81</sub>-HO or XIII<sub>R</sub>-HO strain, respectively. The XIII<sub>R</sub> or TTAGGG repeat (wedge) sequence was integrated centromere-proximal to the TG repeat in TG<sub>81</sub>-HO cells, creating the XIII<sub>R</sub>-TG<sub>81</sub>-HO or TAG<sub>n</sub>-TG<sub>81</sub>-HO strain, respectively. The 22-base pair TG<sub>22</sub> (one arrowhead) sequence was inserted centromere-proximal to the HO cleavage site to generate the XIII<sub>R</sub>-TG<sub>22</sub>-HO-CA or TG<sub>22</sub>-HO-CA strain. The inverted 81-base pair TG<sub>81</sub> (CA) sequence was placed centromere-distal to the HO cleavage site for the XIII<sub>R</sub>-TG<sub>22</sub>-HO-CA, TG<sub>22</sub>-HO-CA, or XIII<sub>R</sub>-HO-CA strain. Centromere is shown as a circle on the left (CEN). The HO primer pair was designed to amplify a region 1 kbp away from the HO site, and the cutting-site primer pair was used to determine the HO cutting efficiency. (B) Effect of the chromosome XIII<sub>R</sub> subteleromic sequence on the association of Mre11 and Tel1 with DNA ends. TG<sub>81</sub>-HO (KSC2849) or XIII<sub>R</sub>-TG<sub>81</sub>-HO (KSC2850) cells expressing myc-tagged Mre11 (Mre11-myc) and HA-tagged Tel1 (Tel1-HA) were transformed with the GAL-HO plasmid. Transformed cells were grown in sucrose and 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 and subjected to CHIP assay to

protein stimulates Rap1-mediated inhibition of MRX binding at DNA ends.

To address whether Tbf1 controls MRX association with DNA ends, we used a heat-inducible *tbf1* degron (*tbf1-d*) construct to examine the effect of Tbf1 depletion. The *TBF1* gene is essential for cell proliferation (Brigati *et al.*, 1993). Correspondingly, *tbf1-d* mutants did not proliferate efficiently at 37°C on galactose medium (Figure 2A). The degron-fused Tbf1 protein was rapidly degraded at high temperatures when the Ubr1 protein was overexpressed from the *GAL1* promoter; Tbf1-d protein was undetectable 3 h after incubation with galactose at 37°C (Figure 2B). Consistent with the Tbf1 degradation patterns, *tbf1-d* mutants essentially stopped proliferation 6 h after incubation with galactose at 37°C (Figure 2C). DNA flow cytometry studies showed that Tbf1 depletion did not result in cell cycle-specific arrest (Figure 2D). We then tested the effect of Tbf1 depletion on MRX or Tel1 accumulation at XIII<sub>R</sub>-TG<sub>81</sub> ends. Tbf1 depletion was found to restore both Mre11 and Tel1 binding at XIII<sub>R</sub>-TG<sub>81</sub> ends (Figure 2, E and F). Thus Tbf1 deficiency reversed the effect of the subteleromic sequence fusion, indicating that subteleromic Tbf1 binding decreases MRX localization to TG<sub>81</sub> ends.

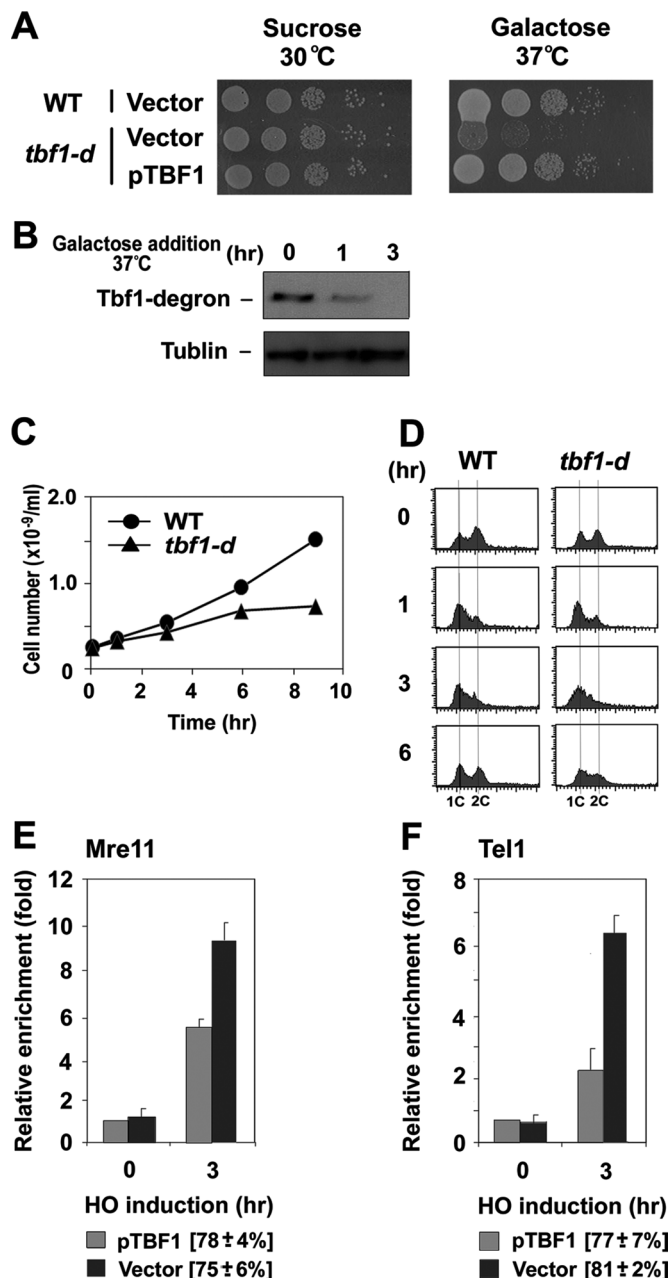
### Effect of copy number of TTAGGG on MRX binding at TG<sub>81</sub> ends

Subtelomeres contain varying numbers of Tbf1 binding motifs, as well as sequences targeted by other proteins. To further evaluate Tbf1 function, we placed various numbers of TTAGGG repeats adjacent to the TG<sub>81</sub> sequence (TAG<sub>n</sub>-TG<sub>81</sub>-HO; Figure 1A). The introduction of the 2x, 4x, or 10x TTAGGG sequences decreased Mre11 or Tel1 localization to nearby TG<sub>81</sub> ends in a copy number-dependent manner (Figure 3). Similar to the XIII<sub>R</sub> sequence, those repeat sequences did not affect Mre11 or Tel1 binding at HO ends (data not shown). These results strongly suggest that subteleromic-binding protein Tbf1 controls the localization of MRX complex to telomeric DNA ends in a dosage-dependent manner.

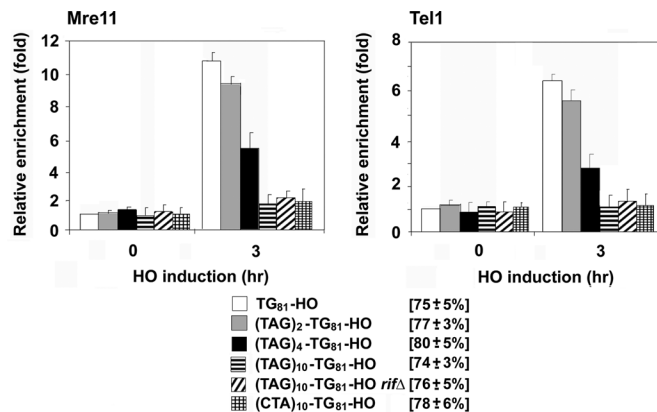
### Effect of Tbf1 tethering on MRX localization to nearby TG<sub>81</sub> ends

Because Tbf1 protein binds to the TTAGGG repeat sequence, Tbf1 function may depend on sequence-specific DNA binding. To address this possibility, we set up a system to tether Tbf1 protein to non-TTAGGG sequences adjacent to the TG<sub>81</sub> repeat (Figure 4A). We constructed a LacI-Tbf1 fusion gene that fully rescues the proliferation defect of *tbf1Δ* mutants (data not shown). To deploy LacI-Tbf1 protein to non-TTAGGG sequence, we placed eight copies of the lacI-binding sequence (lacO) adjacent to the TG<sub>81</sub> sequence. We examined the effect of LacI-Tbf1 tethering on Mre11 or Tel1 binding to nearby TG<sub>81</sub> or HO ends (Figure 4, B and C). LacI-Tbf1 expression inhibited Mre11 or Tel1 binding to TG<sub>81</sub> ends but not

monitor the association of Mre11 or Tel1 with DNA ends. Coprecipitated 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. HO cutting efficiency for each construct is shown in square brackets. (C) Effect of the chromosome XIII-R subteleromic sequence on the association of Mre11 and Tel1 with nontelomeric DNA ends. HO (KSC2851) or XIII<sub>R</sub>-HO (KSC2852) cells expressing Mre11-myc and Tel1-HA were transformed with the GAL-HO plasmid, and association of Mre11 or Tel1 with DNA ends was analyzed by CHIP assay as in B.



