

# Regulation of Telomere Elongation by the Cyclin-Dependent Kinase *CDK1*

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## Summary

Telomere elongation is cell-cycle regulated and requires the coordinated activity of proteins involved in the DNA damage response. We used an assay that detects de novo telomere addition to examine the role of the cyclin-dependent kinase Cdk1 (Cdc28) in cell-cycle-specific telomere elongation. Inhibition of an ATP analog-sensitive allele of Cdk1 completely blocked the addition of telomere repeats. Mutations in Rif2 and DNA polymerase  $\alpha$  that cause increased telomere elongation were unable to compensate for the loss of Cdk1 activity, suggesting Cdk1 activity is required for an early step in telomere addition. Mutations in DNA repair proteins that act with Cdk1 at double-strand breaks also prevented telomere elongation. Cdk1 activity was required for the generation of 3' single-strand overhangs at both native and de novo telomeres. We propose Cdk1 activity controls the timing of telomere elongation by regulating the single-strand overhang at chromosome ends.

## Introduction

Telomeres are nucleoprotein complexes at the ends of linear chromosomes that distinguish natural ends from DNA breaks in the cell. A failure to maintain a functional telomere can result in chromosome fusions and genomic instability (Blasco et al., 1997; Hackett et al., 2001; van Steensel et al., 1998). The basic structure of eukaryotic telomeres is highly conserved and consists of repetitive arrays of G-rich sequences that terminate in a 3' single-strand overhang (Blackburn, 2001). This 3' overhang is a universally conserved feature of telomeres in ciliates, fungi, and mammals (Henderson and Blackburn, 1989; Makarov et al., 1997; Wellinger et al., 1993). This single-strand region allows the binding of sequence-specific binding proteins that play critical roles in protecting telomeres from cellular nucleases and chromosome end-to-end fusions (Baumann and Cech, 2001; Garvik et al., 1995; Gottschling and Zakian, 1986). In at least some organisms, telomeres end in a protective t-loop structure formed by the invasion of the 3' single-strand overhang into double-strand telomere sequence (Griffith et al., 1999; Nikitina and Woodcock, 2004).

Telomere length is maintained by the enzyme telomerase, a reverse transcriptase that uses an intrinsic RNA template to elongate telomeres (Greider and Blackburn, 1987; Lingner et al., 1997). The timing and genetic requirements for telomere elongation have been most thoroughly studied in budding yeast (Lundblad, 2006).

In yeast, telomere elongation by telomerase occurs preferentially on short telomeres, but only a small fraction of short telomeres are elongated during a single cell cycle (Teixeira et al., 2004). In addition, even though an active form of the telomerase enzyme is present in extracts made from cells in various stages of the cell cycle, telomere elongation is restricted to late S phase (Diede and Gottschling, 1999; Marcand et al., 2000). The timing of telomere elongation in late S phase correlates with the binding of many proteins involved in telomere elongation, including the telomerase holoenzyme components Est1, Est2, Cdc13, and the single-strand binding protein RPA (Schramke et al., 2004; Taggart et al., 2002).

Many of the proteins involved in the recognition and repair of DNA damage have important roles in responding to abnormal telomere structures. The loss of telomere function in yeast and mammalian cells signals a DNA damage response similar to that of a double-strand break. In yeast, short telomeres trigger a G2/M cell-cycle arrest through activation of a DNA damage checkpoint (Enomoto et al., 2002; Ijima and Greider, 2003). Telomeres that are uncapped by inactivating the telomere end-binding protein Cdc13 also trigger a DNA damage checkpoint (Garvik et al., 1995). In mammalian systems, the overexpression of a dominant-negative form of the end-binding protein TRF2 leads to telomere uncapping and results in a robust p53- and ATM-dependent DNA damage response (Karlseder et al., 1999). Short telomeres also activate a DNA damage checkpoint in senescent human fibroblasts (d'Adda di Fagagna et al., 2003) and in telomerase null mouse cells (Hao et al., 2004).

In addition to their role at dysfunctional telomeres, evidence is accumulating that DNA damage proteins play important roles in normal telomere function. In yeast, the deletion of the *ATM* homolog *TEL1*, or the deletion of any component of the MRX complex (*MRE11*, *RAD50*, *XRS2*), results in short telomeres (Ritchie and Petes, 2000). In yeast and mammalian cells, the MRX/N complex and the Tel1/ATM proteins localize to telomeres at distinct phases of the cell cycle without eliciting a DNA damage checkpoint (Takata et al., 2005; Verdun et al., 2005). In yeast, the Mre11 protein is required for the normal 5' to 3' processing of telomeres to create a long single-strand overhang in late S phase (Larrivee et al., 2004; Takata et al., 2005). At a de novo telomere, the MRX complex is required to generate a 3' overhang that allows the subsequent binding of Cdc13 (Diede and Gottschling, 2001). The importance of DNA damage proteins at normal telomeres argues for an intimate mechanistic connection between DNA damage signaling and telomere function.

In yeast and mammals, double-strand break repair is regulated by the cell cycle (Ira et al., 2004; Rothkamm et al., 2003). After a break forms, the MRX complex is required for a poorly understood 5' end-resection processing reaction (Ivanov et al., 1994). This activity is thought to allow access for an unknown nuclease that processes the break to create a long 3' single-strand overhang (Lisby et al., 2004). Recent data suggests this 5' end resection requires the kinase activity of the

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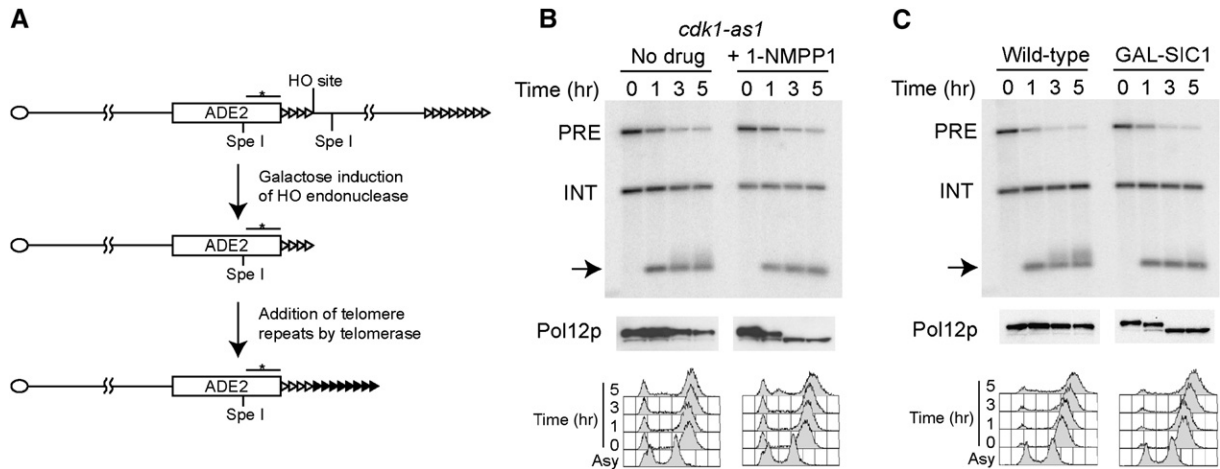


