Tel2 Is Required for Activation of the Mrc1-mediated Replication Checkpoint*1,2

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Proteins belonging to the Tel2/Rad-5/Clk-2 family are conserved among eukaryotes and are involved in various cellular processes, such as cell proliferation, telomere maintenance, the biological clock, and the DNA damage checkpoint. However, the molecular mechanisms underlying the functions of these molecules remain largely unclear. Here we report that in the fission yeast, Schizosaccharomyces pombe, Tel2 is required for efficient phosphorylation of Mrcl, a mediator of DNA replication checkpoint signaling, and for activation of Cds1, a replication checkpoint kinase, when DNA replication is blocked by hydroxyurea. In fact, Tel2 is required for survival of replication fork arrest and for the replication checkpoint in cells lacking Chk1, another checkpoint kinase the role of which overlaps that of Cds1 in cell cycle arrest by replication block. In addition, Tel2 plays important roles in entry into S phase and in genome stability. Tel2 is essential for vegetative cell growth, and the tel2Δ strain accumulated cells with 1C DNA content after germination. In the absence of hydroxyurea, Tel2 is vital in the mutant lacking Swi1, a component of the replication fork protection complex, and multiple Rad22 DNA repair foci were frequently observed in Tel2-repressed swi1Δ cells especially at S phase. In contrast, the cds1Δswi1Δ mutant did not show such lethality. These results indicate that S. pombe Tel2 plays important roles in the Mrc1-mediated replication checkpoint as well as in the Cds1-independent regulation of genome integrity.

There are multiple safeguard systems for maintenance of genome integrity, one of which is the cell cycle checkpoint. When mammalian cells suffer DNA damage or DNA replication fork stalling, members of the phosphatidylinositol 3-kinase-related family, including ATM1 and ATR, are activated and phosphorylate their downstream targets to cause cell cycle arrest until the DNA is fully repaired or until replication can resume (1–5). In the fission yeast, Schizosaccharomyces pombe, among the two phosphatidylinositol 3-kinase-related proteins Rad3 (an ATR homolog) and Tel1 (an ATM homolog), Rad3 plays a major role in both the DNA damage checkpoint and the DNA replication checkpoint. Rad3 constitutively forms a complex with the regulatory subunit Rad26 (a homolog of vertebrate ATRIP) (6, 7) and phosphorylates and activates the effector kinases Chk1 (a homolog of vertebrate Chk1) and Cds1 (a homolog of vertebrate Chk2) (8–10). Crb2 protein mediates the phosphorylation of Chk1 by Rad3-Rad26 kinase in response to DNA damage, whereas phosphorylation of Cds1 in response to replication fork stalling is mediated by Mrcl (11–14). Mrcl is expressed predominantly during S phase and is phosphorylated by Rad3 and Tel1 on HU treatment (12, 15). Phosphorylation of Mrcl is required for its interaction with Cds1 and is vital for HU resistance (15). Chk1 and Cds1 have overlapping roles in both the DNA damage checkpoint and the DNA replication checkpoint; therefore, the chk1 cds1 double mutant is highly sensitive to various forms of DNA damage and to replication block, comparable with rad3 or rad26 mutants, whereas chk1 or cds1 single mutants show only moderate or weak sensitivity (16–18). Chk1 and Cds1 phosphorylate and inactivate the tyrosine phosphatase Cdc25, and activation of Chk1 and Cds1 leads to accumulation of the tyrosine kinase Mkl1, in response to DNA damage or replication block (16, 19–22). This in turn leads to inhibitory phosphorylation of the cyclin-dependent kinase Cdc2 (23, 24). Cds1 also plays a role in stabilization of replication forks. The frequencies of replication fork collapse and arrest are significantly increased in the cds1Δ strain (25).

Another important system for maintaining genome integrity is protection of replication forks. In S. pombe, the Swi1–Swi3 complex is localized at and protects stalled replication forks (25–29). The mutants swi1 and swi3 show accumulation of abnormal DNA structures, such as ssDNA, in the absence of HU, and are defective in activation of Cds1 kinase in response to replication block (25, 28, 29). In the budding yeast Saccharomyces cerevisiae, Tof1, a homolog of Swi1, forms a stable complex with Csm3 (Swi3 homolog); this complex is loaded onto replication forks shortly after initiation of DNA replication and protects them during S phase (30–33). Thus, the presence of the Swi1–Swi3 complex at replication forks is conserved at least in the two yeasts.

