

Requirement of the FATC domain of protein kinase Tel1 for localization to DNA ends and target protein recognition

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ABSTRACT Two large phosphatidylinositol 3-kinase-related protein kinases (PIKKs), ATM and ATR, play a central role in the DNA damage response pathway. PIKKs contain a highly conserved extreme C-terminus called the FRAP-ATM-TRRAP-C-terminal (FATC) domain. In budding yeast, ATM and ATR correspond to Tel1 and Mec1, respectively. In this study, we characterized functions of the FATC domain of Tel1 by introducing substitution or truncation mutations. One substitution mutation, termed *tel1-21*, and a truncation mutation, called *tel1-ΔC*, did not significantly affect the expression level. The *tel1-21* mutation impaired the cellular response to DNA damage and conferred moderate telomere maintenance defect. In contrast, the *tel1-ΔC* mutation behaved like a null mutation, conferring defects in both DNA damage response and telomere maintenance. Tel1-21 protein localized to DNA ends as effectively as wild-type Tel1 protein, whereas Tel1-ΔC protein failed. Introduction of a hyperactive *TEL1-hy* mutation suppressed the *tel1-21* mutation but not the *tel1-ΔC* mutation. In vitro analyses revealed that both Tel1-21 and Tel1-ΔC proteins undergo efficient autophosphorylation but exhibit decreased kinase activities toward the exogenous substrate protein, Rad53. Our results show that the FATC domain of Tel1 mediates localization to DNA ends and contributes to phosphorylation of target proteins.

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INTRODUCTION

The maintenance of genomic stability relies on a network of cellular processes, including DNA replication, DNA repair, and cell-cycle progression (Elledge, 1996; Harper and Elledge, 2007). Two phosphatidylinositol 3-kinase-related protein kinases (PIKKs), ATM and ATR, coordinate the cellular processes and govern the DNA dam-

age response pathway. The DNA damage response pathway, also called the DNA damage checkpoint pathway, is highly conserved in eukaryotes (Harper and Elledge, 2007; Cimprich and Cortez, 2008). In budding yeast, ATM and ATR correspond to Tel1 and Mec1, respectively (Elledge, 1996). ATR/Mec1 recognizes many different types of DNA damage. By contrast, ATM/Tel1 acts specifically in response to DNA double-strand breaks (DSBs; Harper and Elledge, 2007; Cimprich and Cortez, 2008). Telomeres—the ends of linear eukaryotic chromosomes—have a specialized chromatin structure that discriminates chromosome ends from DSBs (Smogorzewska and de Lange, 2004; Wellinger and Zakian, 2012). ATM/Tel1 also maintains telomere length homeostasis (Greenwell et al., 1995; Metcalfe et al., 1996).

Evidence indicates that the Mre11-Rad50-Nbs1 (MRN) complex, which corresponds to the Mre11-Rad50-Xrs2 (MRX) complex in budding yeast, is a sensor that activates ATM/Tel1 (Lee and Paull, 2007). MRN/MRX localizes to DNA ends and plays multiple roles in DNA end processing (Symington and Gautier, 2011). In addition, MRN/MRX recruits ATM/Tel1 to DNA ends via the C-terminus of Nbs1/Xrs2 (Nakada et al., 2003a; Falck et al., 2005). The recruitment of ATM/Tel1 to DSBs is required for ATM/Tel1-mediated DNA damage

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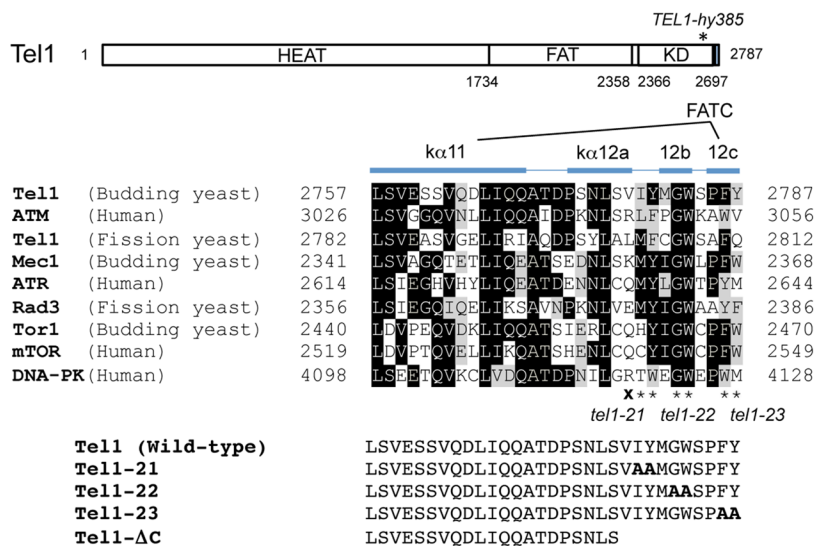
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Abbreviations used: FAT, FRAP-ATM-TRRAP; FATC, FRAP-ATM-TRRAP-C-terminal; HEAT, Huntingtin-elongation factor 3-A subunit of protein phosphatase 2A-TOR1; PI3K, phosphatidylinositol 3-kinase; PIKK, phosphatidylinositol 3-kinase-related protein kinase; TTT, Tel2-Tti1-Tti2.

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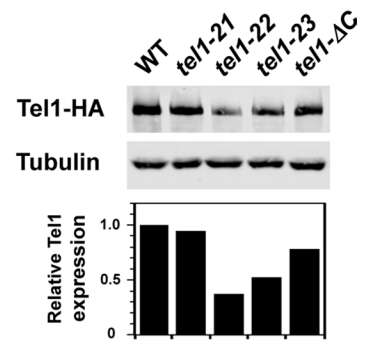


FIGURE 1: Substitution and truncation mutations of Tel1 at the conserved extreme C-terminal region. (A) Schematic of *tel1* mutations at the FATC domain. Tel1 possesses a kinase domain (KD) within the C-terminal portion. The aligned sequences are derived from the FATC domains of ATM-family proteins (Tel1, human ATM, and fission yeast Tel1), ATR family proteins (Mec1, human ATR, and fission yeast Rad3), TOR proteins (budding yeast Tor1 and human mTOR), and human DNA-PK. Identical amino acid residues are boxed in black. Related amino acid residues are highlighted in gray. The α -helix structures $\kappa\alpha 11$ and $\kappa\alpha 12$ (12a, 12b, 12c) are adopted from Yang *et al.* (2013). The *tel1-21*, *tel1-22*, and *tel1-23* mutations change the amino acid residues into alanine at positions 2779–2780, 2782–2783, and 2786–2787 (indicated by asterisks), respectively. The *tel1-ΔC* mutation introduces the termination codon for the amino acid residue at 2778 (indicated by X), truncating the amino acid residues from 2778 through 2787. The HEAT repeats and the FAT domains are shown as well. The residue numbers of the FAT and KD domains for Tel1 are indicated along with the overall Tel1 structure. The mutation site of *TEL1-hy385* is shown on the overall structure (Baldo *et al.*, 2008). (B) Expression level of Tel1 in *tel1-21*, *tel1-22*, *tel1-23*, and *tel1-ΔC* mutants. Top, cells expressing Tel1-HA (KSC1709), Tel1-21-HA (KSC3087), Tel1-22-HA (KSC3088), Tel1-23-HA (KSC3089), or Tel1-ΔC-HA (KSC3549) were subjected to immunoblotting analysis with anti-HA or anti-tubulin antibodies. Bottom, protein levels of Tel1 were normalized to tubulin and expressed relative to the wild type.

