TELOMERE REGULATION IN *Arabidopsis thaliana* BY THE CST CAPPING COMPLEX AND DNA DAMAGE RESPONSE PROTEINS

A Dissertation

by

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ABSTRACT

The ends of chormosomes are capped by telomeres, which distinguish the termini from damaged DNA. Paradoxically, DNA repair proteins are also required for telomere maintenance. How DNA repair pathways are regulated to maintain telomeres while remaining competent to repair DNA damage throughout the genome is unknown. In this dissertation, I used a genetic approach to investigate how critical components of telomerase and the telomere protein complex interact with the DNA damage response (DDR).

In the flowering plant, *Arabidopsis thaliana* telomeres are bound by the CST (CTC1/STN1/TEN1) heterotrimer. Loss of any CST component results in telomere shortening, telomere fusions, increased G-overhang length and telomere recombination.

To understand the phenotypes caused by CST deficiency, I examined telomeres from plants lacking CTC1 or STN1 and TERT or KU. My analysis showed that CST acts in a separate genetic pathway for telomere length regulation from both KU and TERT. Further, I found that KU and CST act in separate genetic pathways for regulation of G-overhang formation. These demonstrate that multiple pathways are used to maintain telomere length and architecture in plants.

My study of the interaction of telomere components with the DDR revealed ATR promotes genome stability and telomere length maintenance in

the absence of CTC1, probably by activating programmed cell death of stem cells with high amounts of DNA damage. I also found that poly(ADP-ribosylation) is not required for maintenance of *Arabidopsis* telomeres, in contrast to human telomeres.

Finally, I found an unexpected connection between the DDR and telomerase. My research showed that ATR maintains telomerase activity levels. Further, induction of double- stranded DNA breaks in seedlings led to a rapid decrease in telomerase activity, which correlated with increased abundance of TER2, an alternate *Arabidopsis* telomerase RNA. I hypothesize that TER2 inhibits telomerase to prevent its inappropriate action at internal sites in chromosomes. These data reveal two ways that DDR pathways work in concert with telomerase to promote genome integrity.

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CHAPTER I

INTRODUCTION

A brief history of telomeres

Telomeres are the nucleoprotein structures at the ends of eukaryotic linear chromosomes. The term telomere was coined by Hermann Muller in the 1930s after he observed that the ends of chromosomes were particularly resistant to X-ray mutagenesis and thus must have unique properties compared to the rest of the chromosome (Muller 1938). At about the same time, Barbara McClintock was studying maize chromosomes. In one maize strain, dicentric chromosomes were frequently formed which would then break apart during cell division as the centromeres were pulled apart during mitosis. She noticed that these broken chromosome ends were unstable and would rapidly join with other broken ends and then break during the next cell division, a phenomenon known as the breakage-fusion-bridge cycle (Fig. 1-1) (McClintock 1939). However, in embryonic cells, broken chromosome ends did not fuse together and were instead “healed” (McClintock 1941).

Further interest in telomeres was limited until the 1970s. As researchers began to understand DNA replication, it became clear that the 3’ end of linear DNA could not be fully replicated. James Watson termed this the end replication

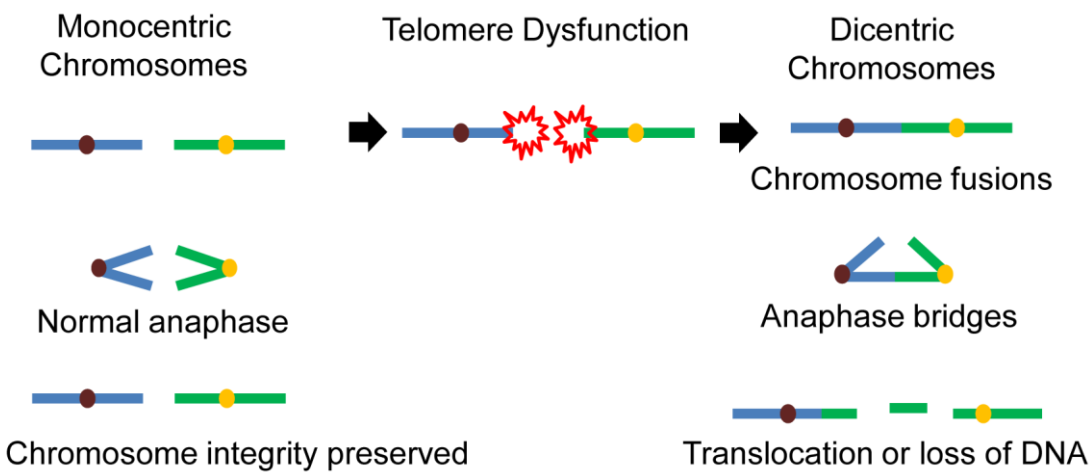

Figure 1-1. The Breakage-Fusion-Bridge cycle. Chromosomes with functional telomeres (left) segregate normally during cell division and the chromosomes remain intact. Telomere dysfunction (center) will trigger DNA repair pathways and result in fusion of chromosome ends. The resulting dicentric chromosomes (right) will be unable to segregate properly during anaphase and will eventually break and result in translocations of DNA from one chromosome to the other. Acentric chromosome fragments will be lost during cell division.

problem (Watson 1972). Alexey Olovnikov went one step further and predicted that the loss of telomeres at each mitosis would lead to the aging and eventual death of the cell (Olovnikov 1973).

In 1978, Elizabeth Blackburn sequenced the ends of *Tetrahymena* rDNA genes and found that they contained 20-70 tandem repeats of 5'-TTGGGG-3' (Blackburn and Gall 1978). Blackburn went on to collaborate with Jack Szostak to show that the *Tetrahymena* telomere sequence could stabilize a linearized plasmid in yeast and therefore established that telomeres could function in two very different eukaryotes (Szostak and Blackburn 1982). Telomerase activity was discovered in *Tetrahymena* extracts by Carol Greider and Blackburn in 1985 (Greider and Blackburn 1985), and then in 1987 they showed that telomerase activity required both a protein and RNA component (Greider and Blackburn 1987). These fundamental discoveries provided the foundation needed to spark an explosion in telomere-related research. The importance of telomeres was further bolstered when the connection was made between telomeres and human health, particularly stem cell maintenance and cancer progression. Ultimately, the discoveries made by Blackburn, Greider, and Szostak were deemed so important that they were awarded the 2009 Nobel Prize in Medicine.

The function of telomeres

Telomeres have two main functions. The first is to differentiate the chromosome ends from double-strand DNA breaks (DSBs). If telomeres are recruited into DNA repair reactions, breakage-fusion-bridge cycles will be initiated and genomic stability will be compromised (Fig. 1-1). To solve this problem, eukaryotes have evolved a cadre of telomere-specific proteins that prevent a DNA damage response.

The second function of telomeres is to solve the end replication problem. After each round of replication, chromosomes shorten because of the loss of the RNA primer at the 5' end of the lagging strand (Fig. 1-2). Telomeres resolve this loss in two ways. First, the telomeric DNA serves as a buffer for the rest of the chromosome so that multiple cycles of replication result in loss of telomeric DNA, but do not result in loss of gene coding regions. The other way telomeres solve the end replication problem is through the action of the telomerase reverse transcriptase, which can elongate chromosomes by adding telomeric repeats at the 3' end of the chromosome.

Features and structure of telomeres

Telomeric DNA consists of G-rich tandem repeats (TTAGGG in humans and TTTAGGG in most plants) which vary in number by species (300 nt in budding yeast, 10-15 kb in human, 2-5 kb in *Arabidopsis*, and up 40 kb in some mice strains) (Riha et al. 2001; Palm and de Lange 2008). Telomeres end in a

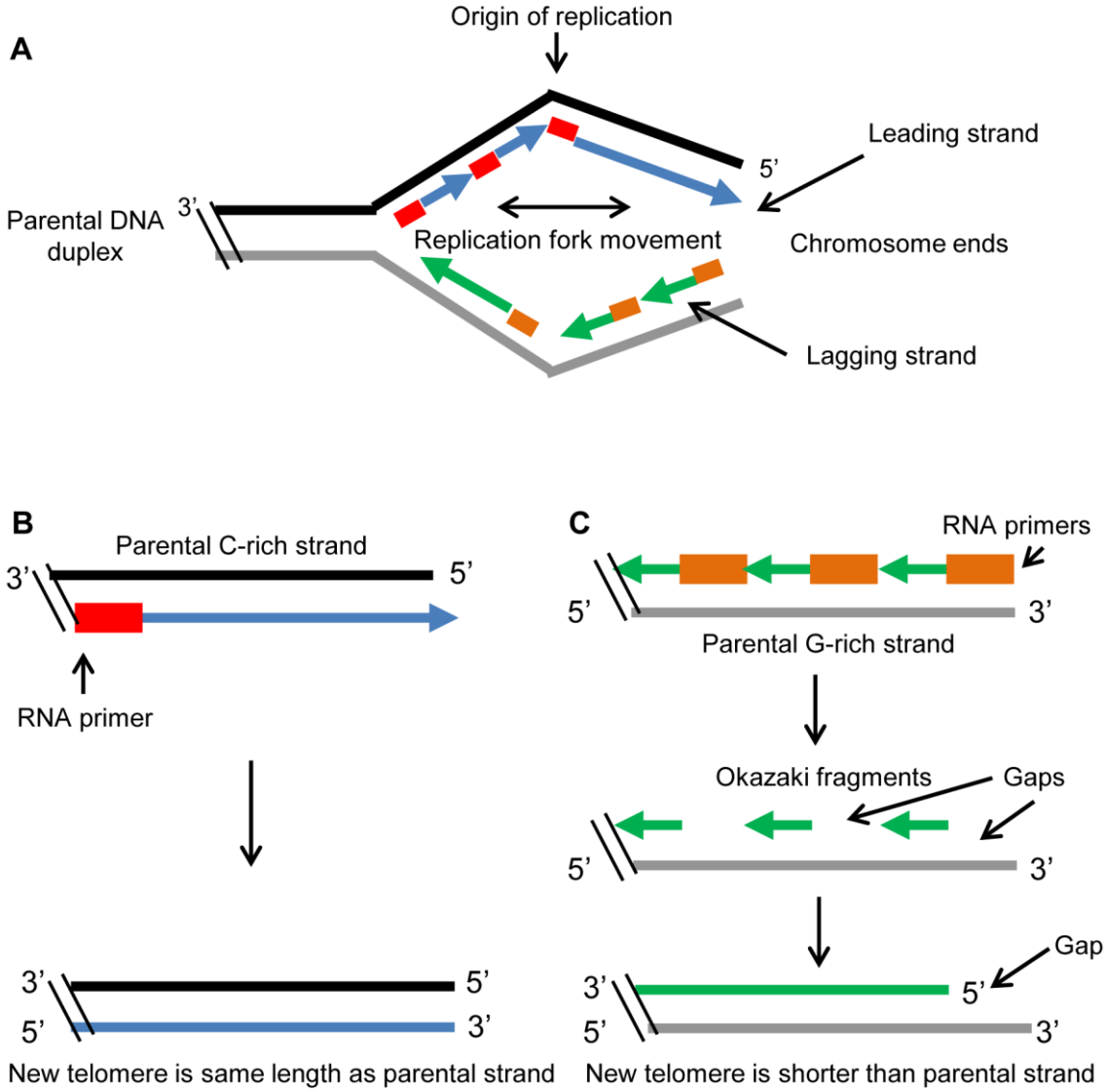

Figure 1-2. The end replication problem. (A) Overview of replication fork passage through the duplex region of the telomere. (B) The parental C-rich telomeric strand is replicated by leading strand replication (top). The telomere of the daughter strand is the same length as the parental strand. (C) The parental G-rich strand is replicated by lagging strand replication (top). Degradation of the RNA primers creates Okazaki fragments with gaps that must be filled in (middle). The telomere of the daughter strand is shorter than the parental strand because DNA polymerase cannot fill in the gap at the very end without a primer (bottom).

3’ G-rich single-stranded DNA (ssDNA) extension known as the G-overhang (Fig. 1-3A). The G-overhang serves as the substrate for telomerase and is also bound by proteins for end protection (discussed below). G-overhangs vary in length depending on the organism (20-30 nt in *Arabidopsis*, 50-500 nt in humans, and 12-14 nt in budding yeast)(Riha et al. 2001; Palm and de Lange 2008). Telomeres in many organisms assume a higher order structure known as a T-loop which is formed when the G-overhang invades the double-stranded (ds) region of the telomere and base pairs with the C-rich strand, thus creating a D-loop with a section of displaced single-stranded G-rich sequence (Fig. 1-3B) (Griffith et al. 1999). T-loops protect chromosome ends by sequestering the G-overhang into a double-stranded structure. T-loops also regulate telomere replication by blocking telomerase access to the G-overhang (Smogorzewska et al. 2000). Telomeres must convert to an open configuration before telomerase can access the G-overhang.

Telomere proteins and telomere end protection

Overview of telomere end protection

Most of the proteins that interact with normal telomeres bind dsDNA or ssDNA, act as bridges between the DNA binding proteins, or function in telomere replication or DNA damage repair. Although some telomere proteins are not conserved among all eukaryotes, most organisms have proteins that

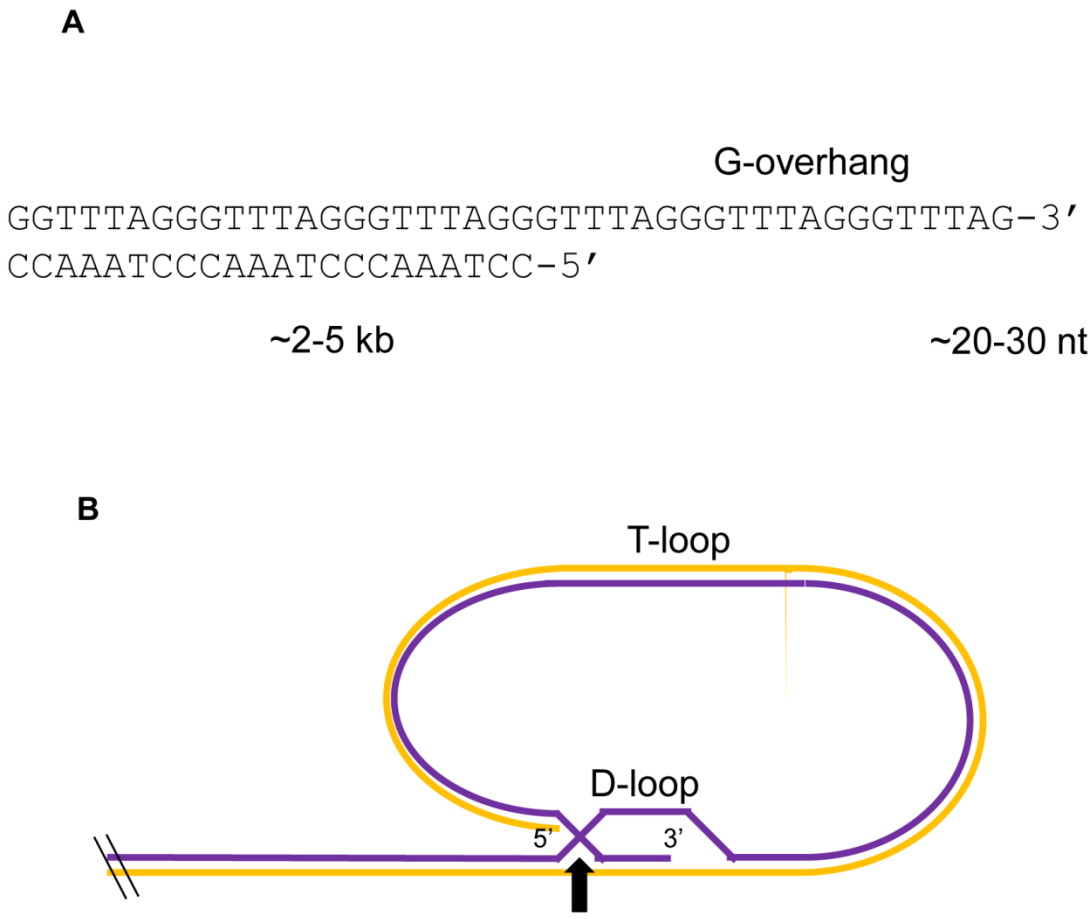

Figure 1-3. Telomere structure. (A) Chromosome ends consist of simple G-C rich repeats and terminate in a single-stranded 3’ G-overhang. In *Arabidopsis* the telomere sequence is TTTAGGG/CCCTAAA and the G-overhang is approximately 20-30 nt in length. The duplex region of the telomere ranges from 2-5 kb in the Col-0 ecotype. (B) The T-loop is formed when the G-overhang invades into the duplex region of the telomere. The arrow indicates the Holliday-like junction where T-loops can recombine.

serve similar functions at the telomeres (Fig. 1-4). Unprotected telomeres shorten because of nuclease attack and inappropriate recombination. When T-loops are unstable because of loss of end protection proteins, they can recombine at the D-loop and form extrachromosomal telomeric circles. The unprotected ends can also be fused together by non-homologous end-joining (NHEJ) or homologous recombination (HR) and enter into breakage-fusion-bridge cycles (Fig. 1-1) (Palm and de Lange 2008). All of these aberrant events can lead to loss of DNA and genomic instability. Short, unprotected telomeres may also lead to cellular senescence and programmed cell death (Garvik et al. 1995; van Steensel et al. 1998). End protection proteins are also important in preventing telomerase access to the G-overhang. When the G-overhang is unprotected, telomerase may extend it beyond optimal lengths. The resulting long telomeres can be unstable and more likely to recombine.

Telomere binding protein

The first telomere end- binding protein, TEBP, was identified in the ciliate *Oxytricha nova* (Gottschling and Zakian 1986). TEBP consists of an alpha and beta subunit that dimerize in the presence of DNA (Fig. 1-4A) (Price and Cech 1989; Fang and Cech 1993). TEBP binds specifically to telomeric ssDNA and its binding requires two telomeric repeats, $(GGGGTTTT)_2$, which corresponds to the length of the G-overhang *in vivo* (Gottschling and Zakian 1986). The crystal structure of TEBP revealed that the alpha subunit contains three

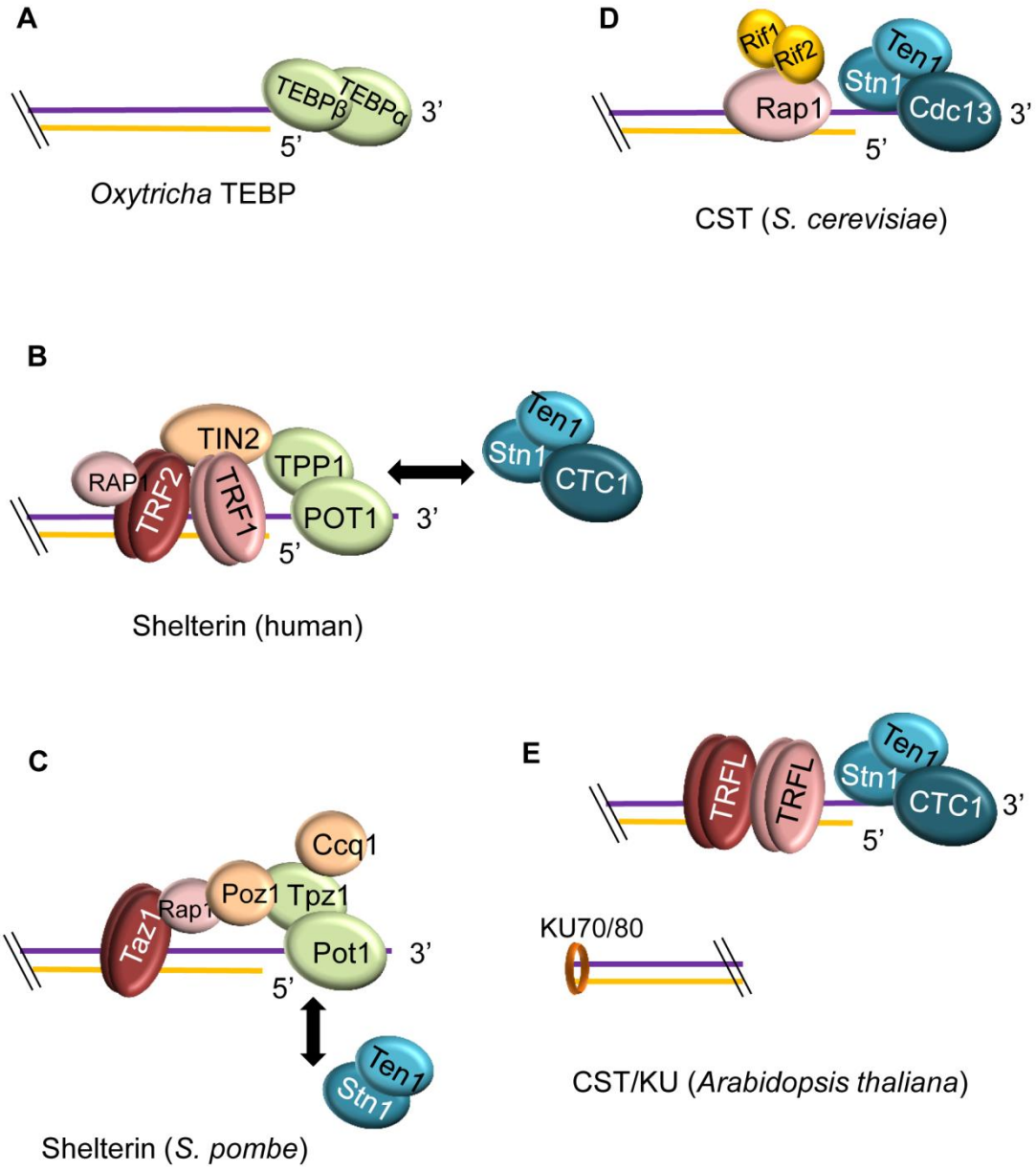

Figure 1-4. Telomere protein complexes. (A) *Oxytricha nova* Telomere End Bindng Protein, (B) Vertebrate shelterin complex and CST complex, (C) Fission yeast shelterin, (D) Budding yeast CST, (E) The *Arabidopsis* capping complex consists of CST and possibly TRFL proteins. One end of the chromosome has CST bound to the G-overhang but the other end is blunt and is protected by KU. The G-rich strand is shown in purple and the C-rich strand is in yellow. In humans and *S. pombe* CST components are shown adjacent to the telomere as they may only transiently associate with telomeres.

oligonucleotide/oligosaccharide binding folds (OB-folds), two of which mediate ssDNA binding while the other is required for binding to the beta subunit. The beta subunit contains one OB-fold which works in conjunction with the two alpha OB-folds for DNA binding (Horvath et al. 1998). The discovery of OB-folds in a telomere-binding protein was an important finding and proved to be a common feature among many telomeric proteins that interact with ssDNA and which led to the identification of telomeric-binding proteins in other organisms (Baumann and Cech 2001).

It has been assumed that TEBP protects chromosome ends from nucleases, but another function of TEBP may be to regulate telomere length by controlling telomerase access to the telomeres (Froelich-Ammon et al. 1998). Telomerase was able to extend telomeric DNA in the presence of either native TEBP proteins (Shippen et al. 1994) or purified recombinant TEBP-alpha (Froelich-Ammon et al. 1998). At higher levels of recombinant TEBP-alpha, or in the presence of both the alpha and beta subunits, telomerase was unable to extend telomeric DNA, suggesting that TEBP can regulate telomere length by altering its affinity for DNA to control when telomerase has access to the telomeres (Froelich-Ammon et al. 1998). The ability of the telomere end-binding proteins to regulate telomerase access to telomeres has proven to be a mechanism used by other eukaryotes (discussed below).

Shelterin

In vertebrates, end protection is provided by a six-member complex known as shelterin (Fig. 1-4B) (Palm and de Lange 2008). TRF1 (Telomeric Repeat binding Factor) and TRF2 bind telomeric dsDNA (Chong et al. 1995; Smogorzewska et al. 2000), POT1 (Protection of Telomeres) binds the ss G-overhang (Baumann and Cech 2001), and TPP1 (TINT1. PTOP, PIP1), TIN2 (TRF1-Interacting Nuclear protein 2), and RAP1 (Repressor/Activator Protein 1) interact with the DNA binding proteins (Palm and de Lange 2008).

TRF1 and TRF2 form homodimers and bind telomeric dsDNA through C-terminal Myb domains (Stewart et al. 2012a) (Fig. 1-5). Loss of TRF1 or TRF2 is embryonic lethal in mice (Karlseder et al. 2003; Celli and de Lange 2005). TRF2 functions in chromosome end protection; it prevents end-to-end chromosome fusions, is a negative regulator of telomere length and maintains the G-overhang (van Steensel et al. 1998; Smogorzewska et al. 2000). In the absence of TRF2, a DNA damage response (DDR) is activated through the ATM kinase (discussed in more detail below) (Denchi and de Lange 2007).

TRF2 contributes to G-overhang formation after leading strand replication (which produces blunt ends) by recruiting the Apollo nuclease (Wu et al. 2010). TRF2 also impacts the formation and stability of T-loops (Stansel et al. 2001; Amiard et al. 2007; Poulet et al. 2009; Nora et al. 2010). TRF2 stimulates strand invasion for formation of the T-loop by promoting positive supercoiling and condensation of DNA (Amiard et al. 2007). Additionally, TRF2 stabilizes the T-

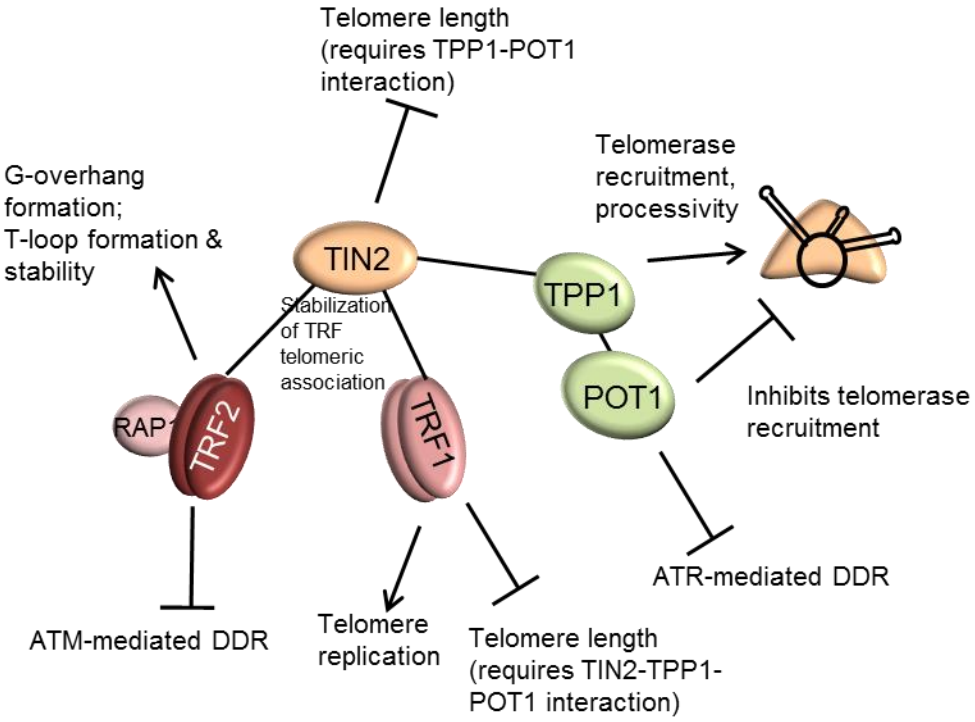

Figure 1-5. Shelterin protein interactions. See text for details.

loop Holliday junction by blocking resolvase mediated cleavage of the junctions and protecting the junctions from Werner helicase (Poulet et al. 2009; Nora et al. 2010). TRF2 thus accomplishes its end protection function by contributing to the formation and maintenance of stable telomeric structures (Fig. 1-5).

TRF1 negatively regulates telomere length; telomeres in TRF1-deficient cells lengthen in a telomerase-dependent manner (van Steensel and de Lange 1997) (Fig. 1-5). In addition, TRF1 is important for semi-conservative DNA replication through the duplex region of the telomeres (Martínez et al. 2009; Sfeir et al. 2009). Telomeres are difficult to replicate efficiently because they are repeat rich and can form secondary structures such as G-quadruplexes, which are formed when guanines create a stacked structure through hydrogen bonding. When TRF1 is deleted from mouse cells, telomeres resemble fragile sites, which are regions of DNA that have breaks or gaps in response to replication stress (Martínez et al. 2009; Sfeir et al. 2009). Depletion of TRF1 also induces an ATR-mediated DDR (ATR is discussed in more detail below) and leads to replication fork stalling within the telomeres. Telomeres in human cells without TRF1 also resemble fragile sites but have normal telomere length (Sfeir et al. 2009). In mice with conditionally-deleted *TRF1*, an epidermal stem cell niche does not develop fully, leading to skin atrophy. If TRF1 is depleted in *p53* mutant mice, which lack the G1-S cell cycle checkpoint, mice survive but develop squamous cell carcinomas (Martínez et al. 2009). The mechanism of TRF1 may be to recruit helicases to telomeres. TRF1 binds the BLM helicase,

which can unwind G-quadruplex structures, and BLM-deficient mouse cells have fragile telomeres. Knockdown of another helicase, RTEL1, which also may act on G-rich DNA, also gave rise to fragile telomeres. For both helicases, the fragile telomere phenotype was epistatic with TRF1 deletion, suggesting that they act in the same pathway for telomere replication (Sfeir et al. 2009). Altogether, these studies suggest that TRF1 plays a vital role in preserving telomere integrity by facilitating replication through the telomeres (Fig. 1-5).

POT1, the ssDNA binding component of shelterin was identified in fission yeast and humans by similarity to the *Oxytricha* TEBPα sequence (Baumann and Cech 2001) (Fig. 1-5). Human POT1 contains two OB-folds which bind the G-overhang (Lei et al. 2004). POT1 caps telomeres and prevents an ATR-mediated DDR (Churikov et al. 2006; Denchi and de Lange 2007; Gong and de Lange 2010). This role of POT1 is discussed in more detail below.

POT1 binds shelterin protein TPP1, which is required for POT1 to localization to telomeres (Liu et al. 2004; Ye et al. 2004b) (Fig. 1-5). Together POT1 and TPP1 regulate telomere length and telomerase access to the telomeres (Liu et al. 2004). POT1 binds TRF1 through its interactions with TPP1 and is required for TRF1 regulation of telomere length (Loayza and De Lange 2003; Ye et al. 2004b). Loss of either POT1 or TPP1 leads to increased telomere length and extended G-overhangs (Loayza and De Lange 2003; Ye et al. 2004b; Churikov et al. 2006). POT1 negatively regulates telomere length by blocking access of telomerase to the G-overhang (Lei et al. 2005; Churikov et al.

2006). Conversely, TPP1, recruits telomerase to telomeres (Abreu et al. 2010) and stimulates telomerase processivity (Wang et al. 2007; Latrick and Cech 2010). It is currently not understood how the seemingly opposing activities of POT1 and TPP1 are coordinated (Stewart et al. 2012a).

TIN2 helps stabilize shelterin and acts as a bridge between the TRF proteins and POT1-TPP1 (Fig. 1-5). TIN2 binds to both TRF1 and TRF2 and stabilizes their association to the telomeres (Ye et al. 2004a). Further, TIN2 interacts with TPP1 and acts as the bridge that connects POT1-TPP1 to TRF1 (Ye et al. 2004b), and like TRF1 and POT1, is a negative regulator of telomere length (Kim et al. 1999). TIN2 is also the only shelterin component that has been implicated in the human telomere disease dyskaratosis congenita (Savage et al. 2008; Walne et al. 2008), perhaps reflecting its central role in the shelterin complex.

The final shelterin component, RAP1, is a homolog of the yeast telomeric protein Rap1 (discussed below) and interacts with TRF2 (Li et al. 2000) (Fig. 1-5). In humans, RAP1 prevents non-homologous end-joining (NHEJ) but not the DDR caused by loss of TRF2 (Sarthy et al. 2009) In mice, however, DNA damage signaling is not activated in *rap1* mutants or cells, but Rap1 does inhibit homologous recombination of telomeres (Sfeir et al. 2010).

The fission yeast *Schizosaccharomyces pombe* has a shelterin-like complex consisting of Pot1, Rap1, Taz1, an ortholog of TRF, Tpz1, an ortholog of TPP1, Poz1, which binds Tpz1 and Rap1, and Ccq1, which binds Tpz1 (Palm

and de Lange 2008) (Fig. 1-4C). Many of the *S. pombe* proteins function similarly to their vertebrate counterparts. Like the TRF proteins, Taz1 binds dsDNA and is a negative regulator of telomere length (Cooper et al. 1997). It is important for both semi-conservative replication through the telomeres and telomere end protection (Ferreira and Cooper 2001; Miller et al. 2006). Rap1 binds Taz1 and works in concert with Taz1 to negatively regulate telomere length, prevent end-to-end chromosome fusions and regulate G-overhang length (Miller et al. 2005). Human and fission yeast Pot1 were discovered at the same time. Pot1 caps telomeres in fission yeast. Loss of Pot1 induces chromosome circularization, which occurs in fission yeast when telomeres are lost (Baumann and Cech 2001). Tpz1, like TPP1, binds to Pot1 and connects Pot1 to Taz1 through the bridging protein Poz1 (Miyoshi et al. 2008). Tpz1 binds to Ccq1, which recruits telomerase to telomeres (Tomita and Cooper 2008). Ccq1 is a positive regulator of telomerase whereas Poz1 is a negative regulator of telomerase (Miyoshi et al. 2008). Thus, Pot1 also plays a role in telomerase regulation like human POT1. The presence of shelterin-type proteins with very similar function in fission yeast and humans suggests that the strategy to protect and maintain telomeres with shelterin is conserved among divergent eukaryotes.

Arabidopsis thaliana also has shelterin orthologs (Fig. 1-4E). Six TRF-like (TRFL) proteins bind telomeric dsDNA *in vitro* (Karamysheva et al. 2004), but their function *in vivo* remains unknown. There are also three POT proteins. However, these proteins bind to the telomerase RNAs instead of the G-

overhang, and likely function in telomerase regulation instead of directly in end protection (Shakirov et al. 2005; Surovtseva et al. 2007; Cifuentes-Rojas et al. 2011; Cifuentes-Rojas et al. 2012). The identification of an *Arabidopsis* ssDNA-binding complex for telomere end protection has been elusive until recently and will be discussed in the next section.

CST

Budding yeast use a different complement of proteins to protect and maintain telomeres (Fig. 1-4D). The CST (Cdc13/Stn1/Ten1) complex binds the ssDNA overhang, and Rap1 binds ds telomeres along with the Rif1 (Rap1 Interacting Factor) and Rif2 proteins (Stewart et al. 2012a). Rap1 was originally described as a transcriptional regulator and was later shown to bind telomeric DNA *in vitro* (Longtine et al. 1989). Mutations in *Rap1* lead to deregulation of telomere length (Conrad et al. 1990; Lustig et al. 1990; Kyrion et al. 1992). The two Rap1 interacting proteins, Rif1 and Rif2, are negative regulators of telomere length (Hardy et al. 1992; Wotton and Shore 1997) (Fig. 1-6). Several studies of Rap1/Rif1/Rif2 have suggested that telomere length is regulated through protein counting (Marcand et al. 1997; Levy and Blackburn 2004). Elongated telomeres will have more Rap1/Rif1/Rif2 molecules bound. One model proposes that the Rif proteins are important for mediating interactions with the telomere cap (Marcand et al. 1997). This interaction would create a folded-back structure that

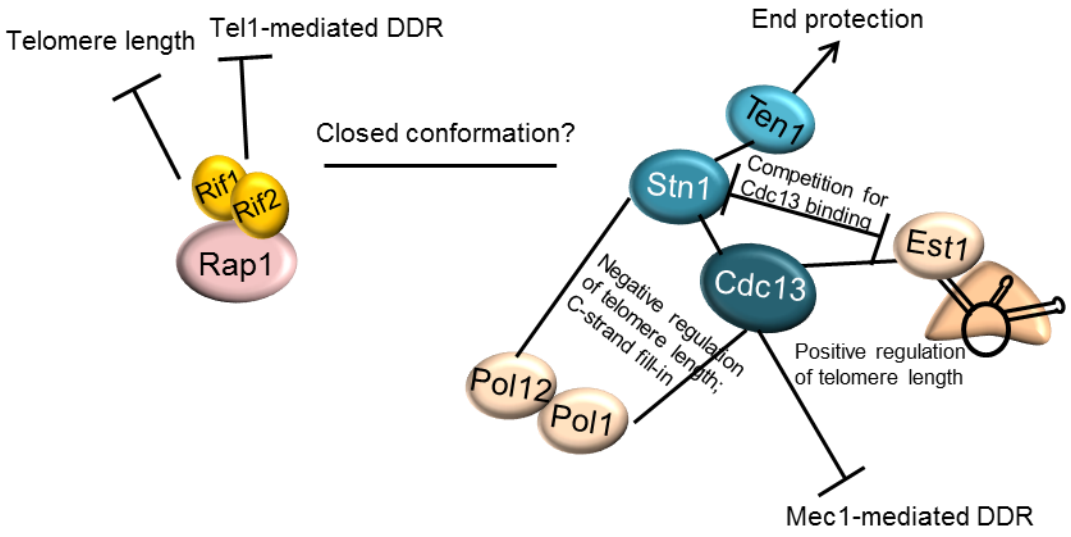

Figure 1-6. Yeast CST interactions. See text for details.

blocks telomerase access to the telomeres. Higher numbers of Rif proteins increase the probability that this “closed” conformation will form and then telomeres will shorten with each cell division. When telomeres shorten, the probability of the closed structure forming is less likely because fewer Rif proteins are at the telomeres. Thus the open structure becomes more likely and increases the chance that telomerase will lengthen those shorter telomeres (Marcand et al. 1997; Levy and Blackburn 2004). The counting model has also been proposed for human telomere length regulation by TRF1 and TRF2 (Smogorzewska et al. 2000).

The *S. cerevisiae* CST complex is structurally different from the POT1-TPP1 complex. POT1-TPP1 are orthologs of the ciliate TEBPα/β proteins (Palm and de Lange 2008), but CST is very similar to the Replication Protein A (RPA) heterotrimeric complex (Gao et al. 2007; Gelinas et al. 2009; Sun et al. 2009), suggesting that CST may function differently than shelterin. Cdc13 is a multifunctional protein with multiple interaction partners that modulate its functions (Fig. 1-6). One function of yeast CST is end protection (Garvik et al. 1995; Lin and Zakian 1996; Nugent et al. 1996; Grandin et al. 1997; Grandin et al. 2001). Loss of function of any one of the CST components leads to loss of C-strand telomeric DNA and extended G-overhangs (Garvik et al. 1995; Grandin et al. 1997; Grandin et al. 2001; Xu et al. 2009). Further, CST prevents a DDR and accumulation of Mec1 (budding yeast ATR) at the telomere (Garvik et al. 1995; Hirano and Sugimoto 2007; Xu et al. 2009). A number of studies have

provided evidence that both Stn1 and Ten1 are important for end protection. Stn1 can rescue the lethality of *cdc13* mutations if it is fused to the Cdc13 DNA binding domain (Pennock et al. 2001). In another study overexpression of Ten1 and a truncated form of Stn1 bypassed Cdc13-mediated end protection(Petreaca et al. 2006). This bypass was dependent on the interaction between Stn1 and Pol12 (this interaction is discussed below). Further, two studies found that the N-terminus of Stn1, which binds Ten1 but not Cdc13, was important for viability and telomere end protection (Petreaca et al. 2007; Puglisi et al. 2008). These studies imply that the end protection function of Stn1 requires the interaction with Cdc13 to bring it to the telomeres, but the interaction itself may not modulate the function of Stn1 in end protection.

The CST complex is also important for telomere length regulation (Nugent et al. 1996; Grandin et al. 2000; Grandin et al. 2001). Interestingly, Cdc13 is both a positive and negative regulator of telomere length (Nugent et al. 1996; Qi and Zakian 2000; Chandra et al. 2001). This dual role for telomere length regulation results from dynamic interactions that occur with Cdc13 during telomere replication. Cdc13 interacts with both telomerase, for G-strand extension, and DNA polymerase α, for C-strand fill-in (Qi and Zakian 2000). In late S phase, telomerase is recruited to the telomeres by the interaction of the telomerase accessory protein Est1 and Cdc13 (Evans and Lundblad 1999; Qi and Zakian 2000) (Fig. 1-6). Mutations that disrupt this interaction result in an EST (Ever Shorter Telomeres) phenotype characterized by progressive telomere

shortening during each cell cycle (Nugent et al. 1996). DNA Polα is recruited to the telomeres after telomerase extension through interactions with both Cdc13 and Stn1; Cdc13 interacts with the catalytic subunit, Pol1, and Stn1 interacts with Pol12, the regulatory subunit (Qi and Zakian 2000; Grossi et al. 2004). Disruption of these interactions leads to telomerase-dependent long telomeres (Qi and Zakian 2000), suggesting that the CST-Polα complex inhibits telomerase recruitment to telomeres. The current model for regulation of switching between telomerase and Polα focuses on post-translational modification of Cdc13. In S-phase the yeast cyclin-dependent kinase (Cdk1/Cdc28) targets Cdc13 threonine 308 and phosphorylation promotes the Cdc13-Est1 interaction, resulting in telomerase recruitment to telomeres. Conversely, absence of phosphorylation by Cdk1 favors the Cdc13-Stn1 interaction (Li et al. 2009) and Stn1 inhibits telomerase association with telomeres (Grandin et al. 2000; Puglisi et al. 2008). Thus, similarly to Pot1, the CST complex functions in both end protection and telomere length regulation through interactions with various proteins that either encourage telomere elongation by telomerase or which favor end protection and exclusion of telomerase.

The notion that CST and shelterin were distinct complexes that had independently evolved to control telomere length and end protection began to fall apart when Stn1 and Ten1 orthologs were identified in *S. pombe*, which was thought to only use a shelterin-like strategy at its telomeres (Martin et al. 2007). *stn1* and *ten1* mutants have telomere detprotection phenotypes similar to *pot1*

mutants, including chromosome circularization. Stn1 and Ten1 interact with each other but not with Pot1, although all colocalize at telomeres (Martin et al. 2007). This suggests that Stn1 and Ten1 may have redundant function with Pot1 or only interact with Pot1 through other proteins.

Chapter II expands on this unexpected finding of both shelterin and CST components in the same organism. This chapter describes how CST was found in both *Arabidopsis* and humans, supporting a new paradigm that shelterin and CST did not evolve independently.

Identification and initial characterization of the *Arabidopsis* CST complex

The belief that shelterin and CST represented two divergent strategies for telomere protection persisted until studies in *Arabidopsis* altered the paradigm of end protection. Conserved Telomere Maintenance Component 1 (CTC1) was discovered serendipitously from a screen of Tilling point mutants for *AtPOT1c* in *Arabidopsis* (Surovtseva et al. 2009). The mutants showed a severe telomere deprotection phenotype, including short telomeres and chromosome fusions. In addition, mutant plants were sterile and displayed a severe fasciated phenotype. However, the developmental phenotypes did not segregate with the *pot1c* mutation. Map-based cloning revealed a mutation in another gene which was then named CTC1(Surovtseva et al. 2009).

At the same time as CTC1 was characterized, an *Arabidopsis* STN1 ortholog of the *S. pombe* Stn1 was found through a bioinformatics search (Song

et al. 2008). The *Arabidopsis stn1* mutant phenotype closely resembled the *ctc1* deprotection phenotype (Song et al. 2008). Moreover, *ctc1 stn1* double mutants displayed a phenotype similar to the phenotype of either individual mutant, suggesting that these genes act in the same genetic pathway. In addition, STN1 physically interacts with a C-terminal fragment of CTC1, consisting of residues 717-990, both *in vitro* and *in vivo* (Surovtseva et al. 2009). Although CTC1 is not a sequence homolog to yeast Cdc13, the deprotection phenotype in *ctc1* mutants in conjunction with the physical and genetic interaction with STN1 suggest that CTC1 and STN1 may form a CST-like complex in plants that plays a significant role in telomere end protection.

The identification of CST outside of yeast was not limited to plants. A human homolog was found bioinformatically using the *Arabidopsis* sequence (Surovtseva et al. 2009) and was also found independently through mass spectrometry of proteins pulled down with human STN1 (Miyake et al. 2009). Human *ctc1* knockdowns in cell culture cause milder telomere phenotypes than *Arabidopsis ctc1* mutants (Miyake et al. 2009; Surovtseva et al. 2009), and HsCTC1 protection of telomeres is independent of Pot1, indicating that shelterin and CST have different functions at the telomeres (Miyake et al. 2009). The discovery of CST in vertebrates, plants, and yeast suggests that CST capping complexes evolved at least as early as shelterin and are likely important for telomere stability in a wide variety of organisms. Further, since shelterin and CST exist in the same organism, cells must coordinate between the two

complexes. Thus, the function and regulation of these complexes is much more complicated than previously thought.

Replication of telomeres: Telomerase and DNA polymerase α

Telomere synthesis by the telomerase RNP

The end of the G-rich strand of telomeres is synthesized by telomerase (Fig. 1-7A). Telomerase consists minimally of a reverse transcriptase protein (TERT) and an RNA (TER), which contains a template of approximately 1.5 telomere repeats which are complementary to the G-overhang (Greider and Blackburn 1985; Greider and Blackburn 1987). To extend the chromosome end, telomerase first aligns itself with the G-overhang through base pairing of the template region of the RNA (Fig. 1-7B). The G-overhang acts as the primer for nucleotide addition, which proceeds in a 5' to 3' direction (Fig. 1-7C). Nucleotides complementary to the RNA template sequence are added, and when the end of the template is reached, telomerase translocates to the newly synthesized chromosome end and then begins another round of synthesis (Fig. 1-7D) (Podlevsky and Chen 2012). The number of repeats added, known as the repeat addition processivity (RAP), varies by organism and cellular context (Lue 2004).

In multicellular organisms, including plants, telomerase is often only expressed during development or in highly proliferating tissue, such as stem cells or cancer cells (Fitzgerald et al. 1996; Wright et al. 1996). Null mutations in

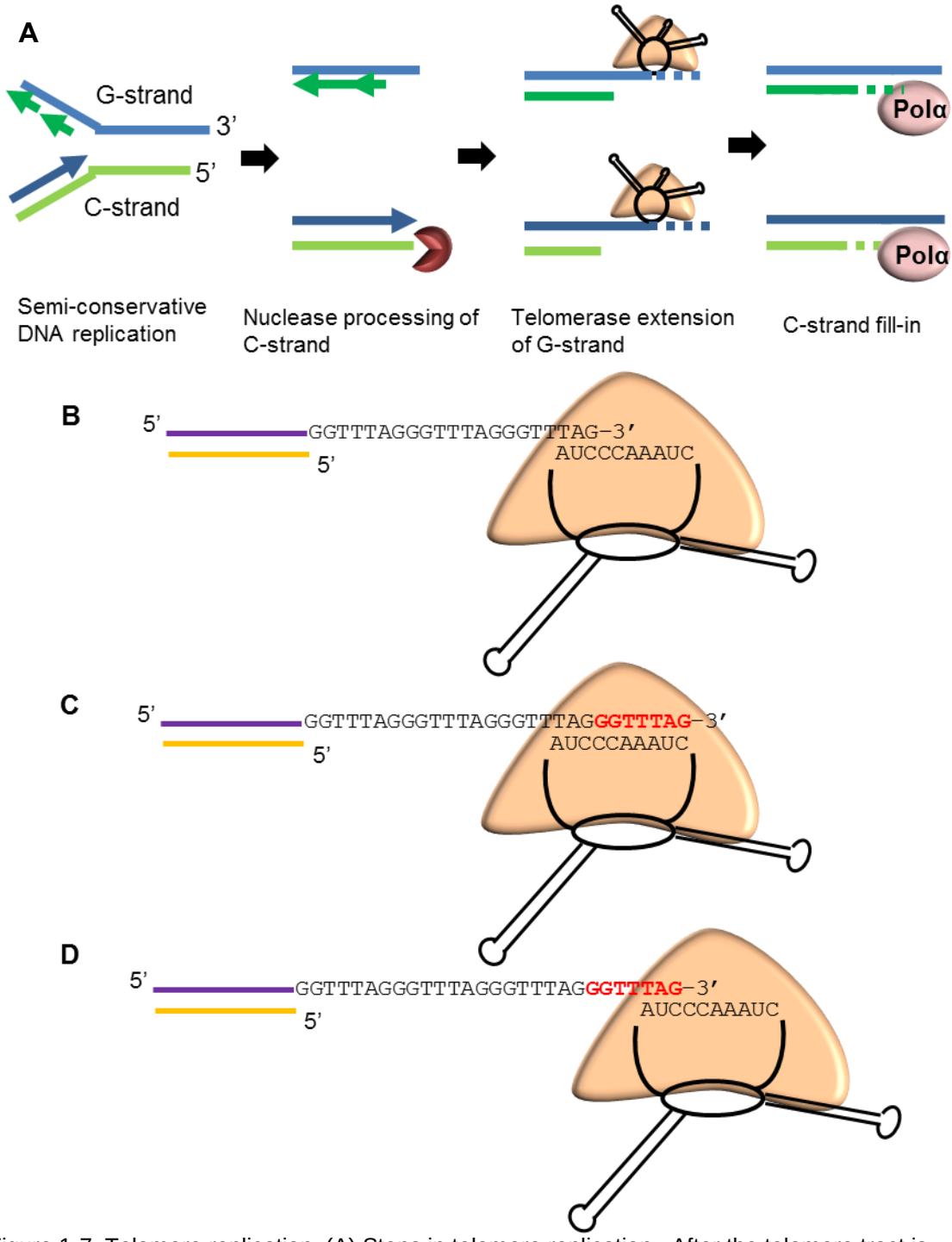

Figure 1-7. Telomere replication. (A) Steps in telomere replication. After the telomere tract is duplicated by semi-conservative replication, blunt ends must be resected to allow telomerase access to G-overhang. Telomerase binds and extends the G-strand. Polα/primase then fills in the C-strand.(B) The telomerase RNP binds to the G-overhang. Base pairing occurs between the 3' terminus and TER. (C) A telomere repeat is added (shown in red) complementary to the TER template. (D) Telomerase translocates to the new 3' end for the next round of synthesis.

telomerase cause progressive shortening of telomeres with each cell division or generation (Lundblad and Szostak 1989; Fitzgerald et al. 1999). In yeast telomerase mutants display an EST (ever shorter telomeres) phenotype. Telomeres gradually shorten until cellular senescence is triggered and ultimately cells die because of loss of chromosome stability (Lundblad and Szostak 1989). Mice deficient in telomerase are viable up to six generations. In each generation, telomeres shorten about 5kb (normal telomeres are 10-40kb in length). By the fourth generation of telomerase defieciency, mice chromosomes display signs of chromosome instability including telomere fusions, aneuploidy, and complete loss of detectable telomeric repeats (Blasco et al. 1997). *Arabidopsis tert* mutants lose approximately 500 bp of telomeric DNA each generation. Mutants begin to show genomic instability by the fifth generation, but can survive up to ten generations (Riha et al. 2001). Telomerase activity is thus an important component of chromosome stability across a wide variety of eukaryotes. Telomerase regulation will be discussed in more detail below.

DNA polymerase α

After telomerase elongates telomeres, the G-overhang must be adjusted to a stable length (Fig. 1-7A). G-overhangs can be too long after telomerase activity if telomerase adds many repeats. Additionally, because telomerase interacts with and extends ss G-rich DNA, the C-strand may be resected to allow

access to telomerase. To fill in the C-strand, cells use DNA Polymerase α (Polα)/primase (Gilson and Geli 2007).

The link between Polα and telomeres was first identified during the characterization of the budding yeast cell cycle-related *CDC17* gene (Carson and Hartwell 1985). *cdc17* mutants showed an increased frequency of mitotic recombination at chromosome termini, which prompted researchers to measure telomere length in these mutants. Telomeres were found to be longer in both *cdc17* alleles tested. Later the *cdc17* alleles were shown to be mutations in the catalytic subunit of DNA Polα (Pol1) (Lucchini et al. 1990).

Additional analyses of *pol1* mutations in budding yeast indicate that the long telomere phenotype is dependent on telomerase (Adams and Holm 1996). Further, *pol1* mutants have extended G-overhangs that appear during S-phase but are processed and gone by G1 (Adams-Martin et al. 2000). The longer G-overhangs occur even without the presence of telomerase, suggesting that Pol1 is important for replication of the lagging strand of telomeres (Adams-Martin et al. 2000). Similar phenotypes were observed in mouse cells harboring a hypomorphic *DNA Polα* allele. As with yeast, longer G-overhangs formed in a telomerase-independent manner and longer telomeres required telomerase. Additionally, the mutant mouse cells showed a high level of Robertsonian chromosome fusions (Nakamura et al. 2005).