The Tel2/Rad-5/Clk-2 family proteins are highly conserved among eukaryotic species. The tel2 mutant in S. cerevisiae has short telomeric DNA (34). S. cerevisiae Tel2 is essential for cell viability and binds to both single-stranded and double-stranded DNA.
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TABLE 1
Yeast strains used in this study

| Strain | Genotype |
|--------|----------|
| JK317  | h+ leu1-32 ura4-D18 |
| TN360  | h+ leu1-32 |
| CS903  | h+ tel2::ura4-1 ade6-M216 ade6-M210 leu1-32 ura4-D18 ura4-D18 |
| CS904  | h+ tel2::ura4-1 ade6-M216 ade6-M210 leu1-32 ura4-D18 ura4-D18 |
| CS1091 | tel2::3HA::ura4+ cdc25-2 ade6-M216 ura4-D18 |
| CS1526 | tel2::myc::kan+ leu1-32 ura4-D18 |
| CS1529 | nmt81::tel2::myc::LEU2::Kan+ leu1-32 ura4-D18 |
| CS1531 | nmt81::tel2::myc::LEU2::Kan+ leu1-32 ura4-D18 |
| CS1539 | nmt81::tel2::myc::LEU2::Kan+ cdc25-2 ade6-M216 ura4-D18 |
| TM1467 | rad22::2YFP::swi1::LEU2::Kan+ leu1-32 ura4-D18 |
| CS1717 | nmt81::tel2::myc::LEU2::Kan+ cdc25-2 ade6-M216 ura4-D18 |
| CS1725 | tel2::myc::kan+ cdc25-2 ade6-M216 ura4-D18 |
| CS1728 | tel2::myc::kan+ cdc25-2 ade6-M216 ura4-D18 |
| YT1738 | nmt81::tel2::myc::LEU2::Kan+ leu1-32 |
| CS1742 | tel2::myc::kan+ mrc1-3::HA::ura4+ leu1-32 ura4-D18 |
| CS1745 | nmt81::tel2::myc::LEU2::Kan+ mrc1-3::HA::ura4+ leu1-32 ura4-D18 |
| CS1746 | nmt81::tel2::myc::LEU2::Kan+ cdc25-2 ade6-M216 ura4-D18 |
| CS1761 | tel2::myc::kan+ cdc25-2 ade6-M216 ura4-D18 |
| CS1773 | tel2::myc::kan+ cdc25-2 ade6-M216 ura4-D18 |
| CS1785 | tel2::myc::kan+ |
| CS1789 | tel2::myc::kan+ rad22::ura4-1 ura4-D18 |
| CS1830 | nmt81::tel2::myc::LEU2::Kan+ rad22::YFP::ura4+ leu1-32 ura4-D18 |
| CS1833 | tel2::myc::kan+ rad22::YFP::ura4+ ura4-D18 |
| CS1837 | nmt81::tel2::myc::LEU2::Kan+ swi1::LEU2::Kan+ swi1::LEU2 leu1-32 ura4-D18 |
| CS1847 | tel2::myc::kan+ swi1::LEU2::Kan+ swi1::LEU2 leu1-32 ura4-D18 |
| CS2045 | tel2::myc::kan+ mrc1-3::HA::ura4+ cdc25-2 ade6-M216 ura4-D18 |
| CS2278 | tel2::myc::kan+ cdc25-2 ade6-M216 ura4-D18 |
| CS2298 | nmt81::tel2::myc::LEU2::Kan+ swi1::LEU2::Kan+ rad22::YFP::ura4+ leu1-32 ura4-D18 |

telomeric DNA in vitro, suggesting that it regulates telomere length by binding to the ends of telomeres (35–37). In Caenorhabditis elegans, the rad-5 mutant was isolated as a radiation-sensitive mutant showing high sensitivity to UV and x-ray irradiation (38), and the clk-2 mutant was isolated as a viable maternal-effect mutant showing alteration of the timing of some developmental and behavioral events, including the embryonic cell cycle and life span (39, 40). Ahmed et al. (41) showed that rad-5 is allelic to clk-2, and cloning of the rad-5/clk-2 gene revealed that it encodes a protein homologous to C. cerevisiae Tel2 (42, 43). The rad-5/clk-2 mutant is also sensitive to HU treatment and is defective in the DNA replication checkpoint (41). Rad-5/Clk-2 has been shown to function downstream of ATL-1 (C. elegans ATR) in the DNA replication checkpoint pathway (44). The human homolog, hCLK2, has also been identified (45). Cells overexpressing hCLK2 are hypersensitive to HU (45). However, the molecular mechanisms of action of Rad-5/Clk-2 or hCLK2 in DNA replication remain unknown.