response in vivo (Nakada *et al.*, 2003a; Falck *et al.*, 2005). Furthermore, purified MRN/MRX complex increases catalytic activity of ATM/Tel1 in the presence of DNA fragments in vitro (Lee and Paull, 2005; Fukunaga *et al.*, 2011). Thus MRN/MRX interacts with and activates ATM/Tel1 at DNA ends. ATM/Tel1 phosphorylates the checkpoint mediators, including MDC1 in mammals and Rad9 in budding yeast, and in turn stimulates the downstream targets, such as protein kinase Chk2 in mammals and protein kinase Rad53 in budding yeast (Vialard *et al.*, 1998; Schwartz *et al.*, 2002; Pellicoli and Foiani, 2005; Harper and Elledge, 2007). In budding yeast, MRX also recognizes short telomeres and recruits Tel1 to the DNA ends (Bianchi and Shore, 2007; Chang *et al.*, 2007; Hector *et al.*, 2007; Sabourin *et al.*, 2007; Viscardi *et al.*, 2007), promoting telomere addition at short telomeres. Recent evidence suggests that Tel1 phosphorylates Cdc13 and stimulates telomerase recruitment (Tseng *et al.*, 2006; Shen *et al.*, 2014). However, this proposed mechanism has been under debate (Gao *et al.*, 2010; Wu and Zakian, 2011; Wellinger and Zakian, 2012).

ATM/Tel1 and other PIKKs, including ATR, DNA-PKcs, mammalian target of rapamycin (mTOR; FRAP), and TRRAP, share a similar domain organization (Lempiainen and Halazonetis, 2009; Figure 1A). The kinase domains of PIKKs are located near their carboxyl termini and are flanked by the conserved FRAP-ATM-TRRAP (FAT) and FAT C-terminal (FATC) domains (Bosotti *et al.*, 2000). Large amino-terminal and internal regions of PIKKs are composed of numerous α -

helical Huntingtin-elongation factor 3-A subunit of protein phosphatase 2A-TOR1 (HEAT) repeats (Perry and Kleckner, 2003). PIKKs share an ~30-residue FATC domain at the far C-terminus. However, the FATC domain is not completely unique to PIKKs; structure analyses indicate that its N-terminal half forms a helix that is also found in phosphatidylinositol 3-kinases (PI3Ks), whereas the C-terminal half forms another helix, which is unique to PIKKs (Walker *et al.*, 1999; Miller *et al.*, 2010; Yang *et al.*, 2013).

In this study, we characterized the PIKK-specific FATC function of Tel1 by introducing substitution or truncation mutations. A substitution mutation (*tel1-21*) and a truncation mutation (*tel1-ΔC*) affected phosphorylation of exogenous substrates but not autophosphorylation. Moreover, the *tel1-ΔC* mutation caused a defect in localization to DSBs. These results show that the FATC domain of Tel1 plays a key role in recognition of target proteins, as well as in accumulation at DNA ends.

RESULTS

Substitution and truncation mutations in the FATC domain of Tel1

PIKKs, including Tel1, share the FATC domain at the C-terminus (Bosotti *et al.*, 2000). Whereas the N-terminal half of the FATC domain forms a helix that is also found in PI3Ks, the C-terminal half that forms another helix ($\kappa\alpha 12$) is unique to PIKKs (Figure 1A; Walker *et al.*, 1999; Miller *et al.*, 2010; Yang *et al.*, 2013). To understand the

significance of the FATC domain, we focused on the last 15 amino acids, which consist of the α 12 helix. We constructed three substitution mutations in which the amino acid residues 2779–2780, 2782–2783, or 2786–2787 were all replaced with alanine. These mutations were termed *tel1-21*, *tel1-22*, and *tel1-23*, respectively (Figure 1A). The human R3047X ATM mutant lacking the last 10 amino acids activates checkpoint signaling in response to DSB induction but fails to respond to oxidative stress (Guo *et al.*, 2010). We also generated an equivalent truncation mutation named *tel1- Δ C* (Figure 1A). Because the FATC domain is required for proper expression of ATM and ATR family proteins (Gilad *et al.*, 1998; Nakada *et al.*, 2005), we first examined the expression level of their gene products (Figure 1B). The *tel1-22* and *tel1-23* mutations decreased the expression level of Tel1 protein (~35 and ~50% of that of wild-type protein, respectively). Unlike the *tel1-22* and *tel1-23* mutations, the *tel1-21* and *tel1- Δ C* mutations did not significantly affect the expression level.

Effect of FATC mutations on the cellular responses to DSB induction

Tel1, like other ATM family proteins, plays a role in the cellular response to DNA damage (Morrow *et al.*, 1995; Sanchez *et al.*, 1996). Although *tel1 Δ* mutation by itself does not confer apparent sensitivity to DNA damage, it does exacerbate the sensitivity of *mec1* mutants to DNA damage (Morrow *et al.*, 1995; Sanchez *et al.*, 1996). We therefore asked whether these *tel1* mutations confer a similar effect (Figure 2, A and B). Because the *mec1 Δ tel1 Δ* double muta-

tion results in a loss of cell proliferation (Chan *et al.*, 2001), we used a weak allele of *MEC1*, *mec1-81*, to assess the effect of *tel1* mutations (Nakada *et al.*, 2003b). As found previously, *mec1-81* single mutants were sensitive to DSB-inducing phleomycin and camptothecin, but introduction of a *tel1 Δ* mutation enhanced the sensitivities to these damaging agents in *mec1-81* mutants (Nakada *et al.*, 2003a). Each of the *tel1-22*, *tel1-23*, and *tel1- Δ C* mutations increased the sensitivities similar to the *tel1 Δ* mutation. However, the *tel1-21* mutation caused a milder effect than the *tel1-22*, *tel1-23* and *tel1- Δ C* mutations.