Figure 1. Cyclin-Dependent Kinase Activity Is Required for De Novo Telomere Addition

(A) Schematic of the de novo telomere addition assay as previously described (Diède and Gottschling, 1999). *ADE2* and the recognition site for the HO endonuclease flank a short stretch of internal telomere repeats (open triangles). Galactose induction of the HO endonuclease exposes this 81 bp telomere "seed" sequence adjacent to the HO site. New telomere repeats are added by telomerase to the exposed sequence, and the elongation is followed by the hybridization of the *ADE2* probe (bar with star) to *SpeI*-cut genomic DNA. New telomere repeats (solid triangles) form a smear that can be visualized by Southern hybridization.

(B) Cells containing the *cdk1-as1* allele (CF246) were arrested and held in G2/M with nocodazole. At  $t = 0$  hr, the culture was shifted to galactose to induce cutting by the HO endonuclease and, at the same time, the culture was divided into halves. One-half was treated with DMSO as the no-drug control, and the other half was treated with the inhibitor 1-NMPP1 to block the activity of Cdk1. Samples of DNA, protein, and fixed cells were collected at each time point. Genomic DNA was digested with *SpeI* and analyzed by Southern hybridization using the *ADE2* probe. The band labeled PRE represents the *SpeI* restriction fragment uncut by the HO endonuclease. The band labeled INT is an internal loading control from the *ade2-101* locus. The arrow identifies the HO-cut de novo telomere that can be elongated by telomerase over time. Protein extracts from each time point were analyzed by immunoblot using a monoclonal antibody recognizing Pol12p (middle panel). Cell-cycle profiles were obtained for each time point by FACS analysis (bottom panel).

(C) The de novo telomere addition assay was repeated as in (B) with a wild-type strain (UCC5706) and a strain containing the galactose-inducible version of the *SIC1* gene (CF206). Shifting CF206 to galactose at  $t = 0$  induces both the expression of HO and the stable version of Sic1.

cyclin-dependent kinase Cdk1, the major regulator of the cell cycle in *Saccharomyces cerevisiae* (Ira et al., 2004).

Telomere maintenance also requires 5' end resection, and here we show that telomere addition requires the cyclin-dependent kinase activity of Cdk1 and the nuclease activity of Mre11. Cdk1 activity is required for the formation of the 3' single-strand overhang structure at both de novo and native telomeres. This requirement for Cdk1 activity may explain the cell-cycle regulation of telomere elongation.

## Results

### Cdk1 Activity Is Required for Telomere Addition In Vivo

To examine the requirements for telomere elongation, we took advantage of a genetic system previously used to visualize telomere addition in vivo (Diède and Gottschling, 1999). In this system, an inducible HO endonuclease is used to create a single double-strand break adjacent to a short stretch of telomere repeats near the end of chromosome VII. The fragment distal to the break is lost, and, over time, the short telomere "seed" sequence is elongated by telomerase (Figure 1A). In this system, *RAD52* is deleted to ensure that all elongation is dependent on telomerase, not on recombination (Diède and Gottschling, 1999).

To determine whether the activity of Cdk1 is required for telomere-repeat addition, we inhibited its activity

while visualizing telomere elongation at the HO endonuclease-derived de novo telomere. To inhibit Cdk1 activity, we replaced the wild-type copy of *CDK1* with the inhibitor-sensitive allele *cdk1-as1* (Bishop et al., 2000). This inhibitor-sensitive protein has an enlarged ATP-binding pocket that specifically sensitizes Cdk1 to the ATP analog inhibitor 1-NMPP1. A strain containing the analog-sensitive allele was grown to mid-log phase and arrested with nocodazole in G2/M. The cells were switched to galactose-containing media to induce HO endonuclease cutting at the telomere seed sequence. The culture was split into halves, and one-half of the culture was treated with the selective kinase inhibitor 1-NMPP1. To confirm the inhibitor was effectively blocking Cdk1 kinase activity, we monitored the mobility of the DNA replication protein Pol12. Pol12 hyperphosphorylation depends on the activity of Cdk1 (Pelliccioli et al., 1999). Treatment with 1-NMPP1 blocked this phosphorylation, confirming that the kinase activity of the *cdk1-as1* allele was effectively inhibited (middle panel of Figure 1B). To ensure any effect was due to the inhibition of Cdk1 activity, and not to a perturbation of the cell cycle, we carried out FACS analysis. The inhibitor 1-NMPP1 did not alter the cell-cycle profile of arrested cells containing the *cdk1-as1* allele (lower panels of Figure 1B). However, this strain did have a higher proportion of cells in G1 both with and without the inhibitor, presumably due to the moderately lower Cdk1 activity of the *cdk1-as1* allele (Bishop et al., 2000). In the untreated culture, elongation of the de novo telomere