Here we report the identification and characterization of tel2+, the gene encoding the fission yeast TEL2/Rad-5/Clk-2-related protein. We found that Tel2 is required for HU-induced activation of the Mrc1-Cds1 DNA replication checkpoint. In addition, the genetic interaction between tel2 and swi1 indicates that Tel2 also plays an important role in maintenance of genome integrity, especially at S phase.

EXPERIMENTAL PROCEDURES

Strains and General Techniques—The S. pombe strains used in this study are listed in Table 1. Yeast extract media YES, SD, MEA, and EMM were used to grow cells. Growth media, basic genetics, and biochemical techniques for fission yeast were described previously (46, 47). UV sensitivity assays and 4′,6-diamino-2-phenylindole staining of nuclear DNA were performed as described previously (28). EMM was supplemented with 14 μM thiamine to repress tel2+ gene expression regulated by the nmt81 promoter (48, 49).

Gene Disruption—For disruption of the tel2+ gene, the tel2+ ORF was amplified by PCR with primers cs11 (5′-GCTTACCGATTACGGGAGGC-3′) and cs12 (5′-CAGCGGACCAACAGCTG-3′) using wild-type genomic DNA as a template and cloned into pBlueScript SK− (Stratagene). The resultant plasmid was digested with HincII and PstI, and a ura4+ cassette was inserted. The resultant plasmid was digested with SpeI and XhoI, and the tel2+::ura4+ fragment was used for transformation.

Flow Cytometric Analysis of Germinated Spores—Diploid cells were induced for sporulation on MEA medium for 2 days at 25 °C and were treated with 0.5% glusulase for 12 h at 30 °C. Cells were washed with water and stored at 4 °C. Spores were germinated in SD medium at 32 °C. Flow cytometric analysis was performed as described previously (50).

Chromosomal Integration of tel2-12myc, tel2-3HA, and mrc1-3HA—To tag genomic tel2+ with a sequence encoding 12 copies of the Myc epitope at the C terminus, the tel2+ ORF was amplified by PCR with primers cs26 (5′-TATAAAGGATCCCTCCAGCGAGTTACTCCAG-3′; the BamHI site is underlined) and cs33 (5′-TATAAAGGATCCCTTAAAGGCTGGGTAACCC-3′; the KpnI site is underlined), cloned into pJK202 (51), and the ura4+ marker was replaced with the Kanr marker. The resultant plasmid was partially digested with HindIII and used for transformation. To tag genomic tel2+ with a sequence encoding three copies of the HA epitope at the C terminus, the tel2+ ORF was amplified by PCR with primers cs13 (5′-TATAAAACTCGAGTCCCAGCCAGTTACTTC-3′; the HindIII site is underlined). The resultant plasmid was digested with HindIII and used for transformation.
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CAG-3’; the XhoI site is underlined) and cs14 (5’-AAATATGGGAGGAAAATCCCTTAATTCAAATC-3’; NotI site is underlined) and cloned into pTN151 (51). The resultant plasmid was partially digested with HincII and used for transformation. To tag genomic tel2, we inserted the ORF, encoding a protein showing high degrees of conservation among species. aa, amino acids. For details, see supplemental Fig. S1. Figure 1. 5% pombe tel2+ is essential for vegetative cell growth. A, schematic alignment of S. pombe Tel2, S. cerevisiae Tel2, C. elegans Rad-5/Clk-2, Mus musculus Tel2, and H. sapiens CLK2. Boxes 1–3 indicate regions showing high degrees of conservation among species. for Cdc2 protein (control). The percentage of cells with septa was determined by counting 200 cells at each time point. C, terminal morphology of the tel2 Δ–deleted spores on the germination plates. Some spores ceased growth immediately after germination (left), whereas others formed microcolonies (middle and right). D, tel2 Δ is essential for cell growth. The tel2+ Δ/Δ+ diploid cells carrying pREP1 (control vector carrying LEU2+) or pREP1-tel2+ were induced for sporulation. Cells were treated with glusulase and ethanol to kill vegetative growing cells, and equal numbers of spores were incubated on 5% lacking uracil and leucine.

GAGTAATTATTC-3’; the NotI site is underlined) and cloned into pTN151 (51). The resultant plasmid was digested with SpeI and used for transformation.