**FIGURE 2:** Effect of Tbf1 depletion on localization of MRX and Tel1 to DNA ends with telomeric or subtelomeric sequences. (A) Effect of *tbf1*-degron mutation on colony formation on galactose medium at 37°C. Wild-type or *tbf1*-degron mutant (*tbf1-d*; KSC2853) cells were transformed with YCpU-TBF1 or the control vector. Cells were serially diluted and spotted on sucrose or galactose plates selectable for the plasmids. Plates were incubated at the indicated temperatures. (B) *UBR1*-induced Tbf1 protein degradation at 37°C. *tbf1*-degron mutant (*tbf1-d*) (KSC2853) cells were initially grown in sucrose at 30°C and were then incubated with galactose and concomitantly shifted to 37°C. Aliquots of cells were collected at the indicated times after incubation with galactose at 37°C and subjected to immunoblotting analysis with anti-myc antibodies. The degron cassette is fused to a myc epitope tag. (C, D) Proliferation of *tbf1*-degron mutants after incubation with galactose at 37°C. *tbf1*-degron mutant (*tbf1-d*) (KSC2853) cells were initially grown in sucrose at 30°C and were then incubated with galactose and concomitantly shifted to 37°C. Aliquots of cells were collected at the indicated times after incubation with galactose at 37°C to determine the cell division rate (C) and DNA content of cells (D). DNA content was determined by flow cytometric



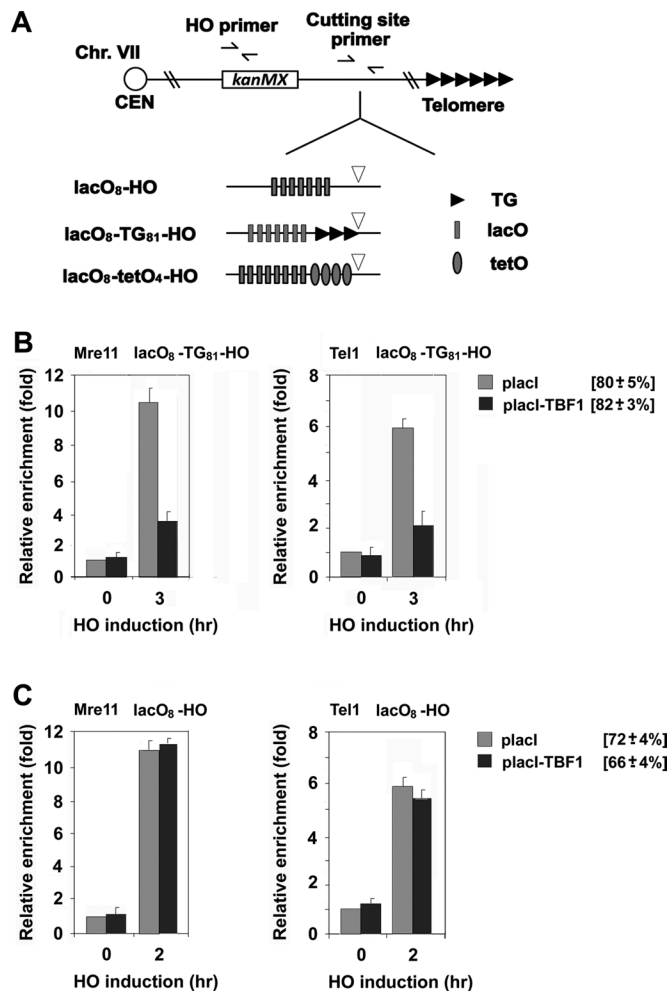
**FIGURE 3:** Effect of TTAGGG repeats on association of MRX and Tel1 with TG<sub>81</sub> ends. TG<sub>81</sub>-HO (KSC2849), TAG<sub>2</sub>-TG<sub>81</sub>-HO (KSC2856), TAG<sub>4</sub>-TG<sub>81</sub>-HO (KSC2857), TAG<sub>10</sub>-TG<sub>81</sub>-HO (KSC2858), TAG<sub>10</sub>-TG<sub>81</sub>-HO *rif1Δ rif2Δ* (KSC2859), or CTA<sub>10</sub>-TG<sub>81</sub>-HO (KSC2860) cells expressing Mre11-myc and Tel1-HA were transformed with the GAL-HO plasmid, and accumulation of Mre11 or Tel1 was analyzed by ChIP assay as Figure 1B.

HO ends. Expression of LacI by itself did not affect Mre11 or Tel1 binding to TG<sub>81</sub> ends (Figure 4B). Thus sequence-specific DNA binding is dispensable for the Tbf1 function. The foregoing findings also support the idea that the Tbf1-mediated inhibition of MRX localization does not depend on direction-specific Tbf1 binding. Consistent with this idea, the 10xCCCTAA repeat inhibited Mre11 or Tel1 localization to TG<sub>81</sub> ends similarly to the 10xTTAGGG repeat (Figure 3).

#### Rap1-dependent, but Rif1- and Rif2-independent, inhibition of MRX localization to DNA ends

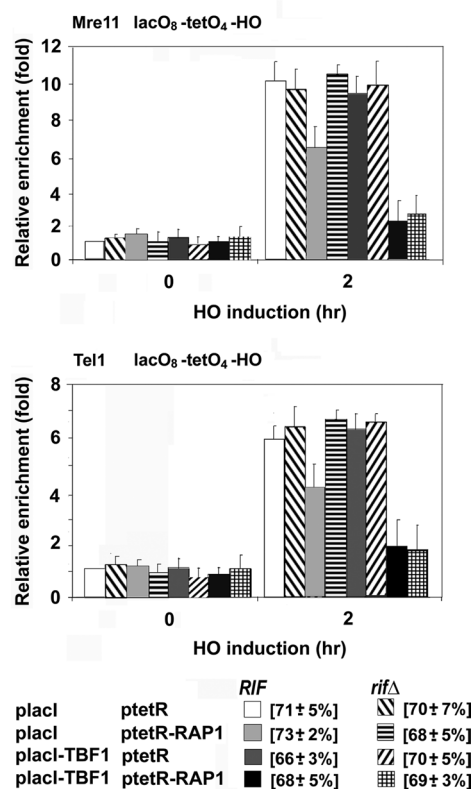
Because telomeric TG repeats are covered with Rap1, it seems likely that Tbf1 function is mediated through Rap1. However, it remains possible that Tbf1 acts on telomeric TG repeat-binding proteins other than Rap1. To address whether Tbf1 collaborates with Rap1, we inserted four copies of the TetR-binding sequence (tetO<sub>4</sub>) between the lacO<sub>8</sub> sequence and the HO cleavage, site generating the lacO<sub>8</sub>-tetO<sub>4</sub>-HO cassette (Figure 4A). We then examined the synergistic effect of LacI-Tbf1 and TetR-Rap1 expression on MRX and Tel1 localization to a DNA end near the lacO<sub>8</sub>-tetO<sub>4</sub> sequence (LacO<sub>8</sub>-TetO<sub>4</sub>) ends (Figure 5). LacI-Tbf1 expression alone did not inhibit Mre11 or Tel1 localization to LacO<sub>8</sub>-TetO<sub>4</sub> ends, and TetR-Rap1 expression moderately decreased Mre11 or Tel1 localization. In contrast, coexpression of LacI-Tbf1 and TetR-Rap1 nearly shut out Mre11 or Tel1 localization. Rap1 interacts with Rif1 and Rif2 through the C-terminus (Conrad *et al.*, 1990; Lustig *et al.*, 1990; Hardy *et al.*, 1992; Wotton and Shore, 1997). Rap1 collaborates with Rif1 and Rif2 to decrease MRX and Tel1 localization to DNA ends (Hirano *et al.*, 2009). Consistent with this view, TetR-Rap1 expression failed to decrease Mre11 or Tel1 localization to LacO<sub>8</sub>-TetO<sub>4</sub> ends in *rif1Δ rif2Δ* double mutants. However, coexpression of LacI-Tbf1 and

analysis. (E, F) Effect of Tbf1 depletion on Mre11 or Tel1 localization to XIII<sub>R</sub>-TG<sub>81</sub> ends. XIII<sub>R</sub>-TG<sub>81</sub>-HO *tbf1-d* cells expressing Mre11-HA (KSC2854) or Tel1-HA (KSC2855) were transformed with the YCpU-TBF1 plasmid or the control vector, together with the GAL-HO plasmid. Transformed cells were treated as in A and analyzed by ChIP assay as in Figure 1B to monitor the accumulation of Mre11 (E) or Tel1 (F) at DNA ends. HO cutting efficiency of cells carrying each plasmid is shown in square brackets.