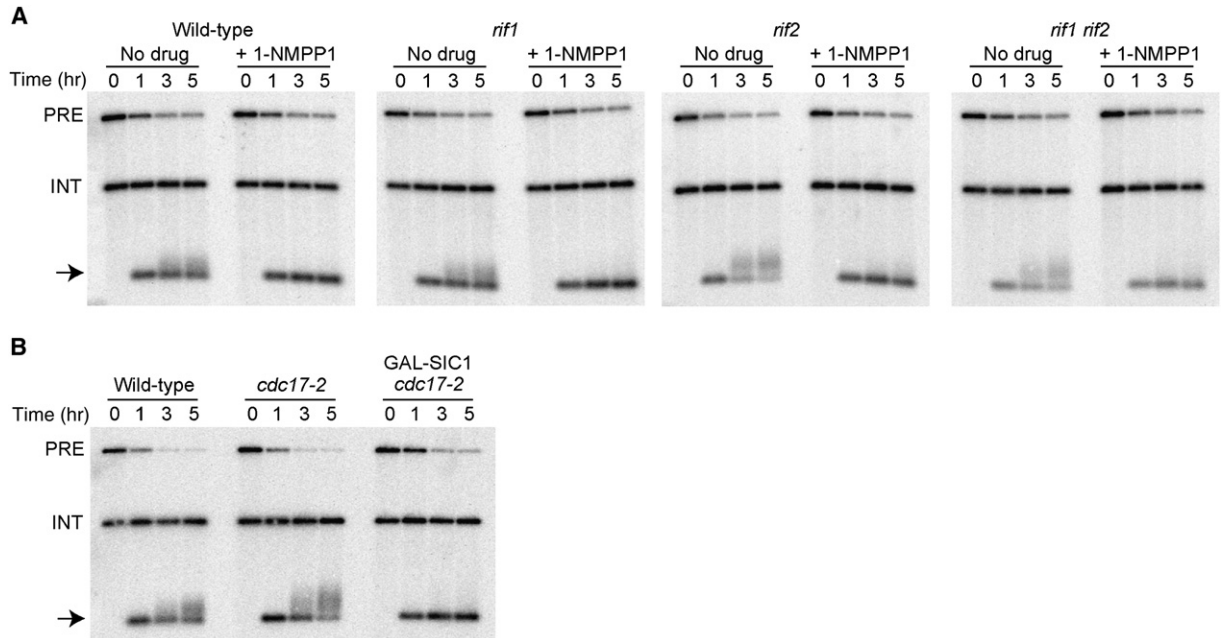


Figure 2. Cdk1 Acts Upstream of Rif2 and DNA Polymerase  $\alpha$  in Telomere Elongation

(A) Strains *cdk1-as1* (CF246), *rif1 $\Delta$  cdk1-as1* (CF318), *rif2 $\Delta$  cdk1-as1* (CF320), or *rif1 $\Delta$  rif2 $\Delta$  cdk1-as1* (CF322) were arrested in G2/M with nocodazole and monitored for de novo telomere addition as described in Figure 1B, either with no drug addition or the addition of 1-NMPP1 as indicated. (B) A wild-type strain (UCC5706), a strain containing *cdc17-2* (UCC5840), and a strain containing *cdc17-2* and the galactose-inducible *SIC1* (CF304) were grown at 23°C, arrested, and held at G2/M with nocodazole. Cell cultures were shifted to galactose at  $t = 0$  to induce HO cutting and the overexpression of Sic1 (in CF304) as in Figure 1C. At the same time ( $t = 0$ ), all three strains were shifted to 30°C, the semipermissive temperature for the *cdc17-2* allele.

occurred as in wild-type cells. In contrast, treatment with 1-NMPP1 completely blocked telomere addition (upper panel of Figure 1B).

To confirm the role of Cdk1 in telomere addition, we repeated the same experiment using a different method to inhibit Cdk1. Overexpression of a stable form of the cyclin-dependent kinase inhibitor Sic1 inhibits Cdk1 activity (Desdouets et al., 1998; Ira et al., 2004), and blocked telomere addition at the de novo telomere in G2/M-arrested cells (Figure 1C). Like treatment with the inhibitor 1-NMPP1, overexpression of Sic1p effectively blocked the phosphorylation of Pol12p and did not alter the cell-cycle profile (Figure 1C). These data show Cdk1 kinase activity is required for de novo telomere addition.

To determine whether Cdk1 activity acts in the same pathway as known telomere-length regulators, we tested the effect of inhibiting Cdk1 in genetic backgrounds that result in excessive telomere-repeat addition. In the absence of the proteins Rif1 and Rif2, native telomere length is greatly increased (Hardy et al., 1992; Wotton and Shore, 1997). Inhibiting Cdk1 kinase activity with 1-NMPP1 blocked telomere addition in *rif1 $\Delta$* , *rif2 $\Delta$* , and *rif1 $\Delta$  rif2 $\Delta$*  mutant strains (Figure 2A). The increase in elongation that is seen when *RIF2* is deleted (Diede and Gottschling, 1999) was blocked by inhibiting Cdk1, suggesting Cdk1 activity acts upstream of *RIF2* in the same genetic pathway. Because the deletion of *RIF1* did not change telomere addition in this assay (Figure 2A), we were unable to assess the epistasis relationship between *RIF1* and Cdk1 activity.

To further define the step that is inhibited by blocking Cdk1, we tested the effect of Cdk1 inhibition in a *cdc17-2*

mutant strain. *cdc17-2* is a temperature-sensitive allele of *CDC17*, the catalytic component of the DNA polymerase  $\alpha$ /primase complex. At the nonpermissive temperature, *cdc17-2* strains are unable to elongate telomeres in the telomere addition assay (Diede and Gottschling, 1999). When shifted to a semipermissive temperature, *cdc17-2* strains show progressive telomere elongation (Carson and Hartwell, 1985; Diede and Gottschling, 2001). We blocked Cdk1 activity by overexpressing Sic1p in a strain containing the *cdc17-2* mutation. At the semipermissive temperature, we observed increased telomere addition over time that was blocked by the overexpression of Sic1 (Figure 2B). This argues that Cdk1 activity is also in the same genetic pathway for telomere addition as *CDC17*. The results of this epistasis analysis suggest that Cdk1 activity is involved in an early step of telomerase-mediated telomere addition.