Construction of the mnt81-tel2+ strain—To insert the mnt81 promoter upstream of the tel2+ ORF, the tel2+ ORF was amplified by PCR using primers cs31 (5’-TATAAACATATGAAATCTTTTGCTAGCGAACC-3’; the Ndel site is underlined) and cs32 (5’-TATAAAGGATCCCTTAAAGTGCCGCTAAATCGAACC-3’; the BamHI site is underlined) and cloned into pRIP81 (49). The upstream region of the chromosomal tel2+ ORF was amplified by PCR using primers cs11 (5’-GCTTACCCATTAGCGGAGC-3’) and cs34 (5’-TATAAAGCTTATCCTTGAGGTAAAGTAAACC-3’; the HindIII site is underlined) and cloned into pRIP81-tel2+. The resultant plasmid was digested with SpeI and SacI and used for transformation of the strain CS1526, yielding the strain CS1531. CS1531 was crossed with JK317 to obtain CS1529, and CS1529 was crossed with TN360 to obtain CS1732.

Cds1 Kinase Assay and Immunoblotting of Chk1 and Mrc1—Cds1 kinase assays were performed as described previously (16). Chk1 immunoblotting was performed as described previously (52). For analyses of Mrc1, cell extracts were prepared, and SDS-PAGE and immunoblotting were performed as described previously (15).

RESULTS

S. pombe tel2+ is Essential for Vegetative Cell Growth—To investigate the molecular mechanisms underlying the function of Tel2/Rad-5/Clk-2 family proteins in DNA replication, we searched for a tel2/rad-5/clk-2-related gene in the Sanger Centre S. pombe genome data base. We identified the ORF SPC458.03 (hereafter referred to as tel2+) that encodes a protein showing 14.9% identity to S. cerevisiae Tel2, 13.8% to C. elegans Rad-5/Clk-2, and 15.7% to Homo sapiens CLK2 throughout the entire sequence. We found three short and unique amino acid sequences highly conserved among Tel2/Rad-5/Clk-2 family proteins (Figs. 1A and supplemental Fig. S1). The Tel2 protein
was expressed throughout the cell cycle (Fig. 1B), and its expression showed no significant changes after UV or HU treatment (data not shown).

The function of Tel2 was investigated by generating a tel2 null mutation. One copy of tel2Δ in a ura4+ diploid strain was replaced with the ura4+ marker gene. Two independent gene disruption transformants (CS903 and CS904) were confirmed by Southern blotting analysis (data not shown). Tetrads analysis of the tel2+/tel2::ura4+ diploids revealed that no more than two spores could form colonies in each set of tetrads and that all the viable cells were Ura−. The terminal morphology of tel2− spores on germination plates was examined. Fifteen percent of the spores ceased growth immediately after germination (Fig. 1C, left), whereas 85% formed microcolonies of 2–50 cells (Fig. 1C, middle and right). To exclude the possibility that the lethality observed in the tel2 null mutant was because of unexpected effects on neighboring genes, tel2+/tel2::ura4+ diploid cells were transformed with pREP1 (control vector) or pREP1-tel2+ (expression plasmid containing the tel2+ gene), and their spores were incubated on plates lacking uracil to select tel2::ura4+ haploid mutant cells. The growth of the tel2 haploid mutant was dependent on pREP1-tel2+ (Fig. 1D). Therefore, we concluded that the tel2 gene function is essential for vegetative cell growth.

The function of tel2+ in DNA replication was investigated by germinating spores from a tel2+/tel2::ura4+ diploid (CS903) in SD medium lacking uracil. The tel2Δ cells completed the first cycle of DNA replication in 8 h and started to form a germ projection at 8 h, as observed in wild-type spores (Fig. 2A and data not shown). The tel2Δ cells progressed to the second cycle of DNA replication (∼8–16 h in Fig. 2C), possibly using inherited Tel2 protein. This and the variety of terminal cell morphology shown in Fig. 1C probably reflect a requirement of only a low level of Tel2 protein for cell growth (see also Fig. 3). At 20 h, a population with 1C DNA content appeared, which was not observed in the wild-type cells, indicating that the entry into S phase was inhibited or delayed in tel2Δ. These observations suggested that Tel2 is required for the normal entry into S phase.