**FIGURE 4:** Effect of Tbf1 or Rap1 tethering on MRX association with nearby DNA ends. (A) Schematic of the HO cleavage site, the LacO<sub>8</sub> array, the TetO<sub>4</sub> array, or the TG<sub>81</sub> repeat at the *ADH4* locus on chromosome VII-L. In LacO<sub>8</sub>-TG<sub>81</sub>-HO or LacO<sub>8</sub>-HO cells, eight copies of the lacO sequence (gray rectangle) were placed centromere-proximal to the TG<sub>81</sub>-HO cassette or the HO cleavage site, respectively. In LacO<sub>8</sub>-TetO<sub>4</sub>-HO cells, eight copies of the lacO sequence and four copies of the tetO sequence (barrel) were placed centromere-proximal to the HO cleavage site. Centromere is shown as a circle on the left (CEN). The HO primer pair was designed to amplify a region 1 kbp away from the HO site, and the cutting-site primer pair was used to determine the HO cutting efficiency. (B) Effect of Tbf1 tethering on association of Mre11 or Tel1 with nearby TG<sub>81</sub> ends. LacO<sub>8</sub>-TG<sub>81</sub>-HO (KSC2861) cells expressing Mre11-myc and Tel1-HA were transformed with placl-TBF1 or the control vector, together with the GAL-HO plasmid. Cells were analyzed by ChIP assay as in Figure 1B to monitor accumulation of Mre11 or Tel1 at DNA ends. (C) Effect of Tbf1 tethering on association of Mre11 or Tel1 with nearby HO ends. LacO<sub>8</sub>-HO (KSC2862) cells expressing Mre11-myc and Tel1-HA were transformed with placl-TBF1 or the control vector, together with the GAL-HO plasmid. Cells were analyzed by ChIP assay as in B to monitor accumulation of Mre11 or Tel1 at DNA ends.

TetR-Rap1 inhibited Mre11 or Tel1 localization in *rif1Δ rif2Δ* double mutants as effectively as in *RIF1 RIF2* wild-type cells. No effect of *rif1Δ rif2Δ* double mutation was observed for LacI-Tbf1 expression alone. We also investigated the effect of *rif1Δ rif2Δ* double mutation on Mre11 or Tel1 accumulation at TAG<sub>10</sub>-TG<sub>81</sub> ends (Figure 3). The *rif1Δ rif2Δ* double mutation did not affect Mre11 or Tel1 accumula-

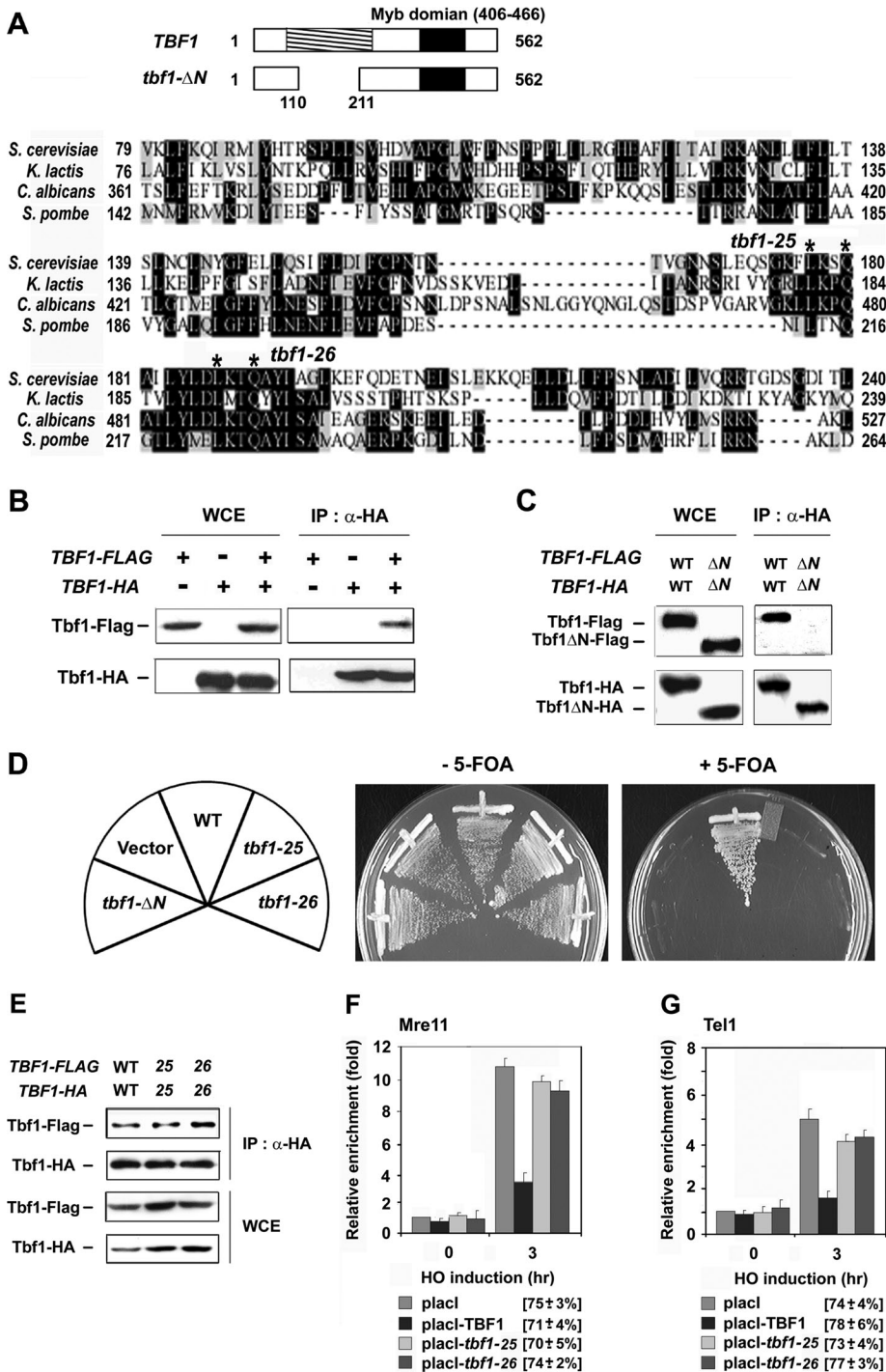


**FIGURE 5:** Effect of Tbf1 and Rap1 cotethering on MRX association with nearby DNA ends. LacO<sub>8</sub>-TetO<sub>4</sub>-HO (KSC2863) and LacO<sub>8</sub>-TetO<sub>4</sub>-HO *rif1Δ rif2Δ* (KSC2864) cells expressing Mre11-myc and Tel1-HA were transformed with placl-TBF1, ptetR-RAP1, or the control vector, together with the GAL-HO plasmid. Cells were analyzed for Mre11 binding or Tel1 binding by ChIP assay as in Figure 1B. The LacO<sub>8</sub>-TetO<sub>4</sub>-HO construct is shown in Figure 4A. The strains used here contained a *sir2Δ* mutation to suppress inefficient HO cleavage resulting from Rap1 tethering nearby (Hirano *et al.*, 2009).

tion. Thus the Tbf1-Rap1 pathway inhibits MRX localization to DNA ends through a Rif1- and Rif2-independent mechanism.

### Requirement of the N-terminus of Tbf1 protein for complex formation and regulation of MRX localization to DNA ends

The family of telomere-repeat binding factors, including TRF1, TRF2, and Taz1, shares a common architecture with two separate structural domains (Palm and de Lange, 2008; Moser and Nakamura, 2009). An N-terminal domain is required for the formation of homodimers, whereas a C-terminal Myb domain recognizes double-stranded TTAGGG repeats (Figure 6A). Tbf1 contains a C-terminal Myb-type DNA-binding domain but does not share similarities with TRF1, TRF2, and Taz1 at the N-terminus (Cockell *et al.*, 2009). It remains possible, however, that Tbf1 functions as a homodimer or homooligomer similar to TRF1, TRF2, and Taz1. We therefore examined protein-protein interaction of Tbf1 protein by coimmunoprecipitation experiments (Figure 6B). Extracts were prepared from cells expressing FLAG-tagged Tbf1 (Tbf1-FLAG) or hemagglutinin (HA)-tagged Tbf1 protein (Tbf1-HA) and subjected to immunoprecipitation with anti-HA antibodies. Immunoprecipitates were then analyzed by immunoblotting with anti-FLAG or anti-HA antibodies. Coprecipitation of Tbf1-HA and Tbf1-FLAG was detected only in cells expressing both tagged proteins (Figure 6B). Thus Tbf1 proteins interact with each other.