#### Telomere Addition Requires Proteins Involved in Double-Strand Break Processing

To further define the genetic requirements for de novo telomere addition in G2/M, we examined the role of specific DNA repair proteins that act early in the response to a double-strand break. Tel1 and Mec1 are PI3-like kinases that are recruited both to telomeres and to double-strand breaks (Lisby et al., 2004; Takata et al., 2004). Tel1 is recruited to a double-strand break before it is processed to form a single-strand overhang, while Mec1 is recruited after the single-strand region is created (Lisby et al., 2004; Zou and Elledge, 2003). In cells arrested in G2/M, deletion of *TEL1* blocked telomere addition but cells lacking *MEC1* exhibited normal telomere

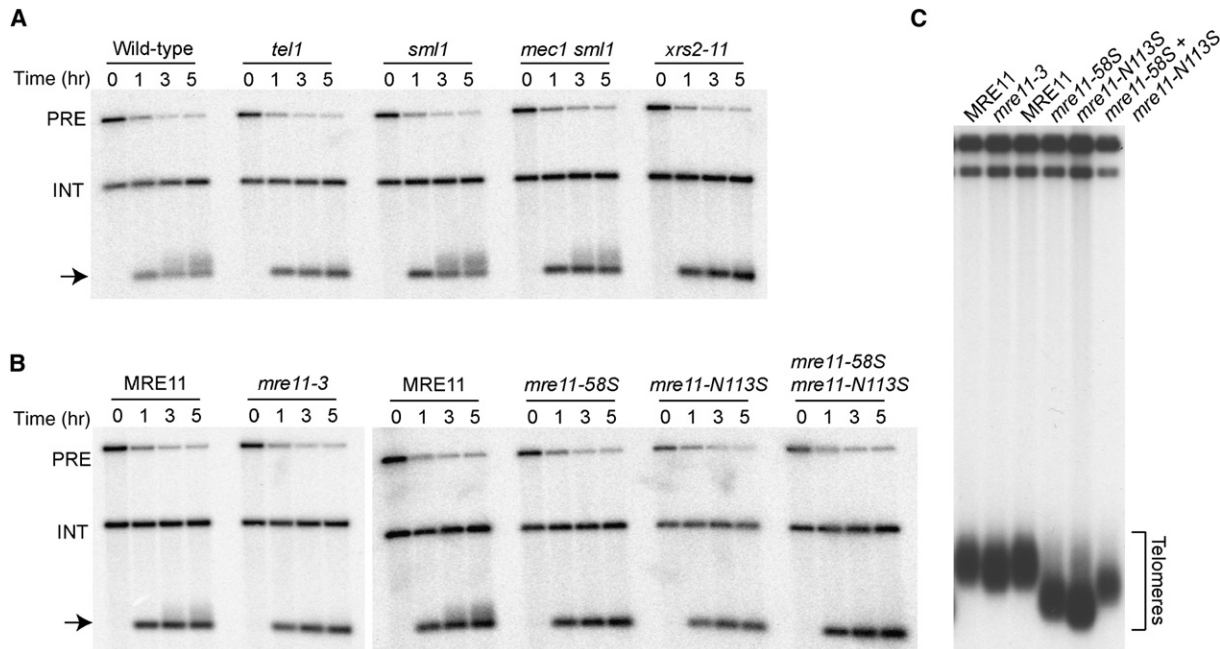


Figure 3. *TEL1* and the Nuclease Activity of Mre11 Are Required for De Novo Telomere Addition

(A) A wild-type strain (UCC5706) and strains containing *tel1* $\Delta$  (CF177), *sml1* $\Delta$  (CF238), *mec1* $\Delta$  *sml1* $\Delta$  (CF239), or an Xrs2 C-terminal truncation, *xrs2-11* (CF179), were arrested in G2/M and monitored for de novo telomere addition as described in Figure 1B. (B) An *mre11* $\Delta$  strain (UCC5969) was covered by CEN plasmids containing different alleles of the *MRE11* gene. From left to right, the first two strains contain either *MRE11* (CF187) or *mre11-3* (CF189) under the control of the constitutive *ADH* promoter. The next four strains express versions of *MRE11* from its native promoter: *MRE11* (CF193), *mre11-58S* (CF195), *mre11-N113S* (CF197), or *mre11-58S mre11-N113S* (CF199). Strains were arrested in G2/M and monitored for de novo telomere addition as described in Figure 1B. (C) The endogenous telomere-length distribution of the strains used in (B) was visualized by Southern blot using a radiolabeled  $\gamma$ -specific subtelomeric probe on *XhoI*-cut genomic DNA. The terminal restriction fragments near the bottom of the gel represent the lengths of native telomeres. An *mre11* $\Delta$  strain had telomeres the same length as *mre11-N113S* (data not shown).

elongation (Figure 3A). *MEC1* is an essential gene; however, the deletion of *SML1* and *MEC1* together allows viability.

Tel1 functions with the MRX complex at both double-strand breaks and at telomeres. It was previously shown that deletion of the MRX complex abolishes telomerase-mediated telomere addition at a de novo telomere (Diede and Gottschling, 2001). To help define the specific role of the MRX complex in telomere elongation, we examined a truncation mutation of *XRS2* and a series of point mutations in *MRE11*. Because a C-terminal deletion of *XRS2* (*xrs2-11*) prevents the interaction of Tel1 and the MRX complex at a double-strand break (Nakada et al., 2003), we tested the effect of the *xrs2-11* mutation on de novo telomere addition. Yeast with the *xrs2-11* mutation were unable to elongate the de novo telomere (Figure 3A). Mre11 itself has several different activities in vitro, including 3' to 5' double-strand exonuclease and single-strand endonuclease activities (Assenmacher and Hopfner, 2004). To examine the role of Mre11 in de novo telomere addition, we tested the effect of different *MRE11* mutants on telomere elongation in G2/M-arrested cells. We first tested two mutant forms of the Mre11 protein, *mre11-58S* and *mre11-N113S*. The *mre11-58S* allele (H213Y) has an impaired interaction with Rad50 and exhibits short telomeres, increased sensitivity to MMS, and severely impaired 5' to 3' resection at a double-strand break (Lee et al., 2002; Tsubouchi and Ogawa, 1998; Usui et al., 1998). The *mre11-N113S* allele is a missense muta-

tion modeling the N117A mutation in human Mre11 that causes the disease ataxia-telangiectasia-like disorder (ATLD) (Stewart et al., 1999). *mre11-N113S* mutants exhibit short telomeres, a moderate sensitivity to MMS, and normal 5' to 3' end resection at a double-strand break (Lee et al., 2002). The *mre11-58S* and *mre11-N113S* alleles complement each other, and yeast expressing both mutant forms of *mre11* have wild-type sensitivity to MMS and nearly normal telomere lengths (Lee et al., 2002). Like *mre11* $\Delta$ , the *mre11-58S* and *mre11-N113S* mutants were defective in the de novo telomere addition reaction (Figure 3B). These results are consistent with the short telomeres seen in these mutants (Figure 3C). When both alleles were expressed, we were unable to observe telomere addition although we observed less complementation in restoring native telomere length than was previously reported (Lee et al., 2002) (Figures 3B and 3C). To specifically test the requirement for the nuclease activities of Mre11, we tested telomere elongation in G2/M cells containing the *mre11-3* allele (H125L/D126V). This allele has a mutation in a conserved active site histidine residue in motif III of Mre11 that is required for in vitro nuclease activity (Hopfner et al., 2001; Moreau et al., 1999). This mutation results in normal-length telomeres, a very mild sensitivity to MMS, and normal 5' to 3' end resection at a double-strand break (Bressan et al., 1998; Lee et al., 2002). Surprisingly, the *mre11-3* mutant was severely impaired in the de novo telomere addition assay even though the native telomeres were nearly

wild-type in length (Figures 3B and 3C). This suggests that, in cells arrested in G2/M, the nuclease activity of Mre11 is required for telomere addition; in actively cycling cells, however, this requirement may be overcome to allow normal telomere-length maintenance.