Characterization of the nmt81-tel2+ Mutant—To further investigate the function of Tel2, we generated a strain in which the thiamine-repressible nmt81 promoter was inserted upstream of the chromosomal tel2+ locus, and the Myc tag was added at the C terminus of the Tel2 ORF (Fig. 3A). In the absence of thiamine, the nmt81 promoter induces expression of the gene located downstream, whereas it "partially" represses the expression in the presence of thiamine (48). The resultant nmt81-tel2+ strain was grown to exponential phase in the absence of thiamine. Before addition of thiamine (time 0), the level of Tel2-myc expression in nmt81-tel2+ cells was approximately double that in wild-type cells (Fig. 3B). After addition of thiamine, the expression level of Tel2-myc in the tel2+ strain decreased gradually (Fig. 3B). This strain showed lower viability than the wild-type strain, although it did not die completely and kept growing slowly even after 60 h of thiamine treatment, indicating that a very small amount of Tel2 protein is sufficient for cell viability (Fig. 3C). Thirty-six hours after addition of thiamine, Tel2-myc was hardly detectable by Western blotting, and the viability started to decrease (Fig. 3, B and C). Therefore, we used cells that had been treated for at least 36 h with thiamine in subsequent experiments to investigate the effects of Tel2 repression.

Tel2 Is Required for Survival of Replication Fork Arrest in chr1Δ—C. elegans rad-5/clk-2 mutants are hypersensitive to HU, IR, or UV treatment (38, 41, 53), and overexpression of human CLK2 causes hypersensitivity to HU (45). Therefore, we examined whether Tel2 is involved in tolerance to DNA damage in fission yeast. Cells were grown in the presence of thia-
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mine for 36 h to repress tel2+ expression and then spotted onto plates containing HU and thiamine (Fig. 4A). Wild-type cells readily formed colonies on plates containing 5 mM HU, whereas the nmt81-tel2+ strain formed a smaller number (~1/20) of colonies, indicating that Tel2 is involved in tolerance to replication fork arrest induced by HU treatment. The nmt81-tel2+ chk1Δ double mutant showed much stronger sensitivity to HU than each single mutant, which was similar to rad3Δ. In contrast, the nmt81-tel2+ cds1Δ double mutant showed only slightly stronger sensitivity than each single mutant. These observations indicated that Tel2 functions in HU tolerance and that this is at least partially independent of Chk1.

Next, we examined whether Tel2 is involved in UV tolerance (Fig. 4A). Exposure of DNA to UV irradiation causes the formation of DNA lesions that block replication forks. The Tel2-repressed nmt81-tel2+ strain and chk1Δ single mutant showed moderate sensitivity to UV irradiation. However, the nmt81-tel2+ chk1Δ double mutant showed acute sensitivity similar to rad3Δ or chk1Δ cds1Δ. In contrast, the nmt81-tel2+ cds1Δ double mutant showed sensitivity similar to that of the nmt81-tel2+ single mutant. These observations indicate that the function of Tel2 in UV tolerance is largely independent of Chk1.

Taken together, these results suggest that Tel2 is required for tolerance to replication block or DNA damage in a pathway that is independent of Chk1 and that partially overlaps with Cds1.

Tel2 Is Required for Cds1-mediated Replication Checkpoint—To analyze the relationship between Tel2 and Cds1, we next investigated the role of Tel2 in HU-induced cell cycle arrest. Both Cds1 and Chk1 are capable of preventing mitosis when replication is blocked by HU (16, 18). When treated with HU, wild-type, chk1Δ and cds1Δ cells arrest division and become elongated, whereas the chk1Δ cds1Δ double mutant, like rad3Δ, continues to divide and shows a lethal nuclear phenotype (a nucleus bisected by a septum or a binucleate cell with unequally segregated chromosomes), indicating a checkpoint failure (18) (Fig. 4B). Cells were grown in the presence of thiamine for 36 h to repress tel2+ expression and were then incubated with HU for 6 h. Division in the nmt81-tel2+ cells was arrested normally after HU treatment. The nmt81-tel2+ cds1Δ cells also showed normal cell cycle arrest, whereas the nmt81-tel2+ chk1Δ cells frequently showed the lethal nuclear phenotype seen in rad3Δ or chk1Δ cds1Δ cells (Fig. 4B). These observations, together with Fig. 4A, indicate that Tel2 is required for the function of Cds1 in HU-induced cell cycle arrest. Consistent with this conclusion, Chk1 phosphorylation was elevated in Tel2-repressed nmt81-tel2+ cells when treated with HU or bleomycin, which produces double-strand DNA breaks and induces efficient Chk1 phosphorylation (Fig. 5, A and B). Robust Chk1 phosphorylation was induced by HU in the nmt81-tel2+ strain as observed in cds1Δ and mrc1Δ cells in which DNA at replication forks has been substantially damaged (12, 17, 25, 54). These observations indicate that Tel2 is not required for the activation of Chk1.