The connection between telomerase and DNA Polα was further supported by studies in the ciliate *Euplotes crassus*. Although the length of the

G-strand telomeres in *Eupolotes* was heterogeneous, the C-strand telomeres were much less variable in length, suggesting that the length of the C-strand is more highly regulated than the G-strand (Vermeesch and Price 1994). When *Euplotes* was treated with the DNA Polα inhibitor aphidicolin, both the G-strand and C-strand lengths were affected, providing evidence that there was coordination between the synthesis of the G- and C-rich strands (Fan and Price 1997). In *Euplotes* cells that were undergoing development after mating, telomerase and DNA primase were found to copurify and pulldown of DNA primase precipitated telomerase activity (Ray et al. 2002). Thus, both telomerase and DNA Polα/primase are important for telomere length regulation and there is interaction and coordination between the two complexes.

Studies in budding yeast suggest the Cdc13 capping protein regulates both telomerase and Polα replication of the telomeres. As discussed above, Cdc13 interacts with both the telomerase component Est1 and the Polα catalytic subunit Pol1 (Qi and Zakian 2000). Mutations which disrupt the Cdc13-Pol1 interaction cause longer telomeres in a telomerase-dependent manner (Qi and Zakian 2000). Stn1 binds the regulatory subunit of Pol α, Pol12, and like *Pol1* mutations in *Pol12* also lead to longer telomeres (Grossi et al. 2004). In humans, CTC1 and STN1 act as Pol α accessory factors *in vitro* (Casteel et al. 2009). The next section will discuss some of the new evidence from vertebrate and plant studies further supporting the significance of CST in telomere replication.

CST function in telomere replication

A number of studies supporting the importance of the CST complex have been published since the initial characterization of CST in plants and human cells. In *Arabidopsis*, the homolog of TEN1 was identified (Leehy et al. 2013). Like *stn1* and *ctc1* mutants, *ten1* mutants exhibit considerable telomere dysfunction. However, *Arabidopsis* TEN1 has an additional function in limiting telomerase processivity (Leehy et al. 2013). It is unknown whether this is a plant-specific phenomenon. CST components in other model organisms have not been shown to directly affect telomerase activity.

In vertebrates, there is a growing body of evidence that the CST complex is dispensable for telomere capping, but is vital to ensuring complete replication of the telomeres (Gu et al. 2012; Huang et al. 2012; Nakaoka et al. 2012; Stewart et al. 2012b; Wang et al. 2012). In human cell culture, STN1 was shown to be important for C-strand fill-in of the G-overhang (Wang et al. 2012). In addition, depletion of STN1 delayed replication through the ds region of telomeres (Stewart et al. 2012b; Wang et al. 2012). Further, after researchers stalled replication forks with hydroxyurea, they discovered that STN1 was important for restarting replication, probably by stimulating the firing of new replication origins (Stewart et al. 2012b). In cells depleted of STN1, DNA Polα levels were increased at the telomeres, suggesting that the role of STN1 is not for Polα recruitment to telomeres, but for modulating Polα function (Huang et al. 2012). Consistent with this idea, research performed with *Xenopus* extracts

found that CST preferentially binds G-rich ssDNA, and further that STN1 functions to promote priming on ssDNA templates (Nakaoka et al. 2012). A conditional knockout of STN1 in mice showed similar results to the human cell culture (Gu et al. 2012). Mice had defects in cellular proliferation, bone marrow failure, catastrophic telomere loss, and accumulation of ss telomeric DNA. As with the human cells, the function of STN1 in mice appears to be primarily in the restart of stalled replication forks rather than telomere capping (Gu et al. 2012). Vertebrate STN1 was originally identified as a Polα accessory factor (Casteel et al. 2009). Thus, the function of STN1 at vertebrate telomeres is in accordance with the initial findings, whereas in plants and yeast, CST is more important for end protection.

Recently, the crystal structure of human STN1-TEN1 was solved (Bryan et al. 2013). The structure was highly similar to both the *S. pombe* and *Candida tropicalis* Stn1-Ten1 structures (Sun et al. 2009) and also resembled RPA (Replication Protein A). STN1 tightly bound ssDNA but TEN1 did not, and TEN1 association with STN1 decreased the STN1 affinity for DNA. Mutations designed to disrupt the STN1-TEN1 interaction resulted in elongation of telomeres, telomere-free chromosome ends, and telomere fragile sites. These phenotypes are consistent with the replication function of STN1 observed in other studies and suggest that TEN1 association with STN1 is required for maintenance of telomeres in humans. Additionally, the structural similarity of human STN1-TEN1 to two different yeast STN1-TEN1 complexes supports the

conclusion that CST complexes have been conserved throughout the eukaryotic lineage.

CST has also been shown to interact with shelterin components in vertebrates. In humans, it interacts and competes with POT1-TPP1 and acts to terminate telomerase activity (Chen et al. 2012). The termination of telomerase activity would be consistent with the long telomeres observed when STN1 and TEN1 binding was disrupted (Bryan et al. 2013). In mice, CST interacts with Pot1b and acts to shorten ExoI-generated G-overhangs, likely by promoting C-strand fill in (Wu et al. 2012). This is evidence that in organisms that have both CST and shelterin, that the two complexes do not exist or function in isolation of the other.

The importance of CST in genome integrity is supported by the recent identification of biallelic CTC1 mutations in patients with the short telomere syndromes Coats plus and dyskeratosis congenita (DC) (Anderson et al. 2012; Keller et al. 2012; Polvi et al. 2012; Walne et al. 2013). DC is a bone marrow failure disease characterized by susceptibility to cancer, premature aging, abnormal skin pigmentation, nail dystrophy, and short telomeres (Nelson and Bertuch 2012). Coats plus patients sometimes have symptoms of DC in addition to intracranial calcification, leukodystrophy (inflammation of the white matter of the brain) and brain cysts (Anderson et al. 2012). To date, mutations in *STN1* or *TEN1* have not been identified in any patients with these syndromes (Walne et al. 2013). This could be because *STN1* and *TEN1* are much smaller genes than

CTC1 and thus there is a lower probability of a mutation occurring in those genes. Alternatively, this may also be evidence of the multifunctionality of CTC1. In all patients, *CTC1* contained at least two mutations, perhaps indicating that multiple functions of CTC1 had to be disrupted before symptoms would be manifested. Further biochemical and genetic investigation of the CTC1 mutations may give insight into the likely complex function of CTC1.

Regulation of telomerase

Telomerase requires multiple layers of regulation to finely control the addition of telomeric repeats to the ends of chromosomes. As discussed above, both long and short telomeres can lead to genomic instability, and misregulation of telomerase activity is linked to human diseases, including cancer and dyskeratosis congenita. In this section I will touch upon transcriptional and posttranslational regulation of telomerase as well as telomerase RNP assembly and inhibition of telomerase at DSBs. Recruitment of telomerase to chromosome ends is also a very important step in telomerase regulation which I discuss in the section above on telomere proteins.

Telomerase biogenesis

Features of both TERT and TER are required for telomerase activity. TERs have little sequence conservation between different organisms, but structural elements required for telomerase function and regulation are

conserved (Egan and Collins 2012). TERs vary in size from 159 bp in *Tetrahymena* to over 1kb in some yeast. In general, TERs contain a template region adjacent to a 5' template boundary region, which is a stem or hairpin that prevents copying the RNA beyond the template sequence. TERs also contain a pseudoknot, the function of which is not known, and a stem-terminus element, which is a hairpin or three-way junction that stimulates telomerase activity. TERs have additional structural elements that vary by organism. These may be binding sites for telomerase regulatory proteins or for proteins that assist in RNP biogenesis (Egan and Collins 2012). For example, in humans, a H/ACA domain consisting of two stem loops separated by the H/ACA box is required for Cajal body localization and for binding proteins necessary for RNP biogenesis (Podlevsky and Chen 2012).

The TERT component of telomerase is better conserved than TER. TERTs are characterized by several conserved domains. The reverse transcriptase domain contains the seven motifs found in canonical reverse transcriptases. The C-terminal extension (CTE) binds telomeric DNA. Two other domains are TERT-specific. The N-terminal (TEN) domain is required for telomerase activity, and the telomerase RNA binding domain (TRBD) binds the TER (Podlevsky and Chen 2012).

The details of telomerase RNP assembly differ between different organisms (Egan and Collins 2012). In humans, TERT is expressed and translated through the normal mRNA pathway. It is then imported back into the

nucleus and stored in the nucleolus. TER is expressed by RNA Polymerase II and then bound by H/ACA box proteins, including dyskerin. The 3' end is then nucleolytically processed and the 5' end is capped by TMG (trimethylguanosine). The processed RNA and TERT are then assembled in the Cajal body. Some patients with the disease dyskeratosis congenita have mutations that disrupt telomerase assembly.

Composition of Arabidopsis *telomerase*

Arabidopsis is unique among organisms studied in telomere biology because it has three different telomerase RNA components (Cifuentes-Rojas et al. 2011; Cifuentes-Rojas et al. 2012) (Fig. 1-8). TER1 acts as a canonical TER to add repeats to chromosome ends (Fig. 1-8A). TER2, conversely, inhibits telomerase activity and does not efficiently add telomere repeats to chromosome ends (Fig. 1-8B). TER2 binds TERT with higher affinity than TER1; both TER2 and TER1 bind TERT with much higher affinity than $TER2_S$, which essentially does not bind TERT (Fig. 2-8C). $TER2_S$ is a processed form of TER2

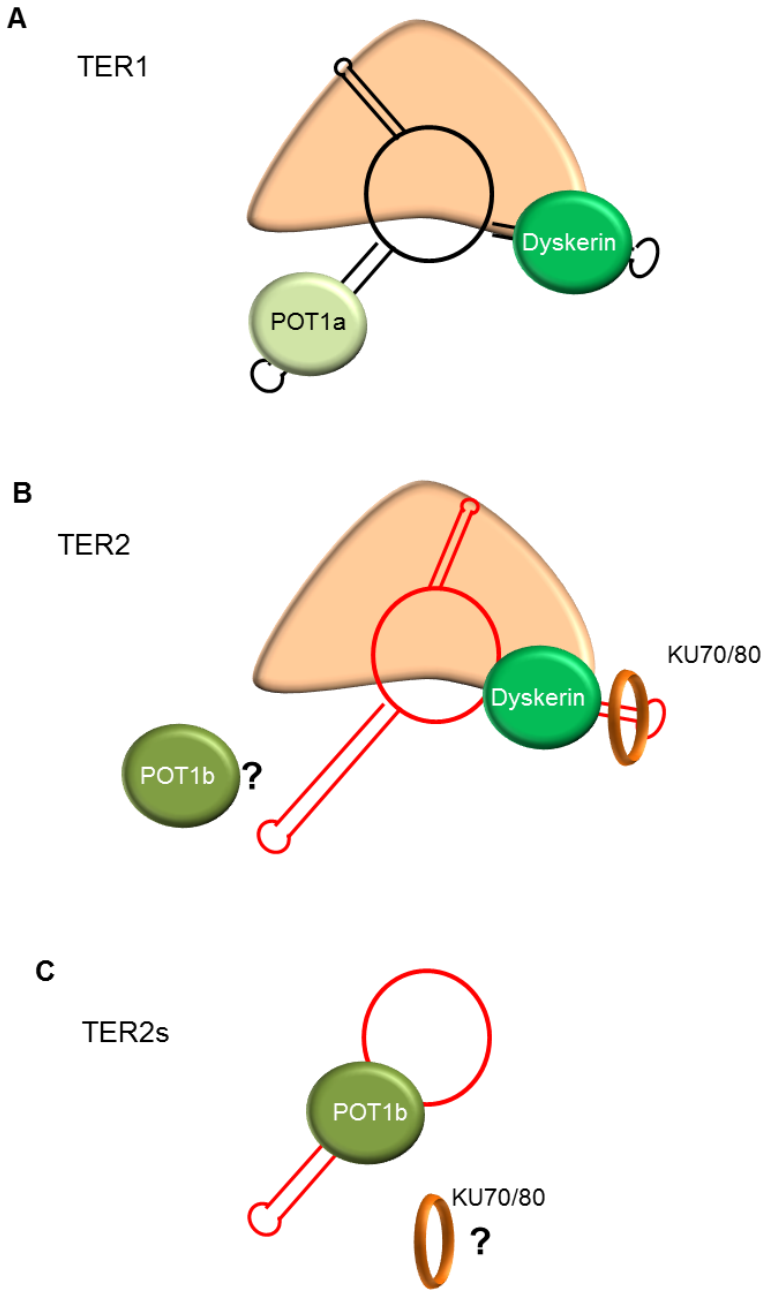

Figure 1-8. Telomerase complexes in *Arabidopsis*. (A) The TER1 telomerase complex adds telomere repeats *in vivo*. POT1a is required for telomerase activity and can bind to CTC1. (B) The TER2 telomerase complex is inhibitory and does not efficiently add telomere repeats *in vivo*. POT1b can bind TER2, but associates primarily with $TER2_S$ so we do not know if it stably associates with the TER2 RNP. (C) TER2s binds very weakly to TERT. KU70/80 can bind to $TER2_S$ but associates primarily with TER2. The *in vivo* function of $TER2_S$ is unknown.

which an intron is removed and the 3' end has been processed. Chapter IV describes the functions of the TERs in more detail.

One way that organisms modulate telomerase function is through the accessory proteins that bind to the RNA component of telomerase. For example, in yeast, Est1 binds the RNA and interacts with Cdc13 to mediate telomerase recruitment to the telomeres and also stimulates telomerase processivity (Evans and Lundblad 1999; DeZwaan and Freeman 2009).

In *Arabidopsis* the presence of three TER subunits has allowed for the evolution of unique combinations of regulatory proteins to associate with the telomerase RNP (Cifuentes-Rojas et al. 2012) (Fig. 1-8). POT1a binds TER1 and acts as a positive regulator of telomerase activity, similar to yeast Est1. POT1b and KU both bind TER2 and $TER2_S$ (Fig. 1-8B and C). It is unknown whether POT1b and KU bind the RNAs simultaneously. In pulldowns of telomerase associated proteins, POT1b was strongly associated with $TER2_S$ whereas KU mostly pulled down TER2 (Cifuentes-Rojas et al. 2012). Dyskerin is important RNP maturation and associates with both TERT-associated TERs (Fig. 1-8A and B). Dyskerin is required for telomerase function in *Arabidopsis* (Kannan et al. 2008).

Transcriptional and posttranslational regulation of telomerase

Transcription is the most common means of regulating telomerase. In humans and *Arabidopsis* TERT expression is restricted to early development,

stem cells, germ cells, and other proliferating cells (Fitzgerald et al. 1996; Wright et al. 1996). A number of proteins bind the TERT promoter in humans (Cifuentes-Rojas and Shippen 2012). These include factors such as E2F transcription factors that are required for cell cycle progression into S phase. Additionally, oncogenes, such as c-Myc, also induce telomerase expression.

In *Arabodopsis*, the hormone auxin can induce TERT expression (Ren et al. 2004; Ren et al. 2007). The transcription factor TAC1 (Telomerase Activator 1) induces expression of multiple auxin-responsive genes, including BT2. TAC1 activation of telomerase requires BT2 activation. BT2 is part of an E3 ubiquitin ligase complex (Figueroa et al. 2005) and may regulate telomerase by destruction of a negative regulator of *TERT* expression.

Posttranscriptional regulation of telomerase includes phosphorylation and ubiquitination of TERT as well as control of TERT localization (Cifuentes-Rojas and Shippen 2012). For example, CHIP (C-terminus of Hsc70-interacting protein) is a co-chaperone protein that mediates ubiquitination of chaperone-bound proteins (Lee et al. 2010). CHIP inhibits telomerase activity by ubiquitinating cytoplasmic TERT. This promotes proteasomal degradation of TERT and also prevents its localization into the nucleus.

TERRA

TERRA is a long non-coding RNA that is expressed from telomeres (Luke and Lingner 2009). It has been implicated in multiple aspects of telomere

regulation, including assisting in recruitment of capping proteins after replication (Flynn et al. 2011) and establishment of telomeric heterochromatin (Deng et al. 2009b; Vrbsky et al. 2010). TERRA may regulate telomerase in multiple ways. In yeast, high levels of TERRA result in short telomeres (Luke et al. 2008). This phenotype is sensitive to RNaseH, indicating that DNA-RNA hybrids are formed, and implying that TERRA may interact with telomeric DNA to inhibit telomerase. TERRA sequence is also complementary to the template sequence of TER. In human cells, TERRA binds and inhibits telomerase through that base pairing as well as directly interacting with TERT (Redon et al. 2010). TERRA thus acts as a competitive inhibitor of telomerase.

Inhibition of de novo *telomere formation*

As mentioned above, one of the functions of telomeres is to differentiate themselves from DSBs in order to prevent activation of DNA repair pathways. Conversely, when a DSB is formed, the cell must prevent telomerase from adding telomere repeats to the break. Additional of a telomere to an internal region of the chromosome can be deleterious because the distal part of the chromosome is no longer connected to the centromere and will be lost during cell division (Fig. 1-9). *de novo* telomere formation is associated with several human diseases, including α- thalassemia, mild forms of mental retardation, and cancer (Flint et al. 1994; Wong et al. 1997).

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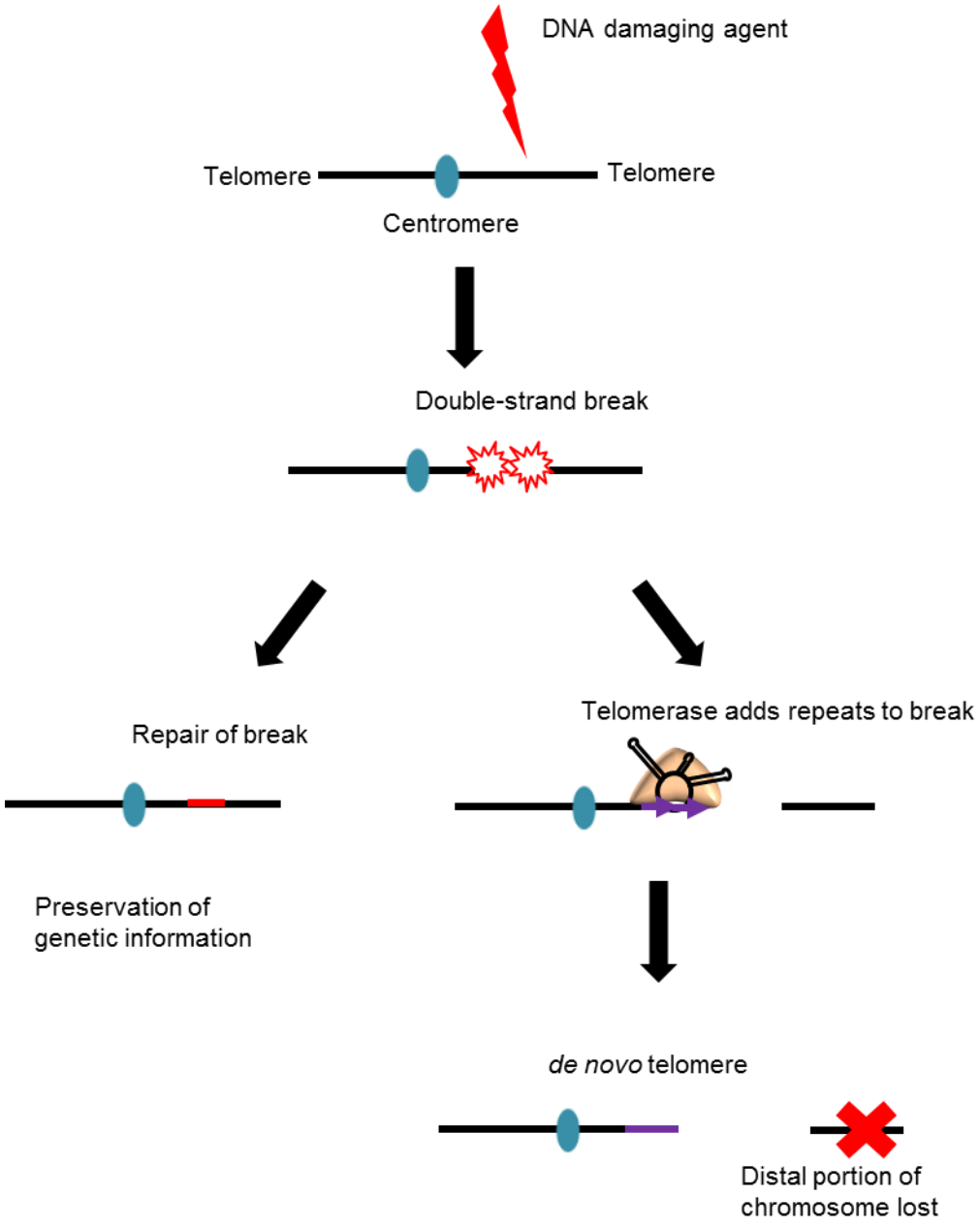

Figure 1-9. *de novo* telomere formation. A DNA damaging agent causes a dsDNA break. Normally the break will be repaired and the chromosome integrity is preserved (left). On rare occasions, active telomerase gains access to the break and adds telomere repeats (right). The distal portion of the chromosome is acentric and will be lost during cell division, along with any genetic information on that portion of the chromosome.

de novo telomere formation has been characterized the most thoroughly in budding yeast. A key enzyme which inhibits telomerase activity at both chromosome ends and DSBs is the Pif1 helicase (Schulz and Zakian 1994).Pif1 acts to unwind RNA-DNA hybrids, thus Pif1 inhibits telomerase by preventing the TER subunit from base-pairing with the DNA (Boule and Zakian 2007). Additionally, Pif1 differentiates between chromosome ends and DSBs. DSBs activate Mec1-Rad53 (Mec1 is ATR; Rad53 is a checkpoint kinase) which phosphorylate Pif1. Phosphorylated Pif1 inhibits telomerase activity at DSBs but not at telomeres (Makovets and Blackburn 2009). Mec1 also inhibits *de novo* telomere formation by phosphorylating Cdc13 (Zhang and Durocher 2010). Phosphorylation prevents Cdc13 accumulation at DSBs. Thus, cells can use the same proteins to regulate telomerase activity at DSBs and telomeres but control their function by post-translational modifications that are DNA damage-specific.

A second pathway for *de novo* telomere formation is thought to be independent from Pif1 (Ribeyre and Shore 2013). The frequency of telomere addition to DSBs increases in the absence of the nucleases Sgs1 and Exo1 (Chung et al. 2010; Lydeard et al. 2010). In these mutants, resection at the DSB is impaired and Cdc13 localization to the break is increased which recruits telomerase to the break. Ku also promotes *de novo* telomere formation by recruiting telomerase (Myung et al. 2001).

In *Arabidopsis*, *de novo* telomere formation has been studied using tetraploid plants (Nelson et al. 2011). Tetraploidy alleviates the detrimental

effects of losing chromosome pieces after telomeres are added at DSBs. In plants, transformation of T-DNAs containing a telomere repeat array (TRA) can cause truncation of the chromosome and induce the establishment of a new telomere using the TRA as a seed sequence. Using this system, researchers found that KU is required for *de novo* telomere formation and DNA Ligase IV is required for efficient *de novo* telomere formation. Unexpectedly, *tert* mutants have the highest rate of chromosome truncation, although these new telomeres are not extended. Both KU and LigIV are components of the NHEJ pathway, so maybe the truncated chromosome is stabilized or processed by an unknown mechanism that favors *de novo* telomere formation in the absence of TERT.

In humans, several other mechanisms to prevent *de novo* telomere formation have been identified. In cells treated with ionizing radiation, telomerase is sequestered in the nucleolus, preventing its access to DNA substrates (Wong et al. 2002). Additionally, the c-Abl protein tyrosine kinase, which is activated by DSBs, phosphorylates TERT, resulting in inhibited telomerase activity (Kharbanda et al. 2000). The presence of multiple protective mechanisms underscores the importance of inhibiting *de novo* telomere formation.

In Chapter IV, I describe the characterization of the *Arabidopsis* telomerase component TER2. This RNA inhibits telomerase activity and its abundance increases after induction of DNA breaks by zeocin. We hypothesize

that TER2 inhibition of telomerase is a novel mechanism to suppress *de novo* telomere formation.

An overview of the DNA damage response and DNA repair

In order to examine how telomeres differentiate chromosome ends from DSBs to prevent inappropriate DNA repair reactions, it is necessary to understand how cells respond to and repair DNA damage. Here I will give a general overview of the DNA damage response (DDR) before I discuss the role of the DDR with telomeres in the next section.

DNA damage can arise from both endogenous and exogenous sources. Environmental causes of DNA damage include toxins, such as arsenic, and radiation (Waterworth et al. 2011). Within cells, normal processes, for instance meiotic recombination, require DNA breaks. Cells also produce metabolites, such as reactive oxygen species, that can lead to DNA lesions or breaks. Because unrepaired DNA damage will lead to genome instability, cells have multiple overlapping repair pathways to ensure chromosome integrity is maintained over many cell divisions. The two main pathways for repair of DSBs are non-homologous end-joining (NHEJ) and homologous recombination (HR). In general, HR is the preferred method of yeast, whereas mammals and plants preferentially use NHEJ (Mahaney et al. 2009; Mladenov and Iliakis 2011). HR requires long stretches of homology, which are typically found in sister chromatids or homologous chromosomes, but can also come from the damaged

chromosome itself. Because of the homology requirement, HR is more accurate than NHEJ and is most commonly used during S and G2 phases of the cell cycle (Mahaney et al. 2009; Mladenov and Iliakis 2011). Conversely, NHEJ is more error prone. DSBs rarely occur as blunt-ended DNA and thus require processing to create ligatable ends, which may lead to loss of nucleotides (Mahaney et al. 2009).

DNA repair is a multifaceted process that requires damage recognition and signaling to recruit repair proteins to the damage. Further, cell cycle checkpoints must be activated to prevent damage from being propagated to daughter cells. Three kinases, ATM (Ataxia Telangiectasia Mutated), ATR (ATM and Rad3 Related) and DNA-PK_{CS} (DNA-dependent Phosphokinase Catalytic Subunit) govern the responses to DNA damage (Fig. 1-10). They are members of the PIKK (phosphoinositide 3-kinase-related kinases) family and all three preferentially phosphorylate serine or threonine residues that are followed by a glutamate. ATM and DNA-PK_{CS} are activated by double-strand breaks (DSB) whereas ATR is activated by single-strand breaks (SSB) (Templeton and Moorhead 2005; Hurley and Bunz 2007; Lovejoy and Cortez 2009). I will discuss these three kinases as well as several other proteins implicated in telomere biology in more detail. For simplicity, most of my discussion will focus on what is known in humans followed by an overview of the DDR in plants. In the next section on DDR and telomeres, I will go into more depth about other organisms.

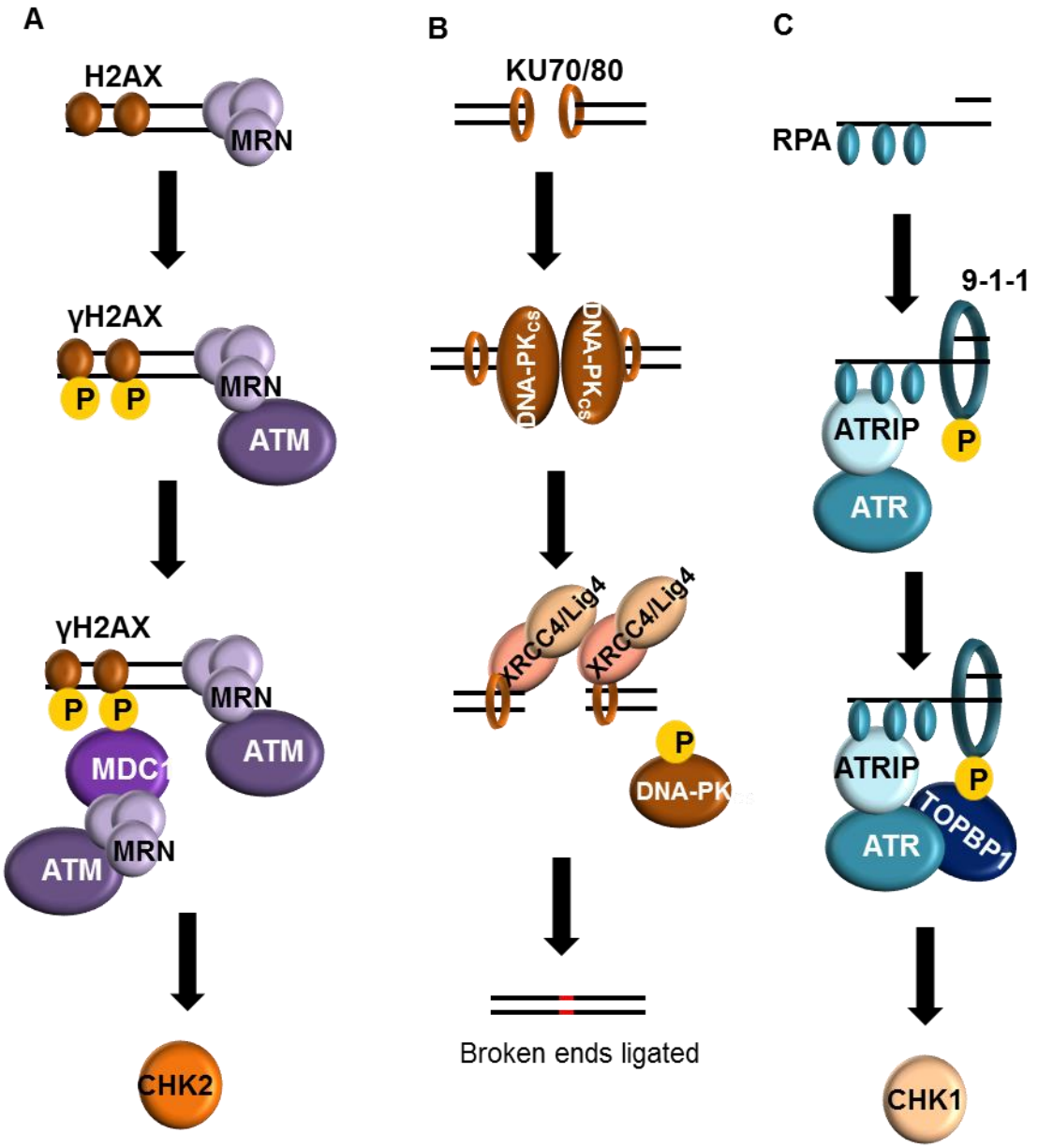

Figure 1-10. The DNA damage response. (A) ATM responds to DSBs. The MRN complex senses the DNA break (top) and recruits and activates ATM (middle), which rapidly phosphorylates H2AX. Phosphorylated H2AX recruits MDC1 (mediator of DNA damage checkpoint 1) (bottom) which amplifies the DDR by binding additional MRN and ATM complexes. ATM phosphorylates many targets, including the checkpoint protein CHK2. (B) The vertebrate non-homologous end joining pathway. KU70/80 senses the DNA break (top). Ku recruits DNA-PK_{CS} and the broken ends are brought together (middle). DNA-PK_{CS} proteins autophosphorylate each other and are replaced by XRCC4/Ligase4 which ligate the ends together. (C) ATR responds to SSBs. RPA senses the DNA damage and binds to ssDNA (top). RPA recruits the ATRIP/ATR complex as well as the 9-1-1 checkpoint clamp (middle). ATR is activated after TOPBP1 (topoisomerase binding protein 1) binding. ATR targets include the checkpoint kinase CHK1.

ATM

ATM was originally identified as the source of mutations leading to the rare genetic disease ataxia-telangiectasia, which is a neurodegenerative disease with additional symptoms of genome instability, sensitivity to radiation and increased susceptibility to cancer (Savitsky et al. 1995). *ATM*, unlike the related *ATR* gene, is a nonessential gene; mouse knockout lines are viable (Hurley and Bunz 2007).

ATM was found to be important in p53-mediated cell cycle checkpoint activation and induction of apoptosis after exposure to ionizing radiation (Banin et al. 1998; Canman et al. 1998; Herzog et al. 1998). ATM activates p53 by phosphorylation, which leads to G1-S checkpoint activation (Banin et al. 1998; Canman et al. 1998). ATM can phosphorylate p53 directly, but also phosphorylates a number of other targets that then either phosphorylate p53 or induce expression of p53 (Shiloh and Ziv 2013). ATM also activates intra-S and G2-M checkpoints by phosphorylating the checkpoint kinase Chk2 (Matsuoka et al. 1998). Another checkpoint effector complex, 38MAPK/MAPKAP-K2, is activated by ATM and ATR (discussed below), and is important for p53-deficient cell survival after DNA damage (Manke et al. 2005; Reinhardt et al. 2007).

Activation of ATM after DSB formation is not completely understood but may require a change in chromatin structure (Reinhardt and Yaffe 2009; Shiloh and Ziv 2013). Optimal activation of ATM requires the MRE11-RAD50-NBS1

(MRN) complex, which is one of the first complexes recruited to DSBs (Reinhardt and Yaffe 2009; Shiloh and Ziv 2013). ATM binds to NBS1 and phosphorylates itself and the variant histone H2AX (γH2AX) (Fig. 1-10A). γH2AX then serves as an anchor for recruitment of other signal transducers and repair proteins. ATM signaling also creates an autoamplification loop where ATM phosphorylation leads to additional ATM recruitment and stability. For example, ATM-and CK-mediated phosphorylation of MDC1 (Mediator of DNA Damage Checkpoint 1), which can bind γH2AX and NBS1, stabilizes the MRN complex and results in increased phosphorylation of H2AX and more stable association of ATM at the DSB (Reinhardt and Yaffe 2009) (Fig. 1-10A). The multiple signals and posttranslational modifications finally recruit repair proteins, including 53BP1 (p53-Binding Protein 1) and BRCA1 (Breast cancer type 1 susceptibility protein), which are important for both NHEJ and HR (Reinhardt and Yaffe 2009).

ATM signaling also occurs outside of its canonical role in DSB repair. ATM can be activated by SSB and replication fork stalling (Cimprich and Cortez 2008; Reinhardt and Yaffe 2009). It is unclear if this ATM activation is always ATR-dependent and if it only responds to DSBs that form during the repair of SSBs (i.e. resection of the SSBs or DSBs that form after replication fork collapse (Cimprich and Cortez 2008; Reinhardt and Yaffe 2009). In addition to DDR signaling, ATM has been implicated in insulin signaling and regulation of oxidative stress (Shiloh and Ziv 2013).

DNA-PK$_{CS}$

DNA-PK$_{CS}$ has only been identified in vertebrates and *Dictyostelium discoideum* (Hudson et al. 2005). In vertebrates, DNA-PK$_{CS}$ is required for the canonical NHEJ pathway (Fig. 1-10B) and also mediates V(D)J recombination (Hill and Lee 2010). Mutations in DNA-PK$_{CS}$ are associated with the disease SCID (severe combined immunodeficiency) in humans and other animals (van der Burg et al. 2009).

DNA-PK$_{CS}$ function in NHEJ requires the protein Ku, and together they form the DNA-PK holoenzyme (Mahaney et al. 2009; Mladenov and Iliakis 2011). NHEJ is initated by Ku localization to chromosome ends and subsequent translocation inwards on the chromosome. Ku recruits DNA-PK$_{CS}$ to the DNA break; DNA-PK$_{CS}$ interacts with the extreme C-terminus of Ku80 (Fig. 1-10B). The two DNA-PK$_{CS}$ molecules on the two broken chromosome ends then interact, tethering the two ends together and activating the kinase activity of DNA-PK$_{CS}$ (Mahaney et al. 2009). Although DNA-PK$_{CS}$ can phosphorylate a number of NHEJ substrates *in vitro*, the only phosphorylation event essential for NHEJ *in vivo* is autophosphorylation (Fig. 1-10B). Structural analysis of the DNA-PK holoenzyme bound to DNA has shown autophosphorylation in *trans* across the DSB induces a conformational change in DNA-PK$_{CS}$, which causes it to release from Ku (Dobbs et al. 2010). The release of DNA-PK$_{CS}$ is required for NHEJ and is thought to allow access for processing and ligation of the

chromosome ends. However, the timing and function of DNA-PK_{CS} release is not clear (Mahaney et al. 2009).

DNA-PK_{CS} may be involved in recruiting and/or activating various DNA processing enzymes to DSBs. XRCC4 (X-ray cross complementation group 4 protein), part of the XRCC4-DNA ligase IV complex that is required for ligation of DNA in NHEJ, interacts with and is phosphorylated by DNA-$PK_{CS.}$ Two of the enzymes which can process DNA ends prior to ligation, Artemis and Werner syndrome protein (WRN), are also targets of DNA-PK_{CS} (Hill and Lee 2010). However, since these phosphorylation events are not required for NHEJ, the actual functions of the phosphorylations are unclear. ATM can target many of the same proteins as DNA-PK_{CS} (Mahaney et al. 2009). DNA-PK_{CS} also phosphorylates p53. In the absence of DNA-$PK_{CS,}$ the p53-mediated apoptosis in response to severe DNA damage is limited but p53-mediated checkpoints are unaffected.

Ku

Ku is a heterodimeric protein complex composed of 70kDa and 80kDa subunits. Ku was first identified as a target of antibodies produced in humans with the autoimmune disease scleroderma-polymyositis overlap syndrome (Mimori et al. 1981). It was then purified from HeLa cells and the complex was found to bind DNA (Mimori et al. 1986). The crystal structure of Ku showed that Ku70 and Ku80 form a ring that encircles dsDNA termini (Walker et al. 2001).

Ku DNA-binding is sequence independent; Ku contacts the sugar-phosphate backbone and not the DNA bases (Walker et al. 2001). Ku can also bind RNA (Peterson et al. 2001; Zhang et al. 2004; Adelmant et al. 2012), and may require a hairpin structure for binding (Dalby et al. 2013). Current evidence suggests that Ku cannot simultaneously bind RNA and DNA (Adelmant et al. 2012; Pfingsten et al. 2012). In the absence of DNA damage, Ku mainly resides in the nucleolus and probably associates mostly with RNAs (Adelmant et al. 2012). In cells that have been UV-treated or that have had sheared DNA injected, Ku moves to the nucleoplasm and mainly interacts with DNA (Adelmant et al. 2012).

In DNA repair, Ku detects DSB and Ku binding to the DNA is the required first step in NHEJ (Fig. 1-10B). As mentioned above, Ku binds and activates DNA-PK_{CS}, and Ku also interacts with and recruits the other essential component of NHEJ, the XRCC4-DNA ligase IV complex (Mahaney et al. 2009).

ATR

ATR is an essential gene in vertebrates (Brown and Baltimore 2000; Cortez et al. 2001). Hypomorphic mutations in ATR cause the rare disease Seckel syndrome, the symptoms of which include growth delays, dwarfism, and mental retardation (O'Driscoll et al. 2003). Unlike ATM, ATR is activated in each S-phase to ensure proper DNA replication (Cimprich and Cortez 2008).

ATR activation requires its localization to sites of DNA damage (Fig. 1-10C). ssDNA is bound by RPA (Replication Protein A), which in turn is bound by

ATRIP (ATR-Interacting Protein). ATR binds ATRIP to localize to ssDNA (Cortez et al. 2001; Zou and Elledge 2003). ssDNA bound by RPA is also required to recruit the 9-1-1 complex (RAD9-RAD1-HUS1) to DNA ends (Cimprich and Cortez 2008). The 9-1-1 complex is related to PCNA , the replicative sliding clamp, and like PCNA forms a ring-structure that loads onto the DNA ends. Finally, the 9-1-1 complex recruits TOPBP1 (Topoisomerase Binding Protein 1) to the DNA to activate ATR (Zou et al. 2002; Kumagai et al. 2006) (Fig. 1-10C).

After activation by TOPBP1, ATR phosphorylates target proteins to initiate cell cycle checkpoints and repair reactions. The best characterized target of ATR is CHK1 (Checkpoint kinase 1). CHK1 causes a cell cycle arrest by phosphorylating CDC25 phosphatase to inactivate it. CDC25 inactivation then prevents activation of CDKs which are required for cell cycle progression into mitosis.

ATR also phosphorylates a number of replication fork proteins, including the MCM2-7 helicase, RPA, and DNA polymerases. The functions of most of these phosphorylation events are unknown, but probably serve to slow down replication and activate dormant replication origin firing (Cimprich and Cortez 2008). ATR also phosphorylates several proteins involved in recombination, including BRCA1, Werner helicase (WRN), and Bloom syndrome protein (BLM). Although the mechanism is unknown, WRN and ATR are thought to work together to suppress DNA fragile sites (Friedel et al. 2009).

Poly(ADP-ribose) polymerases

PARPs (Poly(ADP-ribose) polymerases) function in post-translational modification of proteins. PARPs catalyze the formation of ADP-ribose and nicotinamide from NAD^+, and then transfer the ADP-ribose to target proteins. The transfer of multiple ADP-ribose molecules forms chains of negatively charged poly (ADP-ribose) (PARs). PARs can alter the function of proteins in several ways (Gibson and Kraus 2012). Parylation adds a negative charge to proteins and can cause the protein to dissociate from binding partners, particularly DNA. PARs can also act as a scaffold for protein complex formation by recruiting PAR binding proteins. Finally, PARs can also mark proteins for destruction by recruiting PAR-binding E3 ubiquitin ligases (Gibson and Kraus 2012). PARs are removed from proteins by PARGs (poly(ADP-ribose) glycohydrolases).

PARPs were first identified for their role in DNA repair, but increasing evidence implicates PARPs in many important cellular processes, including mitosis, regulating chromatin state, and transcription (Bai and Canto 2012; Gibson and Kraus 2012; Oka et al. 2012). In vertebrates, tankyrases are PARPs initially identified to interact with and regulate TRF1 function at telomeres (Smith et al. 1998). Various types of stress, including genotoxic, heat, metabolic and oxidative, activate PARPs (Bai and Canto 2012; Gibson and Kraus 2012; Oka et al. 2012). PARPs can mediate cell survival in multiple ways. First, because PARPs use NAD^+ as a substrate, high levels of PARP activity can

rapidly deplete ATP, causing an energy crisis which leads to necrotic cell death (Bai and Canto 2012; Oka et al. 2012). Additionally, overabundance of free PARs induces caspase-independent cell death. The PARs exit the nucleus and enter the mitochondria, which in turn releases AIF (apoptosis-inducing factor). AIF enters the nucleus and causes DNA fragmentation (Bai and Canto 2012; Oka et al. 2012). Thus, low PARP activity promotes cellular survival through resolution of cellular stress, but high levels of PARP activity are an indication that stress levels have overwhelmed the cell.

Three PARPs, PARP1, PARP2, and PARP3 are the only PARPs of the seventeen human PARPs identified as DNA damage-dependent. PARPs function mainly in SSB repair, but, in conjunction with the MRN complex, are also central to an alternative NEHJ pathway for DSB repair (Mladenov and Iliakis 2011). One of the best studied SSB repair pathways with PARP-involvement is Base Excision Repair (BER) (Oka et al. 2012). When DNA has a damaged base, the base is removed by DNA glycosylase, and apurinic/apyrimidinic endonuclease cleaves the DNA backbone 5' of the missing base. The resulting DNA nick is recognized as a SSB by PARP1. PARP1 is activated by its association with DNA and PARylates itself. The PARylated PARP1 then recruits PAR-binding XRCC1 (X-ray repair cross-complementing 1), which recruits DNA polymerase β and DNA ligase III, which repair the lesion (De Vos et al. 2012; Oka et al. 2012).

PARPs in *Arabidopsis* telomere biology are the subject of Chapter 5.

DNA damage responses in plants

Many components of the mammalian DDR are conserved in plants, but less is known about the details of the plant DDR. Certain key proteins, such as DNA-PK_{CS} and p53, are absent. Additionally, few signaling components downstream of ATR and ATM are known. One important feature that differentiates plants from animals is their high tolerance to genome instability. This tolerance may arise from the maintenance of undifferentiated stem cell niches throughout the plant life cycle. DNA damage in somatic tissue may not have as large an impact on the survival and function of the plant because they can compensate by initiating new growth and tissue differentiation. In gamma radiation-treated plants, for example, cell cycle arrest is induced in meristems,but not in somatic cells (Hefner et al. 2006).

ATR and ATM are conserved in plants and respond primarily to ssDNA damage and DSB, respectively (Garcia et al. 2003; Culligan et al. 2004). Both *atm* and *atr* mutants are viable in *Arabidopsis*, providing opportunities for genetic analysis not possible in animal systems (Garcia et al. 2003; Culligan et al. 2004). Similarly to vertebrates, ATM interacts with DSB through the MRN complex (Bundock and Hooykaas 2002; Bleuyard et al. 2004; Heacock et al. 2004; Puizina et al. 2004; Akutsu et al. 2007), and ATR associates with SSB through ATRIP and RPA (Sweeney et al. 2009). Although p53 is absent in *Arabidopsis*, a plant-specific transcription factor, SOG1 (Suppressor of Gamma Response 1), mediates most of the transcriptional responses generated by ATM or ATR

signaling (Yoshiyama et al. 2009). After induction of DNA damage by gamma radiation, ATM, but not ATR, is required for the altered transcription of hundreds of genes (Culligan et al. 2006), and SOG1 is also required for the response (Yoshiyama et al. 2009). SOG1 activity is not restricted to ATM-mediated signaling. In plants lacking the repair endonuclease XPF, gamma radiation causes a temporary arrest in cell division in the apical meristem that results in a delay in development of about 8 days. This arrest requires both ATR and SOG1 (Yoshiyama et al. 2009).

Additional evidence suggests that plant ATR and ATM function in both separate and overlapping pathways. For example, both *atr* and *atm* mutants are sensitive to ionizing radiation, but only ATM is required for the immediate transcriptional response (Culligan et al. 2006). Conversely, the initial changes in expression induced by ATM require ATR for long term maintenance (Culligan et al. 2006). Like vertebrates, *Arabidopsis* phosphorylates H2AX in response to DSBs. This response is primarily governed by ATM, but about 10% of phosphorylation events are ATR dependent (Friesner et al. 2005). The formation of γH2AX foci by either ATR or ATM in response to ionizing radiation requires the MRN complex, suggesting that MRN can activate either ATM or ATR (Amiard et al. 2010). In MRN mutants, γH2AX foci form spontaneously without exogenous DNA damage in an ATR dependent manner (Amiard et al. 2010). This finding indicates that ATM activation requires MRN, whereas ATR activation can occur independently from MRN.

Like their mammalian counterparts, plants can activate cell cycle checkpoints or programmed cell death in response to DNA damage. The WEE1 kinase is currently the only known checkpoint activator in *Arabidopsis (De Schutter et al. 2007).* WEE1 expression is induced by ATR in response to replication stress and by ATM in response to DSBs. WEE1 phosphorylates CDKA;1 (Cyclin Dependent Kinase A;1) which inactivates it and prevents progression of the cell cycle into M phase (De Schutter et al. 2007). In mammals the CDC25 phosphatase counters the activity of the kinase to reactivate CDKs. However, in *Arabidopsis* the CDC25 ortholog does not affect cell cycle (Dissmeyer et al. 2009). Thus, many details of this G2/M checkpoint still need to be identified.

Both ATR and ATM have also been implicated in activation of programmed cell death (PCD) in stem cell niches in response to DNA damage (Fulcher and Sablowski 2009; Furukawa et al. 2010). Because plant stem cells remain active throughout the life of the plant, it is thought that PCD helps preserve genome integrity and plant survival by targeting cells with unrepaired DNA damage. Unlike mammals, which activate apoptosis through p53, PCD in plants is autolytic (Fulcher and Sablowski 2009). Either ATM or ATR can activate PCD in response to either replication stress or ionizing replication, but SOG1 is required for PCD in response to either type of genotoxic stress (Furukawa et al. 2010).

In Chapter III, I describe the role of ATR-mediated PCD in the maintenance of genome integrity in CST mutants.

DNA damage response at dysfunctional telomeres

If one of the functions of telomeres is to differentiate chromosome ends from DSBs, then loss or misregulation of end-protection proteins, disruption of telomere higher order structure, or critically short telomeres would be expected to activate a DDR. This is indeed the case. Appreciation of cellular responses to telomere dysfunction is necessary for understanding how normal telomeres function.

The connection between the DDR and telomeres was made early in the study of telomeres in budding yeast when Tel1 was identified in a screen for mutations leading to short telomeres (Lustig and Petes 1986). However, Tel1 was not identified as the yeast homolog of ATM until almost a decade later (Greenwell et al. 1995).

The inhibition of a DDR is similar in yeast and vertebrates. In yeast, in telomerase deficient cells, short telomeres activate a DDR characterized by phosphorylation of the Rad53 checkpoint kinase (homolog of vertebrate CHK2), increased expression of *RNR3*, a subunit of ribonucleotide reductase that is upregulated in response to DNA damage, and activation of the G2/M checkpoint. The DDR is dependent on Mec1 (yeast ATR) (Ijpma and Greider 2003). The telomeric accumulation of Mec1, but not Tel1 is limited by Cdc13 capping, a

finding that is similar to POT1 inhibition of ATR at vertebrate telomeres (Hirano and Sugimoto 2007) (Fig. 1-6). Another study found that Mec1 associates with dysfunctional, short telomeres in cells that were beginning to senesce due to either telomerase or Ku deficiency (Ku will be discussed in the next section). Tel1 association with short telomeres is limited to functional telomeres. This study also showed that Exonuclease I, which degrades DNA in a 5' to 3' direction, is required for Mec1 telomeric association. Thus, accumulation of ssDNA caused by ExoI action at dysfunctional telomeres enhances Mec1 localization to telomeres (Hector et al. 2012). Interestingly, the absence of Cdc13 in cells arrested in G1 does not alter cell viability or induce formation of single-stranded telomeric DNA when the block was removed. If Cdc13 is eliminated in G2/M arrested cells, however, the cells remain arrested in G2/M and C-strand telomeres are degraded, forming more G-strand ssDNA. If the S-phase CyclinB/CDK is inhibited, very little ssDNA formed in the G2 arrested cells, suggesting that DNA processing due to telomere dysfunction requires completion of S-phase and CDK-dependent activation of nucleases (Vodenicharov and Wellinger 2006). This restriction of the initiation of DNA repair activity until after replication is complete may be one way that telomeres are prevented from entering into repair reactions when they are uncapped in S-phase to allow telomerase access.

Double-stranded telomere-associated proteins also inhibit a DDR in yeast (Fig. 1-6). Rif1 and Rif2 prevent Tel1 localization to telomeres, but the MRX

complex (yeast MRN: Mre11-Rad50-Xrs2) can still associate with telomeres (Hirano et al. 2009; McGee et al. 2010). Rif2 then competes with Tel1 for binding with Xrs2. Once Tel1 has been displaced, MRX no longer associates with telomeres (Hirano et al. 2009). This mechanism is consistent with the counting model for telomere length regulation; more Rif1 and Rif2 would indicate that telomeres are long and no DDR is needed at the telomeres.

In vertebrates, shelterin proteins prevent a DDR (Takai et al. 2003; Denchi and de Lange 2007; Buscemi et al. 2009; Bombarde et al. 2010; Gong and de Lange 2010; Sfeir and de Lange 2012) (Fig. 1-11). When telomeres are dysfunctional and a DDR has been initiated, several proteins rapidly accumulate at the telomeres, including 53BP1 and γH2AX. Immunolocalization of 53BP1 and γH2AX reveals distinct foci, termed telomere dysfunction-induced foci (TIF), that localize to telomeres (Takai et al. 2003). Both the dsDNA-associated proteins and ssDNA-associated proteins prevent TIF formation.