**FIGURE 6:** Role of the N-terminus of Tbf1 protein in inhibiting MRX localization to DNA ends. (A) Deletion or substitution mutations of *TBF1*. The budding yeast *Saccharomyces cerevisiae* Tbf1 protein, consisting of 562 amino acid residues, contains a Myb-related DNA-binding domain at the C-terminus. The N-terminal conserved domain was shaded. Amino acid alignment shows the N-terminal conserved domains of *S. cerevisiae*, *Kluyveromyces lactis*, *Candida albicans*, and *Schizosaccharomyces pombe* Tbf1 proteins. Identical or related amino acid residues are boxed in black or gray, respectively. The *tbf1*-ΔN mutation lacks the N-terminal 111–210 amino acid residues. The *tbf1*-25 or *tbf1*-26 mutation replaces the conserved amino acid residues at 177 and 180 or 187 and 190 (indicated by asterisks) with alanine, respectively. (B) Protein–protein interaction of Tbf1. *tbf1*Δ mutants carrying YCp33-TBF1-HA, YCp22-TBF1-FLAG, or the respective control vector were grown in medium selectable for the plasmids and subjected to immunoprecipitation with anti-HA antibodies. Immunoprecipitates (IP) and whole-cell extracts (WCE) were subjected to immunoblotting analysis with anti-HA or anti-FLAG antibodies. All the cells used here are haploid, which are derived from a *TBF1/tbf1*Δ diploid strain (KSC2505). (C) Requirement of the N-terminus for protein–protein interaction. *tbf1*-d

*Tbf1* shows significant conservation with fungal Tbf1 proteins in the N-terminal region (Cockell et al., 2009; Figure 6A). As shown, Tbf1 inhibits MRX localization independently of sequence-specific DNA binding. We therefore tested the hypothesis that the N-terminus of Tbf1 mediates inhibition of MRX localization to DNA ends. We first investigated the effect of deletion of the N-terminus on protein–protein interaction of Tbf1. To exclusively monitor the protein–protein interaction of mutant proteins, we performed coimmunoprecipitation experiments in the *tbf1*-d mutants at 37°C after depletion of functional Tbf1 protein. Extracts from cells expressing HA- or FLAG-tagged N-terminal truncated Tbf1 protein (Tbf1-ΔN) were subjected to immunoprecipitation and subsequent immunoblotting as described. Protein–protein interaction between Tbf1-ΔN proteins was undetectable (Figure 6C), indicating that the conserved N-terminal domain is required for efficient protein–protein interaction. Because the *TBF1* gene is essential

(KSC2756) cells were transformed with pRS316-TBF1-HA and pRS314-TBF1-FLAG, or pRS316-tbf1ΔN-HA and pRS314-tbf1ΔN-FLAG. Cells were grown in sucrose at 30°C and synchronized at G2/M with nocodazole. After synchronization, the culture was incubated with galactose and concomitantly shifted to 37°C. Aliquots of cells were collected after 3 h of incubation with galactose at 37°C and analyzed as in B. (D) Effect of *tbf1*-ΔN, *tbf1*-25, or *tbf1*-26 mutation on cell proliferation. *tbf1*Δ mutants carrying the *URA3*-marked YCpU-TBF1 plasmid were transformed with pRS314-TBF1-FLAG, pRS314-tbf1-ΔN-FLAG, pRS314-tbf1-25-FLAG, pRS314-tbf1-26-FLAG, or the control vector. Transformants were streaked and grown on plates containing medium with or without 5-FOA. The strain used here is derived from a *TBF1/tbf1*Δ diploid strain (KSC2505). (E) Effect of *tbf1*-25 or *tbf1*-26 mutation on protein–protein interaction. *tbf1*-d cells (KSC2756) were transformed with pRS316-TBF1-HA and pRS314-TBF1-FLAG, pRS316-tbf1-25-HA and pRS314-tbf1-25-FLAG, or pRS316-tbf1-26-HA and pRS314-tbf1-26-FLAG. Protein–protein interaction of Tbf1 was examined as in C. (F, G) Effect of Tbf1-25 or Tbf1-26 protein tethering on Mre11 and Tel1 association with adjacent TG<sub>81</sub> ends. LacO<sub>8</sub>-TG<sub>81</sub>-HO *tbf1*-d cells expressing Mre11-HA (KSC2865) or Tel1-HA (KSC2866) were transformed with the GAL-HO plasmid and placi-TBF1, placi-tbf1-25, placi-tbf1-26, or the empty placi plasmid. Transformed cells were treated as in C and subjected to ChIP assay as described in Figure 1B to monitor the association of Mre11 (F) or Tel1 (G) with DNA ends.

for cell proliferation, we examined whether the N-terminal truncation affects Tbf1 function. Both FLAG-tagged and untagged versions of the *tbf1-ΔN* mutation failed to rescue the lethality associated with the *tbf1Δ* mutation at 25, 30, or 37°C (Figure 6D; data not shown).

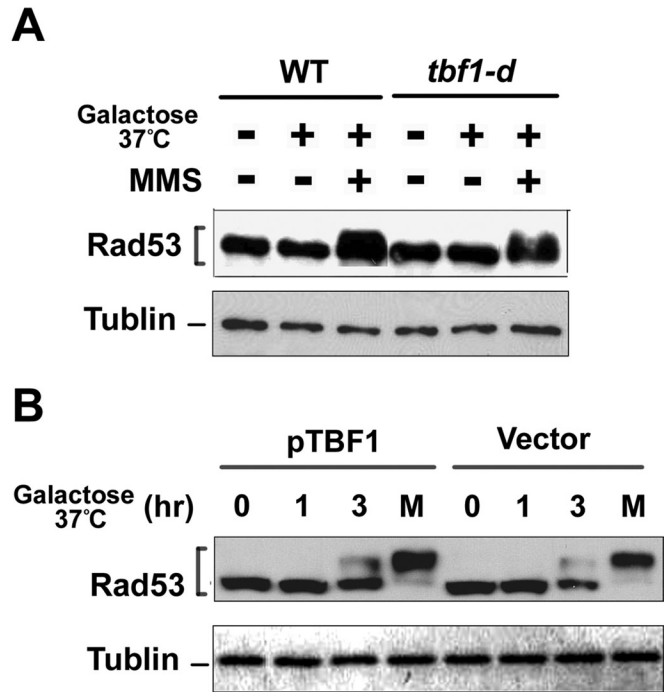
The foregoing result raises a possibility that Tbf1 function requires protein–protein interaction. However, it is equally possible that some Tbf1 functions operate independently of protein–protein interaction. We generated several substitutions by replacing conserved residues with alanine in the N-terminal conserved region and determined whether loss of function is coupled to defective protein–protein interaction. Similar to the *tbf1-ΔN* mutation, two substitution mutations, *tbf1-25* and *tbf1-26*, regardless of whether they were tagged or not, each failed to rescue proliferation defects associated with the *tbf1Δ* mutation at 25, 30, or 37°C (Figure 6D, data not shown). Because the conserved region is required for protein–protein interaction, we determined whether these *tbf1* mutations disrupt complex formation. As described, *tbf1-d* cells were transformed with plasmids carrying HA- or FLAG-tagged wild-type *TBF1* or mutant *tbf1* constructs. After depletion of Tbf1-degrogen protein, cells were subjected to coimmunoprecipitation experiments. Neither *tbf1-25* nor *tbf1-26* mutation affected complex formation (Figure 6E).

We then addressed whether the *tbf1-25* or *tbf1-26* mutation confers defects in inhibiting MRX localization to DNA ends. To examine the function of the Tbf1-mutant protein, we tested the effect of LacI-Tbf1-25 or LacI-Tbf1-26 expression on MRX or Tel1 localization to LacO<sub>8</sub>-TG<sub>81</sub> ends in the *tbf1-d* background after depletion of functional Tbf1 protein (Figure 6, F and G). Cells expressing Mre11-myc or Tel1-HA were transformed with the GAL-HO plasmid and placi-TBF1, placi-tbf1-25, placi-tbf1-26 plasmid or the control vector (placi). Transformants were grown in sucrose at 30°C and then incubated in galactose at 37°C to induce HO expression, as well as Tbf1 degradation. Cells were analyzed by ChIP assay as described, to monitor Mre11 or Tel1 binding at DNA ends. Both *tbf1-25* and *tbf1-26* mutations conferred defects in inhibiting MRX localization; Mre11 or Tel1 binding was increased in cells expressing a LacI-Tbf1-25 or LacI-Tbf1-26 fusion compared with those expressing the wild-type LacI-Tbf1 fusion. Neither the *tbf1-25* nor the *tbf1-26* mutation altered the expression level of LacI-Tbf1 fusion protein (data not shown). These results suggest that the conserved N-terminal region of Tbf1 mediates inhibition of MRX localization to telomeric DNA ends.

### Effect of Tbf1 depletion on checkpoint activation at endogenous telomeres

As shown here, Tbf1 and Rap1 inhibit MRX localization to DNA ends independently of Rif1 or Rif2. In parallel, Rap1 collaborates with Rif1 and Rif2 in inhibiting Tel1 localization. We previously showed that the 162–base pair TG<sub>162</sub> sequence inhibits MRX and Tel1 localization in a Rif1- and Rif2-dependent manner, whereas the 81–base pair TG<sub>81</sub> sequence does not (Hirano *et al.*, 2009). We therefore tested the hypothesis that Tbf1 is required to maintain genome stability of chromosomal ends containing short telomeric sequences.

MRX plays a key role in activating the Mec1-dependent checkpoint pathway, as well as the Tel1 pathway (Nakada *et al.*, 2003a, 2004). We first examined the effect of Tbf1 depletion on checkpoint activation at normal-length telomeres. Checkpoint activation is controlled in a cell cycle–dependent manner (Ira *et al.*, 2004; Vodenicharov and Wellinger, 2006). We therefore monitored phosphorylation of Rad53 in G2/M-arrested cells (Figure 7A). No Rad53 phosphorylation was detected after Tbf1 depletion, whereas MMS treatment induced Rad53 phosphorylation in Tbf1-depleted cells.