Tel2 Is Required for Activation of Mrc1-Cds1 Pathway—Next, we investigated whether Tel2 regulates the kinase activity of Cds1. An in vitro kinase assay was performed using GST-Wee170 protein as the substrate (Fig. 6, A and B). Cds1 protein kinase was activated after HU treatment of wild-type cells, whereas no activation was observed in rad3Δ cells, as reported previously (16). Cds1 activation was markedly inhibited in the Tel2-repressed nmt81-tel2+ strain, indicating that Tel2 is required for efficient Cds1 activation in response to HU treatment. It was suggested that the residual Cds1 activity in the Tel2-repressed nmt81-tel2+ strain alleviated its sensitivity to HU (Fig. 4A).
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Tel2 Is Vital in *swiΔ* Cells—It is possible that the reduction of Mrcl phosphorylation in the Tel2-repressed *nmt81-tel2* strain is because of its delayed entry into S phase or perturbed cell cycle progression (Fig. 6,E). It was reported that Mrcl is localized at replication forks and is required for the stabilization of a pausing replication complex as well as for activation of the replication checkpoint in budding yeast (30, 56). The involvement of Tel2 in activation of the Mrcl-Cds1 pathway suggests that Tel2 is related to stabilization of the replication fork. In fact, the phenotypes of the Tel2-repressed strain described above (i.e. defects in Cds1 activation and in entry into S phase), with the exception of lethality, are similar to those of the strain in which *Swi1*, a component of the replication fork protection complex, is deleted (25, 27, 29). Therefore, we investigated the relationship between Tel2 and *Swi1*. After growth in the presence of thiamine for 36 h to repress *tel2* expression, the viability of the *nmt81-tel2 swiΔ* double mutant in the absence of HU was markedly decreased to <0.1%, whereas the *clds1 swiΔ* strain did not show such a severe growth defect (Fig. 7A). Deletion of *Swi1* did not affect the abundance of Tel2 protein in the presence or absence of thiamine (Fig. 7B), suggesting that the lethality of the *nmt81-tel2 swiΔ* double mutant was not because of down-regulation of Tel2 protein by...
swi1+ deletion. These results indicated that Tel2 has a Cds1-independent role that is vital in swi1Δ cells.

Swi1 prevents replication fork collapse and activates the Cds1 pathway, and swi1Δ cells accumulate spontaneous DNA damage (single strand DNA gaps at replication forks or rearranged replication forks) without fork breakage (25, 28, 29). Therefore, Tel2 was suggested to have some role in genome maintenance in swi1Δ. To explore this possibility, we analyzed the localization of Rad22-YFP fusion protein (57) (Fig. 7C). S. pombe Rad22 is a homolog of S. cerevisiae Rad52 that binds to ssDNA at double strand breaks during homologous recombination (58, 59). As reported previously (25), spontaneous Rad22-YFP foci were observed frequently in swi1Δ cells, whereas only a small population of the wild-type cells had Rad22-YFP foci. The Tel2-repressed nmt81-tel2Δ cells had more Rad22-YFP foci than the wild-type, and the foci were observed frequently during S phase or early G2 phase (apparent “early G2 phase” may be prolonged or delayed S phase). These observations suggested that Tel2 is required for genome stability, especially at S phase. In the nmt81-tel2Δ swi1Δ double mutant, Rad22-YFP foci were observed more frequently than in either single mutant, especially at S phase, which may correspond to the severe growth defect of the double mutant. These results suggested that Tel2 and Swi1 cooperatively play important roles in the suppression of spontaneous DNA damage during S phase.

DISCUSSION

Tel2/Rad-5/Clk-2 family proteins are involved in a variety of cellular functions, including cell proliferation, regulation of the biological clock, telomere maintenance, and the DNA damage

FIGURE 5. Tel2 is not required for the activation of Chk1. A, Tel2 is not required for Chk1 phosphorylation induced by bleomycin treatment. Wild-type (CS1773) and nmt81-tel2Δ (CS1746) cells expressing Chk1-HA were grown in the presence of thiamine for 36 h and then treated with 5 milli-units/ml bleomycin for the indicated times. Whole-cell extracts were prepared, and immunoblotting was performed with anti-HA antibodies for Chk1-HA protein, with anti-Myc antibodies for Tel2-Myc protein, and with anti-PSTAIRE antibodies for Cdc2 protein (control). Reduction in Chk1 electrophoretic mobility caused by phosphorylation (52) was detected in both strains after bleomycin treatment. B, Tel2 is not required for Chk1 phosphorylation induced by HU treatment. The same strains as in A were treated with 12 mM HU for 4 h. Immunoblotting was performed as in A.