Of the shelterin proteins, TRF2's action to inhibit a telomeric DDR is probably the most thoroughly investigated (Takai et al. 2003; Denchi and de Lange 2007; Buscemi et al. 2009; Bombarde et al. 2010; Sfeir and de Lange 2012; Jullien et al. 2013). Cellular deficiency of TRF2 leads to formation of TIFs, Mre11 and ATM localization to the telomeres and phosphorylation of Chk2 (Takai et al. 2003; Denchi and de Lange 2007). When TRF2 was deleted in *atm*

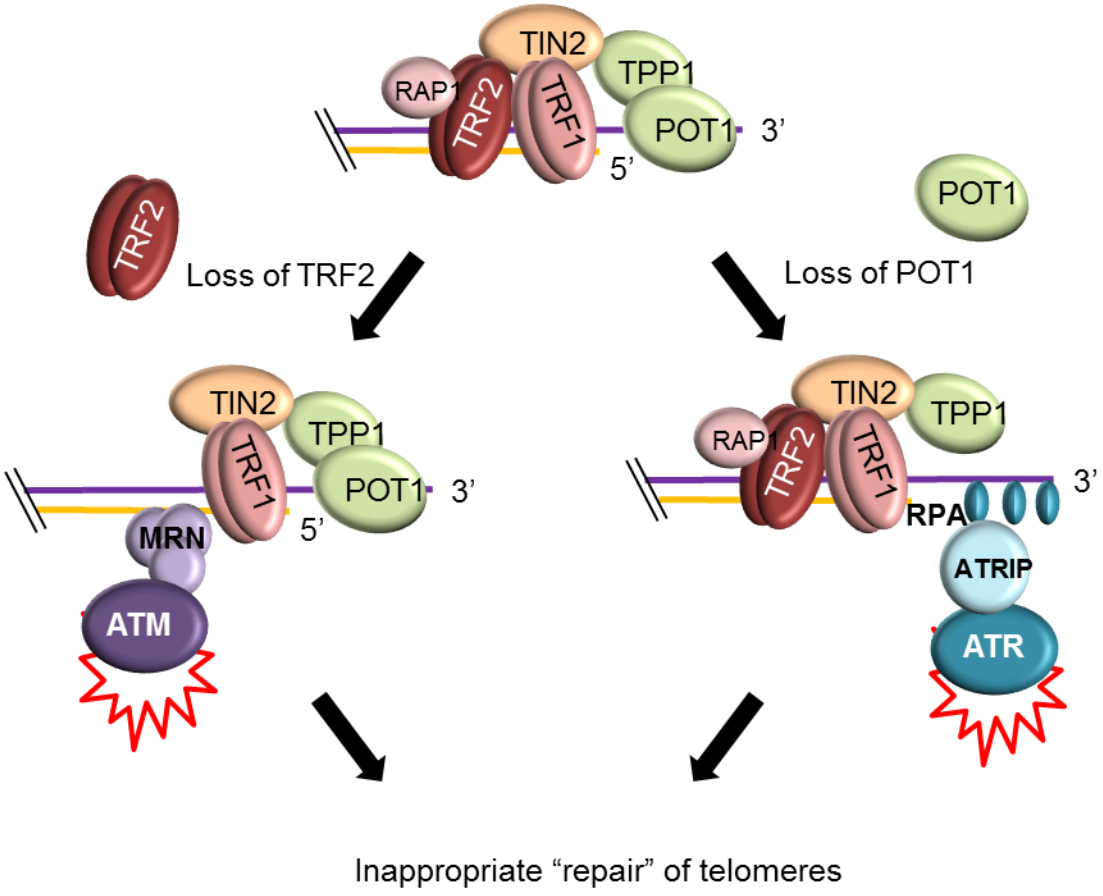

Figure 1-11. DNA damage response at telomeres. The figure depicts the human pathways and proteins. Loss of TRF2 from the double-stranded region activates ATM. Loss of POT1 from the G-overhang activates ATR. The resulting repair reactions cause end-to-end fusions and genome instability. See text for details.

cells, TIFs were not formed and Chk2 was not phosphorylated; deletion of TRF2 in conjunction with ATR knockdown the response was similar to deletion of TRF2 alone (Denchi and de Lange 2007). These observations suggest that ATM, but not ATR, is the primary kinase that responds to TRF2 deficiency, and are also consistent with the expectation that the kinase that responds to DSBs would respond to the loss of a dsDNA-associated protein (Fig. 1-11).

TRF2 does not inhibit the DDR at telomeres simply by hiding telomeric DNA from the repair machinery. TRF2 can bind and inhibit ATM directly by blocking an autophosphorylation site (Karlseder et al. 2004). TRF2 further handicaps the ATM response at telomeres by binding Chk2, which prevents ATM from phosphorylating and activating Chk2 (Buscemi et al. 2009). TRF2 may also prevent telomeric fusions by recruiting the shelterin bridging protein RAP1 to telomeres. When RAP1 was tethered to telomeres in the absence of TRF2, fusions were reduced by 10-fold even though ATM was still activated (Sarthy et al. 2009). Thus, TRF2 protects telomeres both by inhibiting the DDR and by blocking NHEJ.

Because ATM responds to loss of TRF2, the dsDNA-associated shelterin protein, one would expect that ATR would respond to loss of the ssDNA-associated telomeric protein. As predicted, in chicken cells, depletion of Pot1 results in phosphorylation and activation of Chk1 in an ATM-independent manner (Churikov et al. 2006). In both mouse and human cells, Pot1a/b or Pot1 depletion caused TIF formation and Chk1 and Chk2 phosphorylation. The

responses required ATR (Denchi and de Lange 2007) (Fig. 1-11). Because ATR requires RPA for localization to DNA, the researchers hypothesized that RPA levels would increase at telomeres in the absence of Pot1a (Gong and de Lange 2010). Loss of Pot1a specifically activates the ATR pathway and induces TIF formation. Knockdown of RPA subunits RPA32 or RPA70 reduced the incidence of TIFs and degradation of Pot1a leads to RPA colocalization with 53BP1 at telomeres (Gong and de Lange 2010). Thus Pot1 inhibits a DDR by preventing telomeric accumulation of RPA, which, in turn, keeps ATR away from telomeres.

Recently, researchers were able to conditionally remove shelterin from telomeres in mouse cells in order to unambiguously identify which pathways were activated by unprotected telomeres (Sfeir and de Lange 2012). Three pathways are activated if all repair pathways are intact in the cells: ATR signaling, ATM signaling, and canonical NHEJ. As previously reported (Denchi and de Lange 2007), Pot1 inhibits ATR signaling, TRF2 inhibits ATM signaling, and both prevent NHEJ. In the absence of NHEJ component Ku70/80, Rap1 and Pot1 inhibit both homology driven repair and alternative NHEJ. Finally, in the absence of 53BP1, which is required for both ATR and ATM signaling, ends are joined through a process requiring 5' end resection.

Research into role of the DDR at dysfunctional *Arabidopsis* telomeres has been hindered because end protection proteins were only recently identified and the plant DDR has been less extensively studied compared to vertebrates and

yeast. However, a few studies have explored the origin and mechanism of end-to-end chromosome fusions in plants with telomere dysfunction (Heacock et al. 2004; Heacock et al. 2007). In *Arabidopsis*, telomeres are recruited into end-to-end fusions when the shortest telomere in the population reaches about 1kb (wild type telomeres in the Col-0 ecotype, the most commonly used for laboratory studies, are 2-5kb) (Heacock et al. 2007) indicating that there is a length threshold. When telomeres become too short for proper capping (i.e. <1kb), the chance that a DDR will be triggered is increased. Although fusions start to form when telomeres are 1kb, telomeres can continue to shorten until they reach 300-400 bp (Heacock et al. 2004).

Arabidopsis likely uses multiple mechanisms for end joining in response to short telomeres (Heacock et al. 2004). In *tert* mutants, plants deficient in NHEJ proteins KU70, MRE11, or DNA Ligase IV still have telomere fusions, indicating that there are pathways other than the canonical NHEJ pathway for joining telomeres in *Arabidopsis*. There are likely multiple mechanisms because certain mutants, such as *tert ku70* double mutants versus *tert ku70 mre11* mutants, were more likely to show evidence of microhomology-mediated end joining. Analysis of the fusion junctions also showed that different amounts of nucleolytic degradation occurred prior to end joining in the different genetic backgrounds, providing additional evidence for multiple pathways (Heacock et al. 2004; Heacock et al. 2007). The evidence for multiple pathways in *Arabidopsis* is consistent with the six pathways identified in vertebrates (Sfeir

and de Lange 2012). Although analysis of fusions junctions does not give direct evidence for DDR activation in response to dysfunctional telomeres, the detection of nucleolytic degradation prior to fusion of chromosome ends suggests that a DDR is activated.

In Chapter III, I provide evidence that loss of CTC1 initiates a DDR, suggesting that like other organisms, *Arabidopsis* capping proteins prevent activation of a DDR at telomeres.

Functions of DNA repair proteins in telomere maintenance

Although one of the functions of telomeres is to prevent a DDR at chromosome ends, paradoxically, multiple DDR proteins are also required for normal maintenance of telomeres. In this section I will discuss how DNA damage related proteins function to maintain telomeres in vertebrates, yeast, and plants.

ATR and ATM

Studies from yeast, vertebrates and plants have implicated both ATR and ATM in telomere maintenance. In budding and fission yeast, ATR and ATM regulate telomerase recruitment to the telomeres. Tseng et al. reported in 2006 that Mec1 (ATR) and Tel1 (ATM) phosphorylate Cdc13, which then recruits telomerase through its interaction with telomerase accessory protein Est1 (Tseng et al. 2006). Another group of researchers have challenged this finding

(Gao et al. 2010). When Gao et al. mutated all 11 putative Tel1 phosphorylation sites, telomere length was nearly wild type, suggesting that Tel1-mediated phosphorylation is not needed to recruit telomerase to telomeres (Gao et al. 2010). In *S. pombe*, the evidence for Tel1 and Rad3 (ATR) mediated telomerase recruitment is clearer (Moser et al. 2009b; Moser et al. 2011). In the absence of Tel1 and Rad3, telomerase is not recruited for two reasons. First, the accumulation of shelterin components Tpz1 and Ccq1 are diminished in *tel1 rad3* mutants. Additionally, the contact between the Tpz1/Ccq1 proteins and telomerase was lost, resulting in telomere shortening and circularization of chromosomes (Moser et al. 2009b). Tel1 or Rad3 phosphorylation of Ccq1 was essential for the interaction between Ccq1 and telomerase component Est1 and was thus essential for telomerase recruitment (Moser et al. 2011; Yamazaki et al. 2012).

Although its role in budding yeast telomerase recruitment is controversial, multiple studies have shown that Tel1 is required for the preferential elongation of short telomeres by telomerase (Arneric and Lingner 2007; Bianchi and Shore 2007; Sabourin et al. 2007). At critically short telomeres, Tel1 stimulates telomerase repeat addition processivity (Chang et al. 2007). This is consistent with the model discussed above where Tel1 accumulates at shorter telomeres because at longer telomeres more Rif1 and Rif2 are present to weaken the Tel1 association with the telomere.

ATR may also be required to ensure the hard-to-replicate duplex regions of the telomere are efficiently duplicated by semi-conservative replication. In cells from humans with Seckel syndrome that have a hypomorphic ATR mutation or in cells depleted of ATR, telomere instability arises during or right after replication and results in sister chromatid fusions and chromatid-type aberrations like telomere deletions or multiple telomere signals on one chromosome arm (Pennarun et al. 2010). Additionally, when ATR-deficient cells are treated with a G4 ligand that stabilizes G-quadrupexes, a significantly higher number of chromatid-type defects are found in the lagging strand telomeres versus leading strand telomeres (Pennarun et al. 2010). The more severe outcome in the lagging strand telomeres in the absence of ATR is consistent with a role for ATR in replicating the G-rich lagging strand telomeres. A mouse model of Seckel syndrome shows a similar phenotype of telomere fusions and the formation of fragile sites resulting from replication fork stalling in the telomeres (McNees et al. 2010).

ATR and ATM are also required for telomere length maintenance. Mutants for either gene in budding yeast or fission yeast have continually shortening telomeres that result in senescence (Ritchie et al. 1999; Moser et al. 2009b). In *Arabidopsis*, *atr* or *atm* mutants have normal telomeres (Vespa et al. 2005). In the double *atr atm* mutant telomere fusions occur at a low rate, suggesting that telomeres are not completely protected when both kinases are absent. When *atr* or *atm* mutations were combined with a *tert* mutation, the

telomere dysfunction induced by telomerase deficiency was accelerated. Onset of genome instability occurred abruptly in *atm tert* mutants compared to the gradual increase seen in *tert* single mutants. However, telomere length decreased at the same rate as *tert* single mutants (Vespa et al. 2005). When individual chromosome arms were monitored in *atm tert* mutants, the presence of only a single critically short telomere, caused by a telomere deletional recombination, would activate chromosome fusions and developmental defects. Comparison of telomeres in *atm tert* with *tert* single mutants also revealed that homologous chromosome ends had more similar lengths when ATM was present, suggesting that ATM protects telomeres from stochastic telomere loss from recombinational deletion events (Vespa et al. 2007).

Unlike ATM, ATR deficiency affects *Arabidopsis* telomeres immediately. In *atr tert* mutants, telomeres shorten at an accelerated pace compared to *tert* single mutants, and onset of genome instability and developmental defects start in G3 instead of G7 for *tert* mutants (Vespa et al. 2005). These studies suggest that ATR plays an important role in telomere length regulation, while ATM is more important for end protection.

In Chapter III I examine the function of ATR and ATM in CST mutants and Appendix C contains my preliminary analysis on the role of ATR in telomerase activity regulation.

Ku

Like ATR and ATM, Ku is implicated several facets of telomere biology including, telomerase recruitment, telomere length regulation, and chromosome end protection. The telomeric functions of Ku are best studied in budding yeast, which was the first organism for which a telomeric role for Ku was identified (Porter et al. 1996). *ku* mutants have shorter telomeres (Porter et al. 1996) and a persistent increase in the amount of ss G-rich telomeric sequence, which forms in a cell cycle-independent manner. This process is confined to S phase in wild type cells (Gravel et al. 1998; Polotnianka et al. 1998). Ku also localizes to telomeres, which suggests it is important for maintaining the structure of the chromosome end (Gravel and Wellinger 2002). Genetic analysis of cells lacking Ku and either Est2 (reverse transcriptase component of telomerase) or Cdc13 revealed that in both genetic backgrounds, *ku* mutation exacerbates the phenotypes of the single mutants (Nugent et al. 1998), suggesting that Ku acts in a pathway separate from both Cdc13 and telomerase for maintenance of telomeres. Additionally, the generation of the G-tails in *ku* mutants is Exo I dependent, providing evidence that Ku protects the C-strand from nuclease attack (Bertuch and Lundblad 2004). This conclusion is further supported by a study in G1 arrested cells showing that Ku but not Cdc13 is important for capping and protecting telomeres from resection by ExoI (Vodenicharov et al. 2010). Finally, analysis of a *ku* mutant defective in DNA binding, but not RNA binding, showed defects in end protection and telomere length maintenance

similar to a null *ku* mutant (Lopez et al. 2011). In a mutant that retained DNA binding, but not RNA binding, the end protection function of Ku remained intact (Stellwagen et al. 2003). These studies point to a model where Ku is a required chromosome end protection factor in budding yeast.

As mentioned above, Ku can also bind to RNA stem-loop structures, including TLC1, the yeast telomerase RNA (Peterson et al. 2001). If Ku binding to TLC1 is disrupted, telomeres shorten and telomerase addition of DNA to broken chromosomes (*de novo* telomere formation) is greatly diminished (Stellwagen et al. 2003). This result points to a function for Ku in either promoting telomerase activity or telomerase recruitment. The current evidence points towards a recruitment function for Ku. During G1 phase of the cell cycle, no Est2 (Tert) is present at telomeres in *ku* mutants, and during S phase, less telomerase is present compared to wild type (Fisher et al. 2004). Ku telomerase recruitment is independent of Est1/Cdc13 telomerase recruitment and is not required for telomere maintenance (Chan et al. 2008). Ku therefore has two important functions in yeast: end protection and telomerase recruitment. The mechanism for how Ku coordinates between those functions is not clear because it has been recently shown that Ku cannot bind DNA and RNA simultaneously, so the same Ku molecule cannot be involved in both telomere protection and telomerase recruitment at the same time (Pfingsten et al. 2012).

Ku clearly functions in end protection in mammals. Chromosome ends in mouse cells lacking Ku exhibit telomeric fusions (Bailey et al. 1999; Samper et

al. 2000; d'Adda di Fagagna et al. 2001). Additionally, human cells with one Ku86 allele inactivated or with conditional deletion of Ku86 have fusions and genomic instability (Myung et al. 2004; Wang et al. 2009).

Whether Ku plays a role in regulating telomere length in mammals is unclear. Telomeres have been reported to be both longer (Samper et al. 2000) and shorter (d'Adda di Fagagna et al. 2001) in *ku* mutants. In contrast with yeast, there is no deregulation of G-strand length (Samper et al. 2000), which may be because mice, unlike yeast, maintain G-overhangs throughout the cell cycle. A role for Ku in telomere length regulation is much clearer in human cells. The conditional knockout of Ku86 causes complete loss of over half of the telomere signals in human cells analyzed by FISH. Telomere extrachromosomal circles also form (Wang et al. 2009). The rapid telomere loss is suggestive of Telomere Rapid Deletion (TRD) and implies that Ku suppresses HR. Ku's function at human telomeres may be in part mediated by interactions with both TRF1 and TRF2 (Hsu et al. 2000; Song et al. 2000). In Ku-depleted cells, overall levels of TRF2 decrease in cells because of proteasomal destruction, resulting in less TRF2 bound to DNA (Fink et al. 2010). Given the importance of TRF2 in chromosome end protection and suppression of T-loop recombination, this interaction may point to one of the mechanisms of Ku in end protection in humans.

In *Arabidopsis,* Ku is a negative regulator of telomere length, a phenotype that is opposite that found in yeast and human cells (Riha et al. 2002).

Telomeres increase in length with each generation in a telomerase-dependent manner (Riha and Shippen 2003b). Also unlike mammals, telomeres in *ku* mutants do not form fusions (Riha et al. 2002), although there is an increase in extrachromosomal telomeric circles (Zellinger et al. 2007), indicating that Ku represses recombination at *Arabidopsis* telomeres There are a few similarities between plant Ku function and yeast Ku function. *ku* mutants in *Arabidopsis* have increased formation of G-overhangs. In addition, *tert* mutants deficient in Ku display accelerated telomere shortening. Like in yeast, these results suggest a role for Ku in C-strand protection and maintenance of the proper architecture of chromosome ends (Riha and Shippen 2003b). This idea is enforced by the recent discovery that about half of *Arabidopsis* telomeres are blunt-ended and require Ku to protect them from Exo I resection and subsequent HR (Kazda et al. 2012). Maintenance of blunt ends does not require STN1, suggesting that CST and Ku represent two separate end protection mechanisms in *Arabidopsis*.

Finally, as dicussed in Chapter IV, *Arabidopsis* Ku binds one of the telomerase RNA subunits, TER2, which is the inhibitory telomerase RNA in *Arabidopsis* (Cifuentes-Rojas et al. 2012). The function of this interaction is unknown.

PARPs

Because PARPs are not found in either *S. pombe* or *S. cerevisiae*, the study of PARPs in telomere biology has been confined to vertebrate systems.

Mammalian PARPs are implicated in telomere biology, functioning in telomere length regulation, chromosome end protection, and telomerase regulation. PARP proteins appear to mediate their telomere functions primarily via interactions with the shelterin components TRF1 and TRF2. Tankyrase1 (TRF1-interacting, ankyrin-related ADP- ribose polymerase) was discovered as an interaction partner of TRF1 (Smith et al. 1998). Tankyrase1 binds TRF1 and also PARylates it, leading to the dissociation of TRF1 from telomeres (Smith et al. 1998; Smith and de Lange 1999). TRF1 is then ubiquitinated and destroyed by the proteasome (Chang et al. 2003). Because TRF1 is a negative regulator of telomere length, its removal causes telomere lengthening, probably by allowing telomerase access to the chromosome ends (Smith and de Lange 2000; Cook et al. 2002). Overexpression of TankyraseI causes telomere elongation in a telomerase-dependent manner (Cook et al. 2002). PARylation of TRF1 by Tankyrase1 is also essential in resolving sister telomere cohesion during mitosis (Dynek and Smith 2004), because it disrupts the interaction of TRF1 with cohesin subunit SA1 (Canudas et al. 2007).

A second tankyrase, Tankyrase2, shows similar localization and function as Tankyrase1 in human cells. Tankyrase2 interacts with and PARylates TRF1 *in vitro* and *in vivo* and overexpression of Tankyrase2 leads to release of TRF1 from the telomeres and telomere elongation (Kaminker et al. 2001; Cook et al. 2002). Thus, the two tankyrases function redundantly for telomere length

regulation, perhaps reflecting their importance in regulating telomere length in humans.

PARP1, PARP2, and PARP3 may also have telomeric functions. Both PARP1 and PARP2 can bind to TRF2 *in vitro* and *in vivo* in human cells and have demonstrated the ability to PARylate TRF2 *in vitro* (Dantzer et al. 2004; Gomez et al. 2006). Similarly to TRF1, PARylation of TRF2 causes it to dissociate from telomeric DNA (Dantzer et al. 2004; Gomez et al. 2006).

PARP1 may have an important role at damaged telomeres. In HeLa cells, PARP1 colocalization at the telomeres increases after treatment with DNA damaging agents (Gomez et al. 2006), and in mouse ES cells lacking telomerase, more PARP1 accumulates at critically short telomeres (Gomez et al. 2006). PARP1 may also regulate telomere length in human cells (Beneke et al. 2008). Treatment of HeLa cells with a PARP-inhibitor or knockdown of PARP1 causes telomeres to shorten about 500bp per population doubling. PARP2 siRNA shows no effect on telomere length (Beneke et al. 2008). PARP1 has been implicated in regulation of telomerase activity. Immunoprecipitation of TERT pulled down PARP1 (Cao et al. 2002), and PARP1 can bind directly to a TERT peptide *in vitro* (Pleschke et al. 2000), but the *in vivo* significance of this interaction is unknown. One group reported a decrease in telomerase activity in two different studies in multiple cell types treated with PARP inhibitors or PARP1 siRNA (Ghosh and Bhattacharyya 2005; Ghosh et al. 2007). However, multiple studies from other researchers found normal telomerase activity in either human

cells treated with PARP1 or PARP2 siRNAs or in mice deficient in PARP1 or PARP2 (d'Adda di Fagagna et al. 1999; Samper et al. 2001; Tong et al. 2001; Dantzer et al. 2004; Beneke et al. 2008).

The newest PARP to be discovered to affect telomere function is PARP3. Knockdown of *PARP3* in human cells led to detection of sister telomere fusions and sister telomere loss in mitotic spreads (Boehler et al. 2011). PARP3 interacts with Tankyrase1 and probably functions at telomeres by stimulating activation of Tankyrase1 (Boehler et al. 2011).

The telomeric proteins that interact with PARPs seem to dictate the PARP's function. The tankyrases are positive regulators of telomere length and interact with TRF1, which also regulates telomere length. PARP1 functions in end protection and interacts with TRF2, which also important for end protection.

I analyze PARP function at *Arabidopsis* telomeres in Chapter V).

Overview of dissertation

My research began with analysis of CST function in *Arabidopsis*. These studies led me to investigate the DDR in CST mutants, which then changed the course of my research to focus more on the DDR and telomeres and telomerase rather than CST specifically.

Chapter II details the genetic analysis performed with *ctc1* and *stn1* mutants. Crosses were made to mutants that shared at least one phenotype with CST mutants. For example, *tert* mutants were used to determine if

telomere shortening of CST mutants was due to the inability of telomerase to extend telomeres. *ku* crosses were used to examine whether long G-overhangs in CST mutants were formed by the same mechanism as in *ku* mutants. I found that CST functions independently of both TERT and KU for telomere length maintenance and G-overhang regulation.

Chapter III covers my analysis of the DDR in CST mutants. As expected for a capping complex, loss of *ctc1* led to an increase in DDR gene expression. I also found that ATR plays an important role in maintaining genome instability in *ctc1* mutants. *ctc1 atr* mutants had multiple phenotypes that indicated that telomere dysfunction was higher in the double mutant than *ctc1* single mutants. These phenotypes included accelerated telomere loss and increased end-to-end fusions. Additionally, programmed cell death in root meristems was decreased in the *atr ctc1* double mutants compared to *ctc1* single mutants, suggesting that ATR preserves genome integrity in *ctc1* mutants by culling out the most damaged cells. Finally, this research led to the unexpected finding that *atr* mutants, which have wild type telomere length, have very low levels of telomerase activity. Further, induction of DSBs caused a similar decrease in telomerase activity.

Chapter IV is about the characterization of the TER2 telomerase RNA. I contributed to the finding that telomerase activity after induction of DSBs was correlated with an increase in TER2 levels, and this response was specific to DSBs and not replication stress or other types of genome instability.

Finally, Chapter V investigates whether PARPs are important in *Arabidopsis* telomere regulation. We found no difference in the telomeres of *parp* mutants or in seedlings treated with the PARP-inhibitor 3-AB relative to wild type, suggesting that the importance of PARPs in telomere maintenance may be restricted to humans.

CHAPTER II

GENETIC ANALYSIS OF CST FUNCTION IN *Arabidopsis thaliana*

Summary

Recently CST (CTC1/STN1/TEN1) has been identified as a conserved telomere complex in plants and in mammals. In *Arabidopsis*, absence of any of the CST components leads to massive telomere dysfunction. In humans, CST is vital for replication of telomeres, probably through its interactions with DNA polymerase α. Mutations in CTC1 have been linked to several human telomere-related diseases, including dyskeratosis congenita. However, little is known how CST interacts with other telomere components in multicellular eukaryotes. Here we employed a genetic approach to examine the relationship of *Arabidopsis thaliana* CTC1 and STN1 with telomerase, DNA polymerase α, and KU70, factors crucial for telomere maintenance and DNA damage repair. As part of this work, we overexpressed STN1 in *ctc1* mutants to determine if STN1 can function independently from CTC1. We found that STN1 overexpression in *ctc1-1* mutants partially rescued the telomere shortening phenotype caused by loss of CTC1. Furthermore, plants doubly deficient in CST and a telomerase RNP subunit TERT exhibit impaired viability, and harbor telomere tracts markedly shorter than in either single mutant. Thus, telomerase is required to stabilize telomere tracts devoid of the CST complex. Plants lacking both CST and KU70 show severe growth defects and exhibit additively increased G-overhang signals

relative to either single mutant. The data indicate that maintenance of G-overhangs in Arabidopsis is facilitated by at least two different pathways: one requiring CTC1 and STN1, and a second involving KU. Finally, we found that plants with hypomorphic Polα alleles display slightly shorter, heterogeneous telomeres compared to wild type, while plants doubly deficient in STN1 and Polα have telomeres resembling *stn1* single mutants. Co-immunoprecipitation experiments showed that the Polα alleles did not disrupt interactions with CTC1 or STN1, and thus the mutant phenotypes are probably caused by Polα defects and not altered interactions with telomere-specific components.

Introduction

The essential functions of telomeres are to promote complete replication of the chromosome terminus and to distinguish the natural ends of chromosomes from double-strand (ds) breaks. Regulation of telomere length is a complex and dynamic process. Telomerase needs access to shorter telomeres to extend them, but overextension of telomere tracts can be problematic as it leads to increased recombination (Londono-Vallejo et al. 2004). Likewise, G-overhangs must be present for telomerase to extend the telomere tract, but excessive amounts of G-overhangs are deleterious to cells and result in chromosome instability. In addition to length constraints, the telomeres must be protected from nucleases and DNA damage response proteins both during telomere replication and during the rest of the cell cycle.

While many proteins are involved in the dynamic regulation of telomere length, a central regulator of telomere dynamics is the heterotrimeric CST complex (Cdc13/Stn1/Ten1 in budding yeast; CTC1/STN1/TEN1 in plants and vertebrates). Studies in budding yeast revealed that the CST is a multifunctional complex. Loss of function of any one of thethree CST components leads to degradation of C-strand telomeric DNA and extension of G-overhangs (Garvik et al. 1995; Grandin et al. 1997; Grandin et al. 2001; Xu et al. 2009). Further, CST prevents a DDR and accumulation of Mec1at the telomere (Garvik et al. 1995; Hirano and Sugimoto 2007; Xu et al. 2009). Stn1 can rescue the lethality of *cdc13* mutations if it is fused to the Cdc13 DNA binding domain (Pennock et al. 2001) or if it is overexpressed (Petreaca et al. 2006). Together, these studies indicate that CST plays a pivotal role in maintaining the stability of the chromosome end. They also imply that the end protection function of CST requires the Stn1-Cdc13 interaction for localization at telomeres, and it is Stn1 that is crucial for chromosome end protection.

The CST complex is also important for telomere length regulation (Nugent et al. 1996; Grandin et al. 2000; Grandin et al. 2001). Interestingly, Cdc13 is both a positive and negative regulator of telomere length (Nugent et al. 1996; Qi and Zakian 2000; Chandra et al. 2001). This dual role for telomere length regulation results from dynamic interactions that occur with Cdc13 during telomere replication. Cdc13 interacts with both telomerase, for G-strand extension, and DNA polymerase α, for C-strand fill-in (Qi and Zakian 2000). In

late S phase, telomerase is recruited to the telomeres by the interaction of the telomerase accessory protein Est1 and Cdc13 (Evans and Lundblad 1999; Qi and Zakian 2000). Mutations that disrupt this interaction result in an EST (Ever Shorter Telomeres) phenotype characterized by progressive telomere shortening during each cell cycle (Nugent et al. 1996). DNA Polα is recruited to the telomeres after telomerase extension through interactions with both Cdc13 and Stn1. Cdc13 interacts with the catalytic subunit, Pol1, while Stn1 interacts with Pol12, the Polα regulatory subunit (Qi and Zakian 2000; Grossi et al. 2004). Disruption of these interactions leads to telomerase-dependent telomere elongation (Qi and Zakian 2000), suggesting that the CST-Polα complex may inhibit telomerase recruitment to telomeres. Thus, similarly to the mammalian shelterin component Pot1, the CST complex functions in both end protection and telomere length regulation through interactions with various proteins that either encourage telomere elongation by telomerase or which favor end protection and exclusion of telomerase.

The contribution of CST in human telomere biology was originally thought to be less important than in yeast. Recent studies indicate that vertebrate CST functions primarily to promote efficient telomere replication as well as C-strand fill-in (Stewart et al. 2012b; Wang et al. 2012). Knockdown or deletion of CTC1 or STN1 in cell culture causes chromosome fusions, telomere loss, and multitelomeric signals (more than one telomeric FISH signal at a single chromosome end) (Miyake et al. 2009; Surovtseva et al. 2009; Stewart et al.

2012b; Wang et al. 2012). Mutations in CTC1 have recently been linked to a variety of human stem cell-related diseases, including dyskeratosis congenita (Anderson et al. 2012; Polvi et al. 2012), arguing that CST is central for genome stability and extended cell proliferation capacity. The mechanisms behind CST function in multicellular eukaryotes are just beginning to be uncovered. We have previously shown that CST from the flowering plant *Arabidopsis thaliana* also functions in telomere length regulation and end protection (Song et al. 2008; Surovtseva et al. 2009; Leehy et al. 2013). CST mutants have short, deregulated telomeres, abundant chromosome fusions, telomere recombination, and increased G-overhang signal. Despite these very severe phenotypes, plants lacking any of the CST components, allowing us to study genetic interactions between CST and KU, TERT, and Polα mutants. Here we describe the results of this analysis.

Materials and methods

Plants and material

Plants were grown in chambers with 16 hr photoperiod at 22°C. Heterozygotes of *stn1-1* or *ctc1-1* (Song et al. 2008; Surovtseva et al. 2009) were crossed *tert* or *ku70* (Kannan et al. 2008). The *icu2*$^{pol\alpha}$ seeds (Liu et al. 2009) were a gift from Karel Riha and *icu2-1* seeds were a gift from Jose´ Luis Micol (Barrero et al. 2007). *icu2-4* seeds (ICU2_198F5) were from the *Arabidopsis* Biological Resource Center. Mutants and offspring were genotyped

by PCR as previously described. F1 plants heterozygous for mutations in both genes were self-crossed and then F2 siblings were used for telomere analysis.

TRF and PETRA

DNA from whole plants was extracted using hexadecyl trimethyl-ammonium bromide (CTAB) as described (Boltz et al. 2012). TRF analysis was performed using 50 µg of DNA digested with Tru1I (Fermentas) and hybridized with a ^{32}P 5' end–labeled $(T_3AG_3)_4$ oligonucleotide probe (Fitzgerald et al. 1999). The blots were developed using a Pharos FX Plus Molecular Imager (Bio-Rad), and data were analyzed with Quantity One software (Bio-Rad). PETRA was performed as described (Heacock et al. 2004). 2 µg of DNA was used per reaction for telomere extension, followed by PCR amplification. PETRA PCR products were separated on an agarose gel and subjected to Southern blotting using the same telomeric probe mentioned above.

In-gel hybridization

In-gel hybridization was performed as described (Song et al. 2008). A ^{32}P 5’ end-labeled telomeric C-strand probe $(C_3TA_3)^3$ was used for hybridization. The relative amount of G-overhang signal was quantified as the hybridization signal from the native gel and was normalized to an interstitial telomere signal obtained from the same gel under a denaturing condition. The G-overhang signal obtained from wild-type DNA was set to one, and each sample was

compared to this value. Controls were also run with 30U T4 DNA polymerase per µg DNA in the absence of dNTPs to verify the signal seen was from chromosome termini and not internal single-strand telomeric sequences.

Protein expression and co-immunoprecipitation

For *in vitro* studies, full-length or truncated *ICU2* and *POLA2* cDNA were cloned into pET28a and pCITE4a vectors (Novagen) and expressed using rabbit reticulocyte lysate (RRL) according to manufacturer's instructions (Promega). Other constructs have been described previously (Surovtseva et al. 2009). For *in vitro* co-immunoprecipitation, pET28a (T7-tag fusion) and pCITE4a (untagged) constructs were expressed in RRL in the absence or presence of ^{35}[S]-methionine (PerkinElmer), respectively. Co-immunoprecipitation was conducted as described (Karamysheva et al. 2004).

Results

STN1 overexpression partially rescues the ctc1-1 deprotection phenotype

We have shown previously that CTC1 and STN1 interact both physically and genetically (Surovtseva et al. 2009). However, it is not known if the two proteins can function independently of each other. Because CTC1 is a very large, multidomain protein (~142 kD), it has the potential for multiple functions. One possibility is that CTC1 acts as a platform to facilitate localization of other proteins to the telomeres. Studies of Cdc13, the presumed functional homolog

of CTC1, support this model. In yeast, overexpression of Stn1 rescues the telomere length deregulation phenotype of the *cdc13-1* mutant (Petreaca et al. 2006; Puglisi et al. 2008). Based on these studies in yeast, we predicted that if CTC1 functions to bring STN1 to chromosome ends, overexpression of STN1 should bypass the need for CTC1 recruitment and provide sufficient amounts of STN1 to protect the telomeres.

To test our hypothesis, we transformed *ctc1-1* and *ctc1-2* heterozygous *Arabidopsis* plants with STN1-YFP under the control of the 35S CaMV overexpression promoter. The *ctc1-1* allele harbors a point mutation that introduces a premature stop codon in exon 9, whereas *ctc1-2* contains a T-DNA insertion in exon 6 (Fig. 2-1A). We previously showed this STN1-YFP construct can rescue the phenotype of *stn1-1* mutants and thus is fully functional *in vivo* (Song et al. 2008).

To determine the effect of STN1 overexpression on telomere length, we used Primer Extension Telomere Repeat Amplification (PETRA) (Fig. 2-1B). Stn1 overexpression partly rescued the telomere length phenotype in *ctc1-1* but not *ctc1-2* mutants. Although average telomere length was not as long as wild type, the range of telomere lengths was more homogenous than in *ctc1* mutants. Interestingly, STN1 overexpression in *ctc1-1* produced distinct populations of telomeres, a result consistent with telomerase inhibition, which is a function of yeast Stn1 (Puglisi et al. 2008). The different results obtained with the two *ctc1*

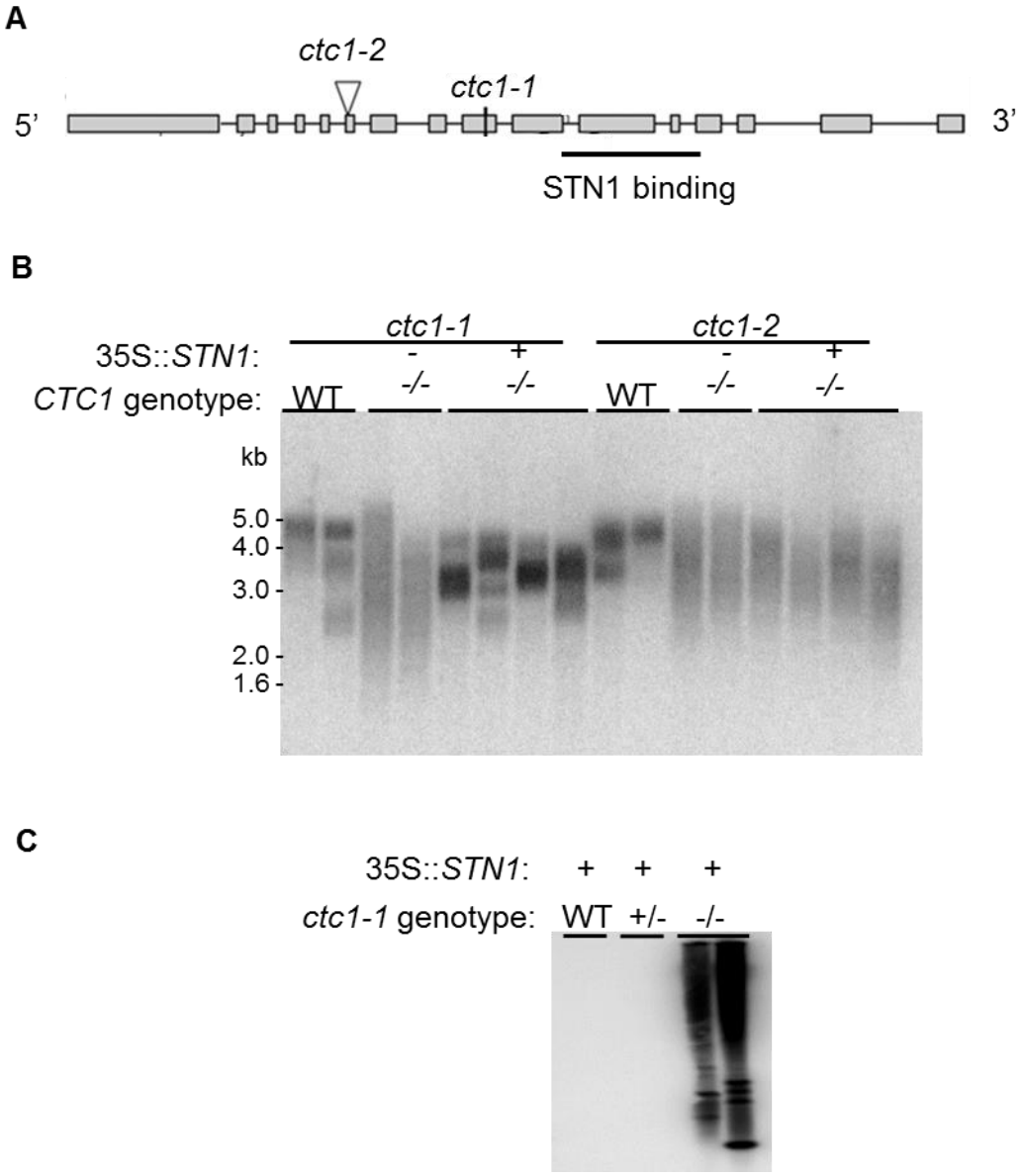

Figure 2-1. STN1 overexpression partially rescues the phenotype of *ctc1* mutants. (A) Schematic of *CTC1* showing the location of the two mutant alleles as well as the approximate region where STN1 binds. (B) PETRA analysis of STN1-YFP overexpression in *ctc1-1* (left) and *ctc1-2* (right) mutants and their wild type siblings. Mutant siblings which were not grown on BASTA (unselected) did not contain the STN1-YFP construct. (C) TF-PCR analysis of *ctc1-1* mutants and heterozygous or wild type siblings with STN1-YFP overexpression.

alleles suggests that the *ctc1-1* allele give rise to a truncated, partially functional CTC1 protein.

Both *ctc1* and *stn1* mutants display extensive end-to-end chromosome fusions (Song et al. 2008; Surovtseva et al. 2009). To determine if STN1 overexpression can inhibit the formation of telomere fusions we used telomere fusion PCR (TF-PCR) (Fig. 2-1C). In contrast to the rescue of the telomere length defect, STN1 overexpression did not eliminate fusions in *ctc1-1* mutants.

Our results of STN1 overexpression in *ctc1-1* mutants indicate that the N-terminal portion of CTC1, which binds DNA, but not STN1, is sufficient for maintaining telomere length, but is unable to protect telomeres from aberrant chromosome fusion events. The results also suggest that STN1 cannot maintain telomere length or prevent telomere fusions with the complete absence of CTC1.

Plants lacking CTC1 or STN1 show reduced viability in the absence of TERT or KU

In most eukaryotes, replication of chromosome ends relies on telomerase. One explanation for the short telomeres in *ctc1* or *stn1* plants is that telomerase cannot efficiently extend telomeres. To determine if CTC1 or STN1 are required for telomerase to act on telomeres *in vivo*, we generated plants doubly deficient in CTC1 or STN1 and the catalytic subunit of telomerase, TERT. During initial genotyping of the progeny of *ctc1*$^{+/-}$ *tert* $^{+/-}$ plants, no *ctc1 tert*

double homozygous mutants could be identified. If double mutants were embryonic lethal, we predict that 25% of the seeds in the siliques of *ctc1*$^{+/-}$ *tert*$^{-/-}$ self-pollinated plants would fail to develop into viable offspring. In siliques from *ctc1*$^{+/-}$ *tert*$^{-/-}$ plants, 21.9% of seeds were aborted (Fig. 2-2A and B). In contrast, only about 2-3% of seeds were aborted in *ctc1*$^{+/+}$ *tert*$^{+/-}$ and *ctc1*$^{+/-}$ *tert*$^{+/+}$ siblings. Moreover, no embryonic lethality was observed in *stn1*$^{+/-}$ *tert*$^{-/-}$ mutants (data not shown). These findings argue that TERT is crucial for viability in plants lacking CTC1, and further that the functions of CTC1 and STN1 are not equivalent.

Despite the severe consequences of the double mutation, eventually several *ctc1 tert* plants were recovered. The plants were small and without reproductive tissue, similar to mutants lacking TERT for 8-10 plant generations (Riha et al. 2001) (Fig. 2-2C). In contrast, although *stn1 tert* mutants displayed a similar morphology, they were found in approximately Mendelian ratios (Fig. 2-2D), indicating that the double *stn1 tert* mutant was not as severe as *ctc1 tert*. Because the morphology was similar between both double mutants, the data suggest that CTC1, unlike STN1, is essential for gametogenesis, pollination, or very early development. Notably, the *ctc1 tert* plants that survive to germination develop similarly as *stn1 tert* plants, indicating that the specific requirement for the CTC1 component of CST is confined to a particular developmental window.

We also made crosses of *ctc1* or *stn1* mutants with *ku70* mutants to determine if CST and KU act in the same pathway to inhibit formation of long G-

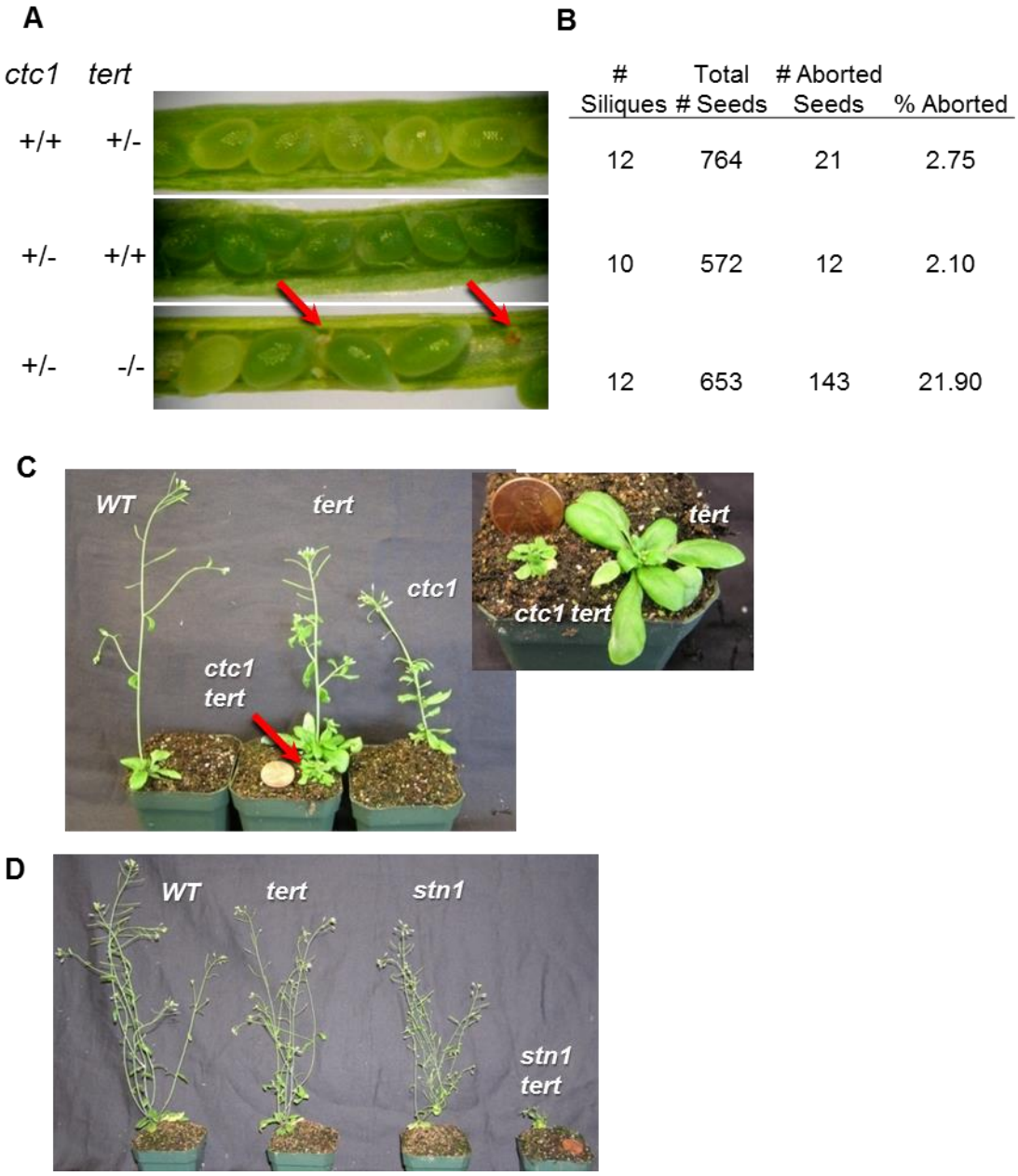

Figure 2-2. TERT is critical for plant growth and viability in *Arabidopsis* lacking CTC1. (A) Siliques from self-crossed parents of the corresponding genotype. Aborted seeds (arrows) were observed in siliques of *ctc1*$^{+/-}$ *tert*$^{-/-}$ plants (bottom panel). (B) Quantification of the aborted seeds in (A). (C) *ctc1 tert* double mutant plants are tiny and do not form reproductive structures. The right panel shows the same *tert* and *ctc1 tert* plants at an earlier age than in the left panel. (D) *stn1 tert* double mutant plants (left) compared to wild type (left), *tert* and *stn1* mutants.

overhangs. As expected, *ku70* mutant plants have wild type morphology (Riha et al. 2002). In contrast, *ctc1 ku70* and *stn1 ku70* double mutant plants displayed similar morphology as *ctc1 tert* or *stn1 tert* mutants (Fig. 2-3 and data not shown). However, *ctc1 ku70* double mutants were present in Mendelian ratios, indicating that KU70 is important for fecundity in *ctc1* and *stn1* mutants, but is not important for early development like TERT.

Telomerase maintains telomeres in the absence of CTC1 and STN1

In yeast, telomerase recruitment to the telomeres requires either Cdc13 or Ku70/80 (Chan et al. 2008). If CTC1 plays a similar role in *Arabidopsis*, some of the telomere shortening seen in *ctc1* or *stn1* could be a result of loss of telomerase recruitment to the telomeres. Alternatively, CTC1 or STN1 could be required for optimal enzyme activity of telomerase *in vivo*. To examine the role of TERT in *ctc1* and *stn1* mutants, we measured telomere length in *ctc1 tert* and *stn1 tert* by TRF (Telomere Restriction Fragment analysis) and PETRA (Primer Extension Telomere Repeat Amplification).

Telomere lengths shortened in the *tert* and *stn1* or *ctc1* single mutants as previously reported (Fig. 2-4) (Riha et al. 2001; Song et al. 2008; Surovtseva et al. 2009). Notably, the *tert* telomeres appeared as sharper bands on both TRF and PETRA, indicating telomeres were present in discreet populations, whereas *ctc1* and *stn1* telomeres were very heterogeneous in length. The sharp banding pattern is an indication of the lack of telomerase activity on telomeres, while

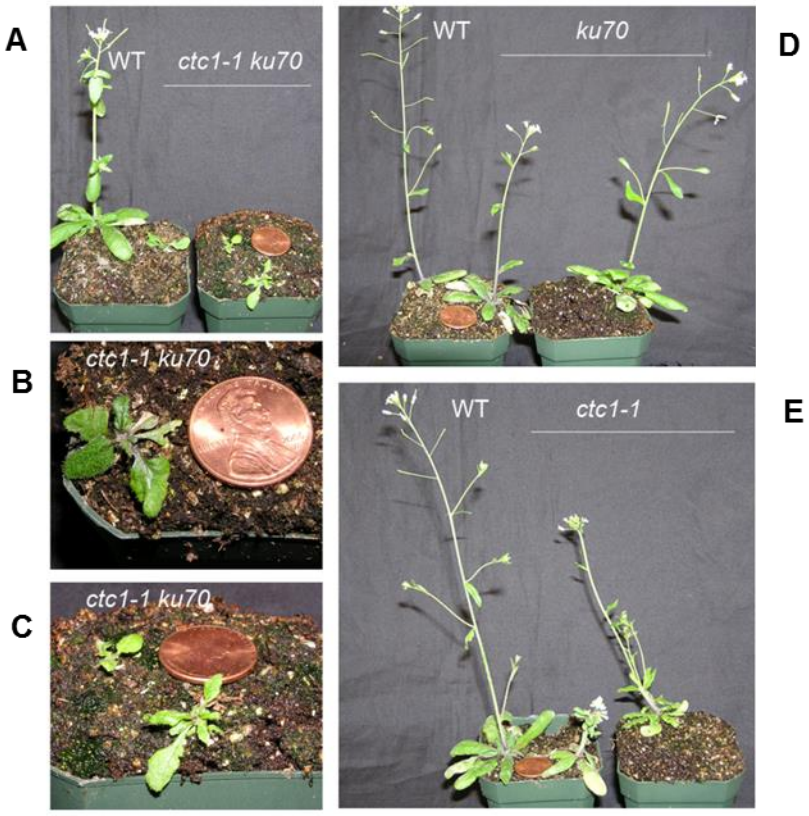

Figure 2-3. Simultaneous loss of CTC1 and KU causes severe developmental defects. (A-C) *ctc1 ku70* double mutants. (D) *ku70* mutant plants. (E) *ctc1-1* mutant plants.

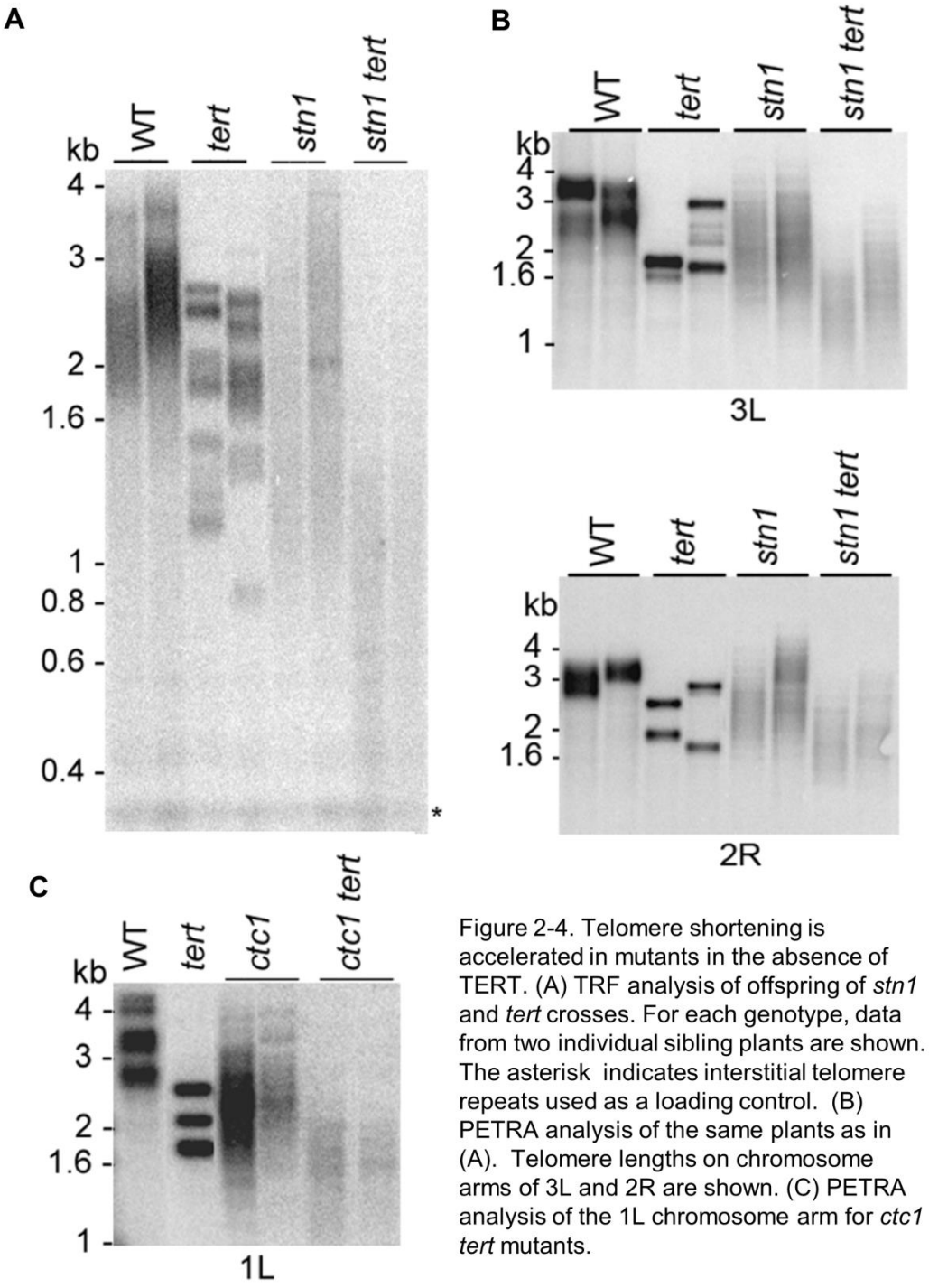

Figure 2-4. Telomere shortening is accelerated in mutants in the absence of TERT. (A) TRF analysis of offspring of *stn1* and *tert* crosses. For each genotype, data from two individual sibling plants are shown. The asterisk indicates interstitial telomere repeats used as a loading control. (B) PETRA analysis of the same plants as in (A). Telomere lengths on chromosome arms of 3L and 2R are shown. (C) PETRA analysis of the 1L chromosome arm for *ctc1 tert* mutants.

heterogeneity reflects stochastic action of telomerase or nucleolytic attack (Riha et al. 2001; Song et al. 2008). *ctc1 tert* and *stn1 tert* telomeres were shorter than those from plants mutant for either *tert* or *ctc1/stn1*. However, the heterogeneity of the telomeres in the double mutants resembled that of *ctc1* and *stn1* single mutants. These results suggest that TERT is necessary for maintaining telomere length in the absence of CTC1 or STN. Importantly, neither CTC1 nor STN1 is required for this function of TERT, which implies that neither is essential for TERT recruitment to telomeres.