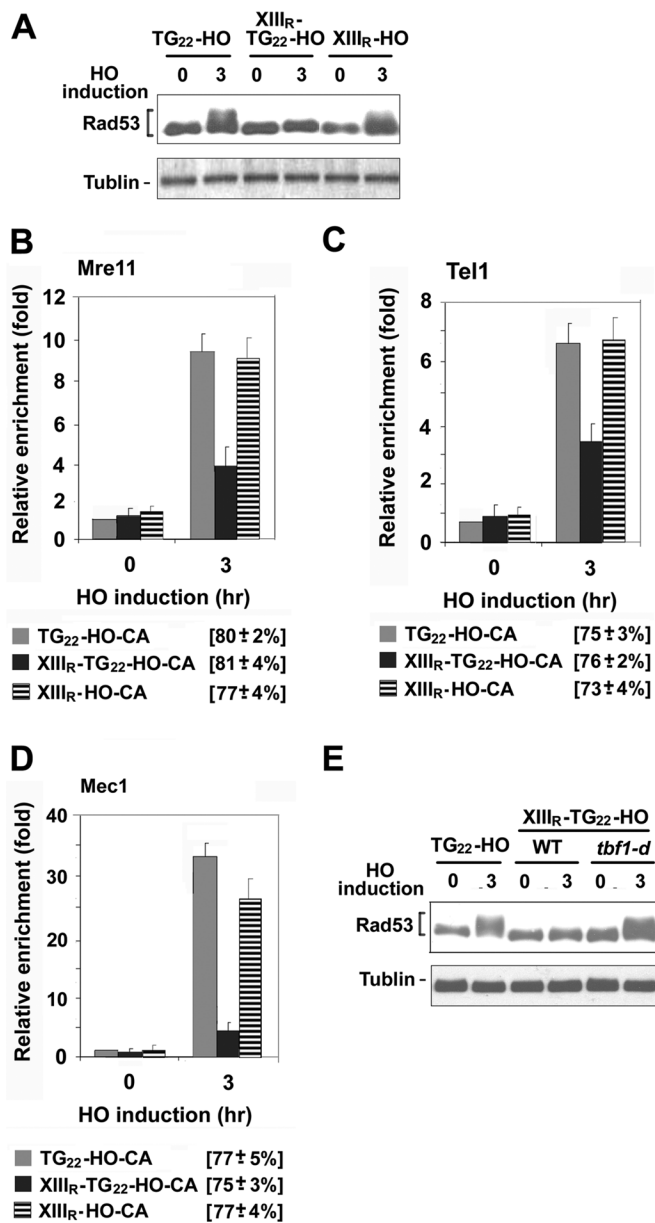


**FIGURE 7:** Effect of Tbf1 depletion on checkpoint response to endogenous telomeres. (A) Effect of Tbf1 depletion on checkpoint activation in cells carrying normal-length telomeres. Wild-type (KSC1602) or *tbf1-d* (KSC2756) cells were cultured as in Figure 6C to degrade Tbf1 protein. Cells were further treated with 0.1% methyl methanesulfonate (MMS) for 1 h at 37°C. Aliquots of cells were collected and subjected to immunoblotting analysis with anti-Rad53 antibodies. Tubulin was detected as a loading control. (B) Effect of Tbf1 depletion on checkpoint activation in *stn1-13* mutant cells. *tbf1-d* (KSC2756) or *tbf1-d stn1-13* (KS2891) cells carrying YCpT-TBF1 or the control vector were grown and treated with nocodazole at 25°C. After synchronization, the culture was incubated with galactose and concomitantly shifted to 37°C. Aliquots of cells were collected at the indicated times after incubation with galactose at 37°C. As a control, aliquots of cells were treated with 0.1% MMS for 1 h at 37°C (labeled as M). Cells were subjected to immunoblotting analysis as in A.

G2/M synchronization with nocodazole did not alter the expression level of Tbf1 protein (Supplemental Figure S1). The Cdc13-Stn1-Ten1 complex acts as a telomere cap to protect telomeres from DNA degradation (Petreaca *et al.*, 2007; Xu *et al.*, 2009). It is possible that Tbf1 functions as a backup for the Cdc13-Stn1-Ten1 complex; therefore, the effect of Tbf1 depletion might be detectable only at uncapped telomeres. We next examined the effect of Tbf1 depletion on checkpoint activation in cells carrying the temperature-sensitive *stn1-13* mutation (Grandin *et al.*, 2001; Figure 7B). Rad53 was phosphorylated in *stn1-13* mutants after shifting to 37°C, but Tbf1 depletion did not affect the phosphorylation status. It should be noted that the *stn1-13* mutation does not cause telomere shortening (Grandin *et al.*, 2001). Thus Tbf1 is dispensable for telomere protection in cells containing normal-length or elongated telomeres.

### Effect of Tbf1 binding on checkpoint activation at DNA ends with the 22–base pair TG sequence

Checkpoint activation occurs at DNA ends adjacent to the 22–base pair TG (TG<sub>22</sub>) sequence although telomeres are added at TG<sub>22</sub> ends (Hirano and Sugimoto, 2007). We next addressed whether Tbf1 attenuates checkpoint activation at DNA ends with the TG<sub>22</sub>



**FIGURE 8:** Effect of Tbf1 binding on checkpoint activation at adjacent DNA ends with the 22-base pair TG<sub>22</sub> sequence. (A) Effect of the XIII<sub>R</sub> and 22-base pair TG<sub>22</sub> sequence on Rad53 phosphorylation after HO expression. TG<sub>22</sub>-HO-CA (KSC2230), XIII<sub>R</sub>-HO-CA (KSC2894), or XIII<sub>R</sub>-TG<sub>22</sub>-HO-CA (KSC2834) cells were transformed with the GAL-HO plasmid. Transformed cells were grown in sucrose and synchronized at G2/M with nocodazole. After arrest, the culture was incubated with galactose to induce HO expression. Aliquots of cells were collected at the indicated times after HO expression, and cell extracts were analyzed by immunoblotting analysis with anti-Rad53 antibodies. (B) Effect of the XIII<sub>R</sub> and 22-base pair TG<sub>22</sub> sequence on Mre11 accumulation at DNA ends. XIII<sub>R</sub>-TG<sub>22</sub>-HO-CA *MRE11-myc* (KSC2892), TG<sub>22</sub>-HO-CA *MRE11-myc* (KSC2901), or XIII<sub>R</sub>-HO-CA *MRE11-myc* (KSC2905) cells carrying the GAL-HO plasmid were analyzed by ChIP assay as in Figure 1B. (C) Effect of the XIII<sub>R</sub> and 22-base pair TG<sub>22</sub> sequence on Tel1 accumulation at DNA ends. XIII<sub>R</sub>-TG<sub>22</sub>-HO-CA *TEL1-HA* (KSC2896), TG<sub>22</sub>-HO-CA *TEL1-HA* (KSC2895), or XIII<sub>R</sub>-HO-CA *TEL1-HA* (KSC2897) cells carrying the GAL-HO plasmid were analyzed by ChIP assay as in B. (D) Effect of the XIII<sub>R</sub> and 22-base pair TG<sub>22</sub> sequence on Mec1 accumulation at DNA ends. XIII<sub>R</sub>-TG<sub>22</sub>-HO-CA *MEC1-HA* (KSC2899), TG<sub>22</sub>-HO-CA *MEC1-HA* (KSC2898), or XIII<sub>R</sub>-HO-CA *MEC1-HA* (KSC2900) cells carrying the GAL-HO plasmid

sequence (TG<sub>22</sub> ends) nearby (Figure 8). The TG<sub>22</sub>-HO-CA cassette was used or further modified to monitor checkpoint activation at a single DNA end after DSB induction (Hirano and Sugimoto, 2007; Figure 1A). To test the effect of Tbf1 binding, we fused the XIII<sub>R</sub> sequence centromere-proximal to the TG<sub>22</sub>-HO-CA sequence, generating the XIII<sub>R</sub>-TG<sub>22</sub>-HO-CA cassette (Figure 1A). We also replaced the TG<sub>22</sub> sequence of the TG<sub>22</sub>-HO-CA cassette, constructing the XIII<sub>R</sub>-HO-CA cassette (Figure 1A). The TG<sub>22</sub> sequence contains a single Rap1-binding motif; ChIP assays confirmed Rap1 binding at the TG<sub>22</sub> sequence (Supplemental Figure S2). TG<sub>22</sub>-HO-CA, XIII<sub>R</sub>-TG<sub>22</sub>-HO-CA, or XIII<sub>R</sub>-HO-CA cells carrying the GAL-HO plasmid were first grown in sucrose and then incubated with galactose to induce HO expression (Figure 8A). Checkpoint activation was examined by monitoring the phosphorylation status of Rad53. Rad53 was phosphorylated in TG<sub>22</sub>-HO-CA cells after HO expression. Rad53 phosphorylation was detected in XIII<sub>R</sub>-HO-CA cells as well. However, no apparent phosphorylation was observed in XIII<sub>R</sub>-TG<sub>22</sub>-HO-CA cells. We also examined the effect of the XIII<sub>R</sub> sequence on accumulation of Mre11, Tel1, or Mec1 at DNA ends (Figure 8, B–D). Accumulation of Mre11, Tel1, or Mec1 was significantly decreased at XIII<sub>R</sub>-TG<sub>22</sub> ends compared with that at TG<sub>22</sub> or XIII<sub>R</sub> ends. Thus the combination of the XIII<sub>R</sub> and the TG<sub>22</sub> sequence inhibits checkpoint activation at nearby DNA ends.