Tel1 controls phosphorylation and activation of Rad53 after DNA damage, and its phosphorylation status is well correlated with activation of DNA damage checkpoint (Elledge, 1996; Longhese *et al.*, 1998). We next determined the effect of these mutations on Rad53 phosphorylation after DNA damage (Figure 2C). To detect Tel1-specific signaling, we monitored Rad53 phosphorylation in a *mec1-81 sae2 Δ* background. Mutations in *SAE2* enhance Tel1-mediated checkpoint signaling (Usui *et al.*, 2001; Fukunaga *et al.*, 2011). Cells expressing hemagglutinin (HA)-tagged Rad53 were grown to log phase and then arrested at G2/M with nocodazole. After arrest, cells were exposed to phleomycin and subjected to immunoblotting analysis with anti-HA antibodies. Rad53 phosphorylation occurred in wild-type cells, whereas phosphorylation decreased in *mec1-81* mutants. However, Rad53 phosphorylation became readily detectable in *mec1-81 sae2 Δ* mutants. Consistent with the current view that the *sae2 Δ* mutation enhances Tel1-mediated checkpoint signaling (Usui *et al.*, 2001; Fukunaga *et al.*, 2011), introduction of the

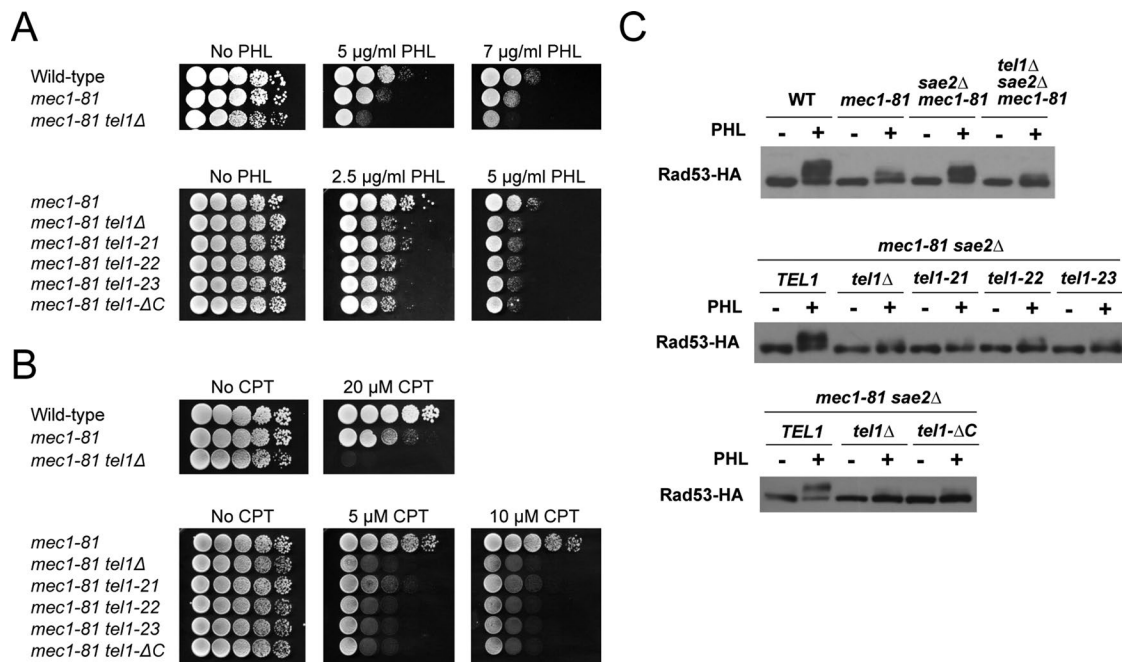


FIGURE 2: DNA damage response of the FATC mutants. (A, B) DNA damage sensitivity of *tel1-21*, *tel1-22*, *tel1-23*, and *tel1- Δ C* mutants. Tenfold serial dilutions of cultures were spotted on yeast extract/peptone/dextrose (YEPD) medium with or without phleomycin (PHL; A) or camptothecin (CPT; B). Plates were incubated at 30°C for 2 or 3 d. Strains used were the wild type (KSC1560), *mec1-81* (KSC1662), *mec1-81 tel1 Δ* (KSC1564), *mec1-81 tel1-21* (KSC3102), *mec1-81 tel1-22* (KSC3100), *mec1-81 tel1-23* (KSC3101), and *mec1-81 tel1- Δ C* (KSC3552). All the strains contain an *sml1 Δ* mutation, which suppresses the lethality of *mec1* mutants. (C) Effect of *tel1* mutations on DNA damage checkpoint signaling. Cells expressing Rad53-HA were arrested with nocodazole (15 μ g/ml) at G2/M and exposed to phleomycin (20 μ g/ml) for 1 h. Cells were harvested and subjected to immunoblotting analysis with anti-HA antibodies. Strains used were the wild type (KSC1560), *mec1-81* (KSC1662), *mec1-81 sae2 Δ* (KSC3140), *mec1-81 sae2 Δ tel1 Δ* (KSC3141), *mec1-81 sae2 Δ tel1-21* (KSC3142), *mec1-81 sae2 Δ tel1-22* (KSC3143), *mec1-81 sae2 Δ tel1-23* (KSC3144), and *mec1-81 sae2 Δ tel1- Δ C* (KSC3555). All the strains contain an *sml1 Δ* mutation.

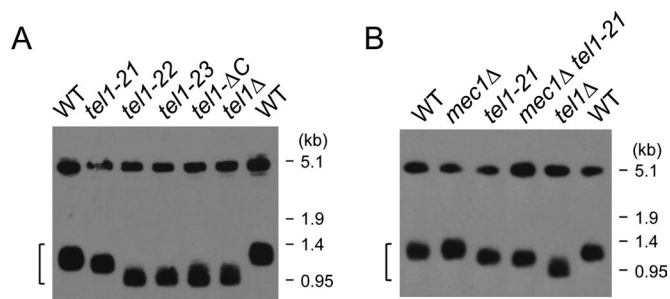


FIGURE 3: Telomere length of the FATC mutants. (A) Genomic DNA prepared from cells was digested with *Xho*I and analyzed by Southern blots to monitor the telomere length. The hybridization probe was a DNA fragment containing ~0.9-kb *Y'* element and ~120-base pair TG repeat sequence. The bracket shows DNA fragments containing the telomere. Strains used were the wild type (KSC006), *tel1Δ* (KSC1057), *tel1-21* (KSC3378), *tel1-22* (KSC3379), *tel1-23* (KSC3377), and *tel1-ΔC* (KSC3556). (B) Southern blotting analysis performed as in A. Strains used were the wild type (KSC1560), *tel1Δ* (KSC1661), *mec1Δ* (KSC1561), *tel1-21* (KSC3629), and *mec1Δ tel1-21* (KSC3630). All the strains contain an *sml1Δ* mutation.

tel1Δ mutation decreased Rad53 phosphorylation in *mec1-81 sae2Δ* mutants. The *tel1-21*, *tel1-22*, *tel1-23* or *tel1-ΔC* mutation also decreased Rad53 phosphorylation in *mec1-81 sae2Δ* mutants.