KU maintains telomeres in the absence of CTC1 and STN1

In yeast, telomerase can be recruited to telomeres by Ku in the G1 phase of the cell cycle (Chan et al. 2008). Moreover, loss of Ku in yeast results in telomere shortening (Porter et al. 1996) In striking contrast, telomeres are elongated in *Arabidopsis ku70* mutants, and this phenotype is telomerase-dependent (Riha et al. 2002; Riha and Shippen 2003a). Thus, KU negatively regulates telomere length in *Arabidopsis* when CST is intact. To determine if KU can serve as a backup mechanism for promoting telomere length maintenance in the absence of *ctc1* or *stn1,* we analyzed telomere length in *ctc1 ku70* and *stn1 ku70* mutants.

Telomere lengths for *ctc1 ku70* double mutants varied from individual to individual (Fig. 2-5). The lengths were highly heterogeneous like *ctc1* mutants, ranging from as short as *ctc1* single mutants to as long as *ku70* mutants.

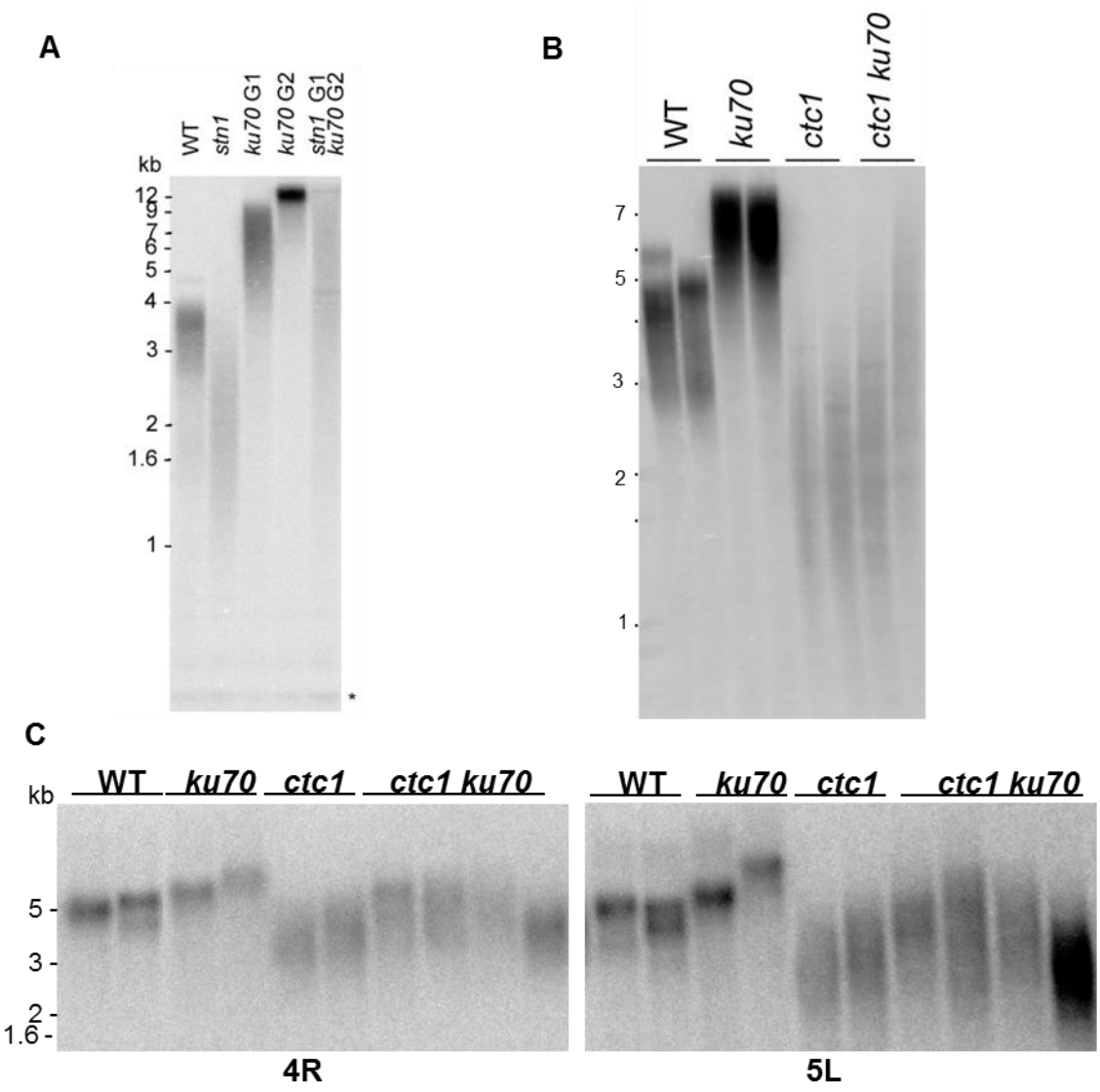

Figure 2-5. Neither KU nor CST is required for telomere elongation in *Arabidopsis.* (A) TRF analysis of *stn1 ku70* mutant (*stn1* G1 *ku70* G2) compared to WT, *stn1*, *ku70* G1 and *ku70* G2 mutants. (B) TRF analysis of WT, *ku70*, *ctc1*, and *ctc1 ku70* mutants. For each genotype, data from two individual plants are shown. Asterisks indicate interstitial telomere repeats used as a loading control. (C) PETRA analysis of WT, *ku70*, *ctc1*, and *ctc1 ku70* mutants for chromosome arms 4R and 5L.

Because *STN1* and *KU70* reside near each other on chromosome one, we could not recover double mutants from a parent heterozygous for both genes. Instead, double mutants were obtained from self-crossed *stn1*$^{+/-}$ *ku70*$^{-/-}$ parents. The *stn1 ku70* telomeres resembled the *ctc1 ku70* telomeres (Fig. 2-5A). We conclude that the telomere length deregulation that occurs in CST and *ku70* mutants is caused by two distinct genetic pathways. In addition, the *ku*-like longer telomeres in the *ctc1 ku70* double mutants argue that neither CTC1 or STN1 nor KU is required for telomerase recruitment to the telomeres.

STN1 acts in a separate genetic pathway from KU for G-overhang regulation

Besides deregulation of the length of the telomeric duplex, the length of G-overhangs can also be perturbed. Both *ku70* and *ctc1/stn1* mutants exhibit increased G-overhang signals. To determine if KU and CTC1/STN1 act in the same pathway for G-overhang maintenance, we measured G-overhangs in *stn1 ku70* double mutants using non-denaturing in-gel hybridization (Fig. 2-6). Strikingly, the *stn1 ku70* double mutants showed a six-fold increase in G-overhang signal compared to wild type, which is higher than the signal for either *ku70* or *stn1* single mutants compared to wild type (four-fold and two-fold, respectively). We conclude that G-overhangs are maintained by at least two different pathways in *Arabidopsis*: one requiring CTC1/STN1 and another involving KU70/80.

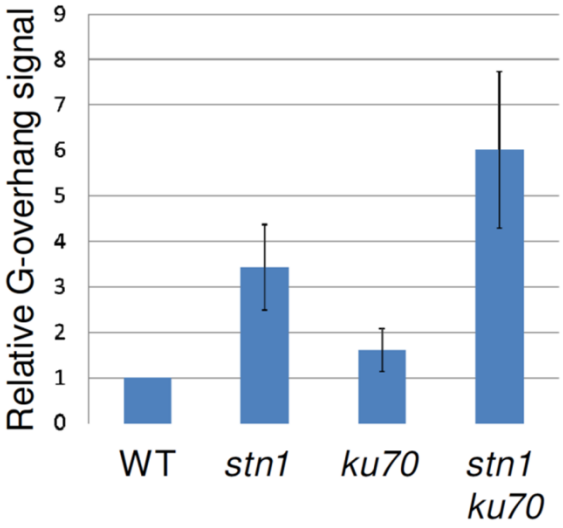

Figure 2-6. STN1 and KU act in distinct genetic pathways to maintain G-overhangs. The average G-overhang signal for three individual plants per genotype is shown relative to WT.

Telomeres are slightly deregulated in ICU2 hypomorphs

In humans, CST functions primarily in telomere replication in conjunction with DNA Polα (Stewart et al. 2012b; Wang et al. 2012). We previously showed that *Arabidopsis* CTC1 interacts with the catalytic subunit of Polα, ICU2, implying that CST and Polα may act in concert for telomere length regulation. Three alleles for *ICU2* were obtained. Each has a point mutation in the catalytic motif (Fig. 2-7A). Because null mutations in ICU2 are lethal (Barrero et al. 2007), all three mutants retain some functionality. The *icu2-1* allele is in the En-2 ecotype, distinct from the Col-0 ecotype where the majority of our *A. thaliana* telomere analysis has been conducted. Since telomere length can vary between *A. thaliana* ecotypes (Shakirov and Shippen 2004), we used PETRA to compare telomere lengths in the En-2 and Col-0 ecotypes as well as the *icu2-1* and *icu2-4* mutants (Fig. 2-7B). The telomere profile of En-2 was similar to Col-0. In both *icu2* mutants, telomeres were about 0.5-1kb shorter than the corresponding wild type ecotype, but were still within the normal size range of 2-5kb for Col-0 (Shakirov and Shippen 2004). Notably, the PETRA products from the two *icu2* mutants were slightly more heterogeneous in size compared to wild type, indicative of some aberration in telomere maintenance.

Crosses were made between *stn1-1* heterozygotes and the *icu2-1* mutants. Siblings from the F2 generation were used for analysis. The double *stn1 icu2* mutant was markedly smaller than either *stn1* or *icu2* single mutants and displayed poorly developed flowers (Fig. 2-7C). The morphological

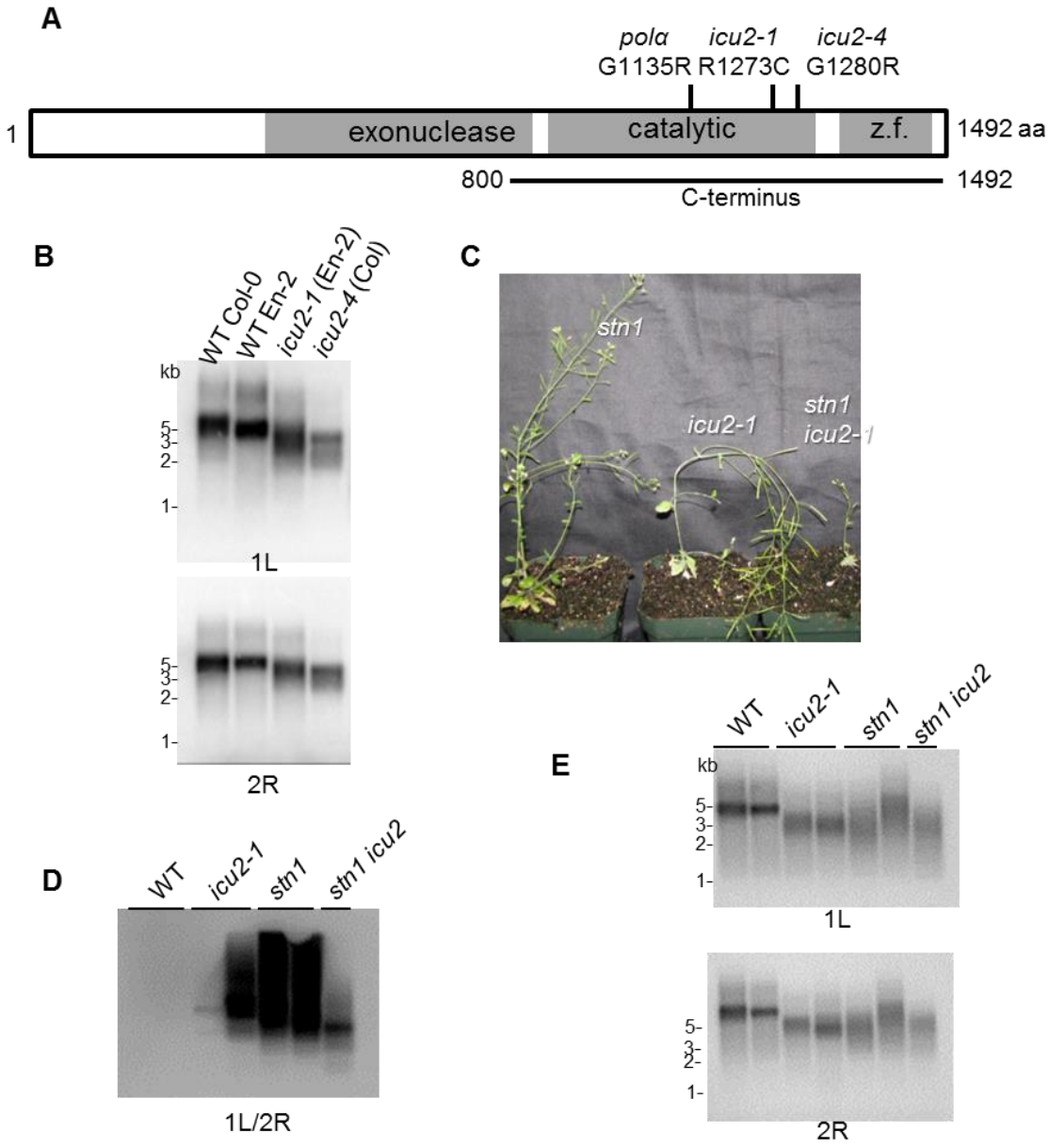

Figure 2-7. Telomeres in *icu2 stn1* double mutants. (A) Schematic of the ICU2 protein. Conserved domains are in gray. The location of the three alleles used in this study is indicated. z.f.: zinc finger. (B) PETRA of *icu2* mutants and their corresponding ecotype. (C) Plant morphology in *stn1 icu2* mutants. (D) Fusion PCR of *icu2* and *icu2 stn1* double mutants using primers for 1L and 2R chromosomes. (E) PETRA analysis of *stn1 icu2* double mutants on two chromosome arms.

phenotypes associated with STN1 deficiency are a direct result of genome instability caused by telomere dysfunction. To investigate whether *icu2* mutation causes telomere dysfunction, we used TF-PCR to assay for end-to-end chromosome fusions (Fig. 2-7D). As expected (Song et al. 2008), *stn1* mutants had abundant fusion PCR products. Although there was TF-PCR signal in both *icu2* single mutants and *stn1 icu2* double mutants, preliminary cloning and sequencing of the products failed to show telomeric sequences. Thus, it is not clear whether ICU2 inhibits telomere fusions.

Telomere analysis by PETRA was also conducted to monitor telomere length on individual chromosome arms (Fig. 2-7E). As expected (Song et al. 2008) , *stn1* telomeres were shorter than wild type and were also very heterogeneous in length. Telomeres in *icu2* mutants were again shorter and more heterozygous than wild type. In comparison to *stn1* telomeres, *icu2* telomeres were not as short and were also intermediate in heterogeneity between wild type and *stn1*. Since telomeres in the double *stn1 icu2* mutant, resemble those of *stn1* single mutants, we conclude that STN1 is epistatic to ICU2 for telomere length regulation.

Hypomorphic alleles of ICU2 do not disrupt CTC1 or STN1 binding in vitro

We previously found that the CTC1 C-terminus interacts with ICU2 *in vitro* (Price et al. 2010). Thus, one possibility for the shorter telomeres in *icu2*

mutants is that the point mutation disrupts binding of Polα with CST, which would be consistent with the epistatic interaction we saw above.

Interactions of CST with the ICU2 point mutants were examined *in vitro* using protein expressed *in vitro* in Rabbit Reticulocyte Lysate (RRL). One protein contained a T7-tag for immunoprecipitation and the other was labeled with ^{35}[S]-methionine to visualize on a gel. For CTC1-ICU2 interactions, pull-downs were performed with unlabeled T7-CTC1 and labeled ICU2 or vice versa (Fig. 2-8A and B). As expected, wild type ICU2 was immunoprecipitated with CTC1. CTC1 was precipitated in all of the pull-downs with mutant ICU2 (Fig. 2-8A, compare the bound lanes 4, 6, and 8). Similarly, when CTC1 was used for the pulldown of the *polα* allele, we found no difference in binding compared to the wild type ICU2 (Fig. 2-8B). Thus, the point mutations do not disrupt the ICU2-CTC1 interaction *in vitro*.

STN1 interactions with ICU2 had not been previously assessed. Budding yeast Stn1 interacts with the regulatory subunit of Polα, Pol12 (Grossi et al. 2004), so I examined whether STN1 could interact with the *Arabidopsis* homolog, PolA2. The STN1-Polα interactions included addition of a radiolabel to the T7-tagged protein to monitor the efficiency of the pulldown. We found a PolA2 STN1 interaction (Fig. 2-8C). In addition, the assay showed that STN1 interacted with wild type ICU2 and all three ICU2 point mutants (Fig. 2-8C). These data suggest that the CST-Polα interactions are intact in all three

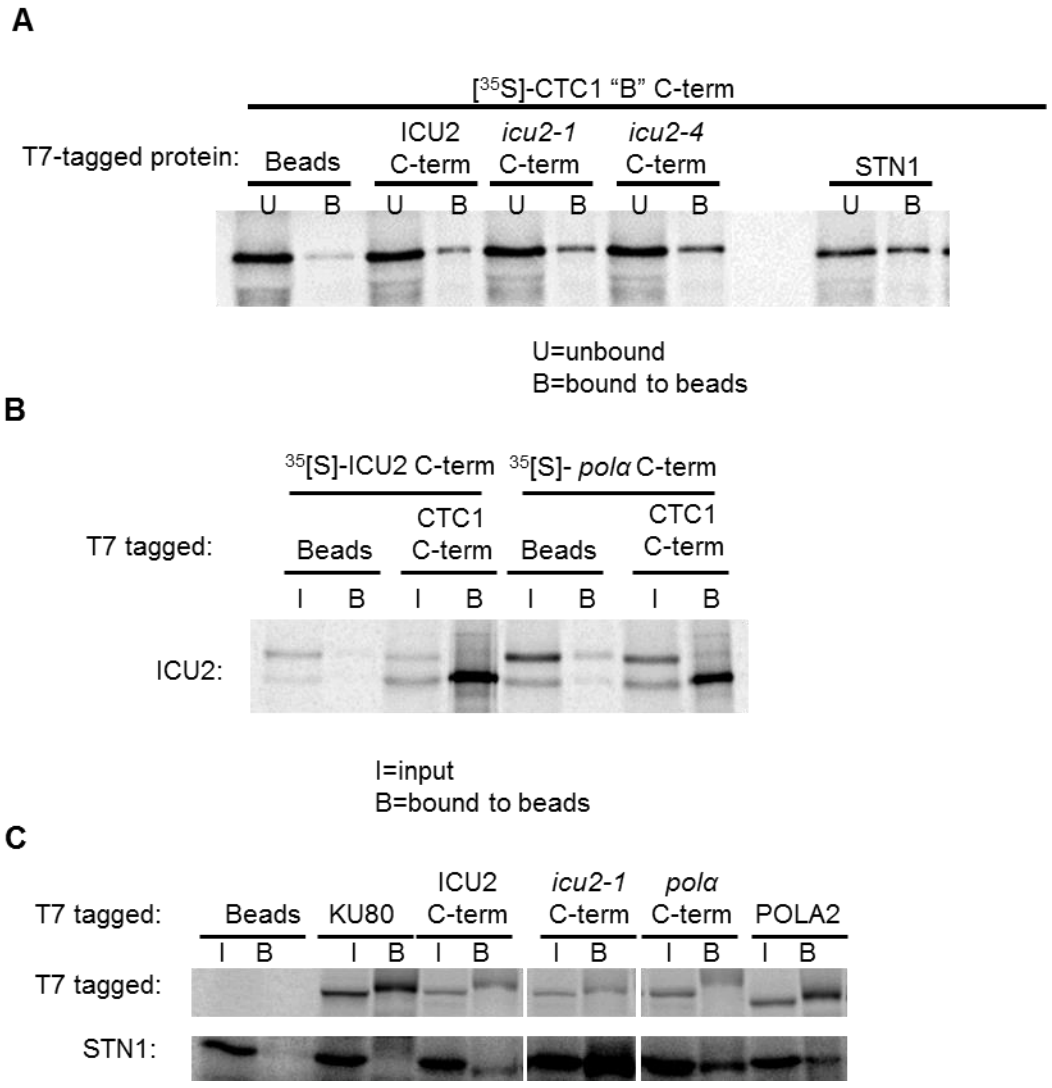

Figure 2-8. Co-immunoprecipitation of CTC1 and STN1 with ICU2 alleles. Beads indicates controls where T7-tagged protein was not included. (A) T7-tagged ICU2 pulldown of radiolabeled CTC1 C-termminus. T7-STN1 is a positive control. (B) T7-tagged CTC1 pulldown of radiolabled ICU2. (C) STN1 interactions with ICU2 and PolA2. Both proteins are radiolabled. The T7-tagged protein is indicated along the top and was used to pulldown STN1. KU80 is a negative control.

mutants, and further, that the phenotypes in *icu2* mutants are not caused by loss of interaction with CST.

Discussion

Eukaryote genome stability relies on intact chromosome ends. In this study, we exploited the extraordinary tolerance of *Arabidopsis* toward telomere dysfunction to examine the genetic interactions of the CST telomere capping complex in a multicellular eukaryote. Although extensive research has been conducted on yeast CST, we still know little about the function of CST in plants and humans. In plants, CST protects telomeres from massive dysfunction (Song et al. 2008; Surovtseva et al. 2009), whereas in humans CST functions primarily in telomere replication (Surovtseva et al. 2009; Stewart et al. 2012b). In yeast CST also protects chromosome ends, suggesting that *Arabidopsis* CST functions more like yeast CST than human CST. Although this study does not precisely define the mechanisms behind CST function, our genetic analysis of the CST complex suggests that *Arabidopsis* employs multiple pathways to regulate telomere dynamics, likely reflecting the importance of telomeres for overall genome stability and plant viability.

CST and TERT or KU act in separate genetic pathways for telomere length regulation

Telomere tracts in CST mutants are highly heterogeneous (Song et al. 2008; Surovtseva et al. 2009), including a subpopulation that falls below the critical 1kb length threshold, which has been shown to mark telomeres for end joining reactions (Heacock et al. 2007). The failure to maintain telomeres in CST mutants could result from the inability of telomerase to act on chromosome ends. We hypothesized that telomerase could affect the telomere length in CST mutants in several ways. First, the heterogeneity of CST telomeres may be indicative of telomerase extension of the telomeres, as telomerase is responsible for the heterozygosity of individual telomere tracts in otherwise wild type cells (Riha et al. 2001). Second, shorter telomeres in CST mutants could indicate a failure of telomerase to be recruited to the telomeres followed by nuclease attack. Indeed, we find that when a TERT deficiency is combined with loss of CTC1 or STN1, telomeres shortened more than either single mutant, indicating that TERT and CST act in separate pathways to regulate telomere length.

In budding yeast, both KU and CST are required for telomerase recruitment; Ku recruits telomerase in G1 phase of the cell cycle, whereas CST recruits telomerase in S phase (Chan et al. 2008). Our telomere length analysis suggests that telomerase can act on *Arabidopsis* telomeres in the absence of both pathways. Our evidence is indirect, however. In *ctc1 tert* mutants,

telomere shortening is accelerated compared to *ctc1* single mutants, which supports the conclusion that TERT can localize to telomeres in the absence of CST. From our analysis of *ctc1 ku* mutants, we surmise that KU and CTC1 both contribute to telomere maintenance. The long telomeres in *ku* mutants are dependent on telomerase (Riha and Shippen 2003a) and thus the long telomeres in *ctc1 ku* double mutants may also be telomerase dependent. Measurement of TERT association with telomeres in *ctc1* mutants will be necessary to determine whether CTC1 is required for telomerase recruitment.

CST and KU act in separate pathways for G-overhang maintenance

Both CST and KU contribute to 3' G-overhang maintenance in *Arabidopsis* (Riha and Shippen 2003a; Song et al. 2008; Surovtseva et al. 2009). Our analysis of *ctc1 ku* and *stn1 ku* double mutants reveals that loss of both proteins results in an additive increase in G-overhang signal, indicating that KU and CST make independent contributions to maintenance of G-overhangs. In *Arabidopsis*, half the telomeres are blunt-ended instead of terminating in a G-overhang (Kazda et al. 2012). KU has been implicated in the protection of blunt-ended telomeres by inhibiting Exonuclease I resection of the C-strand. In the absence of KU, the G-overhang could provide additional substrates for telomerase to extend the telomeres, which is consistent with the telomerase-dependent long telomeres observed in *ku* mutants (Nelson and Shippen 2012). Although we do not know the mechanism for formation of long G-overhangs in

CST mutants, there are several possibilities. The most likely is that C-strand fill-in is deficient in *Arabidopsis* CST mutants. In yeast, CST coordinates telomerase extension of G-strands with Polα C-strand fill-in (Qi and Zakian 2000), while in humans CTC1 and STN1 enhance Polα activity at the telomeres (Nakaoka et al. 2012; Wang et al. 2012). The ability of *Arabidopsis* CTC1 and STN1 to interact with the Polα subunits lCU2 and POLA2 (Price et al. 2010)(this study), argues that the interaction between CST and Polα is highly conserved. Analysis of the G-overhangs in the *stn1 icu2* double mutants may reveal whether deficient C-strand fill-in is the mechanism for G-overhang extension in CST mutants.

A second possibility is that the G-overhangs are extended in CST mutants because telomerase activity at the telomeres is unrestrained. If CST is needed to regulate telomerase access to telomeres, as is seen in yeast (Chandra et al. 2001; Petreaca et al. 2006), then G-overhangs would form in mutants because of overactivity of telomerase. One intriguing possibility is that TEN1 inhibition of telomerase processivity (Leehy et al. 2013) limits telomerase action. This hypothesis can be tested by measuring G-overhangs in *ctc1 tert* and *stn1 tert* mutants.

CST proteins have functions independent from each other

There is a growing body of evidence that the CST components function independently from each other. Here we have shown that STN1 overexpression

partially rescues the phenotype of *ctc1-1* mutants; telomere length is maintained, but chromosome fusions still form. We also found that loss of TERT in *ctc1* mutants is more detrimental to development than when it is lost in *stn1* mutants. Both of these results point to independent contributions for CTC1 and STN1.

The most convincing evidence for independent function of CST components comes from analysis of TEN1. Loss of TERT in *ten1* mutants is much more detrimental than loss of CTC1 or STN1 (K. Leehy, unpublished results). Plants doubly deficient in TEN1 and TERT do not survive long enough after germination to produce enough tissue for DNA analysis. Plants lacking TEN1 exhibit a higher frequency of telomere fusions than *stn1* mutants (Leehy et al. 2013) Additionally, TEN1 localizes to the telomeres at a lower frequency than STN1 or CTC1. Finally, TEN1 homo-oligomers act as molecular chaperones, whereas STN1 and CTC1 do not (J.R. Lee and D. Shippen, unpublished data). Experiments are underway to identify mutations in each of the CST components. Such studies will help to clarify the contributions of CTC1, STN1, and TEN1 in telomere biology.

CHAPTER III

ATR COOPERATES WITH CTC1 AND STN1 TO MAINTAIN TELOMERES AND GENOME INTEGRITY IN *Arabidopsis*[*]

Summary

The CST (CTC1/STN1/TEN1) complex is an essential constituent of plant and vertebrate telomeres. Here we show that CST and ATR act synergistically to maintain telomere length and genome stability in *Arabidopsis.* Inactivation of ATR, but not ATM, temporarily rescued severe morphological phenotypes associated with *ctc1* or *stn1*. Unexpectedly, telomere shortening accelerated in plants lacking CST and ATR. In first generation (G1) *ctc1 atr* mutants, enhanced telomere attrition was modest, but in G2 *ctc1 atr*, telomeres shortened precipitously, and this loss coincided with a dramatic decrease in telomerase activity in G2 *atr* mutants. Zeocin treatment also triggered a reduction in telomerase activity, suggesting that the prolonged absence of ATR leads to a hitherto unrecognized DNA damage response (DDR). Finally, our data indicate that ATR modulates DDR in CST mutants by limiting chromosome fusions and transcription of DNA repair genes and also by promoting programmed cell death in stem cells. We conclude that the absence of CST in *Arabidopsis* triggers a

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multifaceted ATR-dependent response to facilitate maintenance of critically shortened telomeres, and eliminate cells with severe telomere dysfunction.

Introduction

A critical function of telomeres is to differentiate natural chromosome ends from DNA damage. The protective cap that defines the chromosome terminus consists of telomere binding proteins that associate with the double-stranded region, the single-stranded 3' G-rich extension (G-overhang), or that bridge these two domains. The best-characterized telomere capping complexes are shelterin in vertebrates and CST (Cdc13/Stn1/Ten1) in budding yeast. The six member shelterin complex spans both the double- and single-strand regions of the telomere (Palm and de Lange 2008). Within shelterin, TRF2 and POT1 play leading roles in chromosome end protection (van Steensel et al. 1998; Baumann and Cech 2001). The CST complex associates exclusively with the G-overhang (Lin and Zakian 1996), forming a heterotrimeric complex with structural similarity to RPA (Gao et al. 2007; Sun et al. 2009) A null mutation in any CST component is lethal, while other alleles trigger massive degradation of the telomeric C-strand causing grossly extended G-overhangs (Nugent et al. 1996; Grandin et al. 1997; Grandin et al. 2001). Deletion of either the Stn1 or Ten1 ortholog in fission yeast leads to catastrophic loss of telomeric DNA and end-to-end chromosome fusions (Martín et al. 2007).

CST has recently been discovered in plants and vertebrates (Song et al. 2008; Miyake et al. 2009; Surovtseva et al. 2009). STN1 and TEN1 are sequence homologs of the budding and fission yeast proteins (Song et al. 2008; Miyake et al. 2009; Price et al. 2010). The third member of the complex, CTC1 (Conserved Telomere maintenance Component 1), is not a sequence homolog of Cdc13, although it shares functional similarities. Like Cdc13, CTC1 physically interacts with STN1 as well as lagging-strand replication machinery (Casteel et al. 2009; Miyake et al. 2009; Surovtseva et al. 2009; Price et al. 2010). In addition, CTC1 in complex with STN1 and TEN1 binds single-stranded DNA, but in a sequence-independent manner (Miyake et al. 2009).

Ctc1 or Stn1 knockdown in human cells results in an increase in G-overhang signal, sporadic loss of telomeric DNA and aberrant chromatin bridges (Miyake et al. 2009; Surovtseva et al. 2009). Recent studies reveal that mutations in *CTC1* underly the rare human genetic disorder Coats plus, characterized by neurological and gastrointestinal defects (Anderson *et al.*, 2012). Coats plus patients also exhibit shortened telomeres and evidence of an ongoing DNA damage response (Anderson *et al.*, 2012). The major function for vertebrate CST may be related to DNA replication and repair, and not to chromosome end protection per se (Linger and Price 2009; Giraud-Panis et al. 2010; Price et al. 2010). Recent studies show that *Xenopus* CST is required to prime ssDNA for replication (Nakaoka et al. 2012). In addition, genetic data argue that CST and shelterin act in distinct pathways to promote telomere

integrity in human cells. When both Stn1 and Pot1 are depleted, a synergistic increase in telomere dysfunction-induced foci is observed (Miyake et al. 2009).

CST plays a pivotal role in protecting plant telomeres. Although *ctc1* and *stn1* null mutants are viable, they suffer dramatic telomere shortening, end-to-end chromosome fusions, increased G-overhangs and elevated extra-chromosomal telomeric circles, indicative of aberrant telomere recombination (Song et al. 2008; Surovtseva et al. 2009). Genetic analysis of *Arabidopsis thaliana STN1* and *CTC1* confirms that these two components act in the same pathway for chromosome end protection (Surovtseva et al. 2009). Unlike vertebrates, *Arabidopsis* harbors only a subset of shelterin components and thus far, none of these are required for chromosome end protection (Watson and Riha 2010). Moreover, *Arabidopsis* encodes three POT1-like proteins, which associate with telomerase instead of the telomere (Surovtseva et al. 2007; Cifuentes-Rojas et al. 2011). Thus, CST appears to function as the major telomere protection complex in plants (Price et al. 2010). CST is also likely to play a role in DNA replication in *Arabidopsis*, given its interaction with DNA polymerase α (Price *et al.*, 2010) and the results of vertebrate studies described above.

When telomere integrity is compromised due to loss of essential capping proteins, or prolonged inactivation of telomerase, the unprotected chromosome terminus triggers a cellular DNA damage response (DDR) that is mediated by the phosphoinositide-3-kinase-related protein kinases, ATM (Ataxia-

Telangeictasia Mutated) or ATR (ATM and Rad3-related) (Sabourin and Zakian 2008). ATM primarily responds to double-strand breaks, while ATR is activated by excessive single-stranded DNA (Nam and Cortez 2011). As expected for telomere duplex binding components, TRF2 in vertebrates suppresses activation of ATM (Denchi and de Lange 2007), while the single-strand binding proteins, mouse Pot1a (Denchi and de Lange 2007), chicken (Churikov et al. 2006), and yeast Cdc13 (Garvik et al. 1995; Ijpma and Greider 2003; Hirano and Sugimoto 2007), suppress an ATR-dependent DDR.

ATR and ATM are also required to maintain normal telomeres. Neither ATM nor ATR have been shown to affect telomerase enzyme activity levels in yeast or vertebrates (Sprung et al. 1997; Chan et al. 2001; McNees et al. 2010), but in yeast both kinases are implicated in the recruitment of telomerase to chromosome ends. In *Schizosaccharomyces pombe,* Tel1 (ATM) and Rad3 (ATR) are required for Ccq1-mediated interaction with telomerase (Moser et al. 2009b; Moser et al. 2011). Similarly, in budding yeast Mec1 (ATR) and Tel1 (ATM) are each proposed to phosphorylate Cdc13 as a prerequisite for telomerase recruitment (Tseng et al. 2006), although this finding is now controversial (Gao et al. 2010). Nevertheless, a number of studies show that Tel1 facilitates the preferential recruitment of telomerase to critically shortened telomeres (Arneric and Lingner 2007; Bianchi and Shore 2007; Sabourin et al. 2007), and stimulates telomerase repeat addition processivity on these chromosome ends (Chang et al. 2007). Analysis of the ATR-deficient Seckel

mouse indicates that while ATR is not required for telomerase recruitment to short telomeres (McNees et al. 2010), it suppresses telomere fusions and the formation of fragile sites triggered by replication fork stalling in highly repetitive telomere repeat arrays (Martínez et al. 2009; Sfeir et al. 2009; McNees et al. 2010).

Many key components of DDR are conserved in plants, but there is considerable divergence in cell cycle regulated responses relative to vertebrates (Dissmeyer et al. 2009). For example, ATM and ATR null mutations are not lethal in plants (Garcia et al. 2003; Culligan et al. 2004), and there is substantial overlap in the two pathways (Culligan et al. 2004; Friesner et al. 2005; Furukawa et al. 2010). Moreover, plants are extraordinarily tolerant to genome instability, an outcome that may reflect the presence of undifferentiated stem cell niches in the shoot and root apical meristems. Meristematic cells allow for continual growth and tissue differentiation, blunting the effect of DNA damage in somatic tissue. Ionizing radiation, for instance, will induce cell cycle arrest in meristems, but not in somatic cells (Hefner et al. 2006).

Although mutation of either ATM or ATR has no effect on telomere length homeostasis in *Arabidopsis* (Vespa et al. 2005), these kinases act synergistically with telomerase to maintain the telomere tract (Vespa et al. 2005; Vespa et al. 2007). Plants doubly deficient in ATM and TERT, the telomerase catalytic subunit, experience an abrupt, early onset of genome instability compared to *tert* single mutants (Vespa et al. 2005). Analysis of individual telomere tracts

showed that that ATM prevents stochastic deletional recombination events, allowing cells to maintain similar telomere lengths on homologous chromosome arms (Vespa et al. 2007). ATR makes a more immediate contribution to telomere maintenance than ATM (Vespa et al. 2005). From the outset, telomeres in double *atr tert* mutants shorten at a greatly accelerated pace relative to *tert*, so that telomere dysfunction occurs in the third generation of the double mutant, compared to the sixth generation of *tert*.

Here we employ a genetic approach to investigate how CST components interface with ATM and ATR to promote telomere integrity and genome stability in *Arabidopsis*. We demonstrate a pivotal role for ATR in the response to CST abrogation that leads to programmed stem cell death. We also show that the combined absence of ATR and CST results in catastrophic loss of telomere tracts in a biphasic manner. The second, more severe phase of telomere shortening coincides with strong down-regulation of telomerase activity. These findings indicate that ATR and CST act synergistically to maintain genome integrity and telomere length homeostasis.

Materials and methods

Plant lines and growth conditions

Mutant *Arabidopsis thaliana* lines and genotyping have been previously described. The alleles used were *ctc1-1* and *ctc1-3* (Surovtseva et al. 2009), *stn1-1* (Song et al. 2008), *atr-2* (Culligan et al. 2004), and *atm-2* (Garcia et al.

2003). Crosses were made with plants heterozygous for *ctc1* or *stn1* and homozygous mutant for *atr* or *atm*. F1 plants were genotyped to identify plants that were heterozygous for both alleles. These were self-crossed and F2 siblings were used for analysis. Plants were grown on soil at 22°C under 16 h light/8 h dark conditions. For experiments using seedlings, seeds were sterilized in 50% bleach with 0.1% Triton-X 100 and then plated on MS with 0.7% agar (Caisson Labs). Plates were placed in the dark at 4°C for 2-4 days and then moved to long day conditions.

For zeocin treatment, seeds were treated as described above. When seedlings were 5-7 days old, they were transferred to liquid MS culture either with or without 20μM zeocin (Invitrogen). Seedlings were grown in the dark for three days and then harvested for protein extraction.

Quantitative RT-PCR

Total RNA was extracted from G1 flowers using the E.Z.N.A. Plant RNA kit with on-column DNaseI digestion (Omega Bio-tek). To make cDNA, 2μg of RNA was used with the qScript cDNA Supermix (Quanta Biosciences). cDNA was diluted 1:4 in 10μg/ml yeast tRNA (Sigma) and 1μl was used in each qPCR reaction. The SsoFast EvaGreen Supermix (Bio-Rad) was used following manufacturers recommendations. Reactions were run on a Bio-Rad CFX96 thermalcycler using 58°C primer annealing and 10s extension. RNA from at least three individual plants was used for each genotype and two replicates were

run for each reaction. The raw amplification data was imported into LinRegPCR (Ruijter et al. 2009) using the default settings. The window-of-linearity and Cq threshold were calculated for each amplicon group. The resulting Cq values, which had been adjusted for the mean PCR efficiency for each amplicon, were used for calculation of expression levels.

For each run, we measured three reference genes (*GAPDH*, *TIP41L*, and *At4G26410*) reported by Czechowski *et al*. (Czechowski et al. 2005). The geometric mean of the three reference genes was used to calculate expression levels by the ΔΔCt method. Expression levels for each genotype were averaged and compared to wild type.

Primers sequences were 5'-TGCATCCATTAAGTTGCCCTGTG-3' and 5'-TAGGCTGAGAGTGCAGTGGTTC-3' for *BRCA1* (At4G21070), 5'-ATGCTACTCTGGCACGGTTCAC-3' and 5'-AGGAGGAGCTATTCGCAGACCTTG-3' for *PARP1* (At4G02390), and 5'-CGAGGAAGGATCTCTTGCAG-3' and 5'- GCACTAGTGAACCCCAGAGG-3' for *RAD51* (At5G20850).

Telomere length measurement, in-gel hybridization, TF-PCR and TRAP

Genomic DNA was extracted from whole plants or seedlings using 2x CTAB buffer (Vespa et al. 2005) with slight modification. Plant extracts were incubated for 1 h at 50°C, and all mixing was done by inverting tubes rather than vortexing. TF- PCR and PETRA (Heacock et al. 2004) and TRF (Fitzgerald et

al. 1999) were conducted as previously reported. For all three assays, products were detected by Southern Blot with a [^{32}P]-5'-end-labeled $(TTTAGGG)_4$ probe. A [^{32}P]-5'-end-labeled $(CCCTAAA)_3$ probe was used for in-gel hybridization as described previously (Surovtseva et al. 2009). Telomere lengths from PETRA analyses were calculated using QuantityOne software (Bio-Rad). For lanes with multiple bands, the average size was calculated. Protein extracts from 5 to 7 day-old seedlings were used for quantitative TRAP as previously described (Kannan et al. 2008).

Propidium iodide staining and cytogenetics

Five to seven day-old G2 seedlings were gently removed from MS plates and placed in 10 µg/ml propidium iodide solution diluted in water for 10 min at room temperature in the dark. Seedlings were then transferred to water. Roots and shoots were separated and roots were mounted on slides in water. *Arabidopsis* chromosome spreads were prepared from pistils as described (Riha et al. 2001). The spreads were mounted on slides with Vectashield Plus DAPI (Vector Laboratories). All slides were visualized with a Zeiss Axioplan2 epifluorescent microscope using a rhodamine filter for PI slides and a DAPI filter for chromosome spreads. ImageJ (Abramoff et al. 2004) was used to adjust the brightness and contrast of images.

Results

Loss of ATR rescues morphological defects in CST mutants

To explore the role of ATR and ATM in plants lacking CST, we crossed *ctc1* or *stn1* heterozygotes to *atr* and *atm* mutants. F1 plants heterozygous for both mutations were self-crossed and offspring were used for analysis. As previously reported (Garcia et al. 2003; Culligan et al. 2004; Vespa et al. 2005), *atm* (Fig. 3-1A) and *atr* (Fig. 3-1B) mutants were phenotypically indistinguishable from wild type. In contrast, *ctc1* and *stn1* mutants exhibited serious morphological defects (Song et al. 2008; Surovtseva et al. 2009), including fasciated inflorescence bolts and flowers (Fig. 3-1C, arrowheads; Fig. 3-2, white arrows), irregularly spaced siliques (Fig. 3-1C, arrows; Fig. 3-2), and small curved leaves. Although *ctc1* and *stn1* mutants always display morphological abnormalities, the expressivity of the mutant alleles is somewhat variable, with some individuals showing more severe phenotypes than others (Song et al. 2008; Surovtseva et al. 2009). Both *ctc1 atm* and *stn1 atm* double mutants displayed the same range of growth defects as *ctc1* (Fig. 3-1A) or *stn1* mutants (Fig. 3-1A). In contrast, *ctc1 atr* and *stn1 atr* mutants showed only minor perturbations in morphology, mainly irregularly spaced siliques. Approximately 30% of the double mutants appeared wild type (Fig. 3-1B and C, Fig. 3-2B). The apparent rescue of morphological defects in *ctc1 atr* and *stn1 atr* mutants is consistent with the conclusion that CST protects against ATR activation.

Figure 3-1. Loss of ATR rescues the morphological defects of *ctc1* mutants. The morphology of *ctc1* mutants in the presence or absence of ATM or ATR is shown. (A) The phenotype of a *ctc1 atm* double mutant (right) resembles the *ctc1* single mutant. (B and C) Morphological defects of *ctc1* mutants are largely rescued when ATR is lost. Arrowheads indicate fasciated stems and flowers; arrows indicate irregular phyllotaxy. Images of second generation (G2) *ctc1 atr* mutants are presented showing an intact plant (D) with curved, small leaves, or malformed flowers (E) bearing a curved pistil, and stamen and petal deficiency.

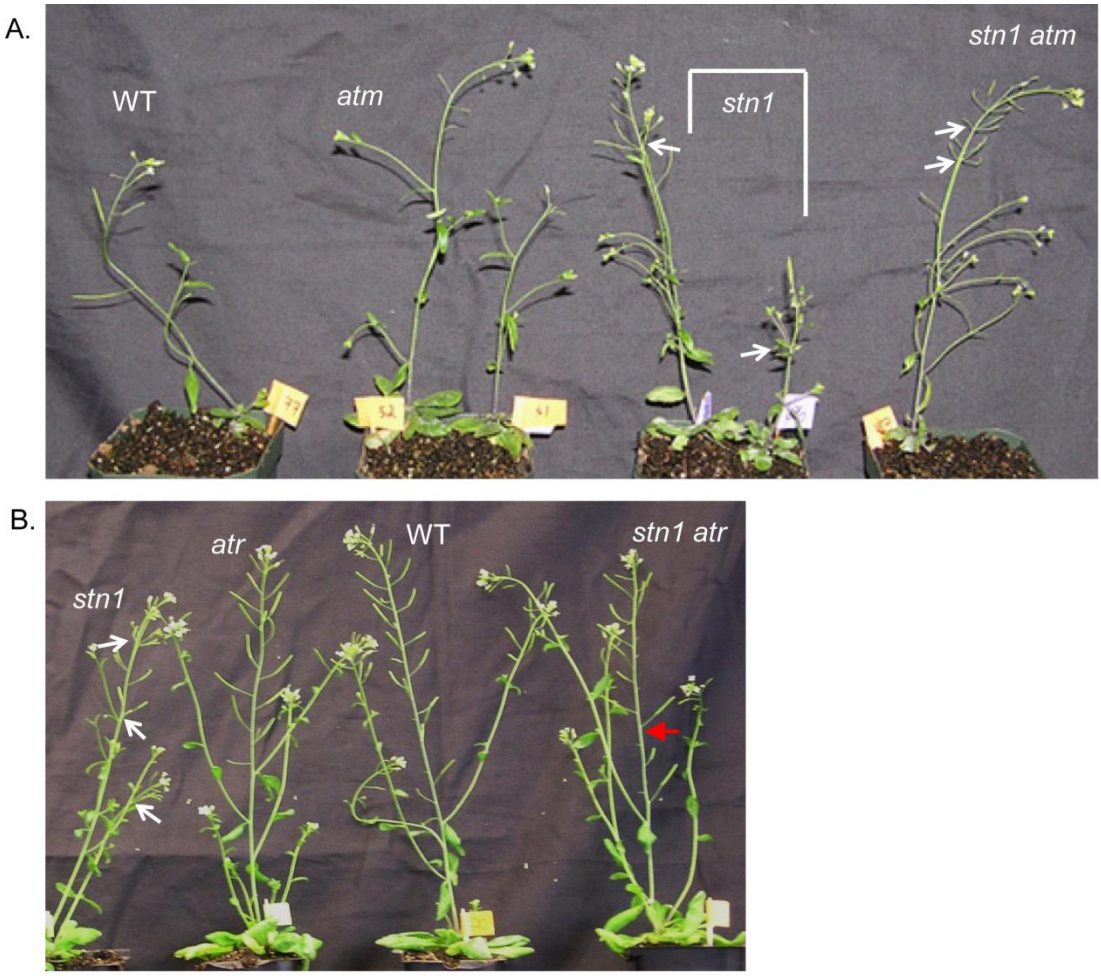
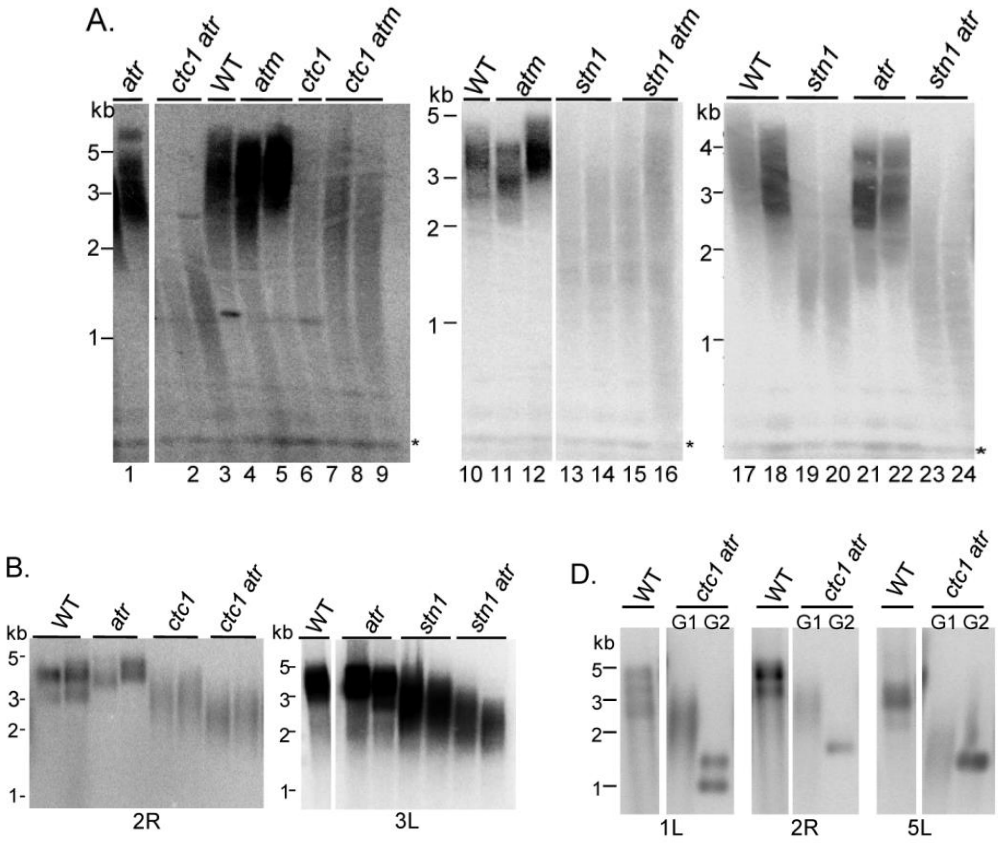


Figure 3-2. Morphological phenotypes of *stn1 atm* (A) and *stn1 atr* (B) mutants. The *stn1* mutants in (A) illustrate the variation in stature associated with CST mutation, ranging from very short to wild type in height. White arrows indicate altered phyllotaxy of siliques; red arrow denotes normal spacing of siliques in the *stn1 atr* mutant.

The improvement of morphological deficiencies in *ctc1 atr* mutants was only temporary. Second generation (G2) *ctc1 atr* mutants showed severe developmental defects, and most died before bolting (Fig. 3-1D and E). Many of the phenotypes associated with G2 *ctc1 atr* resembled G1 *ctc1* mutants (Surovtseva et al. 2009). Defects included curved, misformed leaves and severe floral abnormalities, such as missing anthers, curved pistils, open carpels with seeds exposed and petals that were green like sepals (Fig. 3-1E). We conclude that ATR alters plant growth in response to CST abrogation.

ATR facilitates telomere length maintenance in the absence of CTC1 or STN1

The morphological rescue seen in CST mutants lacking ATR argues that ATR is activated by telomere dysfunction. Given the role of ATR in telomere maintenance in telomerase mutants (Vespa et al. 2005), we considered the possibility that ATR also contributes to telomere maintenance in plants lacking CST. Bulk telomere length was monitored using Terminal Restriction Fragment (TRF) analysis. As previously reported (Vespa et al. 2005), telomere tracts in *atr* and *atm* were similar to wild type (Fig. 3-3A, lanes 1, 4, 6), while G1 *ctc1* telomeres were shorter and more heterogeneous (Fig. 3-3A, lane 7). The absence of ATM did not affect telomere length in G1 *ctc1* mutants (Fig. 3-3A, lanes 8-9). In both G1 *ctc1* and G1 *ctc1 atm* mutants, telomeres ranged from 1-5kb, with a peak signal at 2kb. In contrast, telomeres were consistently shorter in G1 *ctc1 atr* mutants than in G1 *ctc1* (Fig. 3-3A, lanes 2-3 and 7), with some



C.