#### Cdk1 Activity Is Required to Generate Long 3' Single-Strand Overhangs

Based on the role of Cdk1 activity in regulating the 5' to 3' processing of a double-strand break (Ira et al., 2004), we hypothesized that the early requirement for Cdk1 activity in telomere addition could be related to the processing of telomeres to create a 3' single-strand overhang. Telomere processing of the de novo telomere occurs efficiently in G2/M-arrested cells and is required for the binding of Cdc13 (Diede and Gottschling, 2001). We repeated the telomere addition experiment and followed the 5' to 3' degradation of the C-rich strand over time using a single-strand riboprobe. In a strain with the *cdk1-as1* allele, the C-rich strand was present 1 hr after cutting with the HO endonuclease. When no inhibitor was added, the C-rich strand was degraded over time and was almost completely absent after 5 hr (Figure 4A). When the inhibitor 1-NMPP1 was used to block Cdk1 activity, the C-rich strand persisted for the duration of the experiment, indicating that Cdk1 activity was required for its degradation (Figure 4A). To confirm that the loss of the C-rich strand was predominantly due to degradation, and not telomere elongation, we followed the C-rich strand in a strain that lacks telomerase activity due to the deletion of *TLC1*. As expected, there was no elongation of the telomere seed sequence in the *tlc1Δ* strain (Figure 4B), as shown previously (Diede and Gottschling, 2001). The signal from the C-rich strand decreased over time when no inhibitor was added to the *tlc1Δ* strain, although less than when elongation occurred in a *TLC1* strain (Figure 4A). When the inhibitor 1-NMPP1 was used to block Cdk1 activity, there was no degradation of the C-rich strand (Figure 4A). This suggests that Cdk1 activity is required for the creation of a 3' overhang at a de novo telomere, independent of telomerase activity at that telomere.

#### Cdk1 Activity Is Required at Native Telomeres to Generate Long G Strand Overhangs

Previous results have shown that many of the genetic requirements for telomere maintenance are the same at natural telomeres and de novo telomeres (Diede and Gottschling, 1999, 2001; Stellwagen et al., 2003). Because Cdk1 activity was required to generate a 3' overhang at a de novo telomere, we tested the effect of Cdk1 activity on the generation of the long 3' G strand overhang at native telomeres. We synchronized cells containing the *cdk1-as1* allele in G1 using  $\alpha$  factor and then released them into media containing hydroxyurea, so the cells would arrest in early S phase. In early S phase, cells have already passed START and replication initiation, two essential parts of cell-cycle progression that depend on Cdk1 activity (Reed and Wittenberg, 1990). The culture was then released from hydroxyurea and divided, and one-half of the culture was treated with the inhibitor 1-NMPP1. We collected cells every 10 min until the cells had reached G2/M. The cultures both

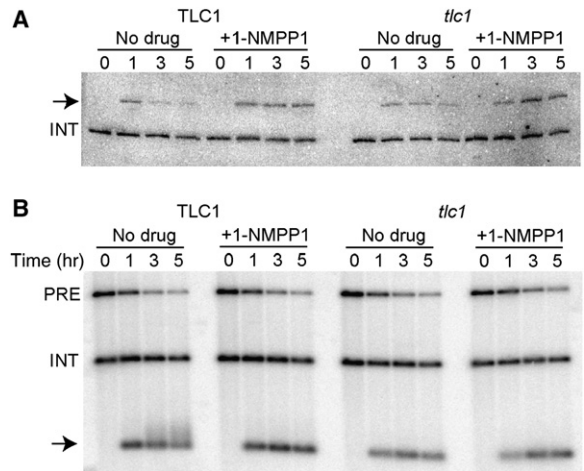


Figure 4. Cdk1 Activity Is Required for 5' End Processing of a De Novo Telomere

(A) The de novo telomere addition assay was carried out using a strain with the *cdk-as1* allele and either a wild-type (*TLC1*) or mutant (*tlc1Δ*) copy of the telomerase RNA. The assay was done as in Figure 1B, and the resulting DNA samples were digested with DdeI, separated on a denaturing 5% polyacrylamide gel, electroblotted, and detected using a radiolabeled single-strand riboprobe that detects the C-rich strand of the de novo telomere (arrow) (Diede and Gottschling, 2001). Loss of this signal represents the disappearance of the C strand of the DdeI restriction fragment. The band labeled INT represents an internal fragment from the *ade2-101* locus and serves as a loading control.

(B) DNA samples from (A) were digested with SpeI, and de novo telomere addition was visualized as in Figure 1B.

contained nocodazole to ensure that the cells could not pass through mitosis and re-enter G1. FACS analysis of the cell-cycle progression showed that the addition of 1-NMPP1 did not prevent the progression from early S to G2/M (Figure 5A). After native gel electrophoresis, we detected the G-rich single-strand telomere overhang by in-gel hybridization with a C-rich radiolabeled oligonucleotide (Dionne and Wellinger, 1996). As a control, the gel was subsequently denatured and rehybridized using the same oligonucleotide to quantify the total amount of telomere DNA in each lane. In the culture with wild-type Cdk1 activity, there was an increase in the single-strand overhang as the cells progressed from S phase to G2/M as expected (Figures 5B and 5C). Inhibition of Cdk1 with 1-NMPP1 blocked this increase in single-strand DNA generation (Figures 5B and 5C). Treatment of the DNA samples with the single-strand exonuclease Exo1 abolished the signal detected in the native gel hybridization (Figure 5D), confirming that the native hybridization only detected single-strand DNA. Two different people independently performed this experiment with very similar results (data not shown). In all cases, the cell-cycle profile of cultures going from S phase into G2/M was nearly indistinguishable, regardless of whether or not the samples were treated with 1-NMPP1. This indicates that the decreased G strand overhang when Cdk1 was inhibited was not due to a delay in S phase. We conclude that Cdk1 activity is required for the generation of the long 3' overhang in late S phase at native yeast telomeres.