Telomere maintenance in FATC mutants

In budding yeast, Tel1 plays an essential role in telomere length maintenance (Wellinger and Zakian, 2012). We further determined the effect of *tel1-21*, *tel1-22*, *tel1-23*, or *tel1-ΔC* mutation on telomere length (Figure 3A). Telomeres become shorter in *tel1Δ* mutants than in wild-type cells (Greenwell *et al.*, 1995; Wellinger and Zakian, 2012). Telomeres in *tel1-22*, *tel1-23*, or *tel1-ΔC* mutants were as short as those in *tel1Δ* mutants. In contrast, telomeres became slightly shorter in *tel1-21* mutants than in wild-type cells. Although *MEC1* is not essential for telomere homeostasis in *TEL1* cells, *Mec1* plays a key role in telomere length regulation in the absence of Tel1 (Wellinger and Zakian, 2012). It is therefore possible that *Mec1* contributes to telomere addition in *tel1-21* mutants. We then examined the effect of *mec1Δ* mutation on telomere length in *tel1-21* mutants (Figure 3B). The telomere length was very similar in *tel1-21* single and *mec1Δ tel1-21* double mutants. Thus the *tel1-21* mutation largely eliminates Tel1 function in DNA damage response but retains some telomere maintenance function. In contrast, the *tel1-22*, *tel1-23*, or *tel1-ΔC* mutation behaves like a *tel1*-null mutant.

Defective localization of Tel1-ΔC protein to DNA ends

Tel1 localizes to DNA ends to activate the signaling pathway and elongate short telomeres (Nakada *et al.*, 2003a; Bianchi and Shore, 2007; Chang *et al.*, 2007; Hector *et al.*, 2007; Sabourin *et al.*, 2007). We thus examined the effect of *tel1-21* or *tel1-ΔC* mutation on localization of Tel1 to HO-induced DSBs by chromatin immunoprecipitation (ChIP) assay (Figure 4A). In budding yeast, HO endonuclease introduces a sequence-specific DSB. We used an experimental system in which cells contain a single HO cleavage site at the *ADH4* locus, and HO is expressed from the GAL-HO plasmid after incubation with galactose (Nakada *et al.*, 2003a). Cells expressing Tel1-HA, Tel1-21-HA, or Tel1-ΔC-HA were transformed with the GAL-HO plasmid. Transformed cells were grown initially in sucrose to repress HO expression and then transferred to medium containing nocodazole

to arrest at G2/M. After arrest, galactose was added to induce HO expression. Cells were then subjected to the ChIP assay. Tel1-21 mutant protein associated with HO-induced DSBs as efficiently as Tel1 wild-type protein did. In contrast, association of Tel1-ΔC protein with DSBs was significantly decreased compared with that of wild-type Tel1 protein.

Because Tel1-ΔC protein does not efficiently accumulate at DNA ends, we determined the effect of *tel1-ΔC* mutation on cellular localization (Figure 4B). Cellular fractionation analysis indicated that Tel1 exists in both nuclear and cytoplasmic fractions. However, the *tel1-ΔC* mutation did not significantly affect the intracellular distribution. Tel1 interacts with the Tel2-Tti1-Tti2 (TTT) complex, which promotes protein stability of PIKKs (Hayashi *et al.*, 2007; Takai *et al.*, 2007, 2010; Anderson *et al.*, 2008; Hurov *et al.*, 2010; Stirling *et al.*, 2011). The TTT complex also controls localization of Tel1 to DNA ends (Anderson *et al.*, 2008). We next examined the effect of *tel1-ΔC* on Tel1-TTT interaction by coimmunoprecipitation assay (Figure 4C). However, Tel1 and Tel1-ΔC were found to interact similarly with Tel2.

Tel1 localizes to DNA ends through an Xrs2-dependent mechanism (Nakada *et al.*, 2003a). We previously showed that Tel1 interacts directly with the C-terminus of Xrs2 in vitro (Hirano *et al.*, 2009). We further examined the effect of *tel1-ΔC* mutation on Tel1-Xrs2 interaction by the pull-down assay (Figure 4D). A glutathione S-transferase (GST) fusion of the Xrs2 C-terminus (GST-Xrs2C) was captured on glutathione-Sepharose beads and incubated with purified FLAG-Tel1 or Tel1-ΔC proteins. Bound proteins were then analyzed by immunoblotting with anti-FLAG antibodies. Although Xrs2 C-terminus was able to bind to the added Tel1 protein, efficient Tel1-Xrs2 interaction did not occur with Tel1-ΔC mutant protein. These results indicate that the FATC domain of Tel1 mediates Tel1-Xrs2 interaction.

Intragenic suppression of *tel1-21* by a hyperactive *TEL1* mutation

Previous studies identified *TEL1-hy* mutations that increase basal catalytic activities (Baldo *et al.*, 2008). Tel1-21 mutant protein localized efficiently to DNA ends, whereas Tel1-ΔC did not. We addressed whether any *TEL1-hy* mutation suppresses the *tel1-21* or *tel1-ΔC* mutation (Figure 5). To this end, we introduced *TEL1-hy* mutations into the *mec1-81 tel1-21* and *mec1-81 tel1-ΔC* strains. One substitution mutation (N2692D) within the kinase domain, termed *TEL1-hy385* (Figure 1A), increased resistance to camptothecin and methylmethane sulfonate (MMS) in *tel1-21* mutant cells but not in *tel1-ΔC* mutants (Figure 5, A and B). We further determined whether the *TEL1-hy385* mutation suppresses telomere defects of *tel1-21* and *tel1-ΔC* mutants. Similar to the *mec1Δ* mutation (Figure 3B), the *mec1-81* mutation had little effect on telomere length regardless of the presence or absence of Tel1 function (Figure 5C). The *TEL1-hy385* mutation elongated telomeres in *mec1-81* single- and *mec1-81 tel1-21* double-mutant cells (Figure 5D). However, the *TEL1-hy385* mutation did not affect telomere length in *mec1-81 tel1-ΔC* cells (Figure 5D). These results are consistent with the finding that Tel1-ΔC protein does not efficiently associate with DNA ends and raise the possibility that Tel1-21 mutant protein has decreased catalytic activity.