Chromosome Arm:	Average Telomere Length (kb) 1L	2R	4R	5L	Avg.	Avg. Difference from WT	Avg. Difference from *atr*	Avg. Difference from *ctc1*
WT	4.57	5.20	3.37	3.21	4.09	0.00	-0.52	0.76
atr	4.68	5.72	4.36	3.67	4.61	0.52	0.00	1.28
ctc1	3.81	4.56	2.67	2.29	3.33	-0.76	-1.28	0.00
ctc1 atr	3.57	4.05	2.54	1.92	3.02	-1.07	-1.59	-0.31

Figure 3-3. ATR, but not ATM, contributes to telomere length maintenance in *ctc1* and *stn1* mutants. (A) TRF analysis of *ctc1* crosses to *atr* and *atm* (lanes 1-9) and *stn1* crosses to *atm* (lanes 10-16) and *atr* (lanes 17-24). (B) PETRA results for the 2R telomere in *ctc1 atr* mutants and the 3L telomere in *stn1 atr* mutants. (C) Quantification of telomere lengths from *ctc1 atr* PETRA analysis shown in panel B. Telomere length was calculated by subtracting the distance of the subtelomeric primer binding site relative to start of the telomere repeat array from the PETRA value. For all genotypes, n=4. (D) Parent-progeny PETRA analysis of telomeres in G1 and G2 *ctc1 atr* mutants. Asterisk indicates interstitial telomeric repeats used as a loading control.

signals trailing below 1kb (peak = 1.5kb). Similar findings were obtained with G1 *stn1 atm* (Fig. 3-3A, lanes 13-16) and G1 *stn1 atr* mutants (Fig. 3-3A, lanes 19-20 and 23-24).

Primer Extension Telomere Repeat Amplification (PETRA) was employed to precisely measure telomere length on individual chromosome arms. In this assay, wild type telomeres range from 2-5kb and typically appear as one to three bands depending on the chromosome arm (Fig. 3-3B) (Heacock *et al.*, 2004). As with bulk telomere analysis, PETRA showed that the telomere profiles of *atr* (Fig. 3-3B) and *atm* (Fig. 3-4A and B) were similar to wild type, whereas telomeres from G1 *ctc1* and G1 *stn1* migrated as a broad smear ranging from 1.5-4kb (Fig. 3-3B). PETRA confirmed that telomere tracts were similar in G1 *ctc1* and G1 *ctc1 atm* mutants (Fig. 3-4A). In contrast, telomeres in G1 *ctc1 atr* mutants were shorter by an average of 300 bp compared to G1 *ctc1* mutants (Fig. 3-3B and C). The same result was obtained for *stn1* mutants in both *atm* (Fig. 3-4B) and *atr* (Fig. 3-3B) deficient backgrounds. Hence, ATR, but not ATM, contributes to telomere length maintenance when CST is compromised.

We examined the status of the G-overhang in G1 *ctc1 atr* mutants using in-gel hybridization. This assay detects single-stranded G-rich telomeric DNA either at the extreme chromosome terminus or within the double-stranded telomere region, if gaps are present in the C-strand. As previously reported (Surovtseva et al. 2009), *ctc1* single mutants showed enhanced G-overhang

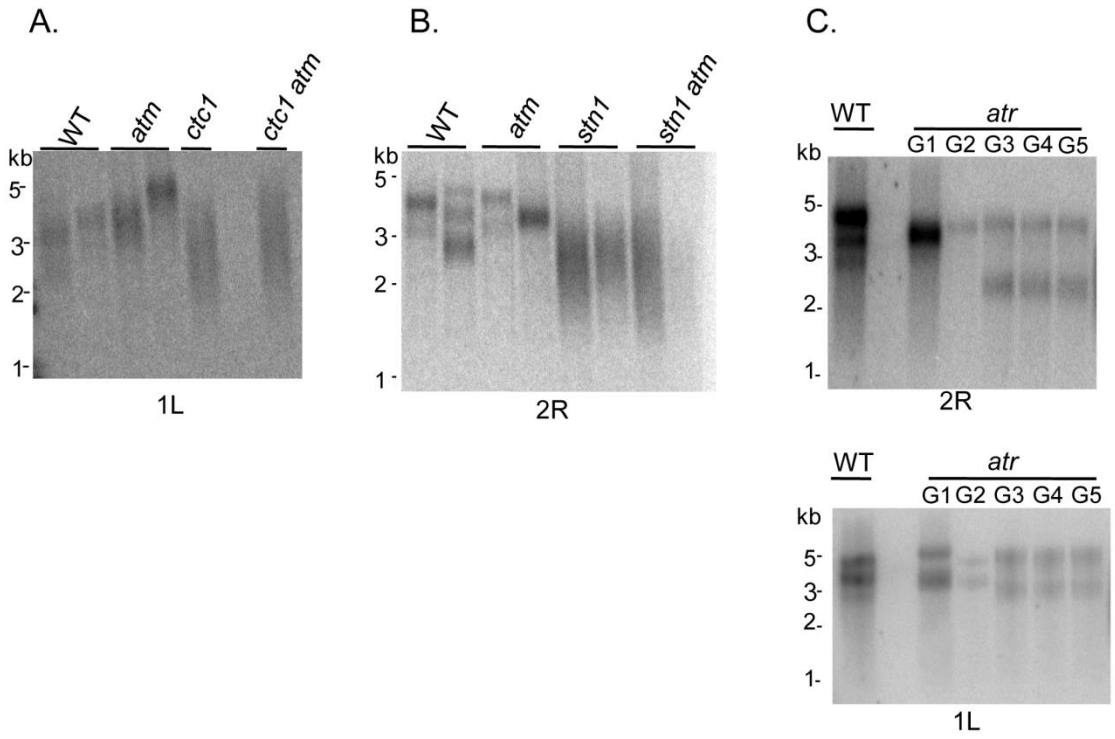


Figure 3-4. Loss of ATM does not affect telomere shortening in *ctc1* or *stn1* mutants. PETRA results for *ctc1 atm* (A) and *stn1 atm* (B) mutants are shown. (C) PETRA results for five generations of *atr* mutants. Telomere tracts are maintained in the wild type range.

signals, three- to six- fold greater than wild type (Fig. 3-5). G-overhang status was wild type in *atr* mutants. Furthermore, the loss of ATR did not exacerbate the G-overhang phenotype in *ctc1* mutants (Fig. 3-5). We conclude that ATR does not play a significant role in G-overhang maintenance, and further that *ctc1 atr* mutants do not carry extensive sections of incompletely replicated telomeric C-strand DNA.

Since G2 *ctc1 atr* mutants have much more severe morphological defects than G1 *ctc1 atr* (Fig. 3-1D and E), we were prompted to examine telomere length in G2 double mutants using PETRA. Telomere tracts in G2 *ctc1 atr* were much shorter (up to 1kb) than their G1 parents (Fig. 3-3D). This attrition is more than three times greater than the telomere shortening in G1 *ctc1 atr* mutants versus their *ctc1* siblings (300 bp) (Fig. 3-3A-C), and more than two times higher than G2 *stn1* mutants versus their G1 parent (~400 bp)(data not shown). In conjunction with telomere shortening, the profile of telomere fragments switched from heterogeneous, smeary bands in the G1 *ctc1 atr* parents to very homogenous, sharp bands in the G2 *ctc1 atr* offspring (Fig. 3-3D). PETRA assays conducted with five generations of *atr* mutants revealed no change in telomere length (Fig. 3-4C), confirming that the telomere maintenance defect in *ctc1 atr* mutants reflects a synergistic effect of both ATR and CST dysfunction. These data further indicate that ATR contributes to telomere maintenance in a biphasic manner. In the first generation of a CST deficiency, ATR makes a modest contribution to telomere

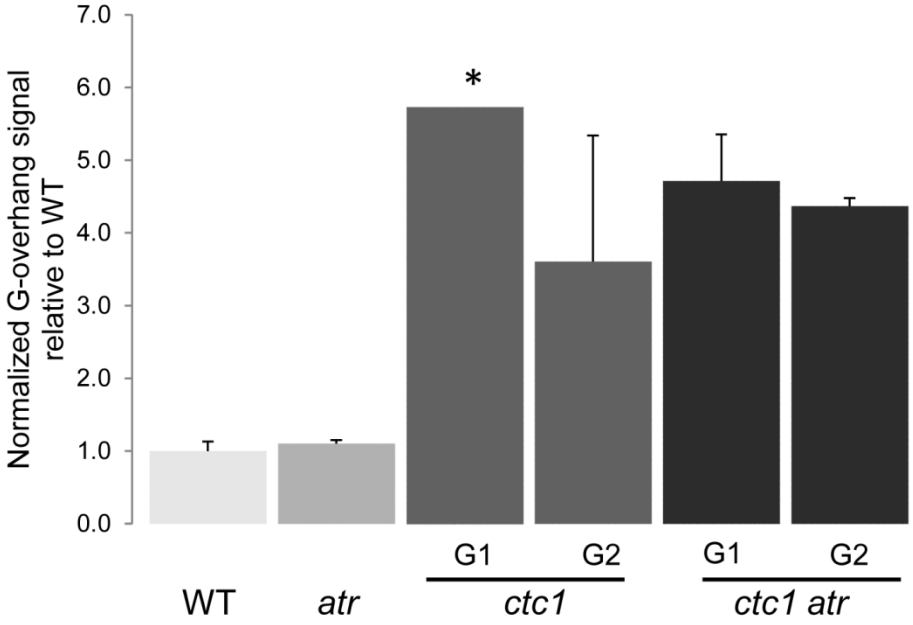


Figure 3-5. Loss of ATR does not alter the status of the 3' G-overhang. G-overhang signals were measured by in-gel hybridization with a (CCCTAAA)3 probe. G1 plants were studied unless otherwise indicated. * n=2 for all genotypes except G1 *ctc1*, n=1. These data are consistent with prior G-overhang analysis for *ctc1* mutants (Surovtseva et al. 2009). Error bars represent standard deviation.
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maintenance. However, the prolonged absence of ATR in plants lacking CST leads to a much more dramatic loss of telomeric DNA.

Inactivation of ATR down-regulates telomerase enzyme activity

A profile of shorter, more homogeneous telomere tracts is consistent with a defect in telomerase-mediated telomere maintenance (Riha et al. 2001; Kannan et al. 2008). Thus, one explanation for the enhanced rate of telomere loss in G2 *ctc1 atr* mutants is that telomerase can no longer act on dysfunctional chromosome ends. To investigate this possibility, we used Quantitative Telomere Repeat Amplification Protocol (Q-TRAP) to measure telomerase enzyme activity levels in consecutive generations of *ctc1 atr* mutants. As expected (Song et al. 2008; Surovtseva et al. 2009), telomerase activity was robust in G1 and G2 *ctc1* and *stn1* seedlings, and indistinguishable from wild type samples (Fig. 3-6). Wild type levels of telomerase activity were also detected in G1 *atr* mutants. Unexpectedly, however, telomerase activity declined by ~15-fold in G2 *atr* mutants (Fig. 3-6). This decrease persisted in subsequent plant generations with G4 *atr* mutants also exhibiting dramatically reduced enzyme activity. The reduction in telomerase activity was not confined to a specific developmental stage; Q-TRAP data obtained from both seedlings and flowers gave similar results (Fig. 3-6). Notably, Q-TRAP revealed the same level of enzyme activity in G1 *ctc1 atr* mutants as in wild type plants, and enzyme activity in G2 *ctc1 atr* decreased by the same amount as in G2 *atr*

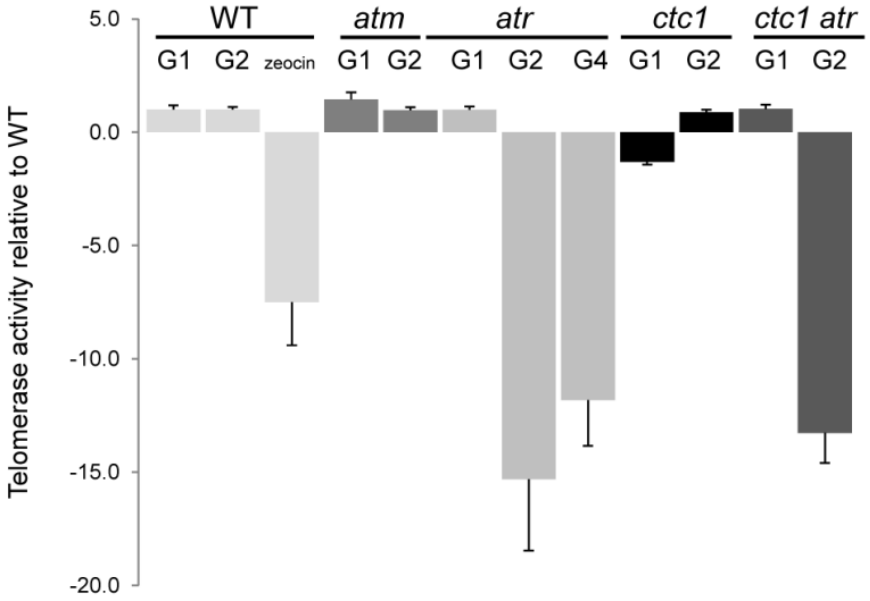


Figure 3-6. ATR stimulates telomerase activity. Quantitative TRAP results for first (G1), second (G2) and fourth (G4) generation mutants of different genotypes are shown. Q-TRAP was also performed on wild type seedlings treated with 20µM zeocin for 3 days. All samples were from flowers except G2 *atr,* G2 *ctc1*, and G2 *ctc1 atr*, which were from seedlings. Telomerase activity is plotted relative to wild type. For zeocin-treated seedlings, telomerase activity is relative to untreated wild type seedlings. Error bars represent standard deviation. n=2 for all genotypes except G1 WT n=5, zeocin-treated WT n=6, G1 *ctc1* n=4, G2 *atr* n=3, and G4 *atr* n=4.

(Fig. 3-6). Hence, loss of ATR, and not CTC1, leads to decreased telomerase activity.

In yeast and vertebrates, disruption of ATR causes genome wide replicative stress (Nam and Cortez 2011), suggesting that the stimulus for reduced telomerase activity in G2 *atr* mutants might be accumulating genome damage. To investigate whether genotoxic stress triggers a decrease in telomerase activity, wild type seedlings were treated with zeocin, which induces double-strand breaks. Q-TRAP revealed ~7.5-fold reduction in telomerase in treated seedlings versus controls (Fig. 3-6). This observation suggests that the repression of telomerase activity in G2 *atr* mutants may reflect the activation of a DDR triggered by replicative stress. Altogether, these results show that the dramatic loss of telomeric DNA in G2 *ctc1 atr* mutants correlates with an abrupt decline in telomerase enzyme activity.

ATR suppresses the formation of end-to-end chromosome fusions in CST mutants

Catastrophic loss of telomeric DNA in *ctc1* and *stn1* mutants coincides with the onset of telomere fusions (Song et al. 2008; Surovtseva et al. 2009). Dysfunctional telomeres are recruited into chromosome fusions through the non-homologous end-joining (NHEJ) pathway, which is activated by ATM and indirectly by ATR (Denchi and de Lange 2007; Deng et al. 2009a). Therefore, we asked if the accelerated telomere shortening in plants lacking CST and ATR

correlates with an increased incidence of telomere fusions using telomere fusion PCR (TF-PCR). TF-PCR employs primers specific to unique subtelomeric sequences on each chromosome arm to amplify junctions of covalently fused telomeres. For these studies, DNA from mature G1 mutants was analyzed. As expected, telomere fusions were not observed in wild type, *atr* (Fig. 3-7B and D) or *atm* (Fig. 3-7A and C) mutants. In contrast, massive chromosome end-joining events, represented by abundant heterogeneous smears, were associated with the loss of CTC1 (Fig. 3-7A and B) or STN1 (Fig. 3-7C-D). When either ATR (Fig. 3-7B and D) or ATM (Fig. 3-7A and C) was absent in *ctc1* or *stn1* mutants, TF-PCR products were still detected.

TF-PCR provides an indication of whether telomeres are prone to end-joining reactions, but it does not give quantitative information about the number of chromosome fusions. To obtain a quantitative assessment of telomere joining events, we monitored the incidence of anaphase bridges in mitotically dividing cells using conventional cytology (Fig. 3-8A). As described previously (Song et al. 2008; Surovtseva et al. 2009), bridged chromosomes were detected in the floral pistils of G1 *ctc1* and *stn1* mutants (23% and 21% of all anaphases, respectively), compared to few or none in wild type, *atr*, and *atm* mutants (Fig. 3-8B). The loss of ATM did not alter the percentage of anaphase bridges in *stn1* mutants. Conversely, there was a dramatic increase in the incidence of anaphase bridges in G1 *stn1 atr* (57%) and G1 *ctc1 atr* (53%) relative to *stn1*

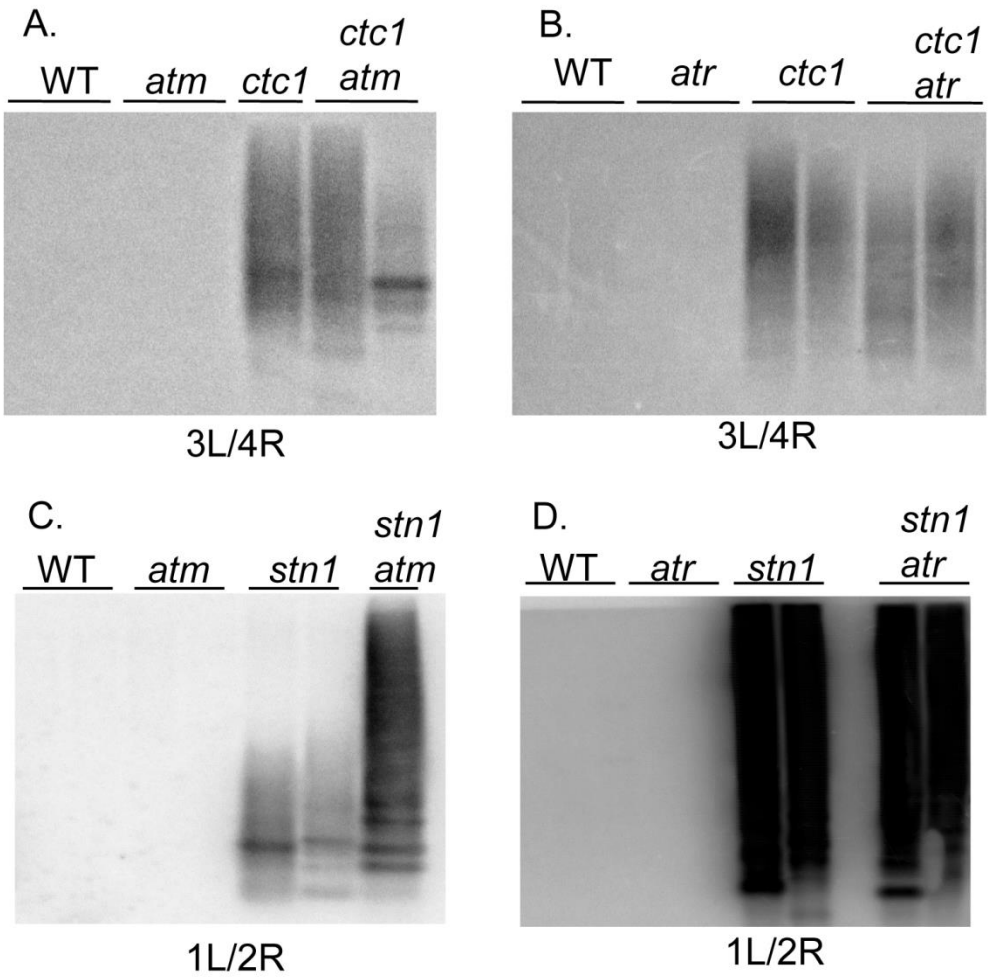
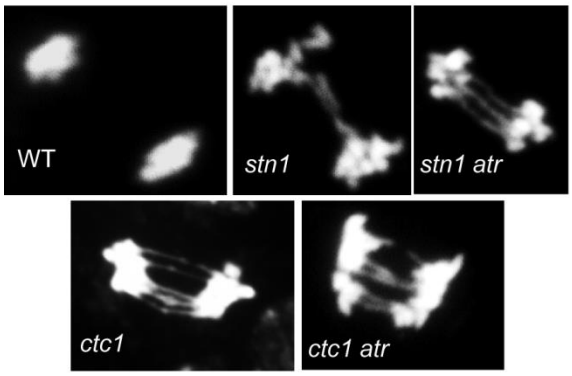


Figure 3-7. TF-PCR results for *ctc1* and *stn1* mutants lacking either ATM or ATR. Telomere fusion products in *ctc1* (panels A and B) or *stn1* (panels C and D) were amplified using primers directed at the chromosome arms indicated below each blot.

A.



B.

	Anaphases		
Genotype	**Total**	**Bridges**	**Ratio %**
WT	69	0	0%
stn1	369	76	21%
ctc1	166	38	23%
atm	202	0	0%
atr	454	6	1%
stn1 atm	137	27	20%
stn1 atr	501	288	57%
ctc1 atr	234	125	53%
stn1 atm atr	323	225	70%

Figure 3-8. End-to-end chromosome fusions increase in plants lacking CST and ATR. (A) Cytology of anaphases from pistils from G1 plants of the genotypes indicated. Spreads are stained with DAPI. (B) Quantification of anaphase bridges from cytology in (A).

and *ctc1* (Fig. 3-8B). Remarkably, 70% of anaphases in the triple G1 *stn1 atr atm* mutants contained bridged chromosomes (Fig. 3-8B). Thus, an ATR- and ATM-independent mechanism can promote fusion of dysfunctional telomeres. The increased incidence of chromosome bridges suggests that ATR inhibits telomere fusion in CST mutants.

ATR attenuates the transcriptional response to DNA damage in plants lacking CTC1

The role of ATR in repressing telomere fusions together with the accelerated telomere shortening, and morphological disruptions in CST mutants argues that loss of CST triggers an ATR-mediated DDR. To investigate this possibility, we monitored the expression of several transcripts implicated in DDR (*RAD51*, *BRCA1* (*BREAST CANCER SUSCEPTIBILITY* 1) and *PARP1* (*Poly [ADP-ribose] polymerase 1*)) (Doucet-Chabeaud et al. 2001; Lafarge and Montané 2003; Yoshiyama et al. 2009). Quantitative RT-PCR was performed using cDNA made from first generation (G1) *ctc1* flowers. Expression of both *PARP1* and *BRCA1* was significantly up-regulated in *ctc1* mutants compared to wild type (3.7- and 1.9-fold, respectively) (Fig. 3-9). In addition, *RAD51* expression was 1.5 times higher in *ctc1* mutants (Fig. 3-9), but the difference was not statistically significant. These results suggest that the CST complex protects against a DDR.

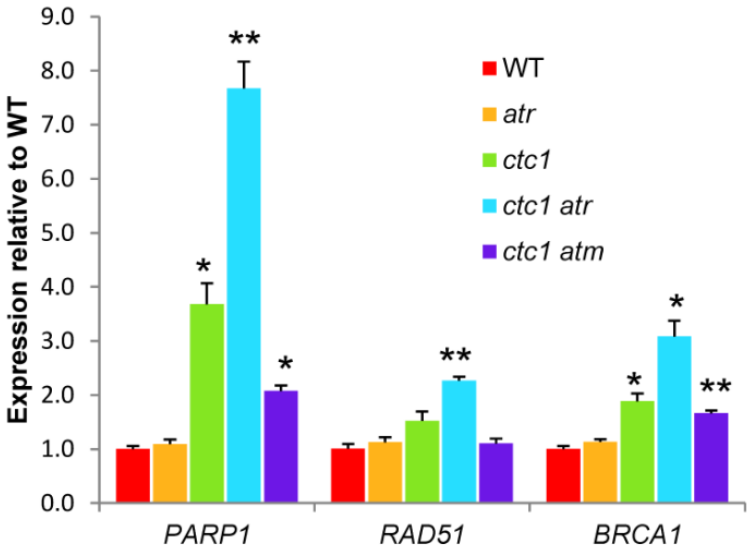


Figure 3-9. Loss of CTC1 activates an ATR-dependent transcriptional response. qRT-PCR results are shown for the DDR transcripts *PARP1*, *BRCA1*, and *RAD51* in floral organs. Expression levels are relative to wild type, and data for first generation (G1) mutants are shown. For each genotype, n=3, except for *ctc1 atm*, n=2. Single asterisk denotes a p-value <0.05 relative to wildtype; two asterisks denote a p-value<0.005 relative to wildtype (Student's T-test). Error bars represent S.E.M.

We next asked if ATM or ATR are necessary to initiate a transcriptional response in plants lacking CST, since in *Arabidopsis*, the response to double-strand breaks is mostly mediated by ATM, but ATR is also required (Friesner et al. 2005). In *ctc1 atm* mutants, *PARP1* and *BRCA1* transcripts were above wild type levels (2.1 and 1.7 times wild type, respectively), but were slightly less abundant than in *ctc1* mutants. This finding suggests that ATM contributes to the activation of a DNA repair transcriptional program in *ctc1* mutants. A more dramatic change in transcript level was observed in plants doubly deficient in CTC1 and ATR. Expression of all three DDR genes was significantly elevated in *ctc1 atr* mutants relative to wild type, *atr* or *ctc1* (Fig. 3-9). Compared to wild type, *ctc1 atr* mutants showed a 7.7 fold increase in *PARP1* expression, a 2.3-fold increase in RAD51, and a 3.1-fold increase in BRCA1. Thus, ATR curbs the transcriptional response to loss of CTC1. This observation is consistent with ATR-mediated suppression of chromosome fusions.

ATR promotes programmed cell death in ctc1 *mutants*

ATR is implicated in programmed cell death signaling in *Arabidopsis* (Fulcher and Sablowski 2009; Furukawa et al. 2010). To further explore the role of ATR in plants lacking CST, we monitored stem cell viability in root apical meristems (RAM) of seedlings using propidium iodide (PI) staining (Fig.3-10A). PI is a membrane-impermeable dye that is excluded from live cells. In dead cells, PI passes through the cell membrane and binds nucleic acids. The limited

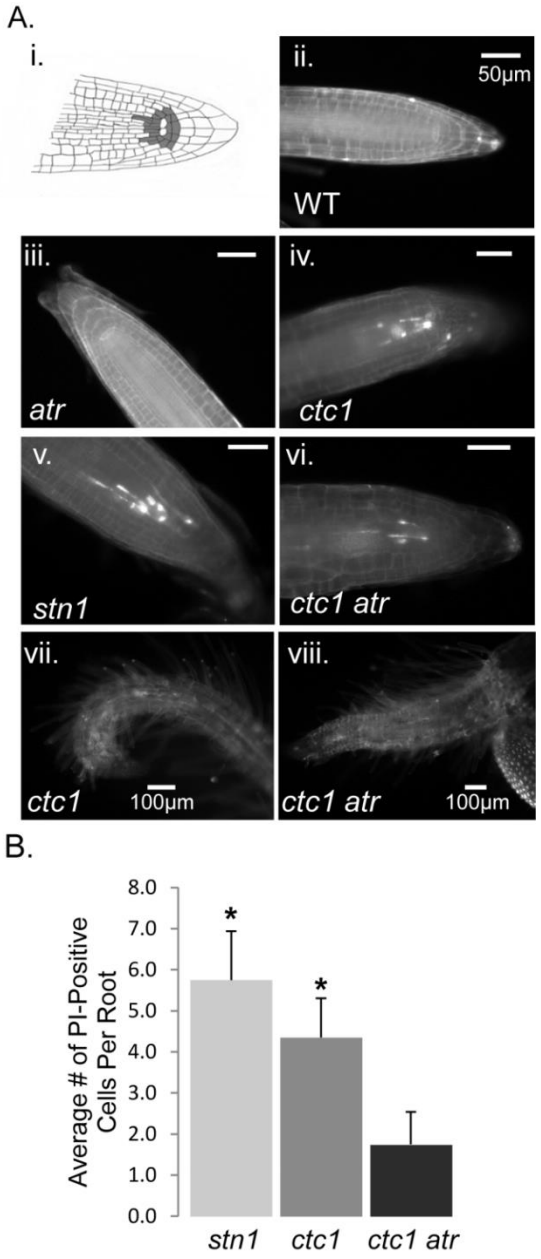


Figure 3-10. ATR activates programmed cell death of the root apical meristem (RAM) of *ctc1* mutants. (A) Representative images of G2 seedling root tips stained with propidium iodide (PI). (i) Diagram of a root tip. Stem cells and adjacent daughter cells are shaded gray. White cells in the RAM center are quiescent center cells. WT (ii) and *atr* (iii) roots are PI-negative, but the RAM of *ctc1* (iv) and *stn1* (v) mutants have numerous PI-positive (dead) cells. (vi) Fewer PI-positive cells are present in *ctc1 atr* mutants. (vi-vii) A subset of *ctc1* or *stn1* roots were PI-negative, but displayed severe morphological defects. (B) Quantification of PI-positive cells in different genetic backgrounds. The average number of PI-positive cells per root tip is shown. *stn1* (n= 12); *ctc1* (n=17); *ctc1 atr* (n=12). Asterisk denotes a p-value <0.05 (Student's T-test). Error bars represent S.E.M.

biomass of young seedlings precluded genotyping to identify G1 double mutants so early in their development. Therefore, we examined the RAM in their progeny, G2 *ctc1 atr* mutants. As expected, PI staining was not associated with the RAM in wild type seedlings (Fig. 3-10A, panel ii). Similarly, G2 *atr* seedlings showed no PI staining (Fig. 3-10A, panel iii). In contrast, strong PI staining was observed in G2 *ctc1* RAM (Fig. 3-10A, panel iv) or G2 *stn1* RAM (Fig. 3-10A, panel v), consistent with activation of a robust DDR. We next asked if ATR is responsible for cell death in CST mutants (Fig. 3-10A, panel vi). Strikingly, the number of PI positive cells in G2 *ctc1 atr* dropped to an average 1.75 cells/root compared to 5.75 and 4.35 cells/root for *stn1* and *ctc1*, respectively (Fig. 3-10A, panel vi and Fig. 3-10B). A subset of mutant seedlings (25% in *stn1,* 35% in *ctc1*, and 67% in *ctc1 atr*) had no PI-positive cells. The short roots from these plants had a high density of root hairs and no obvious RAM (Fig. 3-10A, panels vii-viii). We speculate that in such plants, epithelial precursor cells may be able to differentiate, but other cell types have been eliminated from the RAM or have differentiated inappropriately. These mutant roots are remarkably similar to gamma-irradiated *lig4* roots, where RAM cells are arrested (Hefner et al. 2006). Taken together, these data indicate that ATR activation leads to programmed cell death in plants lacking CST. Further, we speculate that the decrease in PCD in *ctc1 atr* mutants leads to an accumulation of cells exhibiting DDR and increased numbers of end-to-end chromosome fusions.

Discussion

CST protects telomeres from activating ATR

A key function of intact telomeres is to prevent the chromosome terminus from eliciting a cellular DDR that leads to end-to-end chromosome fusions and genome wide instability. Here we show that the *Arabidopsis* CST prohibits the activation of ATR-mediated DDR. We find that the absence of CTC1 results in elevated levels of DDR transcript expression and programmed cell death in the RAM. The sacrifice of stem cells by programmed cell death is a common response to DNA damage in plants (Fulcher and Sablowski 2009; Furukawa et al. 2010), and has obvious benefits for organismal viability. Several observations support the idea that ATR-mediated programmed cell death reduces genome instability in CST mutants. First, expression of DDR transcripts increases in *ctc1 atr* mutants compared to *ctc1* mutants. Second, the incidence of chromosome fusions increases in *ctc1 atr* mutants. Finally, plants lacking core components of CST display severe morphological abnormalities as a consequence of profound genome instability (Song et al. 2008; Surovtseva et al. 2009), and these phenotypes are largely rescued by a deficiency in ATR, but not ATM. The rescue is only temporary, however, and in the next generation (G2), *ctc1 atr* mutants suffer even more devastating developmental defects than G2 *ctc1* single mutants. This observation is consistent with checkpoint bypass, resulting in the accumulation of DNA damage when ATR is lost in *ctc1* mutants. We postulate that the failure to initiate programmed cell death allows *ctc1 atr*

cells with dysfunctional telomeres to continue cycling until rampant genome instability leads to developmental arrest (Fig. 3-11B).

While this manuscript was under review, Amiard *et al.* published a study that verifies and complements our findings concerning the role of CST in suppressing an ATR-mediated DDR (Amiard et al. 2011). These authors show an ATR-dependent induction of γH2AX at telomeres in *Arabidopsis ctc1* mutants, consistent with our transcriptional data showing induction of DDR transcripts in response to loss of CTC1. Amiard and colleagues also demonstrate that ATR and ATM repress formation of anaphase bridges and promote PCD in *ctc1* mutants. They conclude, as do we, that ATR maintains genome stability in CST mutants (Amiard et al. 2011).

Together, these *Arabidopsis* studies highlight the complexity of the DDR in plants and show that multiple, overlapping mechanisms are harnessed to detect and to process dysfunctional telomeres. For example, the increased incidence of telomere fusions in plants lacking CST and ATR could reflect survival of cells with profound telomere dysfunction due to checkpoint bypass, as well as a contribution of ATR in facilitating maintenance of short telomeres (see below). Notably, telomere fusions accumulate even in the absence of both ATM and ATR when CST is compromised (Amiard et al. 2011). A third PIKK family member in vertebrates, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), functions in non-homologous end-joining (NHEJ) (Lieber et al. 2003) and could potentially serve as back-up mechanism to trigger telomere

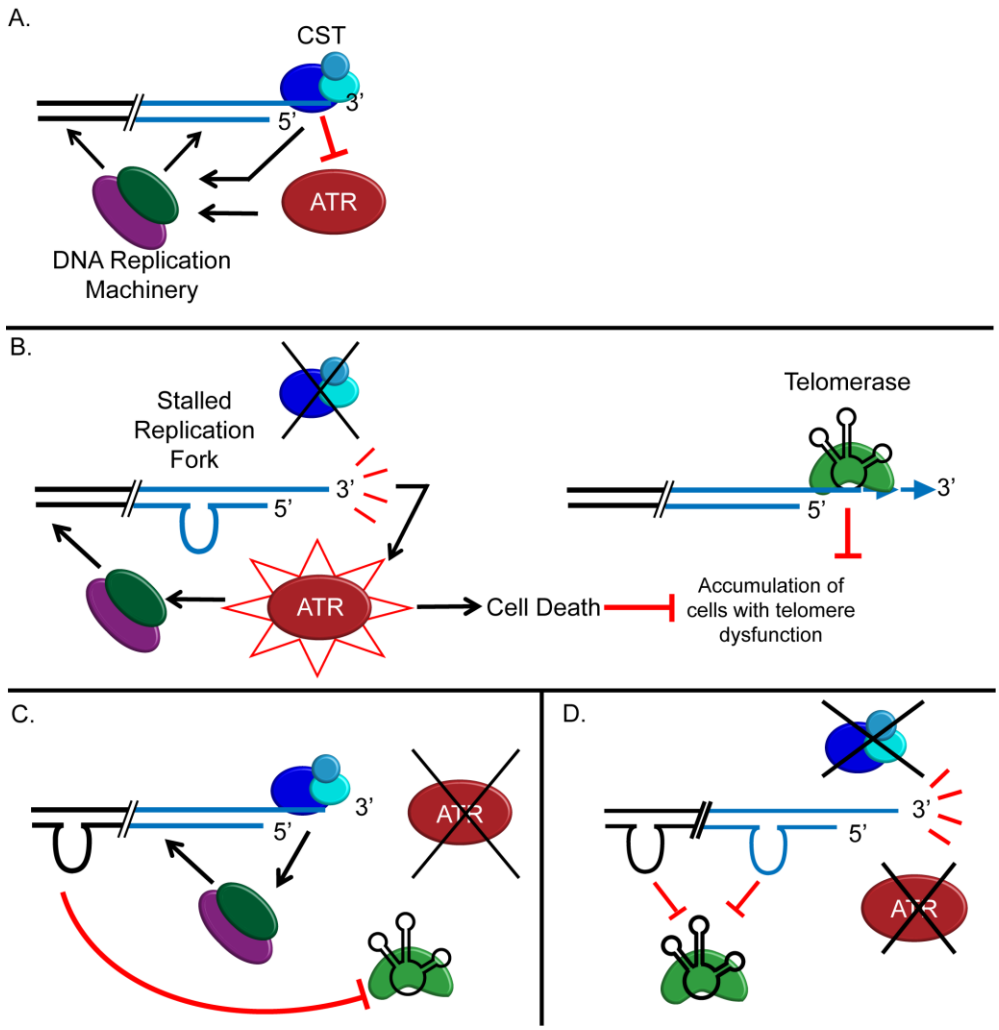


Figure 3-11. Model depicting CST and ATR cooperation in maintaining telomeric DNA and genome integrity in *Arabidopsis*. (A) In wild type plants, CST interacts with the 3' overhang to protect the chromosome terminus from telomere shortening, end-to-end chromosome fusions (Song *et al.*, 2008; Surovtseva *et al.*, 2009) and activation of ATR-dependent DDR (this study). ATR facilitates replication fork progression. Similarly, CST is thought to stimulate replication fork restart within the telomeric duplex via interaction with DNA polymerase alpha (Price et al. 2010; Nakaoka et al. 2012) Telomeric DNA is represented by blue lines. (B) Plants lacking CST activate ATR-dependent DDR, initiating programmed cell death in stem cell niches. Replication fork progression is perturbed in the telomeric duplex, contributing to the loss of telomeric DNA. Telomerase action delays the onset of complete telomere failure. (C) Accumulating replicative stress in *atr* mutants triggers an ATR-independent DDR that results in telomerase inhibition. Telomeres in the wild type size range can be maintained. (D) Catastrophic telomere shortening occurs in plants lacking both CST and ATR due incomplete replication of the duplex and failure of telomerase to act on critically shortened telomeres. See text for details.

fusion. Plants lack an obvious DNA-PKcs ortholog, and thus the ATR/ATM independent response elicited by telomere dysfunction is unknown. Further complicating matters, uncapped telomeres engage both canonical and non-canonical DNA repair pathways in *Arabidopsis*. Critically shortened telomeres fuse in the absence of two core NHEJ repair proteins, Ku70 and Ligase IV (Heacock et al. 2007), and in plants lacking Ku as well as Mre11 (Heacock et al. 2004). In humans, an alternative end-joining pathway, which employs PARP1 and DNA ligase III, is activated if the canonical DNA-PKcs/Ku pathway is non-functional (Audebert et al. 2004; Wang et al. 2006). It is unknown if PARP1 plays a similar role in plants, but it is an intriguing possibility given the induction of *PARP1* expression in *ctc1* and *ctc1 atr* mutants (Fig. 3-9).

Cooperation of CST and ATR in telomere maintenance

Figure 3-11 presents a model summarizing the multifunctional roles of ATR at *Arabidopsis* telomeres. The data presented here showing a central role for ATR in the response to CST abrogation provides additional support for the proposal that CST binds single-stranded DNA at the chromosome terminus in multicellular organisms (Miyake et al. 2009; Surovtseva et al. 2009) (Fig. 3-11A). While our findings do not specifically address whether CST directly contacts the G-overhang, they are consistent with this conclusion and with the current model that single-strand telomere binding proteins protect the chromosome terminus by

excluding RPA from the G-overhang (Gong and de Lange 2010; Flynn et al. 2011).

Our results show that CST and ATR cooperate in the maintenance of telomeric DNA. We found that inactivation of ATR, but not ATM, accelerates the attrition of telomeric DNA at telomeres lacking CST. Multi-generational analysis of *ctc1 atr* mutants demonstrated that ATR makes a biphasic contribution to telomere length homeostasis. Our data indicate that in the first generation of a CST deficiency, the role of ATR is relatively minor. Telomeres are ~300bp shorter in *ctc1 atr* mutants than when ATR is intact. However, in the next generation, telomere shortening is much more aggressive, and up to 1kb more telomeric DNA is lost. We hypothesize that this biphasic response reflects two distinct contributions of ATR in promoting telomere maintenance (Fig. 3-11B and C).

Emerging data indicate that ATR and CST cooperate to facilitate DNA replication through the telomeric duplex (Price et al. 2010) (J. Stewart and C. Price, personal communication). ATR is activated in response to replication fork stalling (Verdun et al. 2005; Miller et al. 2006), and specifically suppresses telomere fragility derived from incomplete replication (Martínez et al. 2009; Sfeir et al. 2009; McNees et al. 2010). Notably, mammalian chromosomes depleted of CTC1 or STN1 display multiple telomere signals, consistent with telomere fragile sites (Price et al. 2010). CST is proposed to participate in replication fork restart via its interaction with DNA polymerase-alpha (Casteel et al. 2009; Price

et al. 2010). Consistent with this model, *Xenopus* CST is required for priming replication of ssDNA (Nakaoka et al. 2012). Altogether these findings indicate CST and ATR cooperate in relieving replicative stress within the telomere duplex (Fig. 3-11B and C). When both CST and ATR are compromised, replication fork stalling is increased (Fig. 3-11D), triggering double-strand breaks, and in turn, deletion of telomeric DNA.

Replicative stress may account for the modest increase in telomere shortening in G1 *ctc1 atr* mutants. While the extent to which ATR and CST modulate replication of the telomeric duplex in plants is unknown, preliminary data suggest that the contribution of these two components could be less significant in plants than in vertebrates. In human cells lacking CST, a small fraction of G-rich telomeric single-stranded DNA signal is resistant to exonuclease treatment (Surovtseva et al., 2009; Miyake *et al.*, 2009), consistent with incomplete replication of internal telomeric DNA tracts. Parallel analysis in Arabidopsis failed to detect exonuclease-resistant G-rich single-stranded DNA (Surovtseva et al. 2009), suggesting that CST acts primarily at the extreme chromosome terminus. We also found no increase in G-rich single-stranded DNA in *ctc1 atr* mutants relative to *ctc1*, implying that loss of ATR does not trigger massive replication fork stalling in CST mutants.

Telomerase and ATR

What accounts for the abrupt and dramatic loss of telomeric DNA in G2 *ctc1 atr* mutants? We propose that this delayed response reflects telomerase inhibition triggered by prolonged ATR inactivation. Depletion of ATR in mice leads to extensive chromosome fragmentation and a null mutation is embryonic lethal (Brown and Baltimore 2000; de Klein et al. 2000). In contrast, plants lacking ATR are viable, fully fertile and morphologically wild type (Culligan et al. 2004). Although no overt genome instability is associated with ATR depletion in *Arabidopsis*, we speculate that accumulating replicative stress elicits a hitherto unrecognized DDR, one consequence of which is telomerase repression (Fig. 3-11C). In support of this hypothesis, we showed that the genotoxin zeocin inhibits telomerase activity in wild type seedlings. Strikingly, telomerase activity is unaffected in plants lacking CST, indicating that telomere dysfunction does not inhibit telomerase. Sustained repeat incorporation onto compromised chromosome ends would be advantageous if it delays the onset of complete telomere dysfunction. Notably, *ctc1 tert* telomeres shorten more rapidly than in either single mutant background (K. Boltz and D. Shippen, unpublished data), arguing that telomerase continues to act on telomeres in the absence of CST.

Although the molecular basis for this ATR-independent pathway of DNA damage-induced telomerase repression is unknown, such a response reduces the potential for telomerase to act at sites of DNA damage, thereby limiting the chance of inappropriate telomere formation. A variety of mechanisms have

been reported in yeast and vertebrates to restrain telomerase action following genotoxic stress (Schulz and Zakian 1994; Kharbanda et al. 2000; Wong et al. 2002; Makovets and Blackburn 2009). The extent to which all of these pathways are conserved bears further investigation.

Finally, it is curious that despite the strong inhibition of telomerase in plants lacking ATR, telomere length homeostasis is unperturbed in the five generations of mutants we monitored (Vespa et al. 2005); this study). One possibility is that DNA damage triggers a qualitative change in telomerase behavior, which is detected in our Q-TRAP assay as a quantitative change in activity. Repeat addition processivity (RAP) is not a property of *Arabidopsis* telomerase that can be assessed in our PCR-based TRAP assay. However, RAP of telomerase influences, and is influenced by, telomere length (Lue 2004). Telomerase RAP is dramatically altered in human cancer cells depending upon whether telomeres are within the normal range, or are artificially shortened (Zhao et al. 2011). Likewise, the RAP of yeast telomerase is enhanced at critically shortened telomeres in an ATM-dependent manner (Chang et al. 2007). Thus, it is conceivable that a crippled telomerase in *atr* mutants is sufficient to maintain telomeres already in the wild type range, but lacks the capacity to act efficiently on critically shortened telomeres in *ctc1* mutants, thereby enhancing the pace of telomere attrition.

CHAPTER IV

AN ALTERNATIVE TELOMERASE RNA IN *Arabidopsis* MODULATES ENZYME ACTIVITY IN RESPONSE TO DNA DAMAGE*

Summary

Telomerase replenishes telomere tracts by reiteratively copying its RNA template, TER. Unlike other model organisms, Arabidopsis thaliana harbors two divergent TER genes. However, only TER1 is required for telomere maintenance. Here we examine the function of TER2. We show that TER2 is spliced and its 3' end truncated *in vivo* to generate a third TER isoform, $TER2_S$. TERT preferentially associates with TER2 >TER1>$TER2_S$. Moreover, TER2 and $TER2_S$ assemble with Ku and POT1b, forming RNP complexes distinct from TER1 RNP. Plants null for TER2 display increased telomerase enzyme activity, while TER2 over-expression inhibits telomere synthesis from TER1 and leads to telomere shortening. These finding argue that TER2 negatively regulates telomerase by sequestering TERT in a non-productive RNP complex. Introduction of DNA double-strand breaks by zeocin leads to an immediate and specific spike in TER2 and a concomitant decrease in telomerase enzyme activity. This response is not triggered by replication stress or telomere

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dysfunction, and is abrogated in ter2 mutants. We conclude that Arabidopsis telomerase is modulated by TER2, a novel DNA damage-induced non-coding RNA that works in concert with the canonical TER to promote genome integrity.

Introduction

Chromosomes must be capped with an ample reserve of telomeric DNA to ensure genome stability. The telomerase reverse transcriptase facilitates telomere homeostasis using its catalytic subunit TERT to reiteratively copy the internal RNA template TER, thereby replenishing terminal sequences lost during DNA replication. Telomerase is a highly regulated enzyme; its action is largely confined to, and essential for, self-renewing cell populations. Inappropriate telomerase expression promotes tumorigenesis, while insufficient enzyme activity triggers genome instability and stem cell-related disease (Artandi and DePinho 2010). Consequently, sophisticated mechanisms have evolved to modulate telomerase activity.

Although TERT is a highly conserved constituent of telomerase, TER subunits have diverged dramatically in length and nucleotide sequence (Egan and Collins 2012). Nevertheless, phylogenetic and mutational analysis reveal functionally conserved elements, including a single-strand templating domain typically corresponding to one and a half telomeric repeats flanked by a 5' boundary element and a 3' pseudoknot domain. Human telomerase activity can be reconstituted with only the TER pseudoknot/template region and the

CR4/CR5 trans-activation domain (Tesmer et al. 1999; Mitchell and Collins 2000). Similarly, yeast and *Arabidopsis* telomerase activity is supported by a "Mini T" version of TER consisting of ~150 nts (Zappulla et al. 2005; Cifuentes-Rojas et al. 2011). Thus, TER is postulated to act as scaffold for telomerase accessory proteins. Such proteins facilitate RNP biogenesis, trafficking and interactions with the chromosome terminus (Egan and Collins 2012). Vertebrate TERs associate with the RNP maturation complex dyskerin, while budding yeast TER assembles with Sm proteins and is processed as an snRNA. Notably, biogenesis of *Schizosaccharomyces pombe* TER involves a novel 3' end "slicing" mechanism (Leonardi et al. 2008) that requires the sequential binding of SM and Lsm complexes (Tang et al. 2012). Other TER binding factors include the KU70/80 heterodimer, which in *Saccharomyces cerevisiae* acts as a positive regulator of telomerase (Boulton and Jackson 1998).

Much of what is known concerning telomerase regulation centers on enzyme activation. In human cells transcriptional regulation of the catalytic subunit TERT is a major point of control, although alternative splicing, post-translational modification and intracellular trafficking of TERT also contribute to enzyme regulation (Cifuentes-Rojas and Shippen 2012). Increased expression of TER is correlated with enzyme activation in some settings, but evidence that TER plays a significant role in modulating enzyme activity is currently lacking.

In conjunction with tight regulation of telomerase activity at natural chromosome ends, the enzyme must also be strictly prohibited from acting at

double-strand breaks (DSBs) to ensure faithful repair, and to prevent "chromosome healing" by *de novo* telomere formation (DNTF). DNTF is a perilous endeavor due to loss of flanking DNA, and in humans is associated with genetic disorders such as α-thalassemia, mental retardation and cancer (Flint et al. 1994; Hanish et al. 1994). A variety of different strategies evolved to curtail telomerase interaction with DSBs. As part of the DNA damage response (DDR) in yeast, the telomere protein Cdc13 is phosphorylated by Mec1 (ATR), blocking its association with a DSB (Pennock et al. 2001) and the subsequent recruitment of telomerase (Zhang and Durocher 2010). Mec1 also stimulates the phosphorylation-dependent activation of Pif1 (Makovets and Blackburn 2009), a helicase that evicts telomerase particles engaged in synthesis by unwinding the TER-DNA hybrid (Boule et al. 2005). While similar mechanisms have not been described for multicellular eukaryotes, human TERT is phosphorylated by c-Abl in response to ionizing radiation, a modification that is associated with telomerase inhibition (Kharbanda et al. 2000). Ionizing radiation also triggers the transient sequestration of hTERT in the nucleolus, a response that would temporarily impede DNTF (Wong et al. 2002).

Plants control telomerase activity in a similar fashion as animals, repressing the enzyme in leaves where cell division is waning and increasing expression in seedlings, flowers and other cells with high proliferation potential (Fitzgerald et al, 1999). As in vertebrates, core components of *Arabidopsis thaliana* telomerase include TERT (Fitzgerald et al. 1999; Cifuentes-Rojas et al.

2011) and likely dyskerin (Kannan et al. 2008). However, *A. thaliana* is unique among model organisms studied to date as it encodes two telomerase RNA subunits, TER1 (748nt) and TER2 (784nt) (Cifuentes-Rojas et al. 2011). TER1 and TER2 share a 220nt highly conserved domain that in TER2 is divided into two segments interrupted by 529nt intervening sequence. Both TER1 and TER2 assemble with TERT to form an active enzyme *in vitro*, but only TER1 is required for telomere maintenance *in vivo* (Cifuentes-Rojas et al. 2011). A null mutation in the template domain of TER2 does not perturb telomere length homeostasis under standard growth conditions (Cifuentes-Rojas et al. 2011), and hence the function of this RNA has been unclear.

Another key component of the TER1 RNP is POT1a (Protection Of Telomeres), one of three POT1 paralogs in *A. thaliana* (Shakirov et al. 2005; Surovtseva et al. 2007; A. Nelson and D. Shippen, unpublished data). Vertebrate POT1 binds the 3' overhang on the chromosome terminus, thereby prohibiting DDR and the inappropriate enzymatic reactions triggered by it (Baumann and Price 2010). In contrast, *Arabidopsis* POT1a is a telomerase RNP constituent that contacts TER1 and acts in the same genetic pathway as TERT for telomere maintenance (Surovtseva et al. 2007; Cifuentes-Rojas et al. 2011). Over-expression studies suggest that POT1b contributes to telomere integrity (Shakirov et al. 2005), and yet like POT1a, POT1b does not interact with telomeric DNA *in vitro* (Shakirov et al. 2009). In addition, POT1b does not

bind TER1 (Cifuentes-Rojas et al. 2011). Thus, TER2 and POT1b do not promote the canonical role of telomerase in telomere maintenance.

Here we describe a new regulatory pathway for telomerase wherein TER2 inhibits telomere synthesis by TER1. Specifically, we show that *Arabidopsis* harbors three different isoforms of TER, including a processed RNA derived from TER2. We demonstrate that telomerase activity is elevated in the absence of TER2, and decreased when TER2 is over-expressed. Additionally, we show that TER2 and $TER2_S$ assemble with POT1b and Ku into alternative RNP complexes that cannot sustain telomere repeats on chromosome ends. Finally, we demonstrate telomerase activity is repressed in response to DSBs, and this regulation is dependent on TER2. We conclude that TER2 is a novel component of the DDR that modulates telomerase activity.

Materials and methods

Plant materials and growth conditions

Plants were grown on soil at 22°C under a 16h light/8h dark photoperiod. For experiments with seedlings, seeds were sterilized in 50% bleach with 0.1% Triton-X 100 and then plated on Murashige and Skoog (MS) medium with 0.7 – 0.8% agar. Plates were kept at 4°C for 2-4 days and then transferred to long day conditions. The MM2d suspension cell culture line (Menges and Murray 2002) was used for all cell culture experiments. Mutant *Arabidopsis thaliana*

lines were *ter2-1* and $TER1_{AS}$ (Cifuentes-Rojas et al. 2011), *tert* (Fitzgerald et al. 1999), *atr-2* (Culligan et al. 2004) and *ctc1-1* (Surovtseva et al. 2009).