FIGURE 6. Tel2 is required for activation of the Mrc1-Cds1 pathway. A, Tel2 is required for efficient activation of Cds1 kinase. Wild-type (CS1785), nmt81-tel2Δ (CS1732), and rad3Δ (CS1789) cells were grown in the presence of thiamine for 36 h and then treated with 12 mM HU for 0, 1, 3.5, or 7 h. Cds1 kinase activity was measured with GST-Wee170 substrate as described previously (16). The amounts of whole-cell extracts used for the in vitro kinase assay were checked by Coomasie Brilliant Blue (CBB) staining and by immunoblotting for Tel2-Myc protein and for Cdc2 protein (PSTAIRE, control). B, relative intensity of GST-Wee170 phosphorylation compared with that in the wild type at 0 h was quantified. C, phosphorylation of Mrc1 is impaired in the Tel2-repressed cells but not in cds1Δ. Wild-type (CS1742), nmt81-tel2Δ (CS1745), and cds1Δ (CS2045) cells expressing Mrc1-HA were grown asynchronously in the presence of thiamine for 36 h and then treated with 12 mM HU for 0, 2, or 4 h. Whole-cell extracts were prepared, and immunoblotting was performed with anti-HA antibodies for Mrc1-HA protein, with anti-Myc antibodies for Tel2-Myc protein and with anti-PSTAIRE antibodies for Cdc2 protein (control). D, Tel2 is required for the efficient phosphorylation of Mrc1. Wild-type (CS1742) and nmt81-tel2Δ (CS1745) cells were grown asynchronously in the presence of thiamine for 36 h and then treated with 10 mM HU for 10 h. Western blotting was performed as in C, E, Tel2-repressed cells showed slow S phase progression. Wild-type (CS1742) and nmt81-tel2Δ (CS1745) cells were grown and treated with HU as in D. The DNA content was analyzed by flow cytometry.
checkpoint. However, the molecular mechanisms of action of the Tel2 family proteins remain largely unclear. In this study, we characterized the functions of Tel2 in the fission yeast, *S. pombe*. First, we showed that Tel2 is required for normal entry into S phase. Second, we found that Tel2 regulates activation of the DNA replication checkpoint. Tel2 is required for efficient phosphorylation of Mrc1, which is essential for the activation of Cds1 kinase. Third, Tel2 was shown to play an important role in genome stability. The Tel2-repressed *nmt81-tel2+* strain is lethal with a significantly increased frequency of DNA damage in the absence of Swi1, a component of the replication fork protection complex. Thus, fission yeast Tel2 performs at least three functions as follows: regulation of entry into S phase, activation of the Mrc1-mediated replication checkpoint, and genome stabilization in collaboration with the fork protection complex.

**Role of Tel2 in DNA Replication**—We found that Tel2 is required for efficient activation of the Mrc1-Cds1 pathway. Swi1 and Swi3 are also required for efficient Cds1 activation in response to HU treatment as well as for replication fork protection (25, 28, 29). The *swi1* and *swi3* cells accumulate abnormal DNA structures that lead to Holiday junction formation without fork breakage (28, 29). As shown in Fig. 7, repression of Tel2 in the *swi1* mutant resulted in lethality and the accumulation of DNA damage, suggesting that Tel2 is required for the stability of abnormal DNA structures in the *swi1* mutant. Therefore, Tel2 may play a role at replication forks in collaboration with the Swi1-Swi3 complex. In budding yeast, the central domain of the Tel2 protein, including the conserved three regions (boxes 1–3), shown in Fig. 1A binds efficiently to telomeric ssDNA in vitro (37). Therefore, it is possible that the nature of binding to ssDNA is conserved in other Tel2 homologs. *S. pombe* Tel2 may bind to ssDNA at replication forks to protect the replication forks. Further analyses of *S. pombe* Tel2 are required to clarify these points.