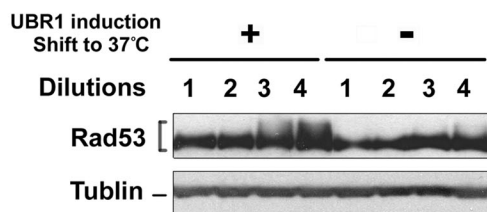
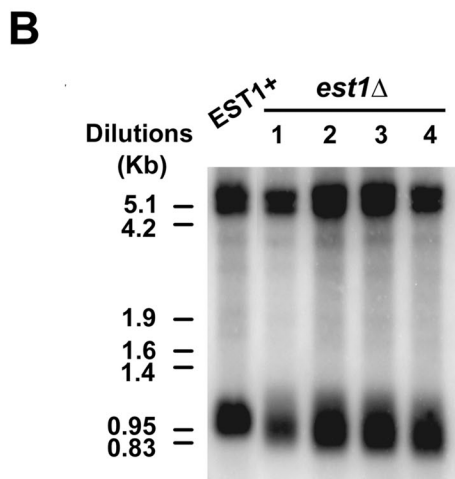
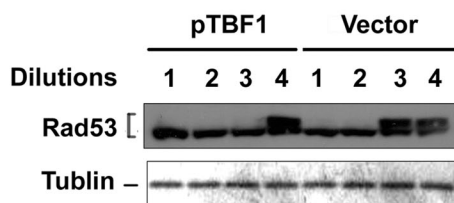
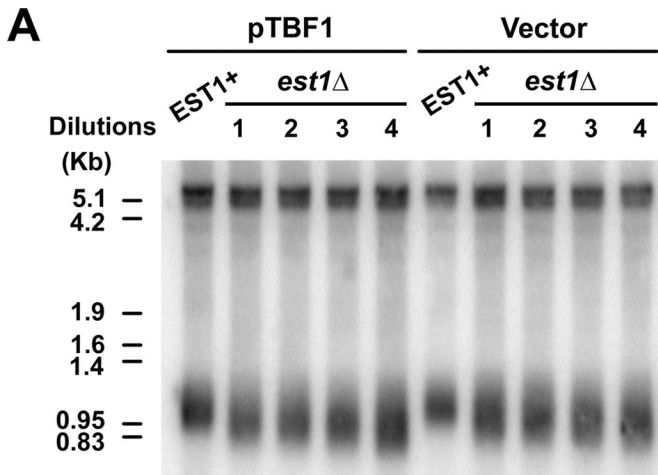
To determine whether Tbf1 indeed contributes to the checkpoint inhibition, we examined the effect of Tbf1 depletion on checkpoint activation at XIII<sub>R</sub>-TG<sub>22</sub> ends (Figure 8E). XIII<sub>R</sub>-TG<sub>22</sub>-HO-CA *TBF1* or *tbf1-d* cells carrying the GAL-HO plasmid were initially grown in sucrose and incubated with galactose at 37°C to induce HO expression and degrade Tbf1-d protein. After 3 h of incubation, phosphorylation of Rad53 protein was monitored by immunoblotting analysis. The *tbf1-d* mutation restored Rad53 phosphorylation in XIII<sub>R</sub>-TG<sub>22</sub>-HO-CA cells after HO expression. These results support the idea that Tbf1 binding inhibits checkpoint activation at DNA ends with short telomeric repeats.

### Effect of Tbf1 depletion on checkpoint activation at short telomeres

We next asked whether Tbf1 depletion triggers checkpoint activation at short telomeres (Figure 9A). To shorten telomeres, we introduced a deletion mutation of *EST1*, which encodes a regulatory protein for telomerase, into *tbf1-d* mutants. Cells carrying an *est1Δ* mutation shorten telomeres gradually and eventually suffer senescence because of the end replication problem (Nugent and Lundblad, 1998). Growth of the *est1Δ* strains was maintained by wild-type *EST1* on a *URA3* plasmid. Cells were transformed with YCpT-TBF1 or the control vector. To select for loss of the complementing *EST1* plasmid, transformants were grown on plates containing 5-fluoroorotic acid (5-FOA). The resulting single colonies were inoculated into liquid culture. The cultures were diluted fourfold and grown for 7 h for successive serial dilutions. Cells from each culture were arrested with nocodazole to block further telomere attrition. Southern blotting analysis confirmed that telomeres in *est1Δ* mutants became shorter after successive dilutions compared with those in wild-type cells (Figure 9A). After

were analyzed by ChIP assay as in B. (E) Effect of Tbf1 depletion on checkpoint signaling from XIII<sub>R</sub>-TG<sub>22</sub> ends. TG<sub>22</sub>-HO-CA (KSC2230), XIII<sub>R</sub>-TG<sub>22</sub>-HO-CA (KSC2839), or XIII<sub>R</sub>-TG<sub>22</sub>-HO-CA *tbf1-d* (KSC2836) cells were transformed with the GAL-HO plasmid. Transformants were treated as in Figure 6C to induce HO expression and Tbf1 degradation. Cells were subjected to immunoblotting analysis as in A.





**FIGURE 9:** Effect of Tbf1 depletion on checkpoint response to short telomeres. (A) Effect of Tbf1 depletion on checkpoint response in telomerase-deficient cells. Telomerase-deficient *est1Δ tbf1-d* (KSC2867) cells carrying YCpT-TBF1 or the control vector were obtained from the strain carrying the *URA3*-marked *EST1* plasmid. Colonies arisen on plates containing 5-FOA were grown to the late log phase. The culture was diluted fourfold to allow cells to undergo cell division twice for 7 h at 30°C. This cycle was repeated four times. Cells from each serial dilution culture were synchronized at G2/M with nocodazole. Aliquots of cells were harvested to monitor the telomere

length by Southern blots (top). Cells were incubated with galactose at 37°C to degrade Tbf1-d protein. After 3 h of incubation, phosphorylation of Rad53 protein was monitored by immunoblotting analysis. Rad53 phosphorylation was detected after three successive dilutions in *tbf1-d* mutant cells but not in cells expressing wild-type Tbf1 protein.

It remained possible that telomere attrition occurs slightly faster in *tbf1-d* mutants than with wild-type *TBF1* cells; therefore, checkpoint is activated earlier in *tbf1-d* mutants than in *TBF1* cells. To exclude this possibility, *tbf1-d est1Δ* cells were grown in sucrose for several generations as described. After nocodazole block, half of the culture was incubated with galactose at 37°C and the other half was grown with glucose at 30°C (Figure 9B). Rad53 phosphorylation was detected after three successive dilutions in Tbf1-depleted cells but not in nondepleted cells. Incubation of cells with galactose at 37°C does not elicit Rad53 phosphorylation (Figure 7; Vodenicharov et al., 2010). Thus Tbf1 depletion accelerated triggering of checkpoint responses in cells containing short telomeres but not normal-length telomeres. Together, our results support the model in which Tbf1 protein plays a pivotal role in maintaining genome stability of short telomeres.

## DISCUSSION

Rap1 binds to double-stranded telomeric sequences and recruits Rif1 and Rif2 proteins via its C-terminal domain (Conrad et al., 1990; Lustig et al., 1990; Hardy et al., 1992; Wotton and Shore, 1997). Both Rif1 and Rif2 inhibit the localization of Tel1, but not the Mre11-Rad50-Xrs2 complex, to adjacent DNA ends (Hirano et al., 2009). Rap1 collaborates with Rif1 and Rif2 and inhibits MRX accumulation at DNA ends (Hirano et al., 2009). The budding yeast Tbf1 protein binds to TTAGGG sequences (Brigati et al., 1993; Koering et al., 2000; Lavoie et al., 2010), which are found at the telomere junction of subtelomeres (Louis, 1995). In this study we show that Tbf1 and Rap1 collaborate to inhibit the localization of MRX to DNA ends independently of Rif1 or Rif2 function. Tbf1 depletion triggers checkpoint activation from short telomeres but not from normal-length telomeres. These observations support a model in which Tbf1 collaborates with Rap1 and circumvents genomic instability at short telomeres. Our results also provide an explanation of why TTAGGG repeats, if placed centromere-proximal to TG repeats at telomeres, are counted as a telomere sequence (Berthiau et al., 2006; Bah et al., 2011).