Template mutation and plant transformation

A PCR product containing the RSA template mutation was generated with $TER2_{RSA}$ fwd and TER2end reverse primers. Table 4-1 lists these and all other primer sequences for this study. The mutation was confirmed by sequencing. $TER2_{RSA}$ was cloned into the destination vector pB7WG2 and transformed into *ter2-1* homozygous mutants as described (Surovsteva et al. 2007). After transformation, seeds were selected in MS agar containing kanamycin at 50µg/ml.

TRF analysis, TRAP and PETRA assays

TRF, TRAP and Q-TRAP assays were performed as described (Shakirov et al. 2005; Kannan et al. 2008). For TRAP reactions with mutant 35S::$TER2_{RSA}$ lines, a specific mutant reverse primer ($TER2_{RSA}$ TRAP) was used. PETRA was performed on either wild type or 35S::$TER2_{RSA}$ lines as described (Heacock et al. 2007). The PETRA-T reaction employed either the standard PETRA-T primer or a modified version ($TER2_{RSA}$ PETRA-T) to amplify repeats generated from $TER2_{RSA}$. The PETRA-A reaction used the standard PETRA-A primer and 1L chromosome arm primer. For sequencing, PETRA products were cloned into the pDRIVE vector (Qiagen).

$TER2_{RSA}$ fwd	5‘-CACCGACGACAACTAGTACCTACGCTTACA-3‘
TER2end rev	5‘-AATTCTGTGTAGCTATGATCTTGTGGCA-3‘
$TER2_{RSA}$ TRAP	5‘-CCTAGTACCTAGTACCTAGTACCTA-3‘
$TER2_{RSA}$ PETRA-T	5’-CTCTAGACTGTGAGACTTGGACTCATGAACCATGA-3’
TER2 3’ adaptor*	5’-pUAUGCACACUGAUGCUGACACCUGCTidT-3’
TER2 forward	5’-GACGACAACTAAACCCTACGC-3’
3’-linker reverse	5’- AGGTGTCAGCATCAGTGTGC-3’
	Quantitative RT-PCR Primers
TER1 Q4F	5‘-CCCATTTCGTGCCTATCAGACGAC-3’
TER1 Q4R	5‘-TCTCCGACGACCATTCTCTCGATAC-3’
TER2#38	5‘-GACGACAACTAAACCCTACGCTTACA-3‘
TER2#40	5‘-CAGGATCAATCGGAGAGTTCAATCTC-3‘
TER2s_QRT_F1	5’-TACGGCAACAGAACCAGAGA-3’
TER2s_QRT_R1	5’-CTCCGACGAGACGACCATAC-3’
TERT Q3F	5‘-AACACTGTCCTGTTCTCTTGCTG-3’
TERT Q3R	5‘-TTTGCCTCCTTGAACTCTGAGAAG-3’
U6-1F	5‘-GTCCCTTCGGGGACATCCGA-3‘
U6-1R	5‘-AAAATTTGGACCATTTCTCG A-3’
GAPDH F	5’-TTGGTGACAACAGGTCAAGCA-3’
GAPDH R	5’-AAACTTGTCGCTCAATGCAATC-3’
BRCA1-F	5’-TGCATCCATTAAGTTGCCCTGTG-3’
BRCA1-R	5’-TAGGCTGAGAGAGTGCAGTGGTTC-3’
PARP1-F	5’-ATGCTACTCTGGCACGGTTCAC-3’
PARP1-R	5’-AGGAGGAGCTATTCGCAGACCTTG-3’

*p=phosphate and idT= inverted deoxythymidine

Table 4-1. Primers used in this study.

RNA analysis

Total RNA was extracted using Tri Reagent (Sigma) and cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) as described (Cifuentes-Rojas et al. 2011). For the DNA damage-related and TER2 mapping experiments, RNA was extracted using the EZNA Plant RNA Kit (Omega Bio-tek). cDNA was generated by using 1-2µg of total RNA with the qScript cDNA supermix (Quanta Biosciences). To map the 3' end and splice junction in $TER2_S$, we used the protocol by Lu et al (Lu et al. 2007) with some modifications. 20µg of purified RNA was separated on a 10% polyacrylamide/urea gel. Using *in vitro* transcribed $TER2_S$ as a marker, a gel slice corresponding to 200-300nt was cut from the gel, pulverized with a pestle, 500µl of 0.3M NaCl was added, and the sample was incubated overnight at RT. Ethanol precipitation followed. Samples were resuspended in a final volume of 10µl. A PAGE-purified 3' RNA adaptor (Dharmacon) was ligated in a 10µl reaction consisting of 5µl of purified RNA, 2µl 3' RNA adaptor (20µM), 1µl 10x RNA ligase buffer, and 2µl of T4 RNA ligase (Ambion). The ligation reaction was incubated at RT for 6h, followed by gel purification. cDNA was generated from the purified RNA and then 3'-tagged $TER2_S$ was amplified using primers TER2 forward and 3'-linker reverse. PCR products were cloned into the pDRIVE vector (Qiagen) and sequenced.

Primer extension and northern blotting were carried out as described (Cifuentes-Rojas et al. 2011). For northern blotting, the probe was a pool of 5'-end ^{32}P-ATP labeled oligos CR1 and CR2 regions of the TERs. qRT-PCR was

run as described (Cifentes-Rojas et al. 2011; Boltz et al. 2012) using SyBr green master mix (NEB and Bio-Rad). GAPDH, actin and U6 served as reference genes. The LinReg PCR software (Ruijter et al. 2009) was used to calculate primer efficiencies and adjust expression levels accordingly.

Immunoprecipitation, RNA-protein binding assays and in vitro *telomerase reconstitution*

Filter binding, EMSA and *in vitro* telomerase reconstitution assays were performed as described (Cifuentes-Rojas et al. 2011) using T7-tagged proteins expressed from pET28a vectors using rabbit reticulocyte lysate (RRL) (T7 TnT Coupled Reticulocyte Lysate System, Promega) and *in vitro* transcribed RNA. For *in vitro* co-IP experiments, recombinant proteins were either co-expressed with TER or TER was expressed separately. After IP, RNA was extracted and RT-PCR was performed. Immunoprecipitation of *Arabidopsis* cell culture, including the antibodies used, was conducted as described (Cifuentes-Rojas et al. 2011).

DNA damage treatments and assays

Arabidopsis seedlings (5-7 day-old) were gently removed from MS plates and incubated in liquid MS media supplemented with 20μM Zeocin (Invitrogen) or 10mM hydroxyurea (Sigma) according to Adachi (Adachi et al. 2011). Seedlings were kept in the dark with gentle agitation for 0.5 -24h. Inflorescences

were removed from flowers and treated in the same manner. Multiple seedlings were combined for either RNA extraction or protein extraction (for Q-TRAP). Each combined sample was counted as one biological replicate. Propidium iodide staining of seedling root tips was conducted as described (Boltz et al. 2012) with the following modification. On day 4 in the light, seedlings were gently removed from the agar, transferred to liquid MS, and slowly shaken overnight in constant light. On day 5 in the light, zeocin was added to the MS.

Results

A third TER isoform is generated by splicing and 3' end cleavage of TER2

We discovered a third isoform of *A. thaliana* TER in experiments designed to examine the expression profile of TER1 and TER2. Primer extension with an oligonucleotide complementary to a region conserved in both TER1 and TER2 (CR2) (Fig. 4-1A) generated the predicted products as well as a smaller species of ~220nt (Fig. 4-1B). This new RNA was amplified by end-point RT-PCR (Fig. 4-2) and was also detected by northern blot analysis in both flowers and cell culture (Fig. 4-1C), ruling out artifactual PCR amplification or bypass reverse transcription. For reasons discussed below, the new RNA was termed $TER2_S$.

Cloning and sequencing revealed that $TER2_S$ is identical to TER2 with two exceptions. First, $TER2_S$ lacks the 529nt segment in TER2 that interrupts the two highly conserved regions shared with TER1 (Fig. 4-1A). In $TER2_S$ CR1

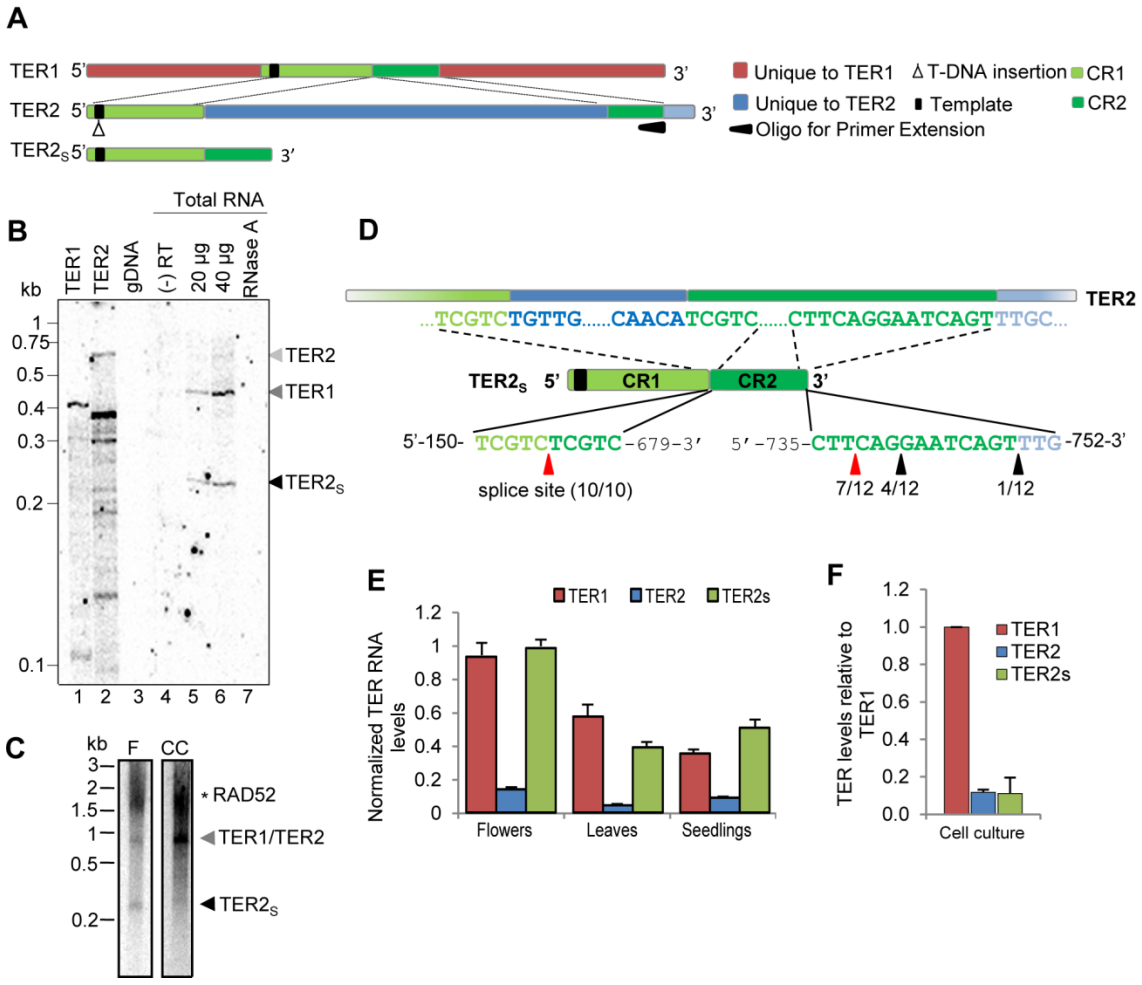

Figure 4-1. *A. thaliana* contains three TER isoforms. (A) Diagram of the three TER isoforms in *Arabidopsis*. (B) Primer extension results of total cellular RNA from cell culture using a primer complementary to a region in CR2 (filled arrow in A). Lane 1, *in vitro* transcribed TER1. Lane 2, *in vitro* transcribed TER2. Lane 3, 40µg genomic DNA (gDNA). Lane 4, control with 40µg of total RNA in the absence of reverse transcriptase. Lane 5, 20µg of total RNA. Lane 6, 40µg of total RNA. Lane 7, 40µg of total RNA pre-treated with RNase A. MW markers are indicated on the left. (C) Northern blot results for using 60mg total cellular RNA from flowers or cell culture. Blot was hybridized using a radiolabeled probe complementary to CR2. Black arrowhead, $TER2_S$. Grey arrowhead, TER1/TER2. Asterisk, RAD52 mRNA is also detected because TER1 CR1 is embedded in the 5' region of this gene (Samach et al. 2011). (D) Sequence analysis of the splice junction and 3' end of $TER2_S$ isolated from flowers. Nucleotide number refers to the corresponding nucleotide within TER2. The number of clones recovered with each sequence is indicated under the vertical arrowheads. qRT-PCR results showing relative levels of TER isoforms in different plant tissues (E) and *Arabidopsis* cell culture (F) are shown. Values in E are relative to TER1 in flowers. Error bars represent S.D. n>5 for all data points.

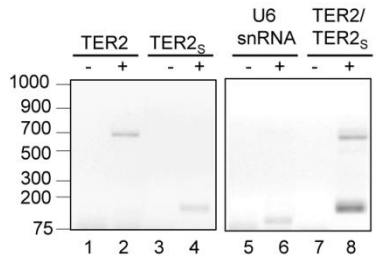

Figure 4-2. $TER2_S$ is a third isoform of TER. RT-PCR results with total RNA from cell culture. cDNA was generated using random pentadecamers. Odd lanes correspond to minus reverse transcriptase controls. Reactions with primers to detect specifically either TER2 or $TER2_S$ are shown in lanes 1-4. Reactions detecting both TER2 and $TER2_S$ are shown in lanes 7-8. U6 snRNA was amplified as a control (lanes 5-6).

and CR2 are precisely joined (Fig. 4-1D) to create a contiguous 220nt stretch with 85% identity to the corresponding region in TER1. The 11nt telomere template sequence is retained in $TER2_S$. BLAST searches failed to identify a locus in the *A. thaliana* genome that could encode $TER2_S$, indicating that this RNA is a processed form of TER2. Second, 3' linker ligation followed by RT-PCR showed that $TER2_S$ is truncated relative to TER2, resulting in elimination of a non-conserved region just downstream of CR2 (Fig. 4-1A). Unlike the internal splice junction, the 3' terminus of $TER2_S$ is somewhat heterogeneous: three different 3' ends were mapped (Fig. 4-1D). Notably, the length of $TER2_S$ differed slightly in flowers versus cell culture (Fig. 4-1C). Whether the size difference is a reflection of alternative 3' ends is unknown.

To assess the steady state levels of the TER RNAs during plant development, quantitative RT-PCR (qRT-PCR) was performed (Fig. 4-1E). The steady state levels of TER1 and $TER2_S$ were nearly equivalent, peaking in flowers, and declining in non-reproductive tissues (Fig. 4-1E). A somewhat different profile of TER expression was observed in *Arabidopsis* cell culture. In this setting, TER1 levels were significantly higher than either TER2 or $TER2_S$, and the latter RNAs were essentially equivalent (Fig. 4-1F). The biological basis for this variation in TER abundance is currently unclear.

Neither TER2 nor $TER2_S$ efficiently incorporate telomere repeats onto chromosome ends

TER2 and an RNA construct corresponding to $TER2_S$ (TER2-B) assemble with TERT to reconstitute telomerase activity *in vitro* (Cifuentes-Rojas et al. 2011). Therefore, we asked if TER2 or $TER2_S$ direct telomere repeat synthesis *in vivo* using site-directed mutagenesis. A similar strategy taken with TER1 demonstrated that this RNA acts as a highly efficient telomerase template *in vivo* (Cifuentes-Rojas et al. 2011). We mutated the templating domain in TER2 from 5'-CUAAACCCUA-3' to 5'-CUAGUACCUA-3' ($TER2_{RSA}$). This mutation, which will direct synthesis of TAGGTAC instead of canonical TAGGGTT repeats, allows us to distinguish synthesis from TER2 versus TER1 *in vivo*. *In vitro* reconstitution with recombinant TERT confirmed that $TER2_{RSA}$ supports telomere repeat incorporation (Fig. 4-3A), and thus the mutation did not significantly alter TER2 function. $TER2_{RSA}$ was then placed under the control of the powerful Cauliflower Mosaic Virus (CaMV) 35S promoter and transformed into plants null for TER2, *ter2-1* (Cifuentes-Rojas et al. 2011). As expected, TER1 levels were wild type in these lines (Fig. 4-4A), while $TER2_{RSA}$ was approximately 30-fold higher than TER2 in wild type. In contrast, $TER2_{S\text{-}RSA}$ increased by only about 2.5 fold, implying that the generation of $TER2_S$ is regulated *in vivo*. TRAP assays were performed on nuclear extracts from the transformants using primers designed to detect RSA-type repeats. Telomerase activity was detected with these primers (Fig. 4-4B), indicating that $TER2_{RSA}$

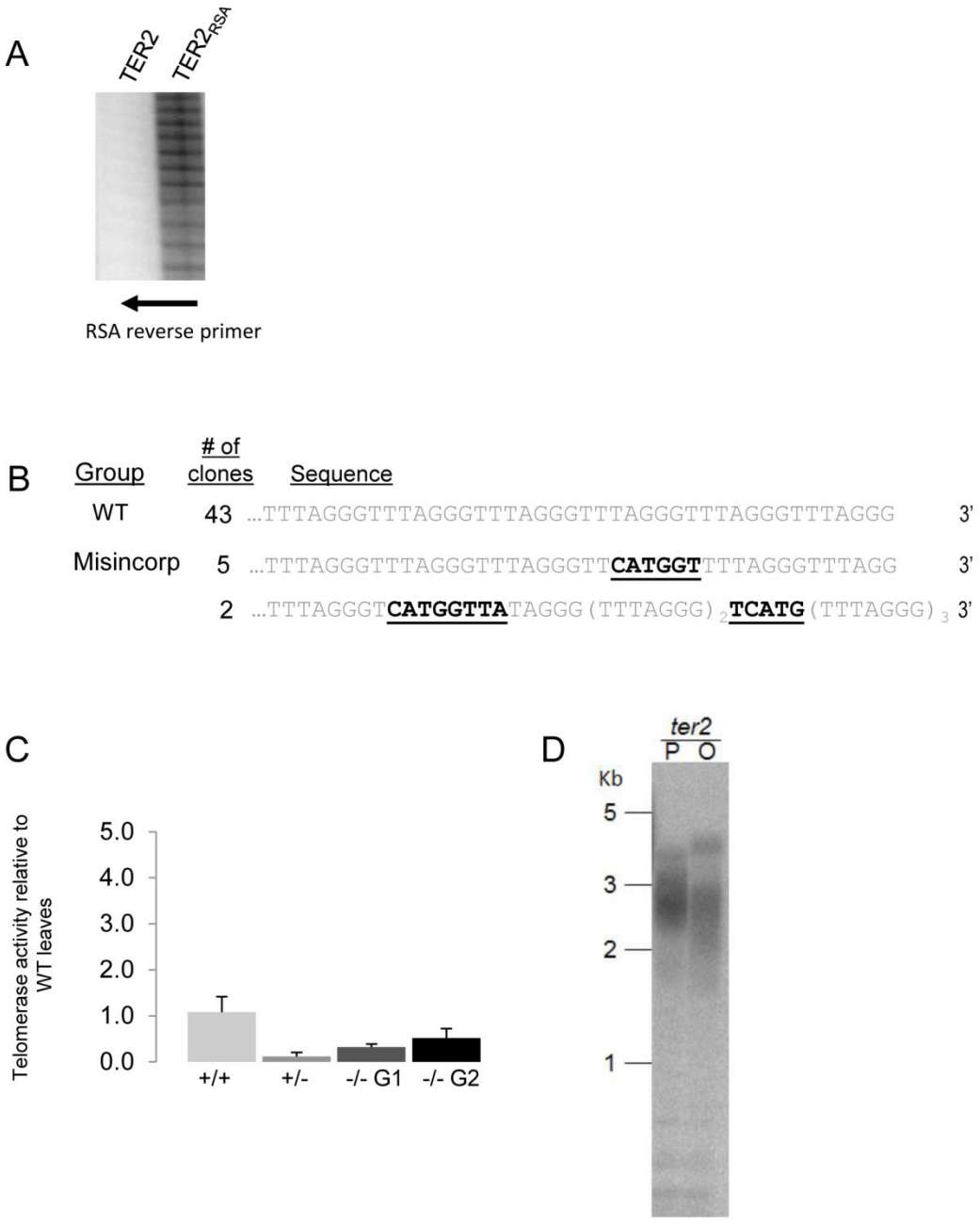

Figure 4-3 TER2 does not contribute to telomere length maintenance. (A) TRAP results with *in vitro* reconstituted telomerase using wild type or $TER2_{RSA}$. Products were amplified using an RSA-specific reverse primer. (B) Sequencing results for PETRA cloned from 35S::$TER2_{RSA}$ transformants. Products were obtained in reactions with wild type PETRA primers. Representative PETRA product sequences depicting single or multiple mis-incorporation events (underlined black font) are shown. (C) Q-TRAP results for leaf tissue extracts from wild type, *ter2-1*+/-, G1 *ter2-1* and G2 *ter2-1* homozygous mutants. (D) TRF analysis of parent (P) and offspring (O) *ter2-1* lines.

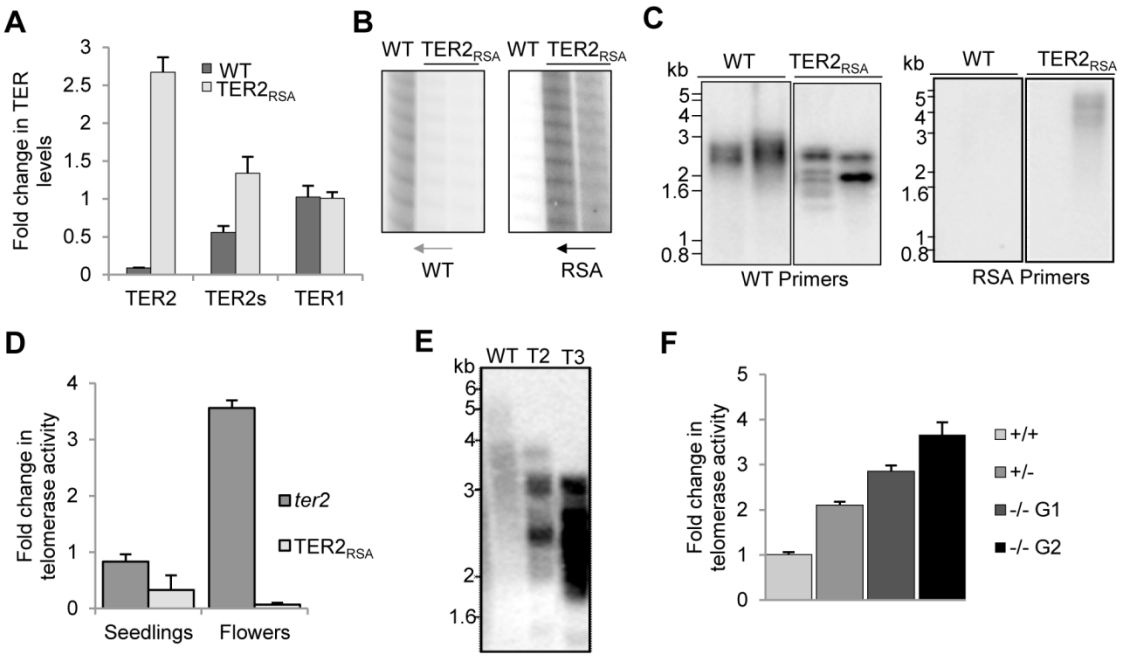

Figure 4-4. TER2 assembles into an active enzyme *in vivo*, but cannot maintain telomere repeats on chromosome ends (A) qRT-PCR results for TER levels in 35S::$TER2_{RSA}$ transformed seedlings. RNA levels are relative to TER1 in wild type seedlings. (B) TRAP results for $TER2_{RSA}$ transformants. Conventional TRAP was performed using a wild type reverse primer (left panel) or a primer specific for the RSA repeat (right panel). Extracts from wild type seedlings (WT lanes) served as a positive or negative control, respectively. (C) PETRA results for 35S::$TER2_{RSA}$ transformants. Reactions were conducted with a reverse primer complementary to wild type telomere repeats or RSA mutant repeats as shown. Results for two independent wild type plants and two 35S::$TER2_{RSA}$ transformants with each primer are shown. (D) Q-TRAP results for the flowers and seedlings of 35S::$TER2_{RSA}$ transformants. Reactions employed a reverse primer complementary to the wild type repeat as in (B). Activity is shown relative to wild type tissue. (E) Terminal restriction fragment (TRF) analysis of the second (T2) and third (T3) generation of 35S::$TER2_{RSA}$ transformants. (F) Q-TRAP results for wild type, *ter2* heterozygotes, and first (G1) and second (G2) generation *ter2* homozygous null mutants. Values were normalized to telomerase activity in wild type plants. In (D) and (F), n≥3.

and/or $TER2_{S\text{-}RSA}$ assemble into an enzymatically active RNP complexes *in vivo*. In addition, 35S::$TER2_{RSA}$ mutants were phenotypically wild type, indicating that either the mutant telomere repeat had not been efficiently incorporated onto chromosome ends, or it had no effect on telomere stability.

Primer Extension Telomere Repeat Amplification (PETRA) was used to assay for RSA-type repeats on chromosome ends. As expected, PETRA reactions with wild type primers yielded PCR products in both transformed and untransformed lines (Fig. 4-4C, left panel). In addition, the PETRA-T_{RSA} primer failed to generate products for wild type plants (Fig. 4-4C, right panel). Despite over-expression of $TER2_{RSA}$, faint products could be detected in reactions with PETRA-T_{RSA} for only one of the two $TER2_{RSA}$ mutant lines (Fig. 4-4C, right panel). Because of the low abundance of these products, we cloned and sequenced PETRA products from $TER2_{RSA}$ transformants that were amplified using wild type PETRA primers. Sequence analysis failed to show any of the predicted TAGGTAC (RSA-type) repeats. However, in 7/50 clones sequenced, one or more nucleotide mis-incorporation events were detected (Fig. 4-3B). In contrast to the results with $TER2_{RSA}$, a significant portion of the telomere tracts cloned from plants expressing a mutant TER1 ($TER1_{CC}$) carried the expected mutant telomere repeat (65/150), even though $TER1_{CC}$ was competing with endogenous wild type TER1 in this earlier experiment (Cifuentes-Rojas et al. 2011). Although we cannot exclude the possibility of selection against mutant repeats generated by $TER2_{RSA}$, in other organisms incorporation of a toxic

telomere repeat sequence leads to profound telomere instability (Yu et al. 1990). This was not observed. These findings and the genetic data presented below argue that neither TER2 nor $TER2_S$ efficiently directs telomere repeat incorporation *in vivo*. Instead, we propose that the low level aberrant repeat incorporation observed is a consequence of TER2 over-expression and resultant TER1 inhibition (see below).

TER2 negatively regulates TER1-directed telomerase activity in vivo

Quantitative TRAP (Q-TRAP) assays unexpectedly revealed that endogenous telomerase activity was reduced by four-fold in 35S::$TER2_{RSA}$ seedlings and ~17-fold in flowers compared to untransformed *ter2-1* controls (Fig. 4-4D). Because this reduction correlates with expression of $TER2_{RSA}$ and not $TER2_{S\text{-}RSA}$ (Fig. 4-4A), the data argue that increased TER2 is responsible for decreased telomerase activity. Furthermore, because 35S::$TER2_{RSA}$ transformants harbor a null mutation at the endogenous TER2 locus, telomere repeat synthesis must be suppressed from the TER1 RNP. Although no change in telomere length was observed in first generation transformants (T1), terminal restriction fragment (TRF) analysis revealed markedly shorter tracts in the second (T2) and third (T3) generations (Fig. 4-4E), consistent with limiting telomerase activity *in vivo*.

If TER2 is a negative regulator of telomerase, enzyme activity should be elevated in plants null for TER2. Indeed, telomerase activity increased 2.1-fold in

first generation (G1) *ter2-1* heterozygotes, 2.8-fold in G1 *ter2-1* homozygotes and 3.7-fold increase in second-generation (G2) *ter2-1* homozygotes (Fig. 4-4F). Telomerase activity was confined to organs where the enzyme is normally expressed; activity was low or undetectable in leaves (Fig. 4-3C). Consistent with previous results showing that increased telomerase activity does not trigger telomere elongation in *A. thaliana* (Ren et al. 2004), telomeres were in the wild type range in *ter2-1* mutants and their offspring (Fig. 4-3D). Taken together, the data indicate that TER2 inhibits the enzymatic activity of TER1 RNP, but does not contribute to telomere length maintenance under standard growth conditions.

TER2 and TER2$_S$ assemble into RNP complexes in vivo

To investigate how TER2 regulates TER1 RNP, we examined TER2-protein interactions, beginning with TERT. TERT was expressed in rabbit reticulocyte lysate (RRL) and a double-filter binding assay was performed with radiolabeled *in vitro* transcribed, 32P labeled TER1, TER2 or TER2$_S$. As expected, none of the TERs bound TRFL4, a double-strand telomeric DNA binding protein (Karamysheva et al. 2004) (Fig. 4-5A). Binding assays with TERT showed a higher affinity for TER2 over TER1, while TERT binding to TER2$_S$ was not detected under these conditions (Fig. 4-5A and B and 4-6A). We next examined TERT-TER interactions *in vivo* by co-immunoprecipitation (co-IP) using anti-TERT antibody on five day-old *A. thaliana* cell culture extracts followed by qRT-PCR (Fig. 4-5C and 4-6B). As expected none of the TER

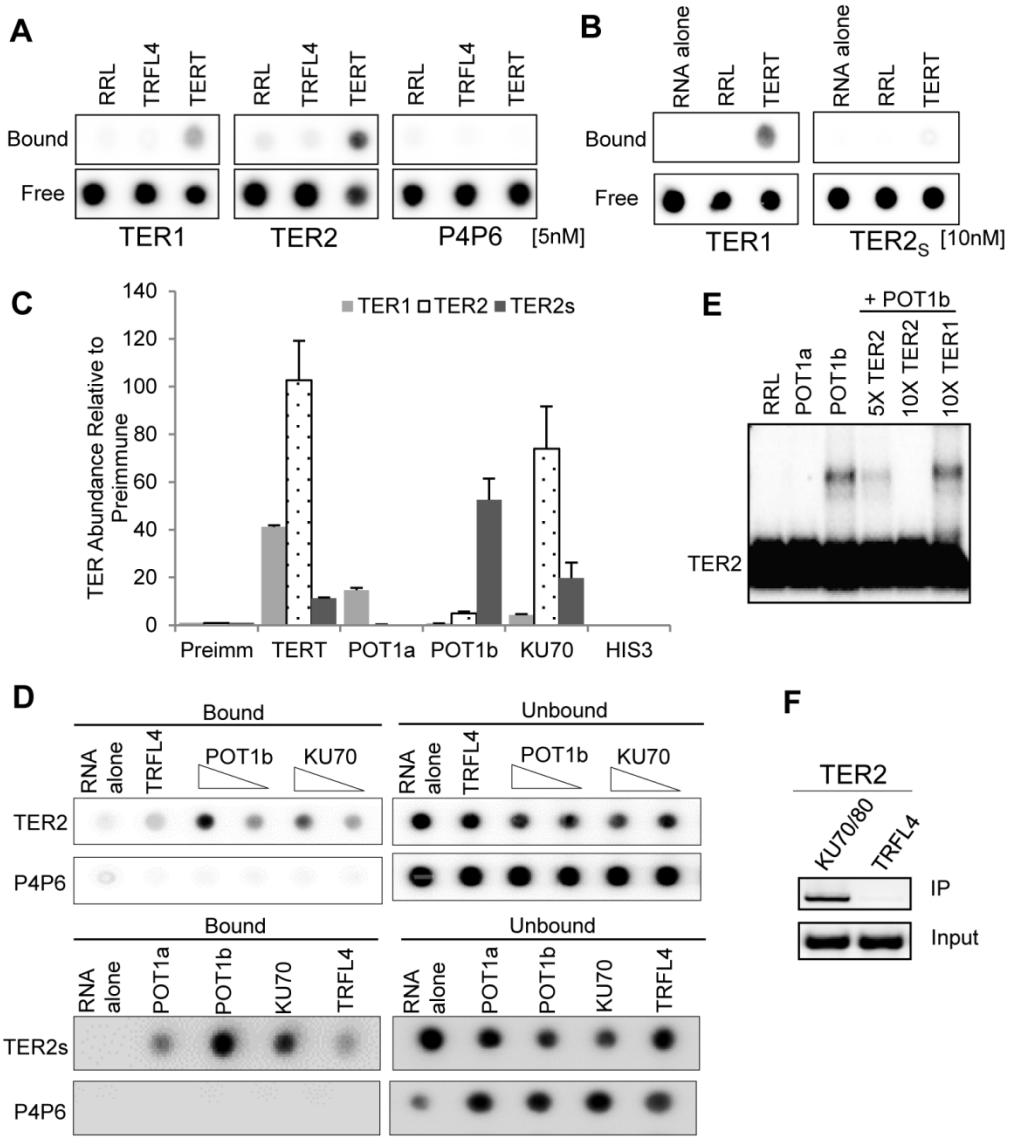

Figure 4-5. The three TER isoforms assemble into distinct RNP complexes. *In vitro* filter binding assay results for TERT with TER1, TER2 and $TER2_S$ are shown in (A) and (B). RRL expressed TERT was incubated with 5nM or 10nM radiolabeled RNA as indicated. TRFL4 protein and P4P6 RNA served as negative controls. (C) Identification TER-associated proteins *in vivo*. *Arabidopsis* cell culture was subjected to IP using the indicated antibodies, and relative TER levels were monitored by qRT PCR. Anti-histone H3 antibody was used a negative control. RNA levels were normalized relative to actin-2 and compared to pre-immune. (D) *In vitro* filter binding results for TER2 and $TER2_S$ with the recombinant proteins indicated. Assays were conducted with 1nM labeled RNA. For POT1b and Ku70 5ml or 7ml programmed RRL were used. (E) Results of TER2 electrophoretic mobility shift assays with recombinant POT1a and POT1b. Competition was performed with cold RNA as indicated. (F) *In vitro* IP-RT PCR analysis using TER2 and recombinant Ku70/80 and or TRFL4.

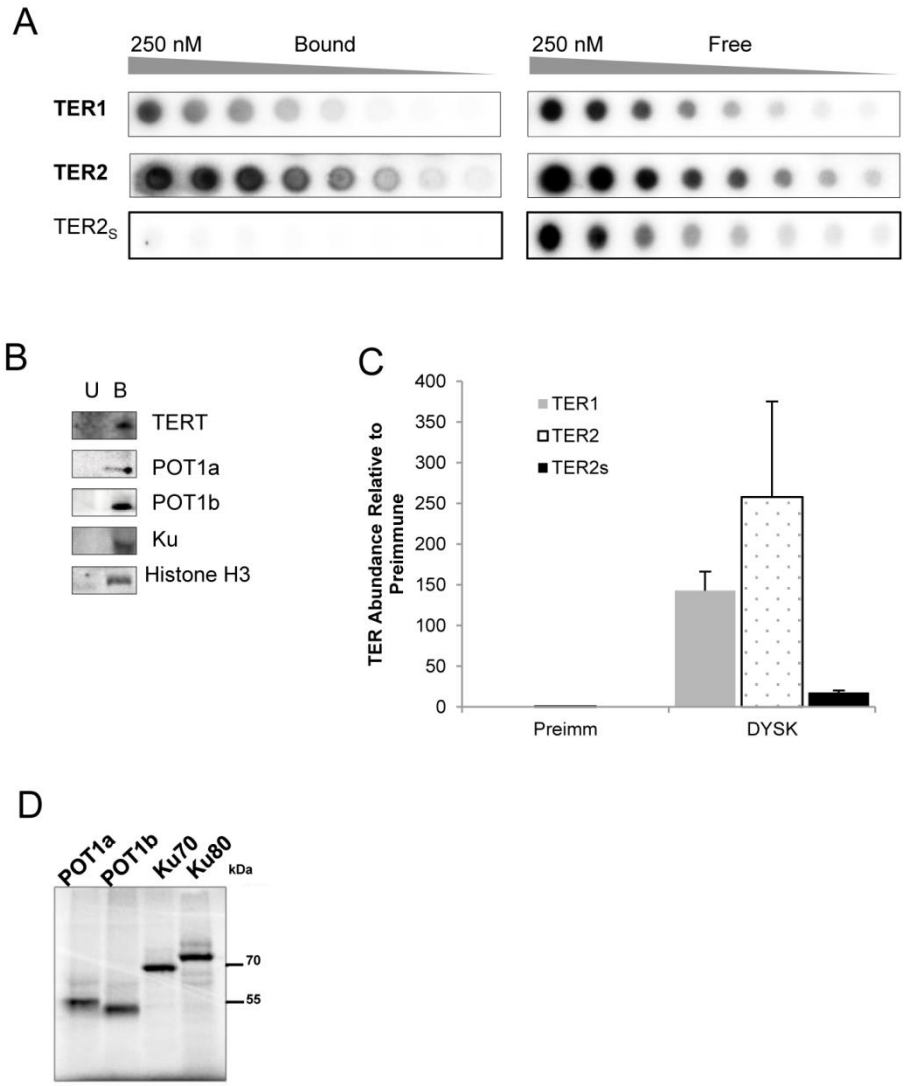

Figure 4-6. Protein interactions with TER2 and $TER2_S$. (A) Filter binding results showing a two-fold dilution series of 32P labeled TER1, TER2 or TER2S (beginning with 250nM RNA) bound by TERT produced from RRL. (B) *Arabidopsis* cell culture was subjected to IP-western using the antibodies shown. (C) The dyskerin IP precipitate was subjected to qRT-PCR to monitor the relative levels of TER1, TER2 and $TER2_S$. Results are compared to the pre-immune control. (D) 35S-labeled proteins expressed in RRL and resolved by SDS-PAGE.

isoforms were detected in Histone H3 or pre-immune IP control reactions (Fig. 4-5C). Although TER1 is approximately 10-fold more abundant than TER2 in cell culture (Fig. 4-1F), TER2 was over-represented in the TERT IP compared to TER1, and enriched by approximately seven-fold relative to the input (Fig. 4-5C). Notably, $TER2_S$ was barely detected in the TERT IP. These findings are consistent with *in vitro* binding data indicating that TERT preferentially binds TER2 > TER1> $TER2_S$.

A candidate approach was used to identify other protein-binding partners for TER2 and $TER2_S$. Although we previously failed to detect *in vitro* binding of Ku with TER1 (Cifuentes-Rojas et al. 2011), filter binding and co-IP followed by RT-PCR revealed an interaction between TER2 and Ku, but not TRFL4 (Fig. 4-5D and F and 4-6D). Another putative TER2 binding protein is POT1b. POT1b displayed the opposite affinity of its POT1a paralog (Cifuentes-Rojas et al. 2011), as it preferentially bound TER2 over TER1 (Fig. 4-5D and E and 4-6D). Gel shift analysis confirmed the interaction between TER2 and POT1b and demonstrated its specificity (Fig. 4-5E). Finally, filter binding showed $TER2_S$ interactions with POT1b and Ku, and a potential weak interaction with POT1a (Fig. 4-5D).

To further evaluate TER2/$TER2_S$ interactions *in vivo*, IP was performed with dyskerin, POT1a, POT1b and Ku70 antibodies. qRT-PCR showed that like TER1 (Cifuentes-Rojas et al. 2011), TER2 is associated with dyskerin (Fig. 4-6C). $TER2_S$, on the other hand, was not enriched in the dyskerin IP, consistent

with the presence of a putative H/ACA box at the 3' terminus of TER2 that is eliminated upon 3' end cleavage of TER2. In the POT1b IP, $TER2_S$ was enriched by 10-fold relative to input. However, only trace amounts of TER2 were detected (Fig. 4-5C), indicating that POT1b has a strong preference for $TER2_S$ *in vivo*. Neither TER2 nor $TER2_S$ were detected in an IP reaction with POT1a antibody (Fig. 4-5C). Finally, although both TER2 and $TER2_S$ were associated with Ku, TER2 was enriched by eight-fold in the IP, and $TER2_S$ by only two-fold. Altogether, the co-IP data indicate that TER2 and $TER2_S$ assemble into RNP complexes that are distinct from TER1 RNP. TER2 associates with TERT, dyskerin, Ku and to a lesser extent POT1b, while $TER2_S$ accumulates in a sub-complex primarily containing POT1b, and to a lesser extent Ku.

DNA damage-induced repression of telomerase activity correlates with TER2 induction

Since a null mutation in TER2 does not affect telomere length homeostasis under standard growth conditions, we asked if TER2 modulates telomerase activity in response to genotoxic stress. We recently discovered that telomerase activity is inhibited in *A. thaliana* seedlings treated with the radiomimetic drug zeocin (Boltz et al. 2012). To further investigate how telomerase activity levels are affected by DNA damage, seven day-old wild type seedlings were transferred to liquid culture containing 20 µM zeocin and Q-

TRAP was performed at different time intervals beginning 30 minutes after transfer to drug. As expected for the *Arabidopsis* DDR (Fulcher and Sablowski 2009), this regime elicited a strong induction of BRCA1 mRNA within four hours of drug treatment (Fig. 4-7A). In addition, staining of the root apical meristem with propidium iodide, a membrane impermeable dye that only enters dead cells, revealed stem cell death (See Fig. 4-9B), consistent with DDR activation (Fulcher and Sablowski 2009; Furukawa et al. 2010). A statistically significant decrease in telomerase activity was observed in seedlings after 30 minutes in zeocin; enzyme activity declined by ~50% relative to untreated samples (Fig. 4-7B). A similar response was obtained for all of the time points tested. Telomerase activity was also significantly reduced in zeocin-treated flowers relative to controls, indicating that this response occurs in both vegetative and reproductive organs (Fig. 4-8A). To ask if the decline in telomerase activity correlated with a change in the steady state level of TER, qRT-PCR was conducted. The abundance of TERT mRNA and TER1 were largely unaffected by zeocin treatment (Fig. 4-7C). In contrast, zeocin triggered a three-fold increase in TER2 after 30 minutes, and a peak consisting of a five-fold increase after one hour, well before the peak of BRCA1 mRNA accumulation (Fig. 4-7C). Although $TER2_S$ levels increased two-fold after 30 minutes in zeocin, this response was transient and not observed with longer treatment. Thus, the induction of TER2 did not result in a concomitant increase in $TER2_S$. Within one

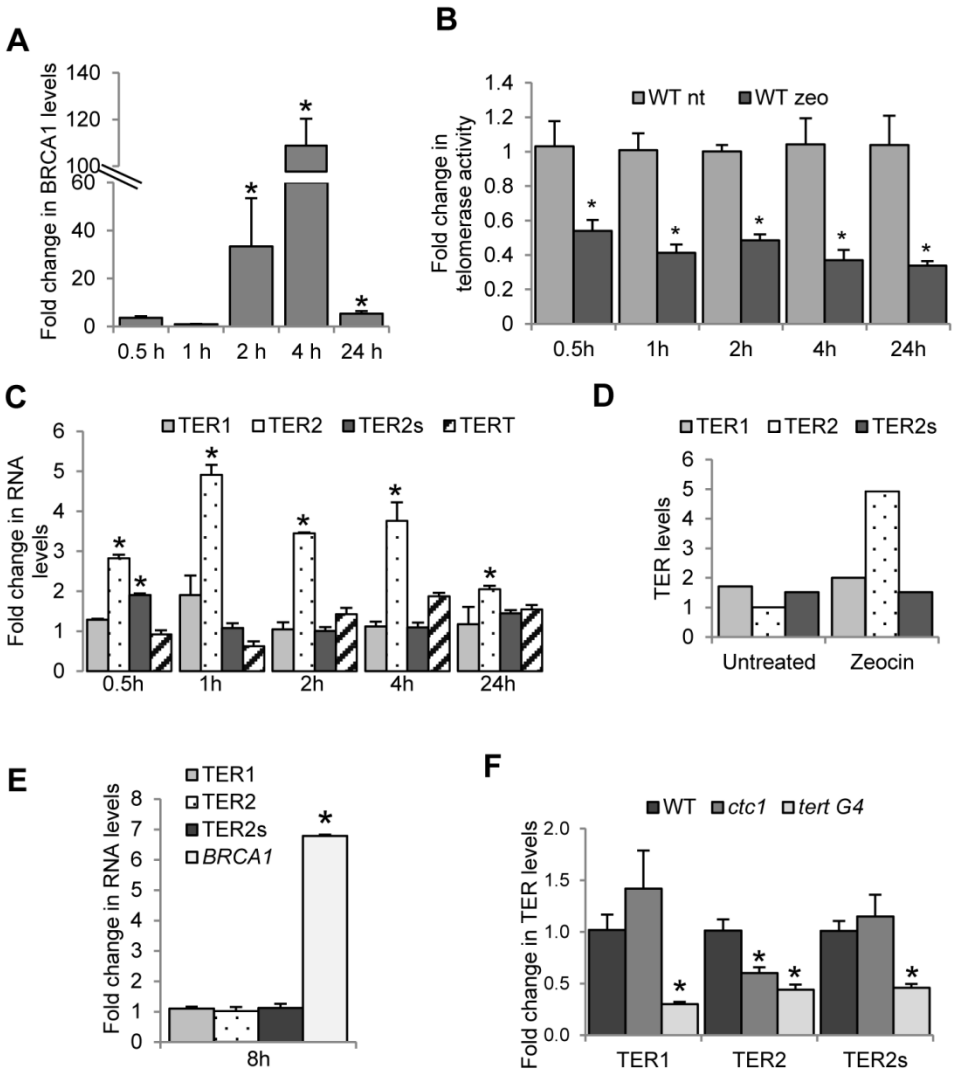

Figure 4-7. TER2 is induced in response to DNA damage. (A) Results of qRT-PCR analysis for BRCA1 mRNA following zeocin treatment. 7 day-old wild type seedlings were treated for the time points indicated. (B) Q-TRAP results for seedlings incubated in water (no treatment, nt) or zeocin (zeo). Fold change in telomerase activity is indicated relative to no treatment. (C) qRT-PCR analysis of TER isoforms and TERT mRNA after zeocin treatment at the time points indicated. RNA levels are shown relative to untreated wild type samples from the same time point. (D) TER levels in wild type seedlings with or without zeocin treatment are plotted. Data were normalized to the TER2 untreated. (E) qRT-PCR analysis of the TER isoforms and BRCA1 mRNA after 8h in HU. (F) qRT-PCR for TER in wild type, *ctc1* and fourth generation (G4) *tert* mutants are shown. RNA levels are plotted relative to TER1 in wild type. Significance for all experiments was calculated relative to untreated at each time point using a Student's t-test (*= p≤0.05).

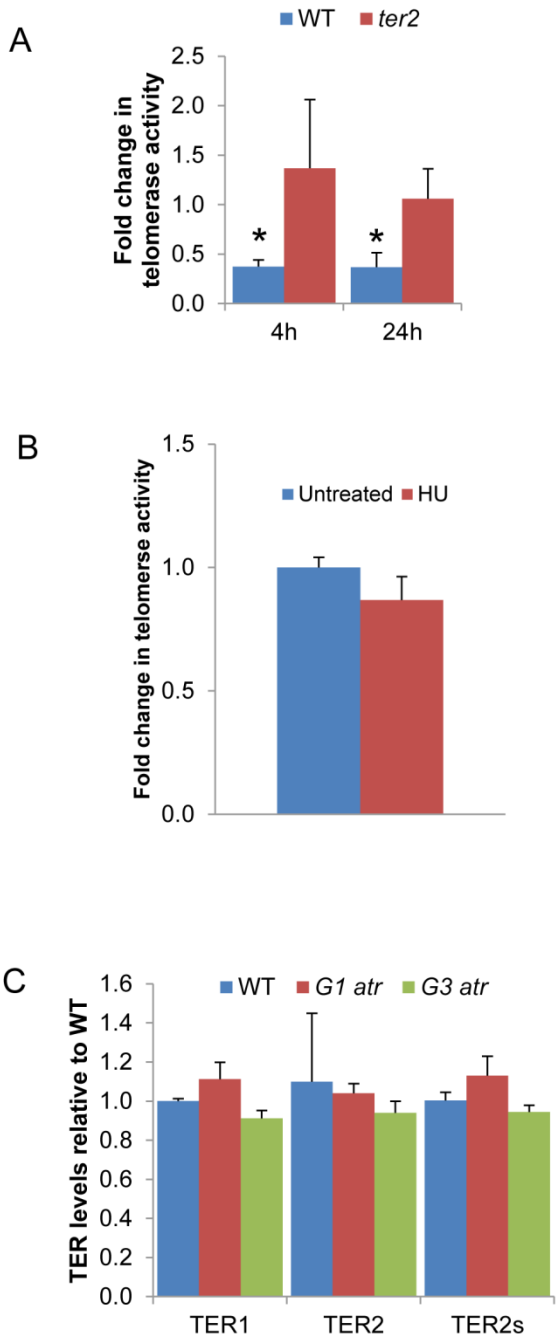

Figure 4-8. Telomerase activity levels and TER induction in response to DNA damage. (A) Q-TRAP results for *Arabidopsis* flowers treated with zeocin for the times indicated. (B) Q-TRAP results for seedings treated with HU for 8 hours. (C) TER levels in first (G1) and third (G3) generation *atr* lines as determined by qRT-PCR.

hour of zeocin treatment, TER2 became the most abundant TER isoform (Fig. 4-7D).

To ask if the decline in telomerase activity correlated with a change in the steady state level of TER, qRT-PCR was conducted. The abundance of TERT mRNA and TER1 were largely unaffected by zeocin treatment (Fig. 4-7C). In contrast, zeocin triggered a three-fold increase in TER2 after 30 minutes, and a peak consisting of a five-fold increase after one hour, well before the peak of BRCA1 mRNA accumulation (Fig. 4-7C). Although $TER2_S$ levels increased two-fold after 30 minutes in zeocin, this response was transient and not observed with longer treatment. Thus, the induction of TER2 did not result in a concomitant increase in $TER2_S$. Within one hour of zeocin treatment, TER2 became the most abundant TER isoform (Fig. 4-7D).

We next asked whether the rapid induction of TER2 is specifically triggered by DSBs or reflects a more general response to DNA damage. Wild type seedlings were treated with 10mM hydroxyurea (HU) to induce replication fork stalling. After 8 hours, BRCA1 mRNA was induced 7-fold, analogous to the 2 hour zeocin time point (Fig. 4-7E). However, in contrast to zeocin, HU did not alter the level of any of the TER isoforms (Fig. 4-7E). Moreover, Q-TRAP revealed no significant change in telomerase activity under these conditions (Fig. 4-8B). In *Arabidopsis*, telomerase activity is wild type in first generation (G1) *atr* mutants, but then declines dramatically in later generations (Boltz et al. 2012). The mechanism underlying this repression is unknown, but it may reflect

accumulating replication stress. As with HU treatment, TER2 was not induced in first or third (G3) generation *atr* mutants (Fig. 4-8C).

Finally, we asked if TER2 induction is associated with telomere dysfunction. TER2 was not induced by loss of CTC1, a core component of the CST complex (Survotseva et al. 2009) or the prolonged absence of telomerase in fourth generation (G4) *tert* mutants (Fig. 4-7F), although both mutations activate DDR (Boltz et al. 2012 and Fig. 4-9A). In plants lacking TERT, all three TER isoforms were reduced, suggesting that TERT promotes their stability. Notably, TER2, but not TER1 or TER2S, declined significantly in *ctc1* mutants (Fig. 4-7F). Taken together, the data indicate that increased accumulation of TER2 reflects an early and specific response to DSBs at internal sites within the genome.