**S. pombe** Hsk1, a homolog of Cdc7, regulates the initiation of DNA replication (60–62). The *hsk1* mutant shows phenotypes similar to those of the Tel2-repressed *nmt81-tel2+* strain. Specifically, the *hsk1-89* mutant is defective in activation of Cds1 kinase after HU treatment and shows a synthetic growth defect in combination with *swi1* mutation with an increased level of Rad22-YFP foci formation (62, 63). Furthermore, the accumulation of the tel2Δ cells with a DNA content of 1C after germination (Fig. 2C) is similar to that observed in *hsk1Δ* cells (60).
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Although it is unclear whether Tel2 physically interacts with Swi1-Swi3, Hsk1 does with Swi1 (63). Thus, Hsk1 is thought to be required for fork stabilization as a subunit of the complex with Swi1-Swi3 as well as for initiation of DNA replication, and maintenance of the structure of replication forks from the initiation of DNA replication may be required for efficient activation of the Mrc1-Cds1 pathway. Therefore, it is speculated that Tel2 is also involved in the stabilization of genome (possibly replication forks) from the initiation and influences activation of the Mrc1-Cds1 pathway. Further analyses on Tel2 localization and its involvement in the maintenance of replication forks are required.

In budding yeast, Mrc1 interacts with Tof1, and the Mrc1-Tof1 complex travels with replication forks in the absence of replication stress and is also required for the stability of stalled replication forks in the presence of replication stress (30, 56). Furthermore, the Mrc1-Tof1 complex is required for normal progression of replication forks (64, 65). In Xenopus, Claspin, an Mrc1 homolog, is loaded onto chromatin just after unwinding of the origin (66, 67). Thus, S. pombe Mrc1 is thought to also function as a component of the replication complex. One possibility is that S. pombe Tel2 regulates the localization of Mrc1 at replication forks. In Xenopus, it is thought that the interaction between Claspin and proteins at the replication fork is necessary to stabilize its association with chromatin, in a process that does not require replication protein A (RPA) (66, 68). Therefore, S. pombe Tel2 may be required for the stable association of Mrc1 at replication forks. Alternatively, Tel2 may regulate the action or activity of Rad3 (and Tel1). However, the latter (regulation of Rad3 activity) is unlikely, because Tel2 repression does not compromise Chk1 phosphorylation by Rad3 in the presence of HU (Fig. 5B). In C. elegans, it has been shown that Rad-5/Clk-2 functions downstream of ATL-1 in the S phase checkpoint (44). Therefore, Tel2 in fission yeast may also function downstream of Rad3.

Evolutionary Changes and Conservation of Tel2 Function—The results of this study demonstrated that S. pombe Tel2 is required for activation of the replication checkpoint. This role in the replication checkpoint is conserved in C. elegans, as worm Rad-5/Clk-2 is required for cell cycle arrest upon HU treatment (41, 44). Furthermore, cells overexpressing human CLK2 are hypersensitive to HU treatment (45). Thus, it seems that the function of Tel2 in the replication checkpoint is evolutionarily conserved, and other Tel2 homologs may also regulate Mrc1 activation as in fission yeast.

S. cerevisiae Tel2 binds to telomeric ssDNA in vitro and regulates telomere length (35, 37). On the other hand, S. pombe Tel2 apparently does not play a critical role in telomere length control, because Tel2 repression did not show any changes in telomere length (data not shown). In C. elegans, rad-5 and clk-2 mutations have little effect on telomere length (41), whereas other reports argued that clear changes in telomere length (both longer and shorter) are observed in clk-2 mutants (42, 43). In humans, overexpression of CLK2 gradually lengths telomere DNA (45), but the effect of inactivation of CLK2 on telomere length is unknown. Thus, Tel2 family proteins may have a conserved function in telomere length control, but further studies are needed to confirm this hypothesis. C. elegans Clk-2/Rad-5 is essential both for generation of circadian rhythm and for the DNA replication checkpoint (40, 41, 44). Interestingly, the homologs of S. pombe Swi1 in C. elegans (TIM-1/Timeless), mice (mTim/Timeless), and in humans (hTim/Timeless) are also involved in developmental timing or generation of circadian rhythm (69–71). It has been shown that C. elegans TIM-1 is involved in chromosome cohesion (72). Furthermore, human Tim interacts with ATRIP (a homolog of S. pombe Rad26) and Chk1 in response to HU treatment and is required for DNA replication checkpoint signaling (71). Taken together, these observations indicate that the homologs of S. pombe Tel2 and Swi1 are involved both in circadian or developmental timing and in the DNA replication checkpoint, although their involvement in growth or developmental timing in fission and budding yeast is currently unknown. Homologs of Tel2 and Swi1 may have acquired additional functions and become key regulators linking the circadian (or developmental) cycle and the cell division cycle during the course of evolution.

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