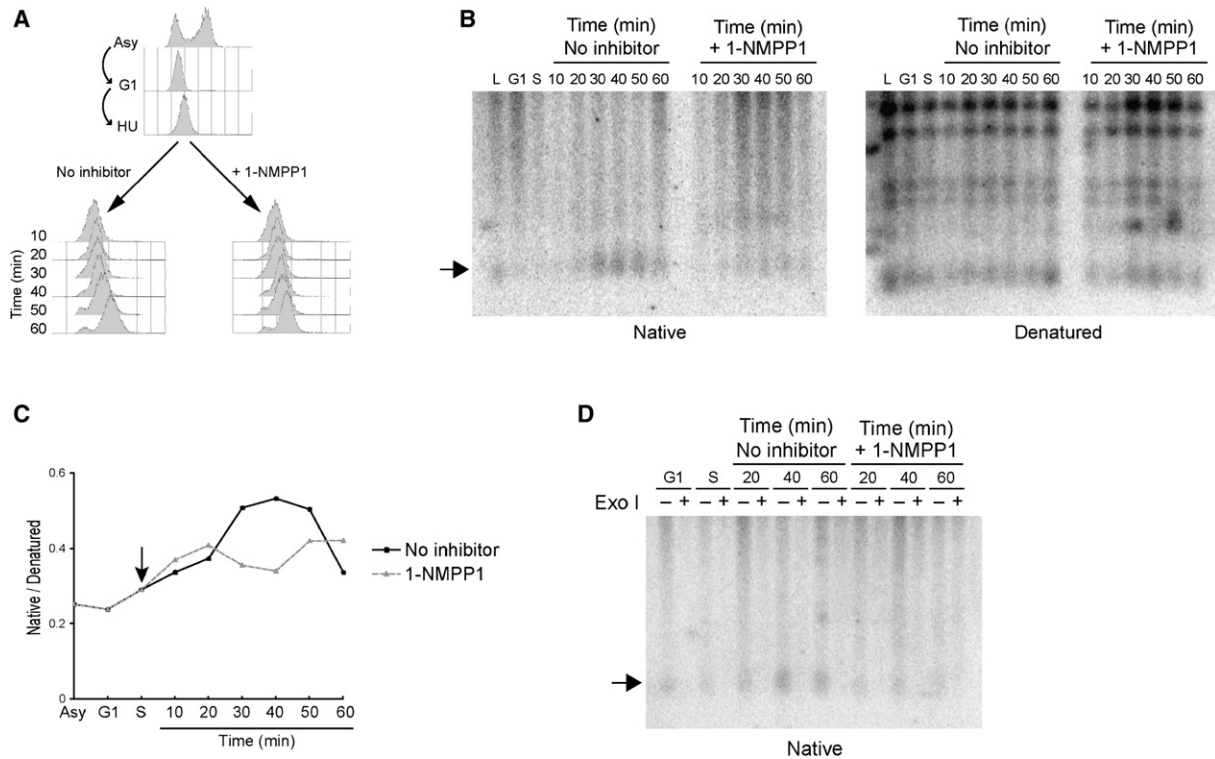


Figure 5. Cdk1 Activity Is Required to Create a Long 3' Single-Strand Overhang at Native Telomeres

(A) Cell-cycle profiles of cells used to monitor the 3' single-strand overhang during the cell cycle. The strain W303 *cdk1-as1* was grown to mid-log phase and arrested in G1 with  $\alpha$  factor. Cells were released from  $\alpha$  factor into media containing hydroxyurea. After arrest in early S phase, the culture was released from the hydroxyurea block and split into media containing nocodazole and either DMSO (no inhibitor) or 1-NMPP1. Cells were collected every 10 min until the cultures reached G2/M.

(B) DNA from the samples in (A) was digested with XhoI, and the single-strand telomere overhang was visualized (arrow) by in-gel native hybridization using an end-labeled C-rich oligonucleotide (Dionne and Wellinger, 1996). The gel was subsequently denatured and probed with the same oligonucleotide to serve as an internal loading control.

(C) The single-strand telomere overhangs detected in (B) were quantified as the ratio of native to denatured signal in the terminal restriction fragment (arrow in [B]). The culture was divided when the cells were released from early S phase (arrow), and DMSO was added to one culture (black solid line), while 1-NMPP1 was added to the other culture (gray dotted line). Two different people independently performed this experiment with very similar results (data not shown).

(D) DNA samples from (B), at time points G1, S, 20 min, 40 min, and 60 min, were treated with or without the single-strand exonuclease ExoI before digestion with XhoI as in (B). The single-strand telomere overhang was visualized after native gel electrophoresis as in (B).

## Discussion

### Telomerase Recruitment Is Cell-Cycle Regulated

Telomerase maintains telomere length by the periodic addition of telomere repeats to short telomeres. Although telomerase activity is present in extracts made from cells in multiple phases of the cell cycle (Diede and Gottschling, 1999), telomere addition is restricted to late S phase (Marcand et al., 2000). The timing of this addition corresponds to the time when telomerase is physically at the telomere (Taggart et al., 2002). Unlike many enzymes, telomerase action seems to be controlled by regulating the interaction of the substrate and the enzyme rather than regulating the activity of the enzyme itself. Mutations in the kinases Tel1 and Mec1 cause telomere shortening without affecting telomerase activity, suggesting that it is the access to the telomere, not telomerase activity, that is regulated (Chan et al., 2001). In this work, we examined the mechanisms that regulate telomere addition during the cell cycle.

In budding yeast, the regulation of the cell cycle is dominated by the periodic expression of cyclins and the activity of the cyclin-dependent kinase Cdk1. In addition to regulating passage through the cell cycle, Cdk1 activity regulates many processes in the cell that may not be directly involved in cell-cycle progression by phosphorylating hundreds of different protein targets (Ubersax et al., 2003). In this study, we used a telomere addition assay to show that cyclin-dependent kinase activity is required for de novo telomere elongation. In addition to Cdk1 activity, elongation in G2/M required the nuclease activity of Mre11, the C-terminal domain of Xrs2, and Tel1. Cdk1 activity was required for the proper formation of G strand overhangs at both de novo and native telomeres.