Tbf1 and Rap1 collaborate to inhibit MRX and Tel1 localization to DNA ends with short TG repeats nearby. Localization of MRX complex to DNA ends leads to accumulation of ssDNA tracts at DNA

length by Southern blots (top). Cells were incubated with galactose at 37°C to degrade Tbf1-d protein. After 3 h of incubation cells were harvested to monitor Rad53 phosphorylation by immunoblotting with anti-Rad53 antibodies (bottom). Tubulin was detected as a loading control. (B) Effect of Tbf1 depletion on checkpoint response in telomerase-deficient cells. Telomerase-deficient *est1Δ tbf1-d* (KSC2867) cells were obtained after streaking the strain carrying the *URA3*-marked *EST1* plasmid. Colonies arisen on plates containing 5-FOA were grown to the late log phase. Cells were continuously grown after dilution and then synchronized with nocodazole at 30°C as in A. Aliquots of cells were harvested to monitor the telomere length by Southern blots (top). After synchronization, half of the rest was incubated with galactose at 37°C to degrade Tbf1 protein. The other half was incubated with glucose at 30°C to retain Tbf1 expression. After 3 h of incubation, cells were harvested to monitor Rad53 phosphorylation as in A.

ends, thereby activating the Mec1-checkpoint pathway as well (Nakada *et al.*, 2003a, 2004). Tbf1 depletion enhances checkpoint activation in cells containing short telomeres but does not stimulate checkpoint activation in cells containing normal-length telomeres. Moreover, Tbf1 is essential for inhibition of checkpoint activation at DNA ends where a subtelomeric sequence is placed together with a short TG repeat sequence. These observations support the model in which Tbf1 plays a critical role in maintaining telomere homeostasis at short telomeres. The presence of the Tbf1-binding TTAGGG sequence at the telomere junctions could reduce the length of the adjacent telomeric TG sequence (Berthiau *et al.*, 2006; Bah *et al.*, 2011). As telomeres shorten, the TG sequence disappears earlier than the centromere-proximal TTAGGG sequence. Binding of Rap1 and Rif2 decreases in proportion as telomeres become short (McGee *et al.*, 2010). Rif1 might remain associated with short telomeres, but Rif1 by itself does not inhibit MRX localization. Both Rif1 and Rif2 require Rap1 to inhibit MRX localization (Hirano *et al.*, 2009). Moreover, Rif1 function is partially dependent on Rif2 function when Rif1 binding near DNA ends is limited (Hirano *et al.*, 2009). Thus Rif1 and Rif2 functions become attenuated at short telomeres. Of note, Tbf1 and Rap1 act independently of Rif1 or Rif2. Not all subtelomeres possess multiple Tbf1-binding consensus motifs at the subtelomere–telomere junctions. A single unreparable DNA break, however, induces the damage checkpoint response (Sandell and Zakian, 1993; Lee *et al.*, 1998). These observations are consistent with the idea that Tbf1 plays a critical role in DNA-end protection at short telomeres adjacent to subtelomeric TTAGGG repeats. Inhibition of MRX and Tel1 recruitment could prevent degradation of DNA ends but might also decrease telomerase recruitment. Previous studies showed, however, that the N-terminus of Tbf1 fused to the Gal4-DNA-binding domain (Gbd-Tbf1N) promotes telomere addition near the Gal4 binding sequence in *tel1Δ* mutants when telomeres are short (Arneric and Lingner, 2007). Tbf1 thus may stimulate telomerase independent of the MRX-Tel1 pathway at short telomeres. Several lines of evidence established that the Cdc13-Stn1-Ten1 complex plays a key role in preventing checkpoint activation at telomeres (Weinert *et al.*, 1994; Garvik *et al.*, 1995; Grandin *et al.*, 2001; Vodenicharov and Wellinger, 2006; Vodenicharov *et al.*, 2010; Petreaca *et al.*, 2007; Xu *et al.*, 2009). Cdc13 can bind to single-strand TTAGGG DNA; however, the affinity to TTAGGG repeat DNA is much weaker than that to TG repeat DNA (Lin and Zakian, 1996). Tbf1 and Rap1 thus appear to function as the last bastion to resist nucleolytic activities that intrude into non-TG sequence.

Rap1 and Tbf1 both contain Myb-related DNA-binding domains, but sequence-specific DNA binding is dispensable for the inhibition of MRX localization. Substitution mutations of *TBF1* at the N-terminal region confer defects in inhibiting MRX localization without affecting protein–protein interaction. These results suggest that the N-terminus of Tbf1 constitutes a domain that mediates inhibition of MRX localization. *TBF1* is required for cell proliferation (Brigati *et al.*, 1993). The substitution mutations disrupt the essential function of Tbf1 as well. Other studies showed that another N-terminal region of Tbf1 regulates telomere length independently of the Tel1 pathway (Berthiau *et al.*, 2006; Arneric and Lingner, 2007). Thus the N-terminus mediates multiple functions of Tbf1 protein. Recent studies indicate that like Rap1, Tbf1 controls the transcriptional activation of genes required for ribosome biosynthesis (Lavoie *et al.*, 2010; Preti *et al.*, 2010). However, it seems less likely that transcriptional activation leads to inhibition of MRX recruitment. Although *lacI*-Gal4 functions as a transcriptional activator similar to *lacI*-Tbf1, no inhibition of MRX localization was observed with the combination of *lacI*-

Gal4 and TetR-Rap1 (data not shown). Further studies will be needed to understand the mechanism of how Rap1 and Tbf1 attenuate MRX binding at DNA ends. At this moment, it is not precisely determined how the MRX complex recognizes and associates with DNA ends.

Budding yeast tolerate vertebrate-sequence telomeres containing only TTAGGG repeats (Alexander and Zakian, 2003). Neither Rap1 nor Rif2 binding was detected at TTAGGG telomeres, indicating that Tbf1 contributes to telomere maintenance independently of Rap1. Tbf1 therefore could protect DNA ends similarly to Rap1. Consistent with the idea, while this manuscript was under revision, Tbf1 was shown to inhibit MRX localization at a DNA end flanked by a 230–base pair TTAGGG repeat (Ribaud *et al.*, 2011). Rap1 collaborates with Rif1 and Rif2 at wild-type TG telomeres (Hardy *et al.*, 1992; Wotton and Shore, 1997), whereas Tbf1 appears to act with other telomere-binding proteins at TTAGGG telomeres. Whereas yeast strains with wild-type TG telomeres undergo cell division to senescence after telomerase loss, telomerase is essential for cell proliferation of strains with TTAGGG telomeres (Bah *et al.*, 2011). Of note, telomere-capping Cdc13 protein was much more abundant at TTAGGG telomeres than at wild-type TG telomeres (Alexander and Zakian, 2003). Thus Rap1 and Tbf1 appear to exert related but different functions at distal telomeric regions.

Mammalian TRF1 and TRF2 and fission yeast Taz1 proteins form homodimers through a homodimerization domain at the N-terminus and bind to telomeric TTAGGG repeats through a Myb-related domain at the C-terminus (Palm and de Lange, 2008; Moser and Nakamura, 2009). Similarly, Tbf1 proteins interact with each other and bind to DNA through a Myb-related domain at the C-terminus. Although the N-terminus of Tbf1 is not structurally related to that of TRF1, TRF2, or Taz1, it is required for protein–protein interaction of Tbf1. Tbf1 does not bind to telomeres; it is concentrated at subtelomeric and intergenic regions (Hogues *et al.*, 2008; Lavoie *et al.*, 2010). Of interest, recent evidence indicates that mammalian TRF2 localizes to TTAGGG-rich extratelomeric regions as well (Martinez *et al.*, 2010; Simonet *et al.*, 2011; Yang *et al.*, 2011). TRF1, TRF2, and Taz1 are involved in telomere protection (Miller and Cooper, 2003; Denchi and de Lange, 2007; Palm and de Lange, 2008; Martinez *et al.*, 2009). TRF2 and Taz1 act in complex with RAP1 and Rap1, respectively (Palm and de Lange, 2008; Moser and Nakamura, 2009). We have shown that budding yeast Tbf1 collaborates with Rap1 to protect DNA ends of short telomeres. Further characterization of Tbf1 should contribute to better understanding of telomere maintenance and telomere evolution.

## MATERIALS AND METHODS

### Plasmids

The pXIII<sub>R</sub>-TG<sub>81</sub>-HO and pXIII<sub>R</sub>-HO plasmid were generated as follows. Complementary oligonucleotides containing the chromosome XIII-R subtelomeric sequence (KS2438 and KS2439) were annealed and digested with *Bam*HI and *Eco*RI, generating the *Bam*HI-*Eco*RI XIII<sub>R</sub> fragment. Two DNA fragments, containing both the TG<sub>81</sub> sequence and the HO cleavage site or the HO cleavage site alone, were obtained by PCR with oligonucleotides (KS1679 and KXS007) using the pTG<sub>81</sub>-HO plasmid (Hirano and Sugimoto, 2007) as a template. A DNA fragment containing both the TG<sub>81</sub> sequence and the HO cleavage site was digested with *Mfe*I and *Not*I and cloned together with the *Bam*HI-*Eco*RI XIII<sub>R</sub> fragment into *Bam*HI/*Not*I-treated pTG<sub>81</sub>-HO, resulting in pXIII<sub>R</sub>-TG<sub>81</sub>-HO. Another DNA fragment containing the HO cleavage site alone was digested with *Eco*RI and *Not*I and cloned together with the *Bam*HI-*Eco*RI XIII<sub>R</sub> fragment into *Bam*HI/*Not*I-treated pTG<sub>81</sub>-HO, resulting in pXIII<sub>R</sub>-HO. The pTAG<sub>2</sub>-TG<sub>81</sub>-HO, pTAG<sub>4</sub>-TG<sub>81</sub>-HO, pTAG<sub>10</sub>-TG<sub>81</sub>-HO, or