Catalytic activity of Tel1-21 and Tel1-ΔC proteins

Mec1 and Tel1 phosphorylate Rad9 to transiently recruit Rad53 nearby at sites of DNA damage (Vialard *et al.*, 1998; Schwartz *et al.*, 2002). In turn, *Mec1* and Tel1 phosphorylate Rad53, thereby increasing the kinase activity of Rad53 (Pelliccioli and Foiani, 2005).

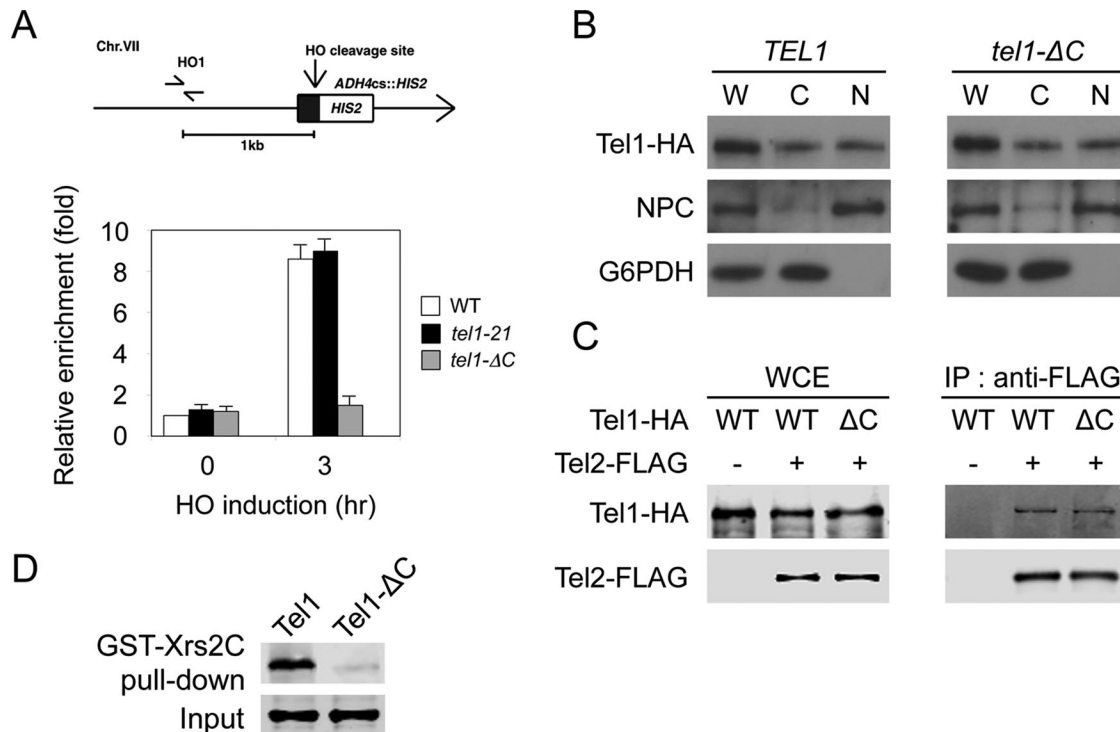


FIGURE 4: Effect of *tel1-21* or *tel1-ΔC* mutation on association with DNA ends. (A) Effect of *tel1-21* or *tel1-ΔC* mutation on Tel1 localization to an HO-induced DSB. Wild-type (KSC1709), *tel1-21* (KSC3087) or *tel1-ΔC* (KSC3549) cells expressing Tel1-HA were transformed with the YCpA-GAL-HO plasmid. Transformed cells were grown in sucrose and treated with nocodazole. After arrest at G2/M, the culture was incubated with galactose for 3 h to induce HO expression. Top, the strains contain an HO cleavage site, marked with *HIS2*, at the *ADH4* locus on chromosome (Chr.) VII. The HO1 primer pair amplifies a region 1 kb away from the HO cleavage site. An arrow represents the telomere. Bottom, cells were subjected to chromatin immunoprecipitation with anti-HA antibodies. Association of Tel1 with an HO-induced DSB was analyzed by real-time PCR. Relative enrichment was determined from three independent experiments. (B) Effect of *tel1-ΔC* mutation on the intracellular distribution. *TEL1*-HA (KSC1709) or *tel1-ΔC*-HA (KSC3549) cells were grown to mid log phase, harvested, and spheroplasted. Spheroplasts were homogenized to prepare whole-cell extracts (W) and then separated into the cytoplasmic (C) and nuclear (N) fractions. Samples from each fraction were separated by SDS-PAGE and immunoblotted with anti-HA, anti-Zwf1 (glucose-6-phosphate dehydrogenase; G6PDH) or anti-nuclear pore complex (NPC) antibodies. (C) Effect of *tel1-ΔC* mutation on Tel1-Tel2 interaction. Whole-cell extracts (WCE) prepared from cells expressing Tel1-HA (KSC1709), Tel2-FLAG Tel1-HA (KSC3633), or Tel2-FLAG *tel1-ΔC*-HA (KSC3634) were subjected to immunoprecipitation (IP) with anti-FLAG antibodies. Samples were immunoblotted with anti-HA or anti-FLAG antibodies. (D) Effect of *tel1-ΔC* mutation on Tel1-Xrs2 interaction. FLAG-Tel1 or FLAG-Tel1-ΔC was incubated with GST-Xrs2C-bound glutathione beads. Proteins bound to glutathione beads were subjected to immunoblotting analysis with anti-FLAG antibodies. The binding assay used 0.5 pmol of FLAG-Tel1 proteins and 70 pmol of GST-Xrs2C.

Several lines of evidence suggest that Mec1 and Tel1 phosphorylate the Rad53 C-terminus after DNA damage (Schwartz *et al.*, 2002; Lee *et al.*, 2003; Smolka *et al.*, 2005; Sweeney *et al.*, 2005). As discussed earlier, the *tel1-21* and the *tel1-ΔC* mutations did not significantly affect the expression level of Tel1. We next tested whether Tel1-21 or Tel1-ΔC protein phosphorylates Rad53 *in vitro* by using GST-Rad53 C-terminus fusion as a substrate (Fukunaga *et al.*, 2011; Figure 6A). Human ATM undergoes autophosphorylation at serine 1981 in response to DNA damage (Bakkenist and Kastan, 2003). S1981 phosphorylation is detected in cells carrying the ATM R3047X gene (Guo *et al.*, 2010), a mutation that is equivalent to the *tel1-ΔC* mutation. We also examined whether Tel1-21 or Tel1-ΔC protein undergoes autophosphorylation (Figure 6A). We used the strain expressing HA-tagged kinase-negative Tel1-KN protein as a negative control (Nakada *et al.*, 2003b). We prepared extracts from cells expressing Tel1-HA, Tel1-21-HA, Tel1-ΔC-HA, or Tel1-KN-HA and immunoprecipitated them with anti-HA antibodies. Immunoprecipitates were then subjected to an *in vitro* kinase assay, in which