DNA damage-induced repression of telomerase activity is dependent on TER2

We investigated the role of TER2 in zeocin-induced telomerase inhibition by monitoring DDR transcript levels and the viability of the root apical meristem (RAM) in seven day-old *ter2-1* seedlings. Unexpectedly, even in the absence of drug, BRCA1 and to a lesser extent PARP1 mRNAs were elevated in *ter2-1* mutants (Fig. 4-9A), consistent with constitutive activation of DDR. As a control, we assessed PARP1 and BRCA1 mRNA levels in third (G3) and fourth (G4) generation *tert* mutants. G3 mutants showed little evidence of DDR, but both BRCA1 and PARP1 mRNAs were elevated in G4 *tert* mutants (Fig. 4-9A),

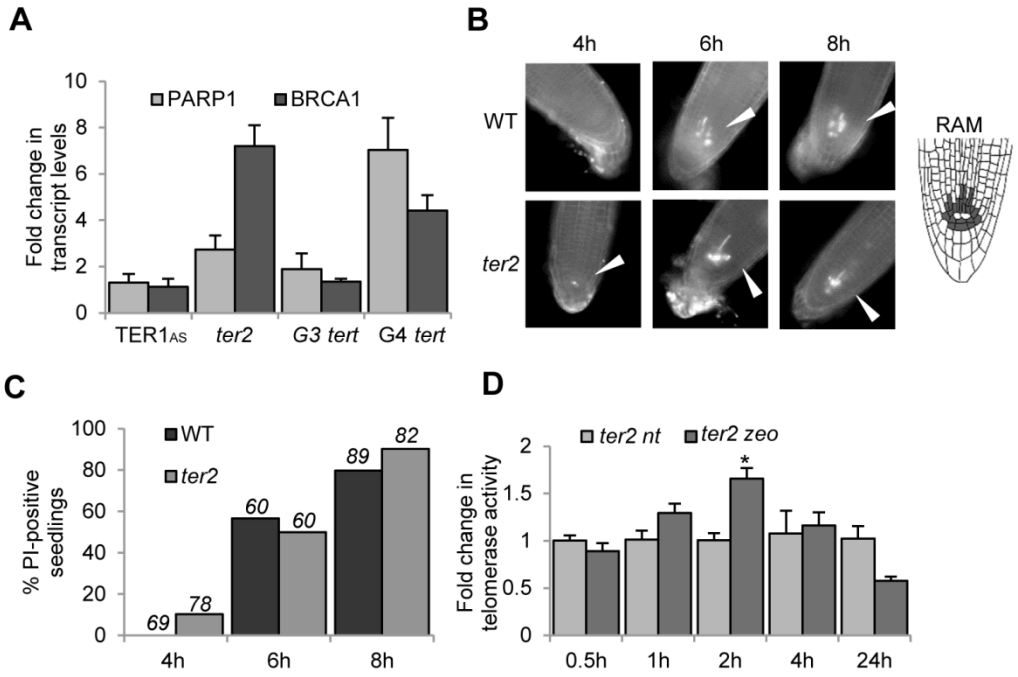

Figure 4-9. TER2 is required for DNA damage-induced repression of telomerase activity. (A) qRT-PCR results for BRCA1 and PARP1 mRNAs in the genetic backgrounds indicated. Fold change is shown relative to untreated seedlings. (B) Representative images of the root apical meristem (RAM) in five day-old seedlings stained with propidium iodide (PI) after zeocin treatment. Arrowheads denote PI-positive stem cells. Right, a schematic diagram of the RAM is shown with stem and progenitor cells in gray surrounding the quiescent center in white. (C) Percent of seedlings containing a PI stained stem cell. Results are for three independent experiments. Number of seedlings examined is indicated above each bar in italics. (D) Q-TRAP results for zeocin-treated and untreated *ter2* seedlings. n≥4 for each time point, in both qRT-PCR and Q-TRAP experiments.

consistent with impending telomere dysfunction (Riha et al. 2001). Despite the apparent constitutive activation of DDR in *ter2-1* mutants, PI staining revealed that these seedlings were slightly more sensitive to DNA damage than wild type. After four hours in zeocin, cell death was detected in the RAM of *ter2-1* root tips, but not in wild type (Figs. 4-9B and 4-9C). The response of *ter2-1* mutants was earlier, but not more robust than wild type. By six hours in the drug, the same number of PI-stained cells was observed in both genetic backgrounds (Fig. 4-10). These findings functionally link TER2 to DNA damage signaling.

In marked contrast to wild type plants, *ter2-1* mutant seedlings did not display a reduction in telomerase activity following zeocin treatment (Fig. 4-9D). Q-TRAP showed no statistical difference in enzyme activity in treated versus untreated *ter2-1* seedlings after one hour in the drug, and after two hours telomerase activity increased compared to untreated controls (Fig. 4-9D). By 24 hours telomerase activity decreased substantially in *ter2-1* seedlings, likely reflecting massive stem cell death associated with protracted genotoxic stress. The failure to down-regulate telomerase activity in response to zeocin was also observed in *ter2-1* flowers, although in this setting the elevated levels of telomerase did not diminish even after 24 hours in the drug (Fig. 4-8A). Taken together, these data indicate that TER2 is a novel component of the DDR required for the immediate reduction in telomerase activity triggered by DSBs.

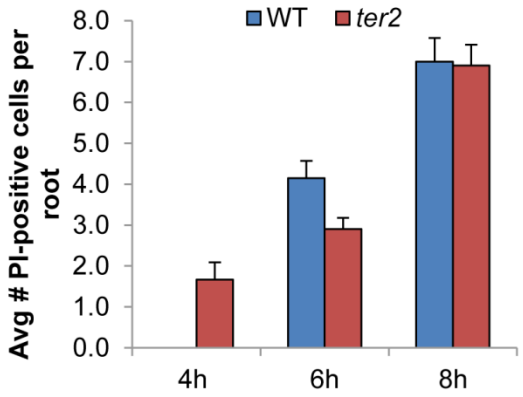

Figure 4-10. Plants lacking TER2 are hypersensitive to zeocin. Wild type and *ter2-1* seedlings were treated with zeocin and the RAM was examined for PI staining. The average number of PI-positive cells per root is shown for three different time intervals of zeocin treatment.

Discussion

Gene duplication is a major driving force for genomic diversity. Within the realm of telomere biology, core components of the vertebrate shelterin complex, TRF1/TRF2 and POT1a/POT1b, exemplify how gene duplication and neo-functionalization shape the interactions and regulation of chromosome ends (Linger and Price 2009). Critical components of telomerase have also been subjected to duplication and diversification. The ciliated protozoan *Euplotes crassus* harbors three divergent TERT genes that prompt a profound switch in telomerase behavior from an enzyme that acts promiscuously for *de novo* telomere formation to an enzyme that maintains telomere length homeostasis (Karamysheva et al. 2003). Here we describe another instance of neo-functionalization of a core telomerase subunit. In this case, TER duplication is linked to the emergence of alternative telomerase RNP complexes, and a novel regulatory pathway that restrains enzyme activity in response to DNA damage.

Three isoforms of TER assemble into distinct RNP complexes

The three TER isoforms we discovered in *A. thaliana* are unprecedented; all other organisms studied to date harbor a single TER gene. *A. thaliana* TER1 and TER2 are encoded by separate genetic loci (Cifuentes-Rojas et al. 2011), while $TER2_S$ is produced via splicing and 3' end cleavage of TER2. Although a bona-fide splicing reaction has not been described for other TER moieties, cleavage of the 3' end of *S. pombe* TER1 is required for telomerase function

(Box et al. 2008). SpTER1 transcripts bearing a poly(A) tail can be detected in fission yeast (Box et al. 2008; Leonardi et al. 2008), but the 3' terminus of the RNA associated with active telomerase is formed by "slicing", a novel mechanism in which the spliceosome carries out only the first transesterification reaction (Leonardi et al. 2008). Unlike *S. pombe* TER, *A. thaliana* TER2 lacks canonical mRNA splicing signals, and hence may be subjected to a different set of unconventional RNA processing reactions. Removal of the intervening sequence in TER2 occurs with high precision, leading to the juxtaposition of CR1 and CR2, the two domains that are conserved with TER1. By contrast, the $TER2_S$ molecules we recovered had slightly different 3' ends. Whether this heterogeneity reflects nucleolytic processing or alternative cleavage site selection is unknown. Intriguingly, the factors necessary for TER2 processing appear to be limiting *in vivo* and subject to environmental cues, because increasing TER2 (by artificial over-expression or induction by DNA damage) does not lead to a parallel increase in $TER2_S$. Thus, the mechanism and regulation of TER2 processing warrant further investigation.

A neo-functionalization model for TER evolution is supported by the fact that *Arabidopsis* not only encodes multiple TER subunits, but it assembles TER2 and its processed product into alternative RNP complexes that are distinct from TER1 RNP (Fig. 4-11A). Both TER1 and TER2 associate with TERT and dyskerin, akin to the core human telomerase RNP (Cohen et al. 2007). However, TER2 interacts with Ku, a protein complex not found in the TER1 RNP

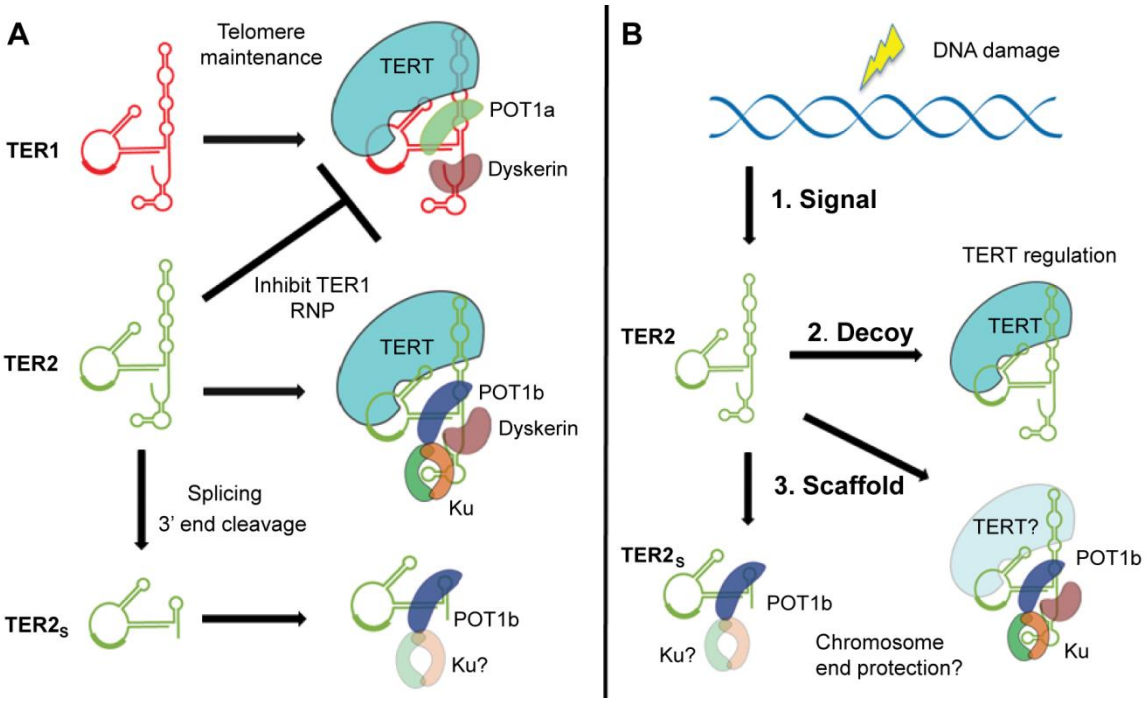

Figure 4-11. Model for *Arabidopsis* TER2: its processing, protein binding partners and possible roles in telomere biology. (A) *Arabidopsis* harbors three distinct TER isoforms. TER1, the canonical TER, is responsible for telomere maintenance and associates with the RNP maturation complex dyskerin as well as POT1a, a positive regulator of telomerase function *in vivo*. TER2 negatively regulates TER1 function, but does not substantially contribute to telomere maintenance. Like TER1, TER2 associates with TERT and dyskerin, but it also interacts with Ku and POT1b, accessory factors that are not enriched in TER1 RNP. TER2 is processed *in vivo* to generate $TER2_S$. $TER2_S$ does not accumulate in an RNP with TERT or dyskerin, but rather forms a sub-complex containing POT1b and perhaps Ku. (B) Three potential modes of TER2/$TER2_S$ action based on the lncRNA archetypes described by Wang and Chang (2011). 1) TER2 is specifically and rapidly induced in response to DNA damage. 2) TER2 has a higher affinity for TERT than TER1, and may sequester TERT in a non-functional complex. 3) The association of POT1b and Ku with TER2/$TER2_S$ may unite these protein factors at telomeres to promote chromosome end protection. See text for details.

(Cifuentes-Rojas et al. 2011). Moreover, the POT1 paralogs, POT1a and POT1b, distribute to TER1 and TER2/TER2$_S$ complexes, respectively. Even though TER2 assembles into an enzymatically active particle *in vivo*, it does not significantly contribute to telomere maintenance. It is conceivable that TER2 RNP lacks a telomerase recruitment factor like Est1 from budding yeast (Evans and Lundblad 1999) to properly position the enzyme at the chromosome terminus. The TER1-associated factor POT1a is postulated to serve this function in *Arabidopsis* (Surovtseva et al. 2007). Our data indicate that TERT does not strongly associate with TER2$_S$ *in vivo*, consistent with its low affinity for this TER isoform *in vitro*. Instead, TER2$_S$ accumulates into a sub-complex enriched for POT1b. Under standard growth conditions, TER2$_S$ is as abundant in plants as the canonical telomerase RNA subunit TER1, arguing that this alternative TER makes a biologically significant contribution to telomere biology (see below).

TER2: a novel non-coding RNA induced in response to DSBs

Genetic analyses reveal that TER2 negatively regulates telomerase activity, and further that this function is triggered by DNA damage. Within 30 minutes of zeocin treatment, well before the peak of BRCA1 mRNA induction, TER2 begins to accumulate and telomerase activity declines. Although a 50% reduction in enzyme activity was measured, this value may be an underestimate of the response since stem cell niches (the shoot and root apical meristems)

where telomerase is most active make up only a tiny fraction of the seedling biomass. For similar reasons, the true extent of zeocin-mediated TER2 induction may be much greater.

TER2 induction appears to be a specific response to DSBs. TER2 expression is unaffected by replication stress or telomere dysfunction, even though DDR is activated in both situations (Boltz et al. 2012; this study). Robust telomerase activity would in fact be advantageous in the latter setting as it could delay the onset of catastrophic telomere failure. In contrast to TER2, the long non-coding RNA (lncRNA) derived from telomere transcription, TERRA, is induced in mammalian cells in response to telomere dysfunction (Caslini et al. 2009), where it is proposed to reinforce the heterochromatic character of the chromosome terminus (Luke and Lingner 2009). Thus, the induction of telomere-related lncRNAs, as well as the fate of telomerase, may hinge on the chromosomal context in which a DDR is elicited.

Our data define TER2 as a new member of an expanding cadre of non-coding RNAs induced by DNA damage (Wouters et al. 2011). The expression, processing and maturation of a variety of miRNAs are altered in response to DSBs in both plants and animals (Hu and Gatti 2011; Wei et al. 2012). Wang and Chang (2011) recently proposed four archetypes to describe the contributions of lncRNAs. Three of these may be instructive for TER2 (Fig. 4-11B). First, lncRNAs can behave as a molecular signal. Several lines of evidence indicate that TER2 acts as a specific biological marker for DNA

damage in Arabidopsis. The induction of TER2 in response to double-strand breaks parallels the transcriptional activation of the major DDR markers BRCA1 and PARP1. In addition, TER2 is functionally linked to DDR: DDR transcripts are constitutively expressed in *ter2-1* mutants, and plants are more sensitive to DNA damage than wild type. DDR is activated in seeds as a normal part germination (Balestrazzi et al. 2011), and it is possible that *ter2-1* mutants are particularly sensitive to the DNA damage associated with development. The net response to zeocin treatment in *ter2-1* mutants is the same as in wild type plants. Although *ter2-1* mutants are capable of mounting a conventional DDR, they cannot down-regulate telomerase activity in response to DNA damage. Thus, TER2 not only heralds DNA damage, but also triggers the reduction in telomerase activity that occurs because of it. Considering the myriad mechanisms that restrict telomerase action at DSBs (Wong et al. 2002; Makovets and Blackburn. 2009; Zhang and Durocher 2010), it is likely that TER2 is only one of several genetic safeguards in Arabidopsis. Notably, TER1 is embedded in the 5' UTR of one of two RAD52 orthologs in Arabidopsis (Samach et al. 2011). Whether this RNA contributes in some way to DDR is an open question.

A second lncRNA archetype is a decoy to sequester protein binding partners (Wang and Chang 2011). Our data indicate that TER2 can sequester TERT in a non-productive complex (Fig. 4-11B). We found that TERT preferentially associates with TER2 over TER1 *in vivo*, and over-expression of

TER2 specifically inhibits TER1-directed telomere repeat incorporation, leading to telomere shortening. Besides curtailing telomerase enzyme activity, TER2 also has the potential to hijack non-telomeric functions of TERT. For human TERT, these functions include stimulation of cell proliferation and repression of apoptosis (Cao et al. 2002; Sarin et al. 2005), activities that would ideally be constrained in response to DNA damage.

A third archetype for lncRNA is a scaffold to bring effector molecules together (Wang and Chang 2011). One interesting possibility is that TER2 and/or $TER2_S$ act a scaffold for a telomere capping RNP complex (Fig. 4-11B). A number of studies in yeast and human cells point to a non-catalytic role for telomerase RNP in chromosome end protection (Zhu et al. 1999; Chan and Blackburn 2003; Hsu et al. 2007). Although TER2 RNP does not effectively maintain telomere repeats, it may engage the chromosome terminus as evidenced by rare telomere repeat mis-incorporation events in plants over-expressing $TER2_{RSA}$. Intriguingly, the two specific binding partners for TER2 and $TER2_S$, Ku and POT1b, are both implicated in chromosome end protection. In contrast to its paralog POT1a, over-expression of the POT1b N-terminus leads to dramatic telomere shortening and end-to-end chromosome fusions (Shakirov et al. 2005). Riha and colleagues recently demonstrated that Ku functions as a critical capping component for blunt-ended telomeres, which constitute ~50% of the chromosome ends in Arabidopsis (Kazda et al. 2012). Thus, TER2 and $TER2_S$ may promote genome integrity by uniting Ku and

POT1b at natural blunt-ended telomeres, or by physically blocking TER1 RNP from accessing DSBs. TER2 also has the potential to influence Ku in its conventional DNA repair capacity. Ku cannot bind DNA and RNA simultaneously (Pfingsten et al. 2012). Remarkably, Ku swaps its RNA binding partner for DNA in response to DNA damage (Adelmant et al. 2012). Thus, dynamic interactions between Ku and TER2 may underlie an additional regulatory mechanism for DNA repair.

Unlike small regulatory RNAs, lncRNAs are very weakly conserved, tending to emerge quickly and evolve swiftly (Ponting et al. 2009). A survey of other members of the *Brassicaceae* family reveals surprisingly rapid evolution of the TER locus, including striking divergence within the telomere templating domain (Beilstein et al. 2012). Furthermore, although putative TER1 orthologs can readily be identified, TER2 counterparts have only been detected in A. thaliana. While it is possible that TER2 is a unique *A. thaliana* invention, none of mechanisms for TER2 action outlined above require an intact telomere templating domain, a defining feature of TER. Thus, TER2-like regulatory molecules may well exist in other organisms, including mammals, where dysregulation of telomerase has dire consequences.

CHAPTER V

POLY(ADP-RIBOSE) POLYMERASES ARE DISPENSABLE FOR TELOMERE REGULATION IN *Arabidopsis thaliana*

Summary

Regulating the length of the telomere tract at chromosome ends is a complex process that is vital to normal cell division. Telomere length is controlled through the action of telomerase, as well as a cadre of telomere-associated proteins which protect the telomeres and modulate telomerase access. In vertebrates, multiple poly(ADP-ribose) polymerases have been implicated in the regulation of telomere length, telomerase activity and chromosome end protection. Here we investigate the role of poly(ADP-ribose) polymerases in plant telomere biology. We analyzed *Arabidopsis thaliana* mutants null for PARP1 and PARP2 as well as plants treated with the PARP inhibitor 3-AB. As expected, *parp* mutants were hypersensitive to genotoxic stress, and expression of *PARP1* and *PARP2* mRNA was elevated in response to genotoxic stress by MMS or by telomere dysfunction. Notably, *PARP1* mRNA was induced in *parp2* mutants, but the reciprocal was not true. *PARP3* mRNA, by contrast, was induced in both *parp1* and *parp2* mutants but not in seedlings treated with 3-AB. Unlike their vertebrate counterparts, PARP mutants and 3-AB treated plants displayed robust telomerase activity, normal telomere lengths, and no end-to-end chromosome fusions. We conclude that PARPs do not play a

significant role in *Arabidopsis* telomere biology. Further, our results add to a growing body of evidence that PARPs play a limited role in telomere regulation in many organisms and that their importance in human telomere biology may reflect a specific adaptation which appeared during human evolution.

Introduction

The essential functions of telomeres are to promote complete replication of the chromosome terminus and to distinguish the natural ends of chromosomes from DNA double-strand breaks (DSBs). Telomeric DNA is synthesized and maintained by the telomerase reverse transcriptase. Telomerase docks on the chromosome end via contacts with telomere binding proteins. The two main telomere protein complexes are shelterin and CST (CTC1/STN1/TEN1). Vertebrate shelterin is composed of six core subunits including double-strand (ds) DNA binding TRF1 and TRF2, and single-strand (ss) DNA binding POT1 (reviewed in (Palm and de Lange 2008)). Although the CST complex was first identified in budding yeast, CST-related components have now been identified in *Schizosaccharomyces pombe*, vertebrates, and plants (Martin et al. 2007; Song et al. 2008; Miyake et al. 2009; Surovtseva et al. 2009; Leehy et al. 2013). *Arabidopsis thaliana* encodes at least six TRF-like proteins (Karamysheva et al. 2004), but CST seems to be the primary end-protection complex. Loss of any of the three CST proteins leads to massive telomere shortening, end-to-end chromosome fusions, and severe

developmental defects (Song et al. 2008; Surovtseva et al. 2009; Leehy et al. 2013). In vertebrates, emerging evidence suggests CST is more important for telomere replication than telomere protection (Gu et al. 2012; Nakaoka et al. 2012; Stewart et al. 2012b). Thus, while core components of the telomere complex are conserved, their specific contributions to telomere biology are evolving.

Although a major function of telomeres is to distinguish chromosome ends from DNA damage (Denchi and de Lange 2007; Sfeir and de Lange 2012), multiple DNA repair-related proteins are vital for normal telomere function. The phosphoinositide-3-kinase-related protein kinase ATM (Tel1 in yeast) responds to DSBs, but is also important for recruitment of telomerase to telomeres (Arneric and Lingner 2007; Sabourin et al. 2007; Yamazaki et al. 2012). Likewise, the related kinase ATR, which is activated by single-stranded DNA breaks (SSB), is implicated in telomerase recruitment (Tseng et al. 2006; Yamazaki et al. 2012) as well as promoting DNA replication through the ds portion of the telomere (Martínez et al. 2009; Sfeir et al. 2009; McNees et al. 2010). The Ku70/80 heterodimer is required for the classic non-homologous end joining (NHEJ) pathway of DSB repair, but also has multiple functions at telomeres. Ku protects chromosome ends, particularly the 5' terminus, in yeast, mammals, and plants (Riha and Shippen 2003a; Kazda et al. 2012). In addition, Ku also can interact with the telomerase RNA subunit and recruits telomerase to budding yeast telomeres in G1 (Chan et al. 2008).

Another group of repair-related proteins required for telomere function in vertebrates are the poly(ADP-ribose) polymerases (PARPs) (Smith 2001). PARPs catalyze the synthesis and transfer of poly ADP-ribose (PAR) from NAD^+ to target proteins. PARs can alter the function of proteins in several ways (Gibson and Kraus 2012). For example, PARylation can cause protein to dissociate from its binding partner, particularly DNA, and can also promote protein complex formation through recruitment of PAR binding proteins. In addition, PARs can also mark proteins for destruction by recruiting PAR-binding E3 ubiquitin ligases. Best known for their role in DNA repair, particularly Base Excision Repair and other types of SSBs, PARPs have also been implicated in other cellular processes, including mitosis, regulating chromatin state, and transcription (Gibson and Kraus 2012). PARPs are important for cellular responses to many types of stressors, including genotoxic, heat, metabolic, and oxidative stress (Bai and Canto 2012).

PARPs have been identified in all eukaryotic supergroups (Citarelli et al. 2010). Current phylogenetic analysis suggests that the common ancestor of all current eukaryotes had two PARPs, which then diverged considerably in numbers and predicted functions. In humans, a superfamily of at least seventeen PARPs has been identified. *Arabidopsis* encodes eight PARPs, including five found in the plant-specific RCD1 clade. The two main model yeast species, *Saccharomyces cerevisiae* and *S. pombe* have lost all PARPs. The large variation in the number and types of PARPs in different organisms could

be an indication that PARP functions have expanded or diverged among eukaryotes.

Several of the mammalian PARPs are implicated in telomere biology, including telomere length regulation, chromosome end protection, and telomerase regulation. PARP proteins appear to mediate their telomere functions primarily via interactions with the shelterin components TRF1 and TRF2. The first identified telomere-associated PARP, Tankyrase1 (TRF1-interacting, ankyrin-related ADP- ribose polymerase), was discovered as an interaction partner of TRF1 (Smith et al. 1998). In human cells, TRF1 is both a binding partner and a PARylation target of Tankyrase1 (Smith et al. 1998; Smith and de Lange 1999). PARylation of TRF1 leads to its dissociation from telomeres (Smith et al. 1998; Smith and de Lange 1999) and subsequent ubiquitination and proteolytic destruction (Chang et al. 2003). Loss of TRF1, a negative regulator of telomere length, then leads to telomere elongation, presumably by increasing telomerase access to the telomeres (Smith and de Lange 2000; Cook et al. 2002). Thus, in human cells, Tankyrase1 is a positive regulator of telomere length. PARylation of TRF1 by Tankyrase1 is also essential in resolving sister telomere cohesion during mitosis (Dynek and Smith 2004), because it disrupts the interaction of TRF1 with cohesin subunit SA1 (Canudas et al. 2007).

The closely related protein Tankyrase2 shows similar localization and function as Tankyrase1 in human cells. Tankyrase2 interacts with and

PARylates TRF1 *in vitro* and *in vivo* and overexpression of Tankyrase2 leads to release of TRF1 from the telomeres and telomere elongation (Kaminker et al. 2001; Cook et al. 2002). The presence of two tankyrases important for regulation of telomeres suggests that, tankyrases have co-evolved with shelterin in humans.

Three other members of the PARP superfamily, PARP1, PARP2, and PARP3, have also been studied in the context of telomere biology. PARP1 and PARP2 can bind to TRF2 *in vitro* and *in vivo* in human cells and have demonstrated the ability to PARylate TRF2 *in vitro* (Dantzer et al. 2004; Gomez et al. 2006). Similarly to TRF1, PARylation of TRF2 causes it to dissociate from telomeric DNA (Dantzer et al. 2004; Gomez et al. 2006). TRF2 is required for end protection and formation of stable telomeric structures.

PARP1 may also have an important role at damaged telomeres. In HeLa cells, PARP1 colocalizes with telomeres, and this colocalization increases after treatment with DNA damaging agents (Gomez et al. 2006). Similarly, in mouse ES cells lacking telomerase, more PARP1 accumulates at critically short telomeres (Gomez et al. 2006). The function of PARP1 at damaged telomeres is further substantiated by a study which looked at the role of PARP1 and PARP2 at G quadruplex (G4) DNA (Salvati et al. 2010). When BJ fibroblasts were treated with a G4 ligand, both PAR and PARP1, but not PARP2, localized to the telomeres. Treatment with both the G4 ligand and a PARP inhibitor abolished PAR localization at telomeres and led to an increase in Telomere

Dysfunction-Induced Foci (TIFs) and aberrant telomeres seen in metaphase spreads (Salvati et al. 2010).

In addition to its role at damaged telomeres, PARP1 also contributes to telomere length regulation in human cells and may modulate telomerase activity (Ghosh and Bhattacharyya 2005; Beneke et al. 2008). Treatment of HeLa cells with 3-AB (3-aminobenzamide) or PARP1 siRNA led to a rapid decrease of telomere length of about 500bp per population doubling. If the 3-AB was removed, then telomeres could recover if telomerase was present. PARP2 siRNA showed no effect on telomere length (Beneke et al. 2008) suggesting that this function is specific to PARP1. PARP1 can bind directly to a TERT peptide *in vitro* (Pleschke et al. 2000), and PARP1 can be pulled down from human cells by immunoprecipitation of the telomerase catalytic subunit, TERT (Telomerase Reverse Transcriptase) (Cao et al. 2002). Whether PARP1 can affect telomerase activity is unclear. One group reported a decrease in telomerase activity in two different studies in multiple cell types treated with PARP inhibitors or PARP1 siRNA (Ghosh and Bhattacharyya 2005; Ghosh et al. 2007). This change was associated with a decrease in expression of the telomerase-associated factor TEP1 and a decrease in TERT PARylation (Ghosh and Bhattacharyya 2005; Ghosh et al. 2007). Conversely, another group detected normal telomerase activity in HeLa cells after treatment with either a different PARP inhibitor or siRNA for PARP1 and PARP2 (Beneke et al. 2008).

The newest PARP to be discovered to affect telomere function is PARP3. Knockdown of *PARP3* in human cells led to sister telomere fusions and sister telomere loss in mitotic spreads (Boehler et al. 2011). PARP3 interacts with Tankyrase1 and probably functions at telomeres by stimulating activation of Tankyrase1 (Boehler et al. 2011).

Although the studies conducted with human cell culture strongly implicate PARPs in human telomere biology, experiments in mice suggest that this function is not conserved across all vertebrates. Mice with gene knockouts of Tankyrase1 (Chiang et al. 2008) or Tankyrase2 (Chiang et al. 2006; Hsiao et al. 2006) have delayed development but normal telomere length and chromosome end protection over multiple generations. This result may reflect the lack of a tankyrase binding motif on mouse TRF1 (Muramatsu et al. 2007). Mouse Tankyrase1 can dissociate human TRF1 *in vitro* and *in vivo* but mouse TRF1 cannot be dissociated by either the mouse or the human Tankyrase1 (Muramatsu et al. 2007). Since double Tankyrase1 and Tankyrase2 null mice die during embryogenesis (Chiang et al. 2008), the possibility that these two proteins act redundantly in telomere regulation cannot be excluded.

A number of studies present contradictory results for the *in vivo* role of PARP1 in mice. One group found that MEFs from PARP1-deficient mice had shorter telomeres and chromosome fusions (d'Adda di Fagagna et al. 1999; Tong et al. 2001), whereas a second group found no evidence of telomere shortening in PARP mutants (Samper et al. 2001). This latter study examined

four generations of *parp1 tert* double mutants and found that telomeres shortened at the same rate as their *tert* single mutant siblings (Espejel et al. 2004). Subsequently, this group showed that *parp2* mutant mice had normal telomeres (Dantzer et al. 2004). Another report found that MEFs from PARP knockout mice had telomere defects, such as telomere signal free chromosome ends, but only if treated with DNA damaging agents (Gomez et al. 2006). Additionally, studies in mice failed to find any change in telomerase activity in either *parp1* (d'Adda di Fagagna et al. 1999; Samper et al. 2001; Tong et al. 2001) or *parp2* (Dantzer et al. 2004) null animals, and the mouse PARP1 does not bind mouse TERT (Samper et al. 2001). As with the tankyrase mutants, the combined contribution of PARP1 and PARP2 at mice telomeres is unknown because the double mutant is embryonic lethal (Menissier de Murcia et al. 2003).

Plants have proven to be an excellent model system for telomere analysis owing to high tolerance to telomere dysfunction. For example, *Arabidopsis* plants lacking the core telomere capping components CTC1 or STN1 are viable and semi-fertile even in the presence of severe telomere dysfunction (Song et al. 2008; Surovtseva et al. 2009). Further, plants can often survive without key DNA damage response proteins. *Arabidopsis* deficient in the DNA damage kinases ATM or ATR are viable and healthy for many generations under normal growth conditions (Garcia et al. 2003; Culligan et al. 2004). In vertebrates loss

of ATR is lethal. *Arabidopsis* is thus a good choice to examine the role of PARPs at telomeres.

Plants have fewer PARP genes than vertebrates. *Arabidopsis thaliana* encodes six PARP proteins, but none bear the signature of tankyrase-like PARPs. Notably, *Arabidopsis* also lacks a homolog to human PARP2. Three of the *Arabidopsis* PARPs have confirmed or predicted poly ADP-ribosylation activity (Jaspers et al. 2010). Of these, AtPARP2 is homologous to HsPARP1 while AtPARP1, while AtPARP3 more closely resemble HsPARP3. Both AtPARP1 and AtPARP2 are ubiquitously expressed, while AtPARP3 expresseion is confined to seeds under standard growth conditions.

Plant PARPs have been studied mostly in the context of biotic and abiotic stress (De Block et al. 2005; Vanderauwera et al. 2007; Adams-Phillips et al. 2008; Adams-Phillips et al. 2010). As in vertebrates, plant PARPs are stimulated by multiple types of stress and, depending on the context, may either promote cell survival or cell death. AtPARP1 and AtPARP2 localize to the mitotic spindle and thus may have similar functions as Tankyrase1 and HsPARP3 in preventing fusion of sister chromatids during cell division (Lamb et al. 2012). There is currently only indirect evidence that AtPARP1 and AtPARP2 function in DNA repair. Both are highly expressed after induced DNA damage and replication stress and AtPARP2 binds to DNA breaks (Garcia et al. 2003; Yoshiyama et al. 2009; Lamb et al. 2012).

Here we examine the role of PARPs in *Arabidopsis* telomere biology. We show that in plants lacking the telomerase catalytic subunit TERT (telomerase reverse transcriptase), PARP transcripts are upregulated, confirming that telomere dysfunction can trigger PARP activation. We also use PARP mutants as well as PARP-inhibitor treated seedlings to determine how PARPs affect telomere maintenance, integrity, and telomerase activity. None of these studies revealed a role for PARPs in *Arabidopsis* telomere biology. These results, in combination with the mouse studies and the complete lack of PARPs in yeast, lead us to conclude that the role of PARPs at human telomeres is recently derived.

Materials and methods

Plant materials and growth conditions

T-DNA lines for AtPARP1 (SALK_ 140400) and for AtPARP2 (GABI_380E06-017222) were obtained from the *Arabidopsis* Biological Resource Center (ABRC). The *tert* mutant (Riha et al. 2001) and *ku70* mutant (Riha and Shippen 2003a) and their phenotypes were described previously. Double *parp1 parp2* mutants were made by crossing a homozygous *parp1* mutant with a homozygous *parp2* mutant. Double heterozygous F1 plants were identified by genotyping and then self-propagated to F2 to obtain double homozygous mutants. Plants were grown at 23°C in an environmental chamber under a 16 h light/8h dark photoperiod.

Chemical treatments

Seeds were sterilized and germinated on solid MS (Murashige and Scoog) medium. Five days after germination, seedlings were transferred to liquid MS medium containing 0, 25, 50, 75 or 100 ppm MMS (Sigma). Seedlings were treated with MMS for five days for measurement of DNA damage response and for one week to score MMS sensitivity.

For 3-AB treatment, seeds were sown directly into liquid MS plus 5mM 3-AB (Sigma)/0.6% DMSO or MS plus 0.6% DMSO and were grown for one week under constant light with gentle shaking. To induce DNA damage in 3-AB treated and control seedlings, 20µM zeocin was added for four hours before harvesting the seedlings.

RNA extraction and RT-PCR analysis

Frozen seedlings were finely ground and RNA was extracted using TRI reagent (Sigma). The extracts were treated with RQ1 DNAse (Promega) for 1hour. cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) with oligo(dT) primer. DNA damage responses were measured in MMS treated wild type and *tert* seedlings by checking the mRNA levels of *PARP2*. The reaction mixture was amplified with Taq polymerase for 20 cycles of PCR at 94°C for 3 min, 55°C for 40 sec and 72°C for 1 min 15 sec with a final extension time at 72°C for 5 min. The entire reaction was resolved on a 1% agarose gel and subjected to Southern blot with a *PARP2* cDNA probe labeled

with [α-^{32}P]-dCTP. As a loading control, RT-PCR was performed with primers specific for *Actin-2*.

For all other RT-PCR experiments, RNA was extracted with the Direct-Zol RNA Miniprep Kit with on-column DNAse treatment (Zymo Research). 1µg of total RNA was used with the qScript cDNA Supermix (Quanta Biosciences). The resulting cDNA was diluted 1:4 in 10µg/ml yeast tRNA and 4µL was used for qPCR. qPCR was run on a CFX Connect Real-Time PCR Detection System (Bio-Rad) using SsoAdvanced SYBR Green Supermix (Bio-Rad) following the manufacturer's suggested protocol. Each reaction was run in duplicate and later averaged, and at least three biological replicates were run for each experiment. Two reference genes that were reported to have steady levels of transcription in many conditions (Czechowski et al. 2005), *PDF2* and At4G26410, were run for each sample. LinRegPCR was used with default settings to calculate initial transcript levels (N_0) that were corrected for PCR efficiency. To correct for loading the target N_0 value was divided by the geometric mean of the two reference genes. This value was then divided by the average corrected value for the control sample (wild type or untreated).

TRAP (Telomere Repeat Amplification Protocol)

Protein was extracted from flowers or seedlings using Buffer W as previously described. qTRAP was performed as previously described (Kannan et al. 2008). For radioactive TRAP extracts were diluted 1:10, and for

quantitative TRAP (qTRAP) 50ng of total protein was used for each sample. The extract, telomere oligo substrate, and α-[^{32}P]dGTP were added to Hot Start GoTaq master mix (Promega) and incubated for 45 minutes at 37°C. TRAP reverse primer was then added to each reaction and then PCR was run. Products were precipitated with ethanol/sodium acetate (pH 5.2)/glycogen and run on a 6% polyacrylamide, 7M urea sequencing gel.

Telomere length measurement and telomere fusion PCR

Genomic DNA was extracted from seedlings or whole plants using 2x CTAB buffer (Vespa et al. 2005). TF-PCR and PETRA (Heacock et al. 2004) and TRF (Fitzgerald et al. 1999) were conducted as previously reported. For all three assays, products were detected by Southern blot with a [^{32}P]-5′-end-labeled $(TTTAGGG)_4$ probe.

Results

Generation of plants null for PARP activity

To examine the role of PARP proteins at *Arabidopsis* telomeres, we sought to identify mutants lacking *PARP1* or PARP2. T-DNA insertion lines were obtained for both *PARP1* (At4G02390) and *PARP2* (At2G31320) (Fig. 5-1A). *PARP1* and *PARP2* transcription was abolished in single *parp1-1* and *parp2-1* mutants as indicated by RT-PCR analysis (Fig. 5-1B). To investigate

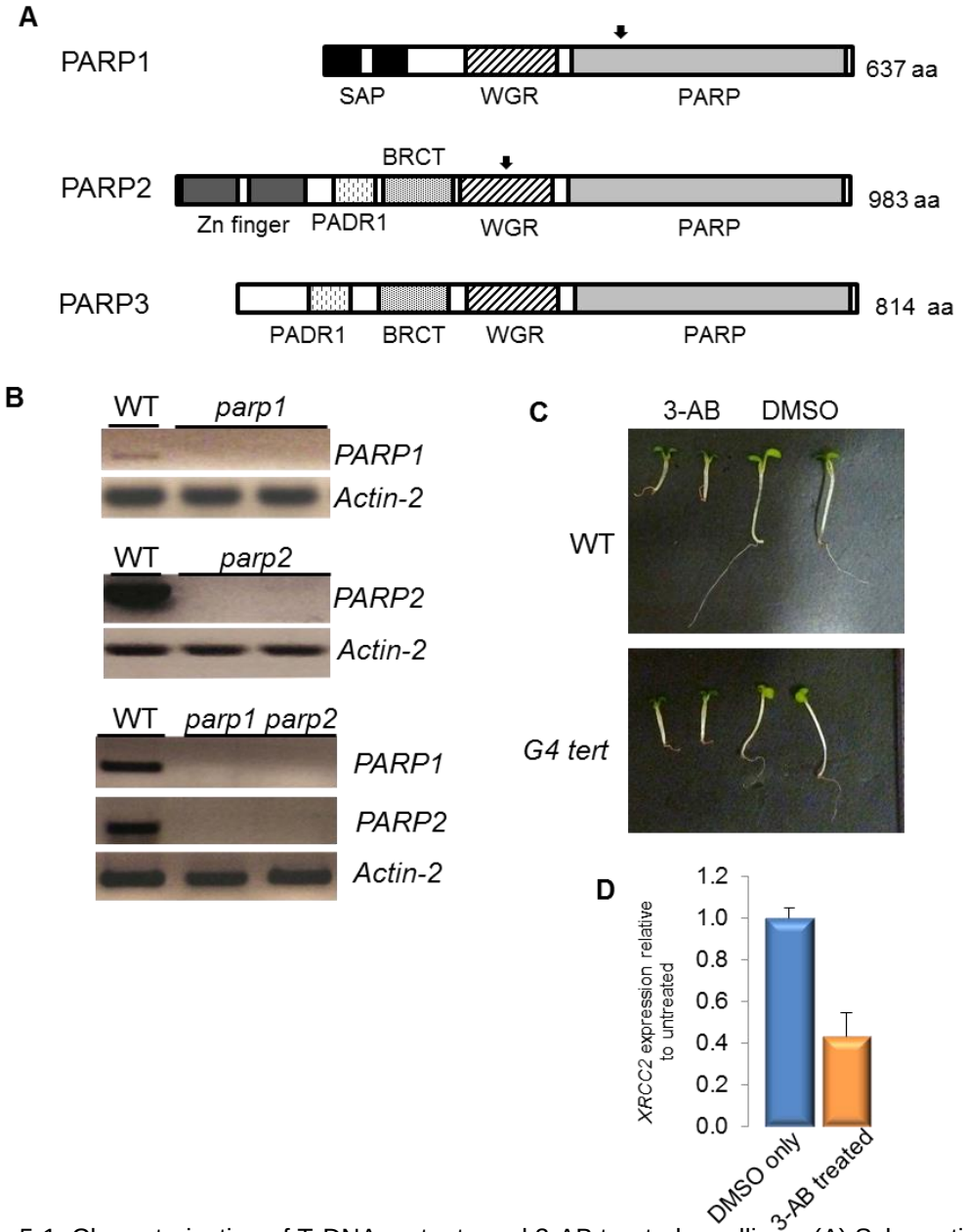

Figure 5-1. Characterization of T-DNA mutants and 3-AB treated seedlings. (A) Schematic of PARP proteins. The arrows indicate the position of the T-DNA insertions. The T-DNA for PARP1 is located in the intron between exons 6 and 7, which are part of the PARP catalytic domain. The T-DNA for PARP2 is located in exon 10, which is within the WGR domain. SAP: SAF-A/B, Acinus and PIAS (nucleic acid-binding domain); WGR: Named after conserved central motif (putative DNA-binding domain); PARP: PARP regulatory and catalytic domain; PADR1: Domain of unknown function found in PARPs; BRCT: BRCA1 C-terminus. (B) Semi-quantitative RT-PCR for *PARP1* and *PARP2* expression levels in *parp1* (top), *parp2* (middle) and *parp1 parp2* double mutants (bottom). *Actin-2* served as a loading control. (C) Wild type (top) and G4 *tert* mutants (bottom) grown in 5mM 3-AB/0.6% DMSO (left) or in 0.6% DMSO (right). (E) qRT-PCR for *XRCC2* expression in 3-AB-treated wild type seedlings relative to untreated seedlings.

the combined contribution of PARP1 and PARP2, we generated a *parp1 parp2* double mutant by genetic crossing. RT-PCR analysis confirmed that expression of both *PARP1* and *PARP2* was abolished in the double mutants (Fig. 5-1B).

During the course of this study, PARP3 was identified in vertebrates (Boehler et al. 2011). A putative ortholog, At5g22470, is also present in *A. thaliana*. Because of the difficulties in generating triple mutants, we instead chose to use the PARP inhibitor 3-AB (3-aminobenzamide) on wild type plants to eliminate all PARP enzymatic activity. 3-AB has been used extensively in plants (Adams-Phillips et al. 2008; Ishikawa et al. 2009) and was employed in several studies of the telomeric function of PARP in mammalian cell culture (Beneke et al. 2008). Seeds were sown in liquid MS with either 3-AB (in DMSO) or DMSO only added. Seedlings were then collected seven days later. In contrast to previous reports of enhanced growth with the PARP-inhibitor 3-MB (3-methoxy-benzamide) (Schulz et al. 2012), our 3-AB treated seedlings had almost no root growth compared to untreated seedlings (Fig. 5-1C). Shoots were also smaller in the 3-AB-treated seedlings, but this could reflect a defect in nutrient uptake caused by the small roots. Because we did not want to rely solely on morphology to verify the action of 3-AB, we also measured levels of *XRCC2* mRNA by qRT-PCR. *XRCC2* is one of the transcripts that was previously reported to be downregulated in response to 3-AB treatment in *Arabidopsis* (Ishikawa et al. 2009). Our 3-AB treated samples showed a similar response in

XRCC2, with the levels reduced by about 60% compared to the DMSO-only treated seedlings (Fig. 5-1D). Thus, the 3-AB treatment worked as expected.

PARP mutants are sensitive to genotoxic stress

Because PARP proteins are important for the response to ssDNA damage, we verified that our mutants were sensitive to genotoxic stress by treating five-day-old seedlings with increasing concentrations of the DNA alkylating agent methyl methane sulfonate (MMS). Growth and morphology of *parp1* and *parp2* mutants were compared to wild type and *ku70* seedlings, which are hypersensitive to MMS (Riha and Shippen 2003a). At all three MMS concentrations tested, *parp1* and *parp2* mutants were smaller and less developed than wild type seedlings but were not affected as much as the *ku70* mutants (Fig. 5-2A). Notably, the *parp1 parp2* double mutants were more sensitive than either single mutant, and the double mutants were similarly or slightly more sensitive to MMS than the *ku70* mutants (Fig. 5-2A). The increased hypersensitivity to MMS in the double mutants suggests that PARP1 and PARP2 may have overlapping but not completely redundant function.

PARPs are upregulated in response to telomere dysfunction in Arabidopsis

We previously reported that *PARP1* mRNA is induced in response to telomere dysfunction triggered by prolonged telomerase inactivation (Cifuentes-Rojas et al. 2012) or by loss of a core component of the CST telomere capping

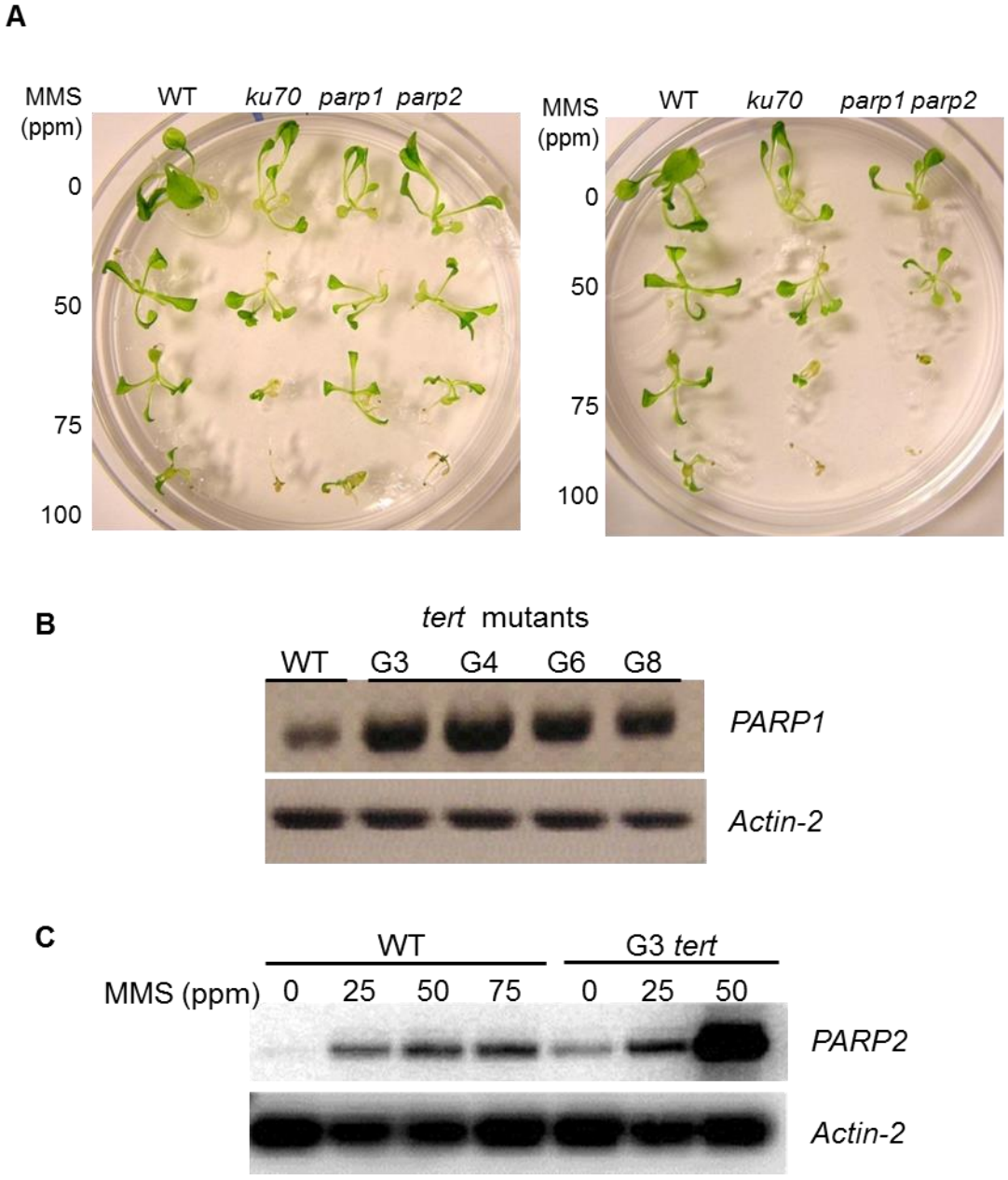

Figure 5-2. *Arabidopsis* PARPs respond to genotoxic and telomeric stress. (A) Morphological and developmental defects of seedlings grown in increasing concentrations of MMS. Left panel: *parp1* and *parp2* single mutants show hypersensitivity to MMS compared to wild type but less sensitivity than *ku70* mutants. Right panel: *parp1 parp2* double mutants show similar or higher sensitivity to MMS compared to *ku70* mutants. (B) RT-PCR of *PARP1* transcript levels in multiple generations of *tert* mutants. (C) RT-PCR of *PARP2* expression in wild type and 3rd generation *tert* mutants at increasing concentrations of MMS.

complex (Boltz et al. 2012). To further explore the PARP1 response, we used RT-PCR to examine *PARP1* expression across four different generations of *tert* mutants. *PARP1* levels remained elevated in all generations tested (Fig. 5-2B). *PARP2* was also upregulated in third generation (G3) *tert* mutants compared to wild type seedlings (Fig. 5-2C). Thus, telomere dysfunction resulting from *tert* mutation leads to a DDR where both *PARP1* and *PARP2* are upregulated. The induction of *PARP2* was even higher in *tert* mutants treated with MMS (Fig. 5-2C). At 50ppm of MMS, *tert* mutants showed a much larger increase in *PARP2* levels compared to wild type (Fig. 5-2C), suggesting that the DNA damage caused by MMS treatment and loss of TERT was greater than either condition alone.

Arabidopsis *PARPs negatively regulate expression of each other*

Because our experiments with MMS suggested that the three PARPs could have overlapping function, we were curious whether the PARPs could regulate each other. We used quantitative RT-PCR to measure levels of all three PARPs in wild type, *parp1, parp2*, and 3-AB treated seedlings. *PARP1* expression in *parp2* mutants was 1.9-fold higher than in wild type plants (Fig. 5-3A) (p-value = 0.001). *PARP2* expression in *parp1* mutants was also increased compared to wild type, however there was large variation among the samples and the difference was not statistically significant (Fig. 5-3A). We conclude that PARP2 negatively regulates expression of *PARP1*.