pCTA<sub>10</sub>-TG<sub>81</sub>-HO plasmid was constructed as follows. First, DNA fragments containing 2xTTAGGG, 4xTTAGGG, 10xTTAGGG, or 10xCCCTAA were generated by PCR using pTG<sub>81</sub>-HO as a template. The PCR primers are KS1255 and KS2324 for 2x TTAGGG, KS2325 for 4xTTAGGG, KS2110 for 10xTTAGGG, or KS2327 for 10xCCCTAA. The resulting PCR fragments were treated with *Nco*I and *Eco*RI and cloned together with the *Mfe*I-*Not*I DNA fragment containing both the TG<sub>81</sub> sequence and the HO cleavage site (see earlier discussion) into *Nco*I/*Not*I-treated pTG<sub>81</sub>-HO. The placO<sub>8</sub>-TG<sub>81</sub>-HO plasmid was generated as follows. First, the placO<sub>8</sub>-HO plasmid containing 8xlacO was generated. The 4xlacO sequence was fused to the KanMX cassette by PCR with the primer pair (KS1255 and KS1746) using pTG<sub>81</sub>-HO as a template, and the resulting PCR fragment was digested with *Nco*I and *Eco*RI and cloned into *Eco*RI/*Nco*I-treated pTG<sub>81</sub>-HO, generating placO<sub>4</sub>-HO. To duplicate 4xlacO, an *Mfe*I-*Not*I fragment containing 4xlacO was cloned into *Eco*RI/*Not*I-treated placO<sub>4</sub>-HO plasmid. Finally, the *Mfe*I-*Not*I fragment containing the TG<sub>81</sub> sequence and HO cleavage site (see earlier discussion) was cloned into *Eco*RI/*Not*I-treated placO<sub>8</sub>-HO. Similarly, an *Mfe*I-*Not*I fragment containing the 4xtetO sequence and the HO site was prepared from pтетO<sub>4</sub>-HO (Hirano *et al.*, 2009) and cloned into *Eco*RI/*Not*I-treated placO<sub>8</sub>, generating the placO<sub>8</sub>-тетO<sub>4</sub>-HO plasmid. The pXIII<sub>R</sub>-TG<sub>22</sub>-HO-CA plasmid was constructed as follows. First, the TG<sub>22</sub> sequence and the XIII<sub>R</sub> sequence were fused by PCR with the KSX006-KS2577 primer set using the pTG<sub>22</sub>-HO-CA plasmid (Hirano and Sugimoto, 2007) as a template. The PCR product was treated with *Eco*RI and *Sal*I and cloned into *Eco*RI/*Sal*I-digested pTG<sub>22</sub>-HO-CA. The *Bam*HI-*Eco*RI XIII<sub>R</sub> fragment (see earlier discussion) was introduced into *Bam*HI/*Eco*RI-treated pTG<sub>22</sub>-HO-CA, generating pXIII<sub>R</sub>-HO-CA. The HO-CA construct was described previously (Hirano and Sugimoto, 2007). All the foregoing plasmids were cleaved with *Not*I and *Sal*I to direct integration into the *ADH4* locus. The XIII<sub>R</sub> and 10XTTAGGG sequence fused to the TG<sub>81</sub> sequence were counted as a telomere sequence after the TG<sub>81</sub> ends were converted to telomeres (Supplemental Figure 3), consistent with the previous findings that TTAGGG repeats, if placed centromere-proximal to TG repeats, behave as a telomere sequence (Berthiau *et al.*, 2006; Bah *et al.*, 2011).

To construct YCp plasmids carrying *TBF1*, the coding region of *TBF1* together with the 5'- and 3'-noncoding region was amplified from genomic DNA by PCR with the KS2330-KS2331 primer set and digested with *Bam*HI and *Sal*I. The resulting fragment was cloned into YCplac22 or YCplac33 (Gietz and Sugino, 1988), generating YCpT-TBF1 or YCpU-TBF1, respectively. To construct HA- or FLAG-tagged *TBF1*, two copies of an HA or FLAG epitope were fused to the N-terminal coding sequence by PCR using the KS2337-2338 or KS2335-2336 primer set, respectively (Wakayama *et al.*, 2001). The tagged constructs were cloned into YCplac22, YCplac33, pRS314, or pRS316 (Sikorski and Hieter, 1989). The N-terminal deletion or amino acid substitution mutations of *TBF1* were introduced by PCR-based methods (Hirano *et al.*, 2009). The oligonucleotides for PCR were KS2446 and KS2492 (*tbf1-ΔN*), KS2497 and KS2498 (*tbf1-25*; L177A, Q180A), or KS2503 and KS2504 (*tbf1-26*; L187A, Q190A). The resulting DNA fragments containing *tbf1* mutations were cloned into pRS314 or pRS316. The plasmids containing *Lacl* or *Lacl*-Tbf1 fusion genes were constructed as follows. A DNA fragment containing the *lacl* coding sequence and the SV40 nuclear localization signal were amplified by PCR with the KS1747-KS1756 primer set from pAFS78 (Straight *et al.*, 1996) and digested with *Sac*I and *Sal*I. The resulting DNA fragment was cloned into YCplac33 or YCplac111 (Gietz and Sugino, 1988) carrying a truncated version of the *ADH1* promoter, resulting in YCpU-*lacl* or YCpL-*lacl*, respectively. The coding se-

quences of *TBF1* wild-type or mutation genes were amplified by PCR with the KS2091-2092 primer set, digested with *Sac*I and *Sac*II, and cloned into YCpU-*lacl* or YCpL-*lacl*. The *Lacl*-Tbf1, FLAG-TBF1, or HA-TBF1 construct fully rescues the lethality of *tbf1Δ* mutants (data not shown). The YCpU-EST1 plasmid was constructed by transferring the *Pst*I-*Xba*I fragment of the *EST1* gene on pVL499 (obtained from V. Lundblad, Salk Institute) to YCplac33. The plasmid pтетR-RAP1 was previously described (Hirano *et al.*, 2009). The *ADE1*- or *TRP1* marked GAL-HO plasmid was described elsewhere (Hirano *et al.*, 2009).

### Strains

The heat-inducible TBF1 degon (*tbf1-d*) was constructed as described previously (Kanemaki *et al.*, 2003). As a result the *tbf1-d* construct is fused to a myc-epitope. A *his3* version of the KSC006 strain (*ade1*, *his2*, *leu2*, *trp1*, *ura3*) (Wakayama *et al.*, 2001) was constructed as follows. First, a *HIS2* fragment (obtained from C. Wittenberg, Scripps Research Institute) was introduced into its own locus to create a His<sup>+</sup> strain. Next the *HIS3* gene was disrupted by a PCR strategy using the *hisG-URA3-hisG* construct (Alani *et al.*, 1987). Finally, Ura<sup>-</sup> cells were selected out on plates containing 5-FOA (Boeke *et al.*, 1987). To delete the *KanMX* marker from the degon construction, *KanMX* was replaced with *URA3* by using the plasmid M4758 (Voth *et al.*, 2003). Cells carrying an *ura3* version of the degon construct were obtained by selecting on plates containing 5-FOA. Epitope tagging and integration or deletion construction were performed by PCR-based methods (Wach *et al.*, 1994; Reid *et al.*, 2002; Janke *et al.*, 2004). The *MEC1-HA*, *MRE11-myc*, *RAP1-HA*, and *TEL1-HA* constructs were as described previously (Wakayama *et al.*, 2001; Nakada *et al.*, 2003a; Hirano and Sugimoto, 2007; Hirano *et al.*, 2009). The *stn1-13* mutation was obtained from M. Charbonneau (Universite de Tours, France; Grandin *et al.*, 2001). Strains used in this study are listed in Supplemental Table S1.

### Telomere shortening by loss of the *EST1* gene

First, *tbf1-d est1Δ* cells carrying YCpU-EST1 were streaked on plates containing 5-FOA to obtain Ura<sup>-</sup> colonies that had lost the *EST1* plasmid. Cells from a single colony were grown in sucrose medium overnight. The culture was then diluted fourfold and grown for 7 h. Similar dilution and culture were repeated. During the fifth serial dilution culture, cells showed decreased growth rate (Ijima and Greider, 2003). Cells from the first, second, third, or fourth serial dilution cultures were grown in sucrose medium for 2 h after diluting to a density of ~5 × 10<sup>7</sup>/ml. Cells were then treated with nocodazole (15 μg/ml) at 30°C for 2 h to block further cell division. The cultures were then shifted to 37°C after addition of galactose (to a final concentration of 2%) to deplete Tbf1 protein or maintained at 30°C after addition of glucose to retain Tbf1 expression.

### Other methods

Southern blotting was performed as described, except for using <sup>32</sup>P-labeled hybridization DNA probes (Hirano *et al.*, 2009). The ChIP assay was described previously (Hirano *et al.*, 2009; Fukunaga *et al.*, 2011). The PCR primers were KS1744 and KS1745 for the HO site and KS2037 and KS2038 for the *SMC2* locus. HO cleavage for each cassette was analyzed by performing real-time PCR with input samples used for the ChIP assay. HO cutting efficiency represents the ratio of the signal from the HO site using the cutting-site primer pair (KS1311 and KS1312) after HO induction to the signal before HO induction. Signals from the HO site were normalized to signals from the control *SMC2* locus. Immunoprecipitation and immunoblotting were performed as described (Fukunaga *et al.*, 2011). Rad53 was

detected with anti-Rad53 antibody (gift from John Diffley, Cancer Research UK London Research Institute). Tubulin was detected with the monoclonal TAT-1 antibody (Woods *et al.*, 1989). DNA flow cytometry analysis was performed as described previously (Burke *et al.*, 2000; Nakada *et al.*, 2003b). Oligonucleotides used in this study are listed in Supplemental Table S2.

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