radioactive phosphorus (^{32}P) was incorporated into GST-Rad53 by immunoprecipitates containing Tel1-HA. The same reactions were also analyzed for ^{32}P incorporation into Tel1 protein. Both Tel1-21 and Tel1-ΔC mutant proteins were significantly defective in phosphorylating Rad53 compared with wild-type Tel1. However, autophosphorylation of Tel1-21 and Tel1-ΔC mutant proteins occurred similarly to that of wild-type Tel1 protein. Autophosphorylation was not saturated in the reaction condition (20-min incubation), as more-pronounced phosphoincorporation was detected after a prolonged incubation (40-min incubation; Figure 6B). Thus the FATC domain is critical for phosphorylation of Rad53 protein but dispensable for autophosphorylation. Taken together, our results support a model in which the FATC domain of Tel1 mediates recognition of target proteins, as well as localization to DNA ends.

DISCUSSION

The C-terminal half of the FATC domain is specifically conserved among PIKKs, including ATM, ATR, DNA-PK, and mTOR

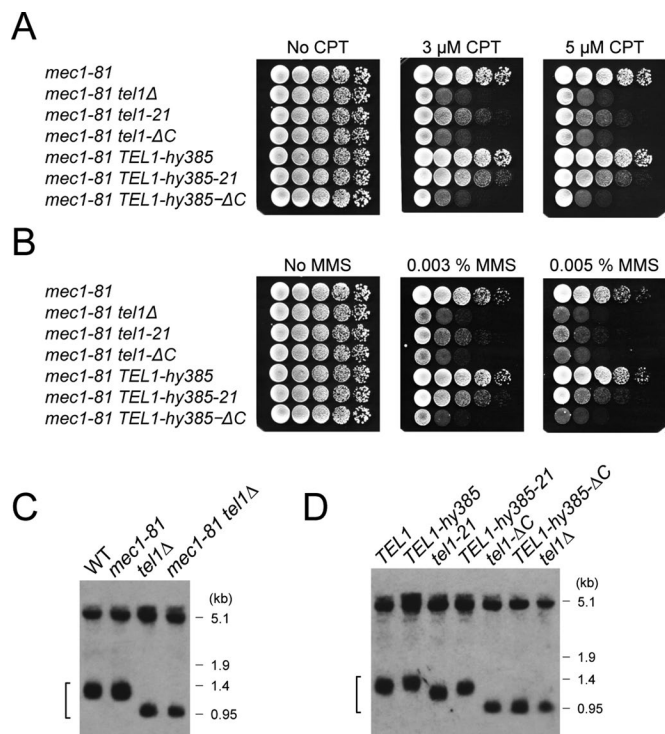


FIGURE 5: Intragenic suppression of *tel1-21* by a hyperactive *TEL1* mutation. (A, B) Effect of *TEL1-hy385* mutation on DNA damage sensitivities of *tel1-21* and *tel1-ΔC* mutants. Tenfold serial dilutions of cultures were spotted on YEPD medium with or without camptothecin (CPT) (A) or MMS (B). Plates were incubated at 30°C for 2 or 3 d. Strains used were *mec1-81* (KSC1662), *mec1-81 tel1Δ* (KSC1564), *mec1-81 tel1-21* (KSC3102), *mec1-81 tel1-ΔC* (KSC3552), *mec1-81 TEL1-hy385* (KSC3635), *mec1-81 TEL1-hy385-21* (KSC3636), and *mec1-81 TEL1-hy385-ΔC* (KSC3637). All the strains contain an *sml1Δ* mutation. (C) Telomere length in *mec1-81* mutants. Telomere length was analyzed by Southern blots as in Figure 3. Strains used were wild type (KSC1560), *mec1-81* (KSC1662), *tel1Δ* (KSC1661), and *mec1-81 tel1Δ* (KSC1564). All the strains contain an *sml1Δ* mutation. (D) Effect of *TEL1-hy385* mutation on telomere length of *tel1-21* and *tel1-ΔC* mutants. The same strains used in A were subjected to Southern blotting analysis to monitor telomere length as in C.

(Bosotti *et al.*, 2000; Yang *et al.*, 2013). We examined roles of the FATC domain of Tel1 by introducing substitution and truncation mutations in the domain. One substitution, termed *tel1-21*, and a truncation mutation, termed *tel1-ΔC*, did not significantly alter the expression level of Tel1. The *tel1-ΔC* mutation behaved like a null mutation and caused defects in both DNA damage response and telomere homeostasis. The *tel1-21* mutation caused less-pronounced defects than the *tel1-ΔC* mutation. Whereas Tel1-21 protein localized to DNA ends as efficiently as wild-type Tel1 protein, Tel1-ΔC did not. Both Tel1-21 and Tel1-ΔC proteins exhibited decreased kinase activities toward Rad53 C-terminus, although they underwent autophosphorylation efficiently. These results suggest that the FATC domain of Tel1 plays a key role in DNA damage recognition, as well as in phosphorylation of target proteins.

Tel1-21 and Tel1-ΔC proteins are defective in phosphorylating Rad53, although they retain activities to undergo overall autophosphorylation. Thus the C-terminal half of the FATC domain is critical for phosphorylating exogenous substrates but dispensable for autophosphorylation. Our finding supports a model in which the FATC domain contributes to recognition of exogenous target proteins.