The working model of telomerase recruitment suggests that G strand overhangs are required for the binding of the single-stranded telomere-binding protein Cdc13. Cdc13 binds to Est1 (Pennock et al., 2001), a protein that in turn recruits the telomerase enzyme through an interaction with a stem-loop structure in the *TLC1*

RNA (Seto et al., 2002). Recent data supporting this model suggest that a properly regulated G strand overhang is a critical requirement for the recruitment of both Cdc13 and Est1 to the telomere in late S phase (Takata et al., 2005). Because the telomerase-associated protein Est1 is recruited to the 3' single-strand overhang at telomeres, it may be this step that is regulated during the cell cycle.

### The Role of Cdk1 at the Telomere

We have shown that Cdk1 activity is required for telomere elongation. The relevant target or targets of Cdk1 at the telomere remain unknown. Cdk1 is not likely to directly regulate the activity of the telomerase enzyme, since telomerase activity is constant in extracts made from different phases of the cell cycle (Diede and Gottschling, 1999). Furthermore, the generation of long G strand overhangs in S phase, which we have shown is regulated by Cdk1 activity, is not dependent on telomerase enzyme activity (Dionne and Wellinger, 1996). The 3' G strand overhang does not represent new telomerase-mediated repeat addition, but rather nucleolytic removal of the C strand.

One possibility is that Cdk1 activity is required to activate a nuclease in late S phase to generate the long 3' single-strand overhang. Mre11 is clearly important in generating proper 3' overhangs at double-strand breaks (Lee et al., 1998) as well as the G strand overhangs at both de novo and native telomeres (Diede and Gottschling, 2001; Larrivee et al., 2004). Paradoxically, the *in vitro* nuclease activity of Mre11 works in the 3' to 5' direction (Paull and Gellert, 1998), which is the opposite polarity that would be needed to create the 3' overhang at the telomere or a double-strand break. Perhaps Mre11 participates in an initial processing reaction that facilitates access for a different, as yet unidentified, nuclease. Either the initial processing reaction by the MRX complex or the activity of an unidentified nuclease could be regulated by the kinase activity of Cdk1. In this regard, Xrs2, a component of the MRX complex, has a number of Cdk1 consensus phosphorylation sites, as does the single-strand endonuclease Dna2, a known Cdk1 target in *S. cerevisiae* (Ubersax et al., 2003). In fission yeast, Dna2 is involved in generating the single-strand overhang at telomeres (Tomita et al., 2004).

Rather than a nuclease being the target, another possibility is that a structural protein at the telomere regulates the access of a nuclease. The end-binding protein Cdc13 acts as a block to 5' to 3' end resection (Garvik et al., 1995), and recent data suggest that Cdc13 may be a target of Cdk1 (Ptacek et al., 2005), although it has no full Cdk1 consensus phosphorylation sites. There are many potential Cdk1 targets that directly or indirectly affect telomere processing, and identifying the relevant ones will be a challenge for the future.

### Telomere Elongation and the DNA Damage Response

The proteins involved in the response to DNA damage are intimately involved in the regulation of telomere function. Cdk1 activity plays an important role in the processing of double-strand breaks in G2/M to create a single-strand overhang that serves as a substrate for homologous recombination (Ira et al., 2004). Cdk1 may

play an analogous role at telomeres, by creating a single-strand overhang that serves as a substrate for the loading of telomerase. The specific phosphorylation target that is essential at a double-strand break may be the same target that is relevant at telomeres. The Cdk1 target essential for processing a double-strand break before homologous recombination remains unknown (Ira et al., 2004; Lisby et al., 2004). It is unlikely that the MRX complex itself is the target because point mutations of potential Cdk1 phosphorylation sites in Mre11 and Xrs2 do not cause defects in homologous recombination as would be expected if they represented the target involved in double-strand break processing (Ira et al., 2004). It will be interesting to see whether telomeres and double-strand DNA breaks share a conserved mechanism of 5' to 3' processing, or whether Cdk1 plays analogous but mechanistically diverse roles at telomeres and double-strand breaks.

The nuclease activity of Mre11 is dispensable for 5' to 3' end resection at a double-strand break in cycling cells (Lee et al., 2002). We observed that a nuclease-deficient allele of *MRE11*, *mre11-3*, blocked de novo telomere addition in G2/M but allowed normal telomere maintenance in cycling cells. This may be analogous to the partially redundant role played by the MRX complex in mating-type switching and double-strand break processing. Mating-type switching is severely impaired when the MRX complex is dysfunctional, but switching still occurs, only at a slower rate (Ivanov et al., 1994). Given enough time, the end result is indistinguishable from a wild-type strain where switching occurred more quickly (Ivanov et al., 1994). Similarly, end resection at a double-strand break is completely dependent on MRX function in G2/M but only partially dependent on MRX in cycling cells (Ira et al., 2004). Similarly, we found that while the nuclease-deficient *mre11-3* cells could not process telomeres for elongation in G2/M, telomeres were wild-type length in cycling cells, suggesting that another nuclease can carry out the critical processing function in other stages of the cell cycle.

At a double-strand break, MRX and Tel1 interact through the C-terminal domain of Xrs2 (Nakada et al., 2003). We found that Tel1 and the C-terminal domain of Xrs2 were required for de novo telomere addition in G2/M. Recent evidence suggests that this domain of Xrs2 is important for both telomere-length regulation and for the translocation of Mre11 into the nucleus (Tsukamoto et al., 2005). Paradoxically, chromatin immunoprecipitation experiments suggest that Tel1 and Mre11 may be at the telomere in different stages of the cell cycle (Tsukamoto et al., 2005). This contrasts with recent data showing that ATM and Nbs1 both localize to telomeres during G2 in human cells (Verdun et al., 2005). It remains unclear whether the interaction of Tel1 and the MRX complex is regulated differently at telomeres and double-strand breaks.

At a double-strand break, in addition to regulating 5' to 3' end resection, Cdk1 plays an important role in a later step of homologous recombination (Ira et al., 2004). Similarly, it is possible that Cdk1 activity is important for more than just the 5' to 3' end resection at the telomere that is required for telomerase-dependent elongation. Interestingly, in cells that lack telomerase, the Cdk1 partner Cib2 is required for a

telomerase-independent telomere elongation pathway that also requires MRX and the Rad51 protein (Grandin and Charbonneau, 2003).