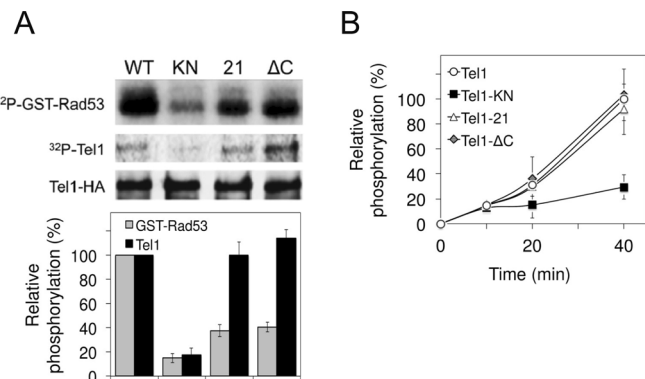


FIGURE 6: Effect of *tel1-21* or *tel1-ΔC* mutation on protein kinase activity in vitro. (A) Effect of *tel1-21* or *tel1-ΔC* mutation on Rad53 phosphorylation and Tel1 autophosphorylation. Extracts were prepared from cells expressing Tel1-HA (KSC1709), Tel1-KN-HA (KSC1751), Tel1-21-HA (KSC3087), or Tel1-ΔC-HA (KSC3549) and subjected to immunoprecipitation with anti-HA antibodies. Immunoprecipitates were assayed for kinase reactions using GST-Rad53 C-terminus as a substrate. Top, Rad53 phosphorylation and Tel1 autophosphorylation were detected by autoradiography, and the amount of Tel1 protein was analyzed by immunoblotting with anti-HA antibodies. Bottom, phosphoincorporation was normalized relative to that from wild-type Tel1 protein (100%). The average intensity calculated from three independent experiments is shown, and the error bars represent the SD derived from these experiments. (B) Tel1 autophosphorylation after incubation for various lengths of time. Tel1 proteins were immunoprecipitated and subjected to the in vitro kinase assay as in A, but the reaction mixtures were incubated for various lengths of time (0, 10, 20, and 40 min). Phosphoincorporation into Tel1 was normalized relative to that for wild-type Tel1 protein at 0 (0%) and 40 min (100%).

Indeed, crystal structures of mTOR reveal that the FATC domain is integrated into the kinase domain structure, and the substrate-holding groove consists of portions of the FATC domain (Yang *et al.*, 2013). Tel1-dependent phosphorylation stimulates telomerase recruitment to short telomeres, although the targets of Tel1 at telomeres have not been precisely determined (Gao *et al.*, 2010; Wu and Zakian, 2011; Wellinger and Zakian, 2012; Shen *et al.*, 2014). The *tel1-21* mutation behaves almost like a null mutation with regard to DNA damage response, whereas it causes a modest defect in telomere length homeostasis. One explanation could be that Tel1-dependent phosphorylation is an essential but not a rate-limiting step during telomere extension. Because telomeres lose only three to five nucleotides per cell division (Wellinger and Zakian, 2012), cells could largely maintain telomere length even if Tel1-21-dependent phosphorylation occurred very slowly. Alternatively, Tel1-21 might largely retain catalytic activities to phosphorylate targets at telomeres. Previous studies showed that ATM R3047X, lacking the C-terminal half of the FATC domain, undergoes autophosphorylation after DNA damage in vivo (Guo *et al.*, 2010) but exhibits decreased kinase activity toward exogenous substrates in vitro (Banin *et al.*, 1998). These observations also support the idea that the FATC domain contributes to target protein recognition, although we cannot exclude the possibility that the *tel1-21* mutation stimulates kinase-independent functions in telomere maintenance (Ma and Greider, 2009). The C-terminal half of the FATC domain is unique to PIKKs, although the N-terminal half is found in PI3K structures (Walker *et al.*, 1999; Miller *et al.*, 2010; Yang *et al.*, 2013). We found that the C-terminal half of the Tel1 FATC domain is not

essential for autophosphorylation in vitro. PI3Ks phosphorylate the 3'-position of the inositol ring in phosphatidylinositol lipids (Fruman *et al.*, 1998). In addition to lipid kinase activities, PI3Ks possess an intrinsic protein kinase activity directed toward the adaptors and the catalytic subunits themselves (Stack and Emr, 1994; Vanhaesebroeck *et al.*, 1999; Foukas *et al.*, 2004). Thus PIKKs might have evolved from PIK3s, acquiring the additional helix domain to phosphorylate exogenous protein substrates.

Tel1 localizes to DNA ends by interacting with the C-terminus of Xrs2 (Nakada *et al.*, 2003a). The current model suggests that the C-terminus of Xrs2/Nbs1 and the HEAT repeats of Tel1/ATM interact with each other (You *et al.*, 2005). Supporting this model, we isolated the C-terminus of Xrs2 from a two-hybrid screen using the N-terminus of Tel1 as bait (Nakada *et al.*, 2003a). In this report, we showed that the FATC domain of Tel1 also mediates Tel1–Xrs2 interaction and contributes to the localization to DNA ends. Similar to Tel1-21 protein, Tel1- Δ C protein retains weak kinase activities. However, unlike Tel1-21 protein, Tel1- Δ C protein did not efficiently accumulate at DNA ends. Consistently, we found that Tel1- Δ C protein interacts with the C-terminus of Xrs2 less efficiently than does wild-type Tel1. Defective localization of Tel1- Δ C to DNA ends should not result from decreased kinase activities, because Tel1 localization to DNA ends does not depend on kinase activity (Fukunaga *et al.*, 2011). The hyperactive *TEL1-hy385* mutation suppressed the *tel1-21* mutation but not the *tel1- Δ C* mutation, supporting the view that Tel1 localization to DNA ends is critical for DSB response and telomere maintenance (Nakada *et al.*, 2003a; Hector *et al.*, 2007; Sabourin *et al.*, 2007). Of note, the FATC domain of Mec1 also has been implicated in localization to sites of DNA damage (Nakada *et al.*, 2005). Mec1 forms a complex with Ddc2 that interacts directly with RPA-coated single-stranded DNA (Zou and Elledge, 2003). Whereas the N-terminal domain of Mec1 interacts with Ddc2 (Wakayama *et al.*, 2001), the FATC domain of Mec1 appears to mediate RPA-Ddc2 interaction (Nakada *et al.*, 2005). How does the FATC domain collaborate with the N-terminal HEAT repeat to localize Tel1 and Mec1 to sites of DNA damage? The crystal structure analysis of mTOR revealed that the FATC domain is integrated into the kinase domain (Yang *et al.*, 2013), although this analysis did not include most of the N-terminal HEAT repeats. For DNA-PKcs, the 6.6-Å x-ray diffraction data suggest that the kinase domain is adjacent to the N-terminal HEAT repeat ring (Sibanda *et al.*, 2010). Thus the FATC domain of PIKKs could affect the overall structure of the HEAT repeats, which may position near the kinase domain.

The other two mutations, *tel1-22* and *tel1-23*, decreased the expression level of Tel1 more significantly than did *tel1-21* or *tel1- Δ C*. We therefore did not extensively characterize molecular details of the Tel1-22 and Tel1-23 proteins. Tel2 interacts with other conserved proteins, Tti1 and Tti2, and controls stability, perhaps by mediating maturation of PIKKs (Hayashi *et al.*, 2007; Takai *et al.*, 2007, 2010; Anderson *et al.*, 2008; Hurov *et al.*, 2010; Stirling *et al.*, 2011). One possibility is that these mutations affect the interaction of Tel1 with the Tel2-Tti1-Tti2 (TTT) complex. However, the *tel1- Δ C* mutation did not affect Tel1–Tel2 interaction, suggesting that the FATC domain is dispensable for Tel1–Tel2 interaction. Previous studies indicated that Tel2 interacts with PIKKs through the HEAT domain (Takai *et al.*, 2007). It is thus less likely that the *tel1-22* or *tel1-23* mutation could interfere with the Tel1–TTT interaction. The TTT complex could control protein stability of PIKKs through the FATC domain; indeed, *tii2* mutations suppress an FATC mutation of *TRA1* (Genereaux *et al.*, 2012). However, the same *tii2* mutation failed to suppress an equivalent mutation of *MEC1* (Genereaux *et al.*, 2012). More-comprehensive studies would be required to determine whether the FATC

domain mediates TTT-dependent function. Although the *tel1-22* or *tel1-23* mutation did not completely abolish the expression, these mutations conferred defects in both DNA damage response and telomere maintenance. Similar to Tel1- Δ C protein, Tel1-22 and Tel1-23 proteins might not efficiently localize to DNA ends.

In summary, our results support a model in which the FATC domain of Tel1 plays a key role in localization to DNA ends and exogenous substrate phosphorylation. Of note, the FATC domain of Mec1 mediates similar functions. Further structure and function studies would help to elucidate the precise molecular mechanism of how PIKKs assemble at specific locations and phosphorylate target proteins.

MATERIALS AND METHODS

Strains and plasmids

The C-terminal region of the *tel1-21*, *tel1-22*, *tel1-23*, *tel1- Δ C*, and *TEL1-hy385* mutations was amplified and fused to the *URA3* marker by PCR using the oligonucleotides KS948 and KS1845, KS1846, KS1140, KS2807, or KS3262, respectively, and integrated into the *TEL1* locus (Reid *et al.*, 2002). The *mec1-81*, *mec1 Δ* , *sae2 Δ* , *tel1-KN*, and *tel1 Δ* mutations have been described (Nakada *et al.*, 2003b; Hirano *et al.*, 2009; Fukunaga *et al.*, 2011). The *TEL1-HA* construct was introduced into the *TEL1* locus as described (Fukunaga *et al.*, 2011). The *TEL2-FLAG* strain was generated by a PCR-based method using the pKL258 plasmid (a gift from M. Kanemaki and K. Labib, University of Manchester, UK) using the primer pair KS2789 and KS2790. To create pRS316-GAL-FLAG-TEL1, the GAL-FLAG-TEL1 construct from pGAL-FLAG-TEL1 (Hirano *et al.*, 2009) was introduced into *Sad1-Sal1*-treated pRS316 (Sikorski and Hieter, 1989). The pRS316-GAL1-FLAG-TEL1- Δ C plasmid was generated from pRS316-GAL1-FLAG-TEL1 by replacing the *SphI-SalI* fragment with that containing the *tel1- Δ C* mutation. All of the strains and oligonucleotides used in this study are listed in Supplemental Tables S1 and S2, respectively. The GST-Rad53 (containing the C-terminal Rad53) and GST-Xrs2C plasmid have been described (Nakada *et al.*, 2003b; Hirano *et al.*, 2009; Fukunaga *et al.*, 2011). YCpT-RAD53-HA has been described elsewhere (Fukunaga *et al.*, 2011).

Tel1 kinase assay

HA-tagged Tel1 protein was immunoprecipitated using protein A–Sepharose beads as described (Fukunaga *et al.*, 2011; Bandhu *et al.*, 2014). Kinase reactions were conducted in 40 μ l of the reaction buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–KOH, pH 7.5, 4 mM MgCl₂, 4 mM MnCl₂, 50 μ M ATP) containing 3 μ Ci of [γ -³²P]ATP (3000 Ci/mmol, PerkinElmer, Melville, NY) for 20 min at 30°C. GST-tagged Rad53 C-terminus (GST-Rad53) was used as a substrate (1 μ g for each reaction) for the in vitro kinase assay (Wakayama *et al.*, 2001; Nakada *et al.*, 2003b). Details of the kinase reaction have been described previously (Fukunaga *et al.*, 2011; Bandhu *et al.*, 2014). The reaction mixtures were electrophoresed on 6% SDS–polyacrylamide gels (30:1) and transferred to polyvinylidene fluoride membranes for immunoblotting analysis to determine the amount of Tel1 proteins. The same membranes were then subjected to autoradiography to detect Tel1 autophosphorylation. To monitor ³²P incorporation into GST-Rad53, aliquots of the reaction mixtures were run on 10% gels. The autophosphorylation time-course experiment was carried out without addition of GST-Rad53. Because the expression level of Tel1- Δ C was lower than that of wild-type cells, the amount of Tel1- Δ C in the immune complex was less than that of wild-type Tel1 protein if immunoprecipitated from the same amount of cells. Amounts of extracts used for immunoprecipitation of Tel1-HA, Tel1-KN-HA, and Tel1-21-HA were adjusted by including the extract from the isogenic nontagged strain.

Phosphorylation was quantified with a phosphorimager system (Typhoon 8600; GE Healthcare, Buckinghamshire, UK).

Telomere Southern blots

Telomere length was monitored by Southern blotting analysis (Hirano *et al.*, 2009; Fukunaga *et al.*, 2012). Genomic DNA was digested with *Xho*I, electrophoresed, and transferred to nylon membranes. Membranes were hybridized with a DNA fragment containing a Y' element and telomeric TG repeat. The DNA probe was labeled by the DIG labeling system (Roche, Basel, Switzerland).

GST-Xrs2 pull-down assay

The pull-down assay was done as described previously (Hirano *et al.*, 2009), with some modifications. GST-Xrs2C was bound to glutathione-Sepharose beads and then incubated with purified FLAG-Tel1 protein in the binding buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.01% Triton X-100) at 4°C for 90 min. FLAG-tagged Tel1 or Tel1-ΔC protein was purified from cells carrying pRS316-GAL-FLAG-TEL1 or pRS316-GAL-FLAG-TEL1-ΔC plasmid, respectively, as previously described (Hirano *et al.*, 2009; Fukunaga *et al.*, 2012).

Other methods

Chromatin immunoprecipitation assay was carried out as described (Nakada *et al.*, 2003a; Fukunaga *et al.*, 2011). The DNA damage sensitivity assay was as described (Nakada *et al.*, 2003a). Cellular fractionation, immunoprecipitation, and immunoblotting were performed as described previously (Wakayama *et al.*, 2001; Fukunaga *et al.*, 2012). Immunoblots were quantified using an Odyssey imager (LI-COR, Lincoln, NE). Anti-Zwf1 (glucose-6-phosphate dehydrogenase) and anti-nuclear pore complex antibodies were purchased from Sigma-Aldrich (St. Louis, MO) and Covance (Princeton, NJ), respectively.

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