The regulation of telomere elongation by cyclin-dependent kinase activity represents another example of the diverse cellular functions regulated by the kinase Cdk1. In addition to identifying the downstream targets of Cdk1 that regulate telomere end processing, it will be exciting to see whether cyclin-dependent kinase activity plays a similar role in telomere elongation in mammalian cells.

## Experimental Procedures

### Strains and Plasmids

Yeast strains used to monitor telomere addition at a de novo telomere were derivatives of UCC5706 (wild-type), UCC5824 (*rif2* $\Delta$ ), UCC5840 (*cdc17-2*), or UCC5969 (*mre11* $\Delta$ ) (Diede and Gottschling, 1999, 2001). *RIF1* deletions were created in UCC5706 and UCC5824 by PCR-mediated gene disruption (Brachmann et al., 1998) to create CF229 and CF311, respectively. CF246 (wild-type *CDK1::cdk1-as1*), CF318 (*rif1* $\Delta$  *CDK1::cdk1-as1*), CF320 (*rif2* $\Delta$  *CDK1::cdk1-as1*), and CF322 (*rif1* $\Delta$  *rif2* $\Delta$  *CDK1::cdk1-as1*) were created by replacing the *CDK1* gene with *cdk1-as1* in UCC5706, CF229, UCC5824, and CF311 using the plasmid pJAU (F88G) (a gift from K. Shokat) as described (Bishop et al., 2000). A galactose-inducible *SIC1* was integrated into the *URA3* locus of UCC5706 and UCC5840 using the plasmid pLD1 (a gift from J. Diffley) (Desdouets et al., 1998) to create CF206 and CF304. Starting with UCC5706, *tel1* $\Delta$ , *smi1* $\Delta$ , *mec1* $\Delta$  *smi1* $\Delta$ , and *xrs2-11* (Nakada et al., 2003) were created by PCR-mediated gene disruption to create CF177 (*tel1* $\Delta$ ), CF238 (*smi1* $\Delta$ ), CF239 (*mec1* $\Delta$  *smi1* $\Delta$ ), and CF179 (*xrs2-11*).

To assess the effect of different *MRE11* point mutations on de novo telomere addition, UCC5969 (*mre11* $\Delta$ ) was transformed with the CEN plasmids pRS314-*ADH-MRE11* or pRS314-*ADH-mre11-3* (gifts from J. Petrini) to create CF187 and CF189, respectively. UCC5969 was transformed with CEN plasmids pJH1414 (*MRE11*), pJH1767 (*mre11-58S*), pJH1768 (*mre11-N113S*), or both pJH1767 and pJF1768 (gifts from J. Haber) to create CF193, CF195, CF197, and CF199, respectively.

To monitor end processing at a de novo telomere, we took a *tlc1* $\Delta$  haploid spore from UCC5695, a diploid strain heterozygous for the *TLC1* deletion, and mated it to CF246. The resulting diploid, CF328, was sporulated to obtain haploids with the *cdk1-as1* allele and either *TLC1* or *tlc1* $\Delta$ . The strain used for monitoring single-strand DNA formation at a native telomere was W303 *cdk1-as1* (a gift from K. Shokat) (Bishop et al., 2000).

### Cell-Cycle Analysis by Flow Cytometry

Cells were analyzed on a FACScan flow cytometer (Becton Dickinson) using the CELLQUEST software package and the method as described (Haase and Lew, 1997). The FACS staining protocol was modified so that cells were not treated with pepsin before the incubation with RNase A.

### Inhibition of Cdk1 Activity

To inhibit Cdk1 activity in strains containing the *cdk1-as1* allele, we added the inhibitor 1-NMPP1 (a gift from K. Shokat) to a final concentration of 5  $\mu$ M. Pol12 immunoblots were done with the 6D2 mouse monoclonal antibody (a gift from M. Foiani) at a 1:10,000 dilution (Foiani et al., 1994).

### Telomere Addition Assay

The telomere addition assay was done as described (Diede and Gottschling, 1999). For *cdk1-as1* experiments, 1-NMPP1 was added when the cells were shifted into galactose at  $t = 0$  hr.

### Monitoring End Processing at a De Novo Telomere

Visualization of the degradation of the C-rich strand at a de novo telomere was done as described (Diede and Gottschling, 2001). DNA samples collected from the de novo telomere addition assay were digested with DdeI and separated on a denaturing 5% poly-

acrylamide gel. After electroblotting to Hybond-N+ (Amersham Biosciences), the membrane was hybridized with a single-strand riboprobe made using T7 RNA polymerase (Invitrogen) and the PCR product from primers 5' ADE2, 5'-ATTTACAGTTTTGATATCTTGGC-3' and T7-3' ADE2, 5'-ATCGATAATACGACTCACTATAGGGTTCTAATGTAGATTCTTGTGTTCCG-3'.

### Measuring Single-Strand Telomere DNA across the Cell Cycle

The strain W303 *cdk1-as1* was grown at 30°C in YPD to mid-log phase and arrested in G1 with  $\alpha$  mating factor at 5  $\mu$ g/ml (Sigma) for 2 hr. The cells were released from G1 by washing with 5 culture volumes of YPD, and then resuspended in 0.1 M hydroxyurea (Sigma) for 2.25 hr. The cells were released from the hydroxyurea block by washing with 4 volumes of YPD. The culture was then resuspended in YPD with 15  $\mu$ g/ml nocodazole (Sigma) and divided into halves. DMSO was added to one culture, while the inhibitor 1-NMPP1 was added to the other culture. Samples were collected every 10 min for FACS analysis and DNA extraction. The in-gel native hybridization technique was a slight modification of the published protocol (Dionne and Wellinger, 1996). We followed the modified protocol available from the Wellinger lab at <http://pages.usherbrooke.ca/wellinger/>. Telomere signals were imaged using a Typhoon 9410 (GE Healthcare) and quantified using the ImageQuant software package.

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#### Note Added in Proof

While this work was under review, a paper was published by Vodenicharov and Wellinger that looked at the role of Cdk1 in degradation at uncapped telomeres (Vodenicharov, M.D., and Wellinger, R.J. [2006]. DNA degradation at unprotected telomeres in yeast is regulated by the CDK1 [Cdc28/Clb] cell-cycle kinase. *Mol. Cell* **24**, 127–137.). This group also found that G strand overhang generation requires Cdk1 activity.