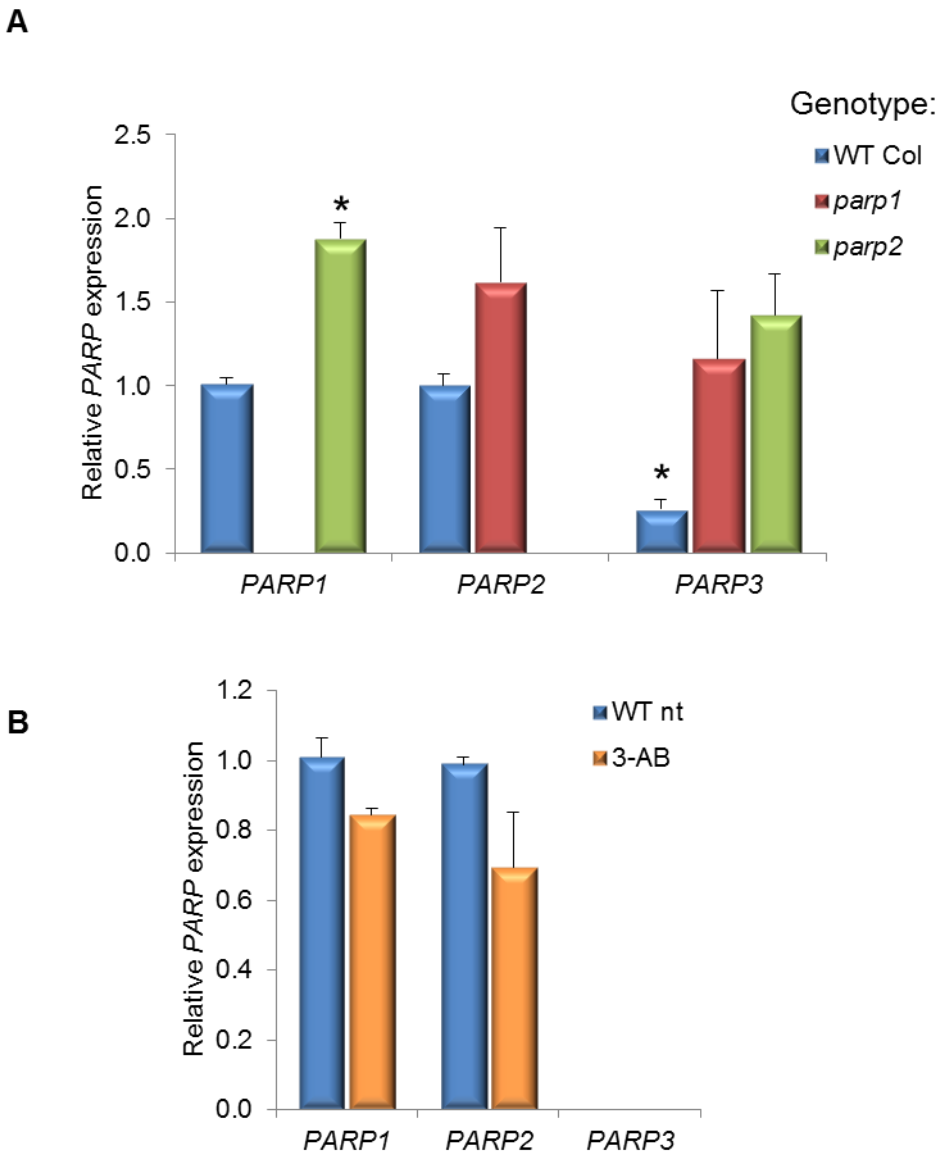

Figure 5-3. Expression of *PARP1* and *PARP3* increases in PARP mutants. (A) qRT-PCR of *PARP* mRNAs in *PARP* mutants relative to WT. *PARP3* expression levels were relative to the level in *parp1* mutants because wild type *PARP3* expression levels were barely detected. (B) *PARP1* and *PARP2* levels in 3-AB-treated seedlings compared to untreated seedlings. *PARP3* was not detected in either treated or untreated seedlings. Asterisk denotes p-value ≤ 0.05 measured by Student's two-tailed T-test.

Although *PARP3* is normally only expressed in *Arabidopsis* seeds, it is possible it functions redundantly with PARP1 or PARP2. To assess whether *PARP3* expression changes in the absence of PARP1 or PARP2, we monitored *PARP3* expression in *parp1* and *parp2* mutants by qRT-PCR. In *parp1* and *parp2* seedlings, the levels of *PARP3* transcript increased 4-5-fold compared to wild type (Fig. 5-3A). However, the qRT-PCR signal was barely detected in wild type which made it difficult to determine an accurate measure of fold change. We can conclude however, that the absence of PARP1 or PARP2 leads to an induction of *PARP3* expression in seedlings. Thus, PARP3 may have some redundant functions with PARP1 and PARP2 that are dormant in wild type seedlings.

PARP transcript levels were also measured in 3-AB-treated seedlings and compared to untreated seedlings. The levels of *PARP1* and *PARP2* were slightly decreased in the 3-AB-treated samples, but the small difference was not statistically significant (Fig. 5-3B). *PARP3* was not detected in either the untreated or treated samples.

Our transcriptional analysis suggests that the PARPs can negatively regulate each other. These results could indicate that transcription of the *PARP* genes is regulated by ADP-ribosylation of factors influencing *PARP* expression. Alternatively, the increase in *PARP* expression may reflect an increase in background levels of DNA damage caused by the absence of one or more *PARPs*. In the 3-AB treated samples, where PARPs were still expressed at wild

type levels, ADP-ribosylation should be absent, suggesting that ADP-ribosylation is not required for *PARP* expression. The possibility that background DNA damage in PARP mutants induces expression of other PARPs is unlikely since the 3-AB-treated seedlings had normal *PARP* expression. Other models could explain the difference in 3-AB-treated seedlings. For example, ADP-rybosylation may not be needed for PARP expression, and instead the PARPs have non-catalytic functions that regulate expression of each other. Alternatively, because all of the PARPs are inactive in 3-AB-treated seedlings, cells may rely on other DDR pathways to deal with background DNA damage caused by the loss of PARP activity and no longer need to upregulate PARP expression.

PARPs do not modulate telomerase activity in Arabidopsis

In human cells, PARP activity is reported to promote optimal telomerase activity (Ghosh and Bhattacharyya 2005; Ghosh et al. 2007). We asked whether PARP proteins are required stimulate telomerase activity in *Arabidopsis* using the Telomeric Repeat Amplification Protocol (TRAP). Telomerase activity was detected in seedlings treated with 3-AB (Fig. 5-4A) as well as in plants doubly deficient for *parp1* and *parp2* (data not shown), arguing that PARP activity is not required for telomerase activity. To further evaluate the contribution of PARPs to telomerase activity we performed quantitative TRAP (qTRAP) on seedlings treated with 3-AB. Telomerase activity was slightly increased (1.4-fold, p-

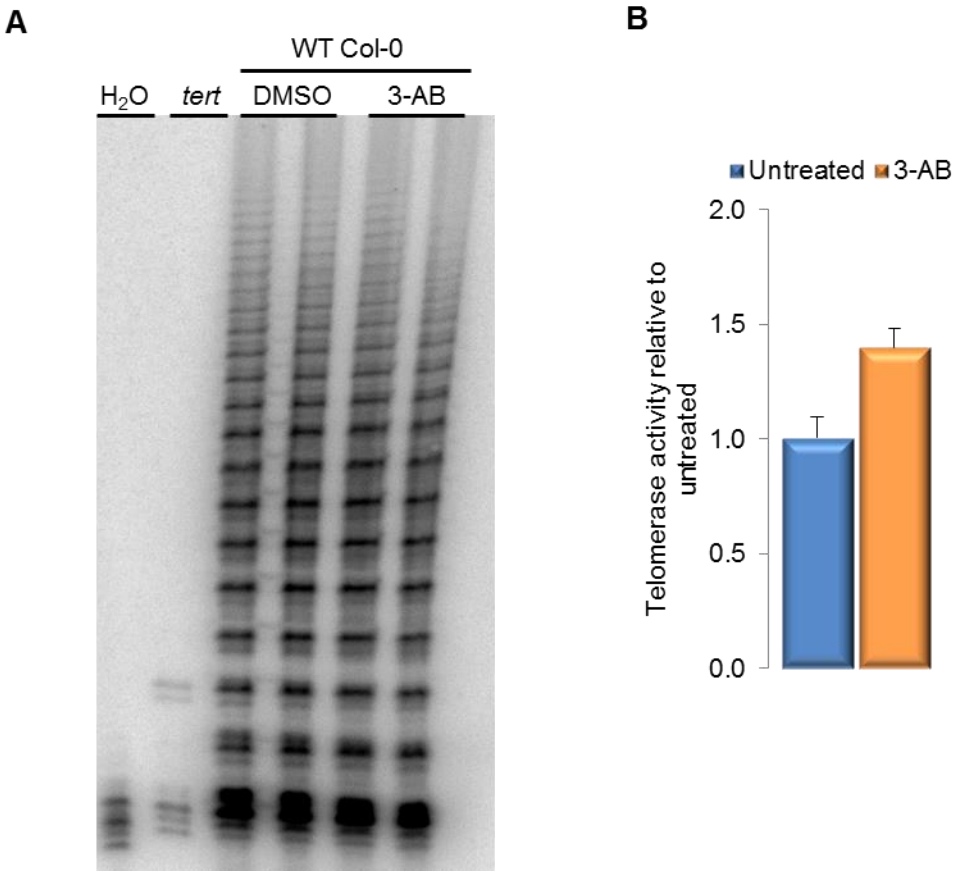

Figure 5-4. PARP activity is not required for telomerase activity in *Arabidopsis*. (A) TRAP analysis on seedlings. Seedlings were treated with either 3-AB (WT) or DMSO (WT and G4 *tert* mutants) (B) Quantitative TRAP results for 7-day-old 3-AB-treated wild type seedlings relative to untreated seedlings. P-value= 0.03 by Student's two-tailed t-test.

value=0.03) relative to untreated seedlings (Fig. 5-4B), but this increase may not be biologically important since higher levels of telomerase activity do not substantially not alter telomere length in *Arabidopsis* (Ren et al. 2004). The presence of telomerase activity in plants lacking PARP activity is in accordance with the majority of studies in mammalian systems which found no change in telomerase activity when PARPs were inhibited or mutated (d'Adda di Fagagna et al. 1999; Samper et al. 2001; Tong et al. 2001; Dantzer et al. 2004; Beneke et al. 2008).

PARPs are not required for telomere end protection in Arabidopsis

We next asked whether PARPs contribute to chromosome end-protection and genome stability in *Arabidopsis*. Cytogenetic analysis was conducted on dissected pistils from *parp1* and *parp2* single and double mutants. Anaphase bridges are the hallmark of dysfunctional telomeres, reflecting the formation of dicentric chromosomes that arise from the fusion of deprotected chromosome ends. No mitotic abnormalities were observed in either *parp1* or *parp2* single mutants or the double mutant (data not shown). A more sensitive assay to detect end-to-end chromosome fusions is telomere fusion PCR (TF-PCR) (Heacock et al. 2004). This method also failed to reveal evidence for telomere fusions in either the *parp1* and *parp2* mutants (data not shown).

We next asked if PARPs act synergistically with telomerase to promote telomere stability. TF-PCR was conducted with 3-AB-treated wild type and G4

tert seedlings. The 3-AB-treated wild type seedlings had no fusions, indicated by the lack of telomeric signal above the background seen in the untreated seedlings (Fig. 5-5). As expected, TF-PCR products were evident in untreated and 3-AB-treated G4 *tert* mutants (Fig. 5-5). Notably, two of the *tert* samples treated with 3-AB did not have fusion products (Fig. 5-5). This result may indicate telomeres in these plants are above the critical length threshold and hence not yet dysfunctional. On the other hand, it is possible that PARP activity may promote some degree of end-to-end chromosome fusions in *Arabidopsis.* In humans, PARPs contribute to an alternative pathway for NHEJ (Mahaney et al. 2009).

PARPs are not required to maintain telomere length in Arabidopsis

Telomeres in plants lacking telomerase do not undergo end-joining reactions until they reach a critical length threshold of approximately 1kb (Heacock et al. 2004). Thus, our negative results for TF-PCR do not preclude the possibility that telomeres were moderately shortened in PARP mutants. To test this possibility, we monitored bulk telomere length in *parp1 and parp2* single mutants and the double mutant using terminal restriction fragment (TRF) analysis. There was some variability in telonmere length between individual plants, but telomeres remained within wild type range (Figure 5-6A). We then used Primer Extension Telomere Length Amplification (PETRA) to examine telomere length on specific chromosome arms. As with TRF analysis, telomere

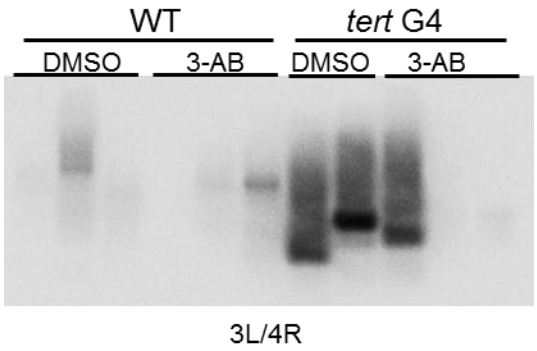

Figure 5-5. PARPs are not required to prevent end-to-end chromosome fusions. Telomere fusion PCR using primers for chromosome arms 3L/4R for WT and *tert* mutants with or without 3-AB treatment.

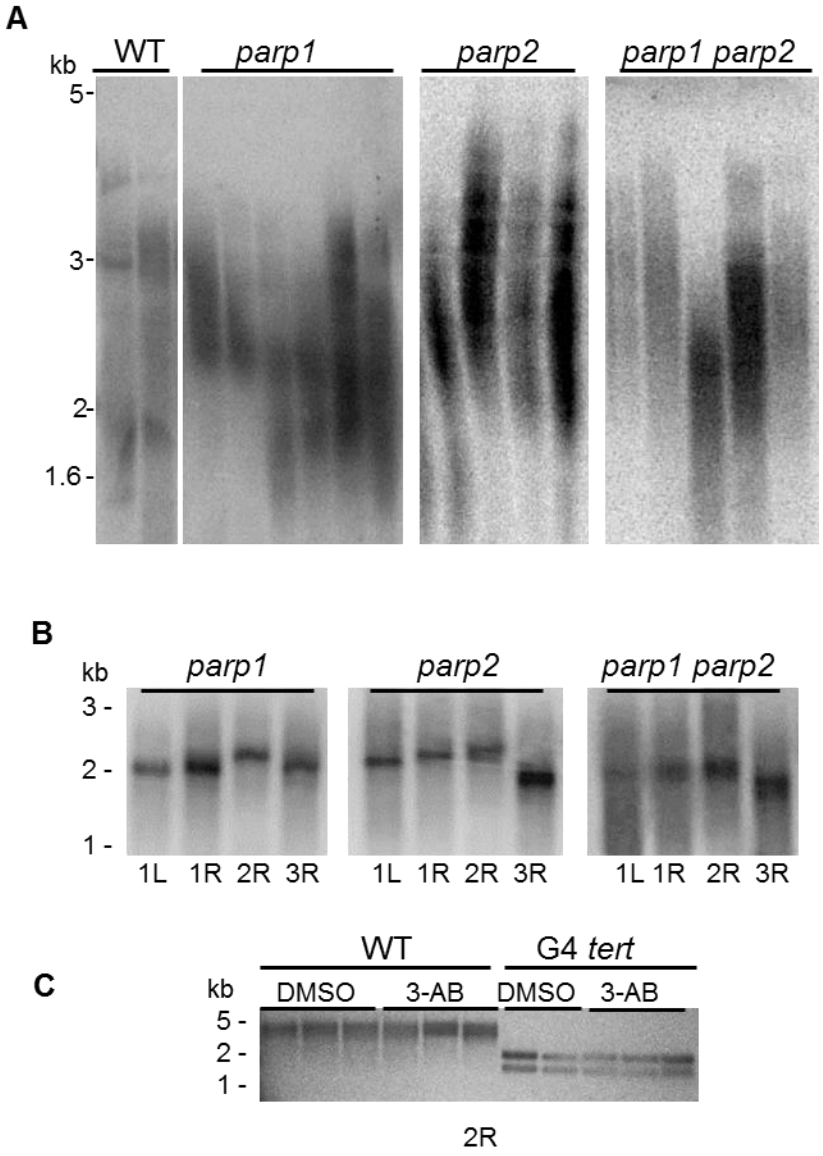

Figure 5-6. PARPs are not required to maintain telomere length in *Arabidopsis*. (A) TRF analysis of bulk telomeres in wild type, *parp1*, *parp2,* and *parp1 parp2* mutants. (B) PETRA analysis of four different chromosome arms for *parp1*, *parp2,* and *parp1 parp2* mutants. (C) PETRA analysis of telomere lengths on arm 2R for wild type and G4 *tert* seedlings grown in either 0.6% DMSO or 5mM 3-AB/0.6% DMSO. Molecular weight markers are shown to the left of each gel.

length for the single or double mutants did not differ from the normal wild type range (Fig. 5-6B). PETRA conducted on wild type and G4 *tert* 3-AB-treated seedlings showed no significant size difference from the corresponding DMSO controls (Fig. 5-6C). Taken together, our data indicate that PARPs do not contribute to telomere length maintenance in *Arabidopsis.*

Discussion

The role of DNA repair proteins in telomere biology is paradoxical. Although one of the main functions of telomeres is to hide chromosome ends from the DNA repair machinery, telomere maintenance requires multiple repair proteins. This situation raises questions about the evolutionary origins for the telomeric role of DNA damage proteins. There are likely multiple scenarios, but one possibility is that eularyotes have co-opted the DDR proteins during evolution as a way to adapt to the evolving roles of telomeres.

Because we were unable to detect any telomere defects in the absence of PARPs, we conclude that PARPs are not essential in *Arabidopsis* telomere biology. This contrasts with the required role of PARPs in vertebrate telomere maintenance and could reflect of fundamental differences in the evolution of PARP function between plants and animals. *Arabidopsis* has six PARPs versus at least seventeen in humans. Several of the PARPs that are important in vertebrate telomere maintenance, including tankyrases and PARP2, have no obvious orthologs in *Arabidopsis.* Thus, it is possible that the larger number of

PARPs in vertebrates allowed them to evolve new functions in relation to telomeres. PARPs, particularly the tankyrases, may be the most important in human telomere biology. In human cells the knockdown or inhibition of PARPs led to telomere length defects which have not been reliably observed in other organisms. Notably, the function of PARPs at mouse telomeres remains unclear. Further, two of the model organisms used to study telomeres, *S. cervisiae* and *S. pombe,* do not have any PARP genes, further supporting that PARPs are not important for telomere regulation in all eukaryotes.

Finally, a role for PARPs at telomeres may also be influenced by the mechanisms used by the organism to regulate telomerase activity and telomere length. For example, the CST complex in *Arabidopsis* is vital for chromosome end protection (Song et al. 2008; Surovtseva et al. 2009), but in humans, telomere protection appears to be afforded by the shelterin complex while CST functions primarily in DNA replication (Nakaoka et al. 2012; Stewart et al. 2012b). PARPs and Tankyrases function mainly through interaction with and modification of the shelterin components TRF1 and TRF2. *Arabidopsis* has six putative TRF-like proteins. Although they can each can bind ds telomeric DNA *in vitro* (Karamysheva et al. 2004), their *in vivo* functions are largely unknown, but appear to be somewhat redundant (L. Vespa, Z. Karamysheva, and D. Shippen, unpublished data). Thus PARP function in *Arabidopsis* may not be apparent because there are multiple TRFs that can compensate for loss of another at telomeres due to PARylation.

Despite their relatively short telomeres, humans must repress telomerase activity as a mechanism to induce replicative senescence to keep mutations from accumulating as cells age (Gomes et al. 2011). Mice, on the other hand, have long telomeres and no repression of telomerase occurs, presumably because their short lifespan does not require as much protection from mutation as humans (Gomes et al. 2011). The telomeric function of human PARPs may reflect an additional layer of fine-tuning for telomere length regulation. The need for human cells to tightly regulate telomerase access to the telomeres could thereby explain why PARPs play a more critical role in humans compared to mice or plants. The variation of PARP importance in telomere biology among eukaryotes illustrates the plasticity of DNA repair proteins in telomere function.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Telomeres are essential for both chromosome end protection and replication of the chromosome terminus. The functions of telomeres require regulation of a diverse assortment of proteins. Defects or misregulation of either end protection or end replication can cause genome instability, cellular senescence, and cell death. An interesting paradox in telomere biology is the conflicting function of the DNA damage response at telomeres. The telomeres must differentiate themselves from DSBs to avoid recruitment into repair reactions. At the same time, a number of DNA repair proteins are required for maintaining telomeres. As more details of telomere regulation are uncovered, it has become clear that telomere end protection is not a simple model where capping proteins hide the chromosome ends from DNA repair reactions. Two important, but unanswered questions are: How do cells distinguish between DNA damage and telomeres, and how are proteins that act in both pathways differentially regulated to maintain genome stability? In my dissertation research I have attempted to address these questions by studying the function of the CST complex in *Arabidopsis* and how it interacts with the DDR to maintain telomeres.

CST functions in telomere maintenance

What is the definition of end protection? The simple response is that chromosome ends are protected from aberrant DNA repair reactions. Thus, many researchers use the presence of a DDR at telomeres as evidence that end protection is compromised (Sfeir and de Lange 2012). Telomeres are capped by CST or shelterin and ends are protected. However, both CST and shelterin have functions beyond simple capping. Both regulate telomerase access to telomeres and regulate telomerase repeat addition processivity (Qi and Zakian 2000; Lei et al. 2005; Latrick and Cech 2010; Leehy et al. 2013). Additionally, if one compares the phenotypes that arise from telomere deprotection, telomerase misregulation, or deficient telomere replication it is not clear how they are different. All can cause changes in telomere length, a DDR, and chromosome fusions. How to reconcile the complexity of end protection is currently a major controversy in the field of telomere research that has been fueled by the discovery of CST in organisms outside of budding yeast.

In humans, CST seems to primarily function in telomere replication. Even phenotypes, such as chromosome fusions, that initially pointed to an end-protection role for human CST can be explained by replication defects in the telomeres (Miyake et al. 2009; Surovtseva et al. 2009). First, CST binds non-specifically to ssDNA. In the absence of CTC1 or STN1, γH2AX foci localize throughout the genome, not just at telomeres. CTC1 and STN1 also promote Polα processivity (Casteel et al. 2009; Nakaoka et al. 2012). Further, replication

through telomeres is slowed in the absence of STN1 or CTC1, and STN1 promotes the genome-wide firing of new origins after replication restart following HU treatment (Stewart et al. 2012b).

In *Arabidopsis*, conversely, the phenotypes in CST mutants are typical of the telomere deprotection phenotypes with significant telomere shortening, chromosome fusions, and aberrant telomere recombination. We do not know how much of a contribution CST makes to Polα-mediated telomere replication in *Arabidopsis*. My research has shown that *Arabidopsis* CTC1 and STN1 interact with DNA Polα, so they could function in telomere replication or at least in C-strand fill in (Chapter II and Appendix B). We have assumed that the primary function of *Arabidopsis* CST is end protection because of the severity of the phenotypes. However, it is possible CST is as important in replication as it is in humans, but the replication phenotypes are masked by the strong deprotection phenotypes. The importance of CST for telomere maintenance suggests that *Arabidopsis* CST may function more similarly to yeast CST than vertebrate CST. In vertebrates POT1 and shelterin are more important for end protection, and the role of CST may have been reduced to replication with the emergence of a protective shelterin complex in vertebrates. In *Arabidopsis* and budding yeast, CST presumably performs both replication and protective functions.

CTC1 as a platform to recruit proteins to the telomeres

In budding yeast, Cdc13 is proposed to be a landing platform to coordinate the telomeric functions of a variety of proteins (Pennock et al. 2001).

The functions of CST are just beginning to be elucidated. I propose that *Arabidopsis* CTC1 is a functional homolog of Cdc13.

The end-protection function of Cdc13 can be bypassed if Stn1 is overexpressed (Petreaca et al. 2006), suggesting that Cdc13 is more important for enriching Stn1 localization to the telomeres than in directly functioning in end protection. When I examined telomere length and telomere fusions in *ctc1* with STN1 overexpression, telomere length was rescued in the *ctc1-1* allele (Chapter II). Although *ctc1-1* plants do not produce a full length CTC1 protein, they may produce a truncated, N-terminal CTC1 because a premature stop codon is present in the middle of the gene. When CTC1 is expressed recombinantly in *E. coli* an N-terminal fragment of CTC1 accumulates but not the full length proteins. The N-terminus is the region of CTC1 that binds to DNA (J.R. Lee and D. Shippen, unpublished data), while the STN1 binding site is in the C-terminus. Thus, the putative truncated CTC1 from *ctc1-1* mutants also probably contains the DNA binding domain, but has lost the STN1 binding domain in its C-terminus. Overexpression of STN1 may allow telomere localization in the absence of CTC1. STN1 binds tightly to DNA, but with less sequence specificity than CTC1 (J.R. Lee and D. Shippen, unpublished data). Overexpression of STN1may create a situation where both it and the N-terminus of CTC1 are simultaneously bound to the telomeres. To develop this model, a number of experiments must be undertaken. First, we need to verify by Western blot that a truncated CTC1 exists in these plants. Also, *in vitro* translated truncated CTC1

should be used for EMSA experiments to show that this fragment can still bind DNA. Finally, a prediction of this model is that more STN1 would bind telomeric DNA. This can be measured using ChIP (chromatin immunoprecipitation) of STN1 in *ctc1-1* mutants with and without STN1 overexpression.

The rescue of *ctc1-1* mutants was incomplete. In the *ctc1-1* 35S::STN1 plants, telomere fusions were still present, although telomere length was within the wild type range. This suggests that STN1 alone is not sufficient to prevent activation of a DDR at telomeres. Could this mean another factor is more important for end protection?

The obvious candidate is TEN1. STN1 overexpression would result in an imbalance in the stoichiometry of STN1 and TEN1 so some portion of telomere-bound STN1 may have lacked TEN1. This hypothesis is supported by preliminary evidence in point mutants where STN1 and TEN1 binding has been disrupted (K. Leehy and D. Shippen, unpublished results). In these mutants, STN1 can still bind to CTC1, suggesting that these are separation of function mutants. When STN1 and TEN1 binding is disrupted, telomere length is rescued to wild type lengths, but telomere fusions still occur.

Several approaches could be used to further test the possibility that TEN1 is the protein needed to prevent a DDR response. First, TEN1 could be overexpressed along with STN1 and then fusion PCR or cytogenetic analysis could be used to determine whether STN1 and TEN1 are both needed to prevent fusions. Additionally, the DDR could be measured and compared in

STN1 overexpression versus STN1-TEN1 overexpression. Expression analysis of transcripts known to be upregulated in CST mutants, such as *BRCA1* and *PARP1*, can be compared to see if addition of TEN1 inhibits the expression. A better, more direct, method would be to look at localization of γH2AX localization to telomeres. This is the standard method used in vertebrate systems because γH2AX is one of the first indicators of a DDR.

My results suggest that the three CST proteins do not have completely redundant function. In Chapter II I show that loss of CTC1, but not STN1, in a *tert* mutant background is detrimental to development. The most important evidence pointing towards independent functions for CST components was provided by analysis of TEN1 (Leehy et al. 2013). *ten1* mutants have more fusions and increased telomerase processivity. TEN1 also localizes to telomeres less frequently than CTC1 or STN1. Additionally, unlike *stn1 tert, ten1 tert* double mutants cannot be recovered (K. Leehy and D. Shippen, unpublished data). Finally, we have evidence that large homo-oligomers of TEN1 can function as molecular chaperones (J.R. Lee and D. Shippen, unpublished data). This is not the case for STN1. If STN1 is needed for telomere length regulation and TEN1 is needed for end protection, does this indicate that the phenotypes seen in *ctc1* mutants are because STN1 and TEN1 cannot stably associate with the telomeres? This is a question that can be addressed in future studies.

The role of CST and KU in protecting the two ends of Arabidopsis *telomeres*

While I do not have an answer to the larger question of what constitutes end protection in telomere biology, my research allows us to conclude that CST is important for telomere maintenance, probably through multiple mechanisms. *Arabidopsis* is unique among model organisms in that the two ends of a chromosome have different architectures (Kazda et al. 2012). One end, capped by CST, ends in a G-overhang; the other end is blunt, and is capped by KU. We do not know how CST and KU may coordinate or compete for telomere binding, but this difference from other organisms could indicate that *Arabidopsis* uses different mechanisms for regulation of telomere capping and telomerase recruitment compared to yeast and humans. In my genetics experiments, for example, my results suggest that neither CST nor KU is required for telomerase recruitment to telomeres in *Arabidopsis*, whereas in yeast, both pathways are required (Chan et al. 2008). Hence there is a third pathway for telomerase recruitment in *Arabidopsis*. One intriguing possibility for such a factor is POT1b. Overexpression of the N-terminus of POT1b causes severe growth defects, telomere fusions, and telomere shortening (Shakirov et al. 2005). POT1b also strongly binds the spliced isoform of TER2, $TER2_S$, which makes it an interesting candidate for both telomerase recruitment or as a third capping complex (Cifuentes-Rojas et al. 2012).

The presence of at least two distinct telomerase complexes with different TER subunits further complicates the comparison of *Arabidopsis* with other

model systems. We currently do not have additional candidate genes for telomere capping or telomerase recruitment. Several people in the Shippen lab are undertaking protein purification experiments for mass spectrometry to identify novel proteins associated with *Arabidopsis* telomeres. Thus, one of these may be an *Arabidopsis* telomerase recruitment protein.

I have presented only a few of the unanswered questions about the function of CST because there are too many for discussion here. However, the identification of human patients with stem cell disorders arising from CTC1 mutation has increased interest in this complex. *Arabidopsis* can make an important contribution to understanding the basis of such diseases because plants null for any of the CST components are viable. Thus, there is the potential to study mutations that cause stem cell disease in humans in this genetically tractable model system.

ATR has multiple functions at *Arabidopsis* telomeres

My study on the role of ATR and ATM with CST in *Arabidopsis* (Chapter III) confirmed several predictions, but also raises new questions to pursue. My initial rationale for investigating *ctc1 atr* and *ctc1 atm* mutants was to verify that CST inhibits a DNA damage response at telomeres. Prevention of a DDR is the hallmark of end protections proteins, so these experiments were important to the larger goal of characterizing CST function. I showed that several known DDR genes were upregulated in *ctc1* mutants, which was the expected result. We

had previously published that ATR was important for telomere length regulation in the absence of TERT (Vespa et al. 2005). I also wondered if ATR played a similar role in *ctc1* mutants. As expected, I saw accelerated shortening in the *ctc1 atr* double mutants, confirming a role for ATR as a positive regulator of telomere length in backgrounds where telomere dysfunction had already occurred.

These experiments also gave unexpected results. I hypothesized that *Arabidopsis* would be similar to vertebrates in the telomeric DDR. In mouse cells knockdown of both POT1, the ssDNA binding shelterin component, and ATR abrogates the DDR caused by the loss of POT1 (Denchi and de Lange 2007). However, I found that absence of ATR and CTC1 led to more genome instability, including increased telomere fusions and an increase in the DNA damage transcriptional response. *ctc1 atm* double mutants were similar to *ctc1* single mutants. These results argue that ATR protects plants against genome instability caused by loss of CTC1.

One finding, even more unexpected, and which did not seem to fit with a protective role for ATR, was the plant morphological phenotypes in *ctc1 atr* mutants. *ctc1* mutants have severe morphological defects including fasciated stems and flowers and irregular phyllotaxy (the pattern of leaves on the stem) (Surovtseva et al. 2009). *ctc1 atr* plants appeared almost wild type, with only an occasional misplaced silique. Why were plants healthier looking without ATR even though their genomes were severely disrupted?

The most likely answer is that ATR promotes programmed cell death of stem cells in *ctc1* mutants. My findings indicate that ATR, and, to a lesser extent, ATM, monitor the levels of genome instability in stem cell niches in *ctc1* mutants. Cells that reach a critical level of DNA damage are culled out of the stem cell population to prevent propagation of the damage to more cells and plants are thus plants are given a greater chance to survive and reproduce. I hypothesize that the increase in genome instability in the *ctc1 atr* mutants reflects the accumulation of cells with high levels of genome instability. The loss of the fasciated phenotype can also be explained by the activation of cell cycle checkpoints and programmed cell death. The fasciated phenotype is associated with an expansion of the meristem caused by an accumulation of cells in G2 phase of the cell cycle, an outcome that was previously observed in *ten1* mutants (Hashimura and Ueguchi 2011). In the absence of ATR-mediated cell death or checkpoint activation, cells would not overaccumulate in the meristem.

Was all of the genome instability in *ctc1 atr* mutants a result of decreased programmed cell death, or could ATR have additional functions in *ctc1* mutants? For example, we do not understand why telomere length drops precipitously in the second generation of *ctc1 atr* mutants. Such a phenotype could be explained by recombination of telomeres, but when we looked for extrachromosomal telomeric circles, the typical indicator of telomeric recombination, we did not find a consistent increase in the double mutant. However, I did find that the drop in telomere length correlates with an abrupt

decrease in telomerase activity in G2 *atr* mutants. We have previously showed that ATR can interact with telomerase components POT1B and KU80, and IP of ATR can pull down telomerase activity as well as TER2 (L. Vespa, M. Jasti, K. Kannan, unpublished results). We do not know the significance of these interactions, however. My study shows that telomerase activity is very low starting with the 2nd generation of *atr* mutation, but telomere length in *atr* mutants is stable over many generations (Chapter III). How much ATR-maintained telomerase activity contributes to the slowing of telomere loss in *ctc1* mutants is unknown.

A few scenarios could explain this phenotype. First, telomerase could act on more telomeres per cell cycle or telomerase recruitment to telomeres could be increased. In both cases, the prediction is that more TERT should be bound to telomeres in *atr* mutants compared to wild type. ChiP of TERT could be used to measure the level of TERT association with telomeres. Another possibility is that telomerase is highly processive in *atr* mutants. The overall level of telomerase activity measured by TRAP could be down, but each telomerase complex could work more efficiently to add more repeats in each cell cycle. Kyle Renfrew has developed a processivity TRAP assay in our lab that can be used to look for longer TRAP products compared to wild type. A final explanation could be that in wild type cells, the amount of telomerase activity measured in our protein extracts by TRAP does not reflect the actual activity at the telomeres. It is possible that *atr* mutants have enough telomerase activity to maintain wild

type telomeres, and in wild type, there is much more telomerase activity than is needed for telomere maintenance.

TER levels are wild type in *atr* mutants (Chapter IV) (Cifuentes-Rojas et al. 2012). Therefore the zeocin-induced decrease in telomerase activity must occur by a different mechanism than the one causing the decrease in *atr* mutants. One possible explanation is that many types of DNA damage cause decreases in telomerase activity, and the severity or type of damage dictates how urgently telomerase must be downregulated. In seedlings, we saw a decrease in telomerase activity within 30 minutes of zeocin treatment, suggesting that the massive induction of DSBs by zeocin required immediate cessation of telomerase activity to prevent further genome instability caused by *de novo* telomere formation at the breaks. We hypothesize that TER2-dependent inhibition of telomerase activity is a rapid response to DSBs. Replication stress caused by hydroxyurea or ATR-deficiency would not immediately lead to large numbers of DSBs so the TER2 mechanism would not get activated. Instead, when DNA damage accumulates slowly over time the need to regulate telomerase is less urgent and other mechanisms are used. In *atr* mutants, the switch to telomerase inhibition in G2 suggests that meiosis is important for the mechanism for inhibition.

Although TER2 levels are wild type in *atr* mutants, we do not know if the amount of TER2 bound with TERT has changed. Preliminary data suggests that zeocin treatment results in increased association of TER2 with TERT (H. Xu and

D. Shippen, unpublished results). It is therefore possible that ATR helps to promote TER1 association with TERT. TER2 has a higher binding affinity for TERT than TER1 (Cifuentes-Rojas et al. 2012) so it would be reasonable to assume that there are factors that facilitate the switching of TERs with TERT. If this is the case, then an IP of TERT in *atr* mutants would be expected to show an increase in TER2 bound to TERT, which would account for the decrease in telomerase activity in these mutants. However, my preliminary data shown in Appendix C, suggests that ATR signaling is required for the downregulation in telomerase activity because the absence of SOG1, the downstream effector of ATR and ATM signaling, also results in a decrease in telomerase activity. Thus, if ATR affects the abundance of TER2 bound to TERT, it is likely indirectly through the upregulation of some unknown protein.

How does TER2 inhibit telomerase activity in response to DSBs?

In our lab's characterization of TER2, we discovered a novel mechanism for telomerase regulation: a long non-coding RNA that competes with the canonical TER1 telomerase RNA for TERT binding. Further, we suggest that one function of TER2-mediated inhibition of telomerase is to prevent telomerase from adding telomere repeats *de novo* to DSBs.

Many questions remain unanswered about TER2-mediated inhibition of telomerase activity. As discussed in Appendix C, I have begun to investigate whether DDR signaling causes the increase in TER2 abundance after zeocin

treatment. My preliminary results show that ATM is not required for the decrease, but the role of ATR and SOG1 is less clear because telomerase activity is low even without zeocin treatment. These experiments will need to be repeated in first generation *atr* and *sog1* mutants, which have wild type telomerase activity. We also do not know if the increase in TER2 is the result of increased transcription or increased stability. Graduate student Hengyi Xu is currently addressing this question by treating seedlings with cordycepin, which inhibits RNA synthesis, and then measuring the levels of the TERs over time.

Finally, our analysis of TER2 showed that it does not serve as an efficient template for telomere addition *in vivo*. This implies that the template region of the RNA may not be essential for the function of an inhibitory RNA. Indeed, TERT association with TER does not require physical contacts with the templating domain sequence (Egan and Collins 2012). According to the paradigm we established in Arabidopsis, such RNAs would not have been identified previously because the general lack of sequence conservation among this class of RNAs, their potential lack of a template region, and the fact that such RNAs may only be induced under certain environmental conditions.

Plants as a model to study the evolution of telomere proteins

Although I have not studied the evolution of telomere proteins directly, my research leads me to ask a number of questions about the origin of telomere proteins and their functions. For example, numerous DDR proteins are required

for telomere maintenance and some telomere proteins may have functions away from the telomeres or may be homologous to non-telomeric proteins. Did the development of linear chromosomes require organisms to co-opt DDR proteins for the new function of maintaining those ends? How did the evolution of telomere protein complexes influence the evolution of DDR proteins at the telomeres? PARP is a good example. PARPs are absent from the *S. cerevisiae* and *S. pombe* genomes, suggesting that organisms can thrive without PARP. In my PARP research I found that PARPs are not important for *Arabidopsis* telomere maintenance (Chapter V). In contrast, in human cells they are critical for viability (Smith and de Lange 2000; Gomez et al. 2006). Do these findings indicate a change in PARP function in humans that evolved along with shelterin, or did PARPs become less important in *Arabidopsis* after CST became the predominant end protection complex? Perhaps the difference lies in the end protection complexes that have evolved in humans and plants. In *Arabidopsis* CST is vital for end protection, whereas in humans it is more important for telomere replication. If shelterin became more important over time for end protection in humans, then PARPs may have evolved to modulate the function of shelterin.

Finally, CST provides a fascinating opportunity to explore the evolution of telomere maintenance and to gain insight into the evolution of protein complexes in general. In humans CST functions mainly in telomere replication while POT1 is a shelterin component that protects telomere ends. While we have not directly

analyzed the contribution of CST to telomere replication in *Arabidopsis*, it is clear that the CST has become important for end protection and the POT1 proteins have evolved from binding telomeric DNA to binding telomerase RNAs. An interesting analysis would be to determine the timing that some of these divergent functions appeared or disappeared. Another former postdoc from our lab, Eugene Shakirov, has developed the moss *Physcomitrella patens* as a new model in which to study plant telomeres. Interestingly, *Physcomitrella* has only one POT1 protein that binds telomeric DNA (Shakirov et al. 2010). *Physcomitrella* also has CST. If we compare the functions of CST in *Physcomitrella* with *Arabidopsis* we may be able to determine whether the ancestral function of CST was as a replication factor or an end protection factor and if its importance in end protection corresponds to the loss of POT1 DNA binding. If we can identify plants, such as *Physcomitrella*, that have ancestral phenotypes, we can then take a biochemical/biophysical approach to understand how changes in the proteins contributed to changes in their functions over time.

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APPENDIX A

CONSERVED TELOMERE MAINTENANCE COMPONENT 1 INTERACTS WITH STN1 AND MAINTAINS CHROMOSOME ENDS IN HIGHER EUKARYOTES*

Summary

Orthologs of the yeast telomere protein Stn1 are present in plants, but other components of the Cdc13/Stn1/Ten1 (CST) complex have only been found in fungi. Here we report the identification of conserved telomere maintenance component 1 (CTC1) in plants and vertebrates. CTC1 encodes a novel ~ 140 kDa telomere-associated protein predicted to contain multiple OB-fold domains. *Arabidopsis* mutants null for *CTC1* display a severe telomere deprotection phenotype accompanied by a rapid onset of developmental defects and sterility. Telomeric and subtelomeric tracts are dramatically eroded, and chromosome ends exhibit increased G-overhangs, recombination, and end-to-end fusions. AtCTC1 both physically and genetically interacts with AtSTN1. Depletion of human CTC1 by RNAi triggers a DNA damage response, chromatin bridges, increased G-overhangs and sporadic telomere loss. These data indicate that

*Reprinted with permission from Surovtseva YV, Churikov D, Boltz KA, Song X, Lamb JC, Warrington R, Leehy K, Heacock M, Price CM, Shippen DE. 2009. Conserved telomere maintenance component 1 interacts with STN1 and maintains chromosome ends in higher eukaryotes. *Mol Cell* **36**: 207-218. Copyright © 2009 by Elsevier.

CTC1 participates in telomere maintenance in diverse species and that a CST-like complex is required for telomere integrity in multicellular organisms.

Introduction

The terminus of a linear chromosome must be distinguished from a double-strand (ds) break to avoid deleterious nucleolytic attack and recruitment into DNA repair reactions. Telomeres prevent such actions by forming a protective cap on the chromosome end. This cap consists of an elaborate, higher-order, DNA architecture and a suite of telomere-specific proteins. The formation of a t-loop of telomeric DNA is thought to play an important role in sequestering the terminal single-strand (ss) G-overhang from harmful activities (Wei and Price 2003; de Lange 2004), while ds and ss telomeric DNA binding proteins coat the chromosome terminus to further distinguish it from a ds break (Palm and de Lange 2008).

In *Saccharomyces cerevisiae*, telomeres are bound by a trimeric protein complex, termed CST, composed of Cdc13, Stn1 and Ten1 (Lundblad 2006; Gao et al. 2007). The three proteins interact to form an RPA-like complex with specificity for ss telomeric DNA. Cdc13 and Stn1 harbor at least one oligonucleotide-oligosaccharide binding (OB) fold, which in the case of Cdc13 is exploited to bind to the G-overhang (Mitton-Fry et al. 2002; Guo et al. 2007). Stn1 and Ten1 associate with the overhang primarily via interactions with Cdc13. The CST complex plays a key role in telomere length regulation (Bianchi

and Shore 2008). Cdc13 recruits the telomerase RNP via a direct interaction with the Est1 component of telomerase (Chandra et al. 2001; Bianchi et al. 2004), while Stn1 is thought to inhibit telomerase action by competing with Est1 for Cdc13 binding (Puglisi et al. 2008; Li et al. 2009). In addition, Cdc13 and Stn1 contribute to coupling of G- and C-strand synthesis through interactions with DNA polymerase α (Qi and Zakian 2000; Grossi et al. 2004).

The CST complex is also essential for chromosome end protection. Mutations in any one of the CST components result in degradation of the C-strand, accumulation of ss G-rich telomeric DNA and late S/G2 cell-cycle arrest (Garvik et al. 1995; Grandin et al. 1997; Grandin et al. 2001). Telomere protection appears to be facilitated primarily by Stn1 and Ten1, and overexpression of Stn1 or Ten1 can rescue the lethality of Cdc13 depletion. Finally, Cdc13 and Stn1 also inhibit telomere recombination.

Mammalian telomeres are bound by Shelterin, a six-member complex that, unlike CST, binds both ss and ds telomeric DNA (Palm and de Lange 2008). The Shelterin proteins TRF1 and TRF2 coat ds telomeric DNA, while POT1 binds the ss G-overhang. The TRF1/TRF2-interacting protein TIN2 and the POT1-interacting protein TPP1 associate with each other, providing a bridge between the duplex and ss regions of telomeric DNA. RAP1 associates with telomeres via interaction with TRF2. The majority of Shelterin components are implicated in telomere capping, although TRF2 and POT1 appear to play pivotal roles in this process. TRF2 associates with telomeric DNA via a myb-like DNA

binding domain. Loss of telomere-bound TRF2 results in immediate degradation of the G-overhang and end-to-end chromosome fusions (Celli and de Lange 2005), while certain dominant negative alleles cause rapid telomere shortening with extrusion of extra-chromosomal telomeric circles (ECTCs) via homologous recombination (Wang et al. 2004).

Like components of the CST complex, POT1 and its partner TPP1 harbor OB-folds. POT1 binds directly to the overhang through two adjacent OB-folds, thus sequestering the DNA 3' terminus and reducing access to telomerase (Lei et al. 2004; Lei et al. 2005). TPP1 does not bind DNA directly, but dimerization with POT1 increases the DNA-binding affinity of POT1 by ~10 fold (Wang et al. 2007). Knockdown of human POT1 by RNAi causes a fairly mild phenotype characterized by impaired proliferation, an increase in chromosome fusions, decreased G-overhang signals and an increase in telomere length. Disruption of the *POT1* gene leads to more dire consequences (Churikov et al. 2006; Hockemeyer et al. 2006; Wu et al. 2006) including activation of a strong ATR-mediated DNA damage checkpoint, G-overhang elongation, rapid telomere growth, elevated telomere recombination and ultimately cell death (Denchi and de Lange 2007; Guo et al. 2007; Churikov and Price 2008).

Telomere protein composition may be more conserved than previously surmised (Linger and Price 2009). At least one Shelterin component, Rap1, is present in *S. cerevisiae*, although unlike vertebrate RAP1, ScRap1p directly binds ds telomeric DNA through two myb-like DNA binding domains and

contributes to telomere length regulation and telomere silencing (Lundblad 2006). Likewise, fission yeast contain several Shelterin orthologs including Taz1, an ortholog of mammalian TRF1/TRF2 proteins (Cooper et al. 1997), and Pot1 (Baumann and Cech 2001). Furthermore, recent purification of SpPot1-associated proteins identified Tpz1, a presumed ortholog of vertebrate TPP1 (Miyoshi et al. 2008). Like TPP1, Tpz1 contains an OB-fold, and physical association of SpPot1 and Tpz1 is required for chromosome end protection (Xin et al. 2007; Miyoshi et al. 2008). The Pot1-Tpz1 complex recruits two additional proteins, Ccq1 and Poz1. Poz1 serves as a bridge linking the Pot1-Tpz1 complex to the ds telomere proteins Rap1 and Taz1 in a manner similar to the Shelterin component TIN2 (Miyoshi et al. 2008). Altogether, these findings argue that the core components of the Shelterin complex are evolutionary conserved.

Emerging data indicate that components of the CST complex are also widespread. Although Cdc13 orthologs have yet to be uncovered outside of *S. cerevisiae*, a Stn1/Ten1 capping complex was recently described for *S. pombe* (Martin et al. 2007). Both proteins localize to telomeres and are essential for chromosome end protection from exonucleases and telomere fusions. Notably, no direct physical association between Stn1/Ten1 and Pot1 has been observed (Martin et al. 2007) and mass spectrometry of SpPot1-associated factors failed to identify Stn1 or Ten1 (Miyoshi et al. 2008). These findings suggest that CST and Shelterin components may constitute distinct telomere complexes.

Plants also appear to harbor both Shelterin and CST components. Several Myb-containing TRF-like proteins bind telomeric dsDNA *in vitro* (Zellinger et al. 2007) and in rice genetic data implicate one of these, RTBP1, in chromosome end protection (Hong et al. 2007). *Arabidopsis* encodes three OB-fold bearing POT1-like proteins (Shakirov et al, 2005; Surovtseva et al, 2007; A.D.L. Nelson and D.E. Shippen, unpubished work). Interestingly, while over-expression of a dominant negative allele of AtPOT1b or depletion of AtPOT1c lead to a telomere uncapping phenotype similar to a *pot1* deficiency in yeast and mammals (Shakirov et al. 2005) (A. Nelson, Y. Surovtseva and D. Shippen, unpublished data), AtPOT1a is dispensable for chromosome end protection and instead is required for telomerase function (Surovtseva et al. 2007). Currently, orthologs for TIN2, RAP1 and TPP1 cannot be discerned in any plant genome.

Recently, a distant homolog of the CST component STN1 was uncovered in *Arabidopsis* (Song et al. 2008). AtSTN1 bears a single OB-fold and localizes to telomeres *in vitro*. Deletion of AtSTN1 results in the immediate onset of growth defects and sterility, coupled with extensive exonucleolytic degradation of chromosome ends, increased telomere recombination, and massive end-to-end chromosome fusions (Song et al. 2008).

Here we report the identification of a novel telomere protein, termed CTC1 (conserved telomere maintenance component 1), that physically and genetically interacts with AtSTN1. We show that AtCTC1 localizes to telomeres *in vitro* and, as for AtSTN1, that loss of AtCTC1 triggers rapid telomere deprotection resulting

in gross developmental and morphological defects, abrupt telomere loss, telomere recombination, and genome instability. Although not as severe as an *Arabidopsis ctc1* null mutant, the consequences of CTC1 knockdown in human cells include a DNA damage response, formation of chromatin bridges, increased G-overhang signals and loss of telomeric DNA from some chromosome ends. Altogether, these data argue that CTC1 is a component of a CST-like complex in multicellular organisms that is needed for telomere integrity. Notably, we have found that mammalian CTC1 and STN1 correspond to the two subunits of alpha accessory factor (AAF), a protein complex previously shown to stimulate mammalian DNA pol α-primase (Goulian and Heard 1990; Casteel et al. 2009). Thus, the CST-like complex from plants and mammals may resemble the *S. cerevisiae* CST by providing a link between telomeric G- and C-strand synthesis.

Materials and methods

Mutant lines and CTC1 localization

The *ctc1-1* line was identified in the TILLING collection (Till et al. 2003). *ctc1-2* and *ctc1-3* lines were found in the SALK database (stock lines SALK_114032 and SALK_083165, respectively). Genotyping is described in supplemental methods. The *stn1-1* line was previously described (Song et al. 2008). A genetic cross was performed between plants heterozygous for *stn1-1* and for *ctc1-1*. For localization studies, a genomic copy of *CTC1* was cloned into

the pB7WGC2 Gateway vector (Karimi et al. 2005). The resulting N-terminal CFP fusion was transformed into wild type *Arabidopsis* (Surovtseva et al. 2007).

Map-based cloning

Map-based cloning was performed essentially as described (Lukowitz et al. 2000). Briefly, a mutant line (*Columbia* ecotype) was out-crossed to wild type *Arabidopsis Landsberg erecta* ecotype. F1 plants were self-propagated to F2. Pools of wild type and mutant plants were generated (~ 50 plants in each pool) for bulked segregant analysis. CIW5 and CIW6 markers were identified as markers linked to the mutation. 150 individual mutant plants were used to find recombinants in the genomic interval between CIW5 and CIW6. The region containing the mutation was mapped by creating and analyzing new markers. Primer sequences of mapping markers are available upon request.

siRNA-mediated knockdown of HsCTC1

HeLa, MCF7 or 293T cells were subject to two rounds of transfection 24 hrs apart using Lipofectamine2000, Oligofectamine CaPO4. The final concentration of siRNA duplex (see supplemental methods for sequences) was 50 mM (Ambion) or 100 nM (EZBiolab) for each transfection. The efficiency of CTC1 knockdown was assessed using quantitative real-time RT-PCR with SYBR Green. Regions corresponding to CTC1 and GAPDH mRNAs were amplified for each RNA sample. The GAPDH mRNA level was used as an endogenous

control to normalize the level of CTC1 mRNA for each RNA sample. The normalized values were plotted relative to the mock-transfected control that was set to 100%. All reactions were performed in duplicate.

Genotyping of Arabidopsis *mutant lines, DNA and RNA extraction, and RT-PCR*

To genotype the *ctc1-1* line, a genomic region flanking the *ctc1-1* point mutation was amplified with CTC1_M2 fwd (5'-GTAATGCCCATCTCAAGTTTTG) and CTC1_M2_rev (5'-CAGCACACGCATAGCACTATG) primers and sequenced with the CTC1_M2 rev primer. Genotyping of the *ctc1-2* and *ctc1-3* lines was performed with T-DNA and gene-specific primers.

DNA was extracted from plants as previously described (Cocciolone and Cone 1993). RNA samples were prepared using Plant RNA Purification Reagent (Invitrogen) and reverse transcription was performed using 2 µg of RNA, as described (Shakirov et al. 2005). AtCTC1 cDNA was amplified in the PCR reaction with primers CTC1_start_fwd (5'-ATGGAGAACACCACAATTCTCAC) and CTC1_stop_rev (5'-TCAGCTATTTAGCAAACCTTGGAG). To evaluate expression of the region flanking the T-DNA insertion in the *ctc1-2* allele, primers 5'-GTCACGCTTTTGAGAGGTCTG and CTC1_M2_rev were used. For the *ctc1-3* allele, primers CTC1_M2_fwd and 5'-CACTTGAGGAACTTATCCTCTG were used.

Protein expression and co-immunoprecipitation

For *in vitro* studies, full-length CTC1 cDNA or its truncated versions were cloned into pET28a and pCITE4a vectors (Novagen) and expressed using rabbit reticulocyte lysate according to manufacturer's instructions (Promega). For *in vitro* co-immunoprecipitation, pET28a (T7-tag fusion) and pCITE4a (untagged) constructs were expressed in rabbit reticulocyte lysate in absence or presence of ^{35}S-Methionine (PerkinElmer), respectively. Coimmunoprecipitation was conducted as described (Karamysheva et al. 2004).

TRF analysis, PETRA, telomere fusion PCR, quantitative TRAP, and in-gel hybridization

TRF analysis of *Arabidopsis* telomeres was conducted as previously described (Fitzgerald et al. 1999). Subtelomeric TRF analysis was performed using a 1L probe (Surovtseva et al. 2007), or 5R probe (Shakirov and Shippen 2004). For PETRA (Heacock et al. 2004), 2 µg of DNA was used. An adapter primer was hybridized to the G-overhang and extended with ExTaq polymerase (Takara), followed by a specific chromosome arm amplification with unique subtelomeric primers as described in (Heacock et al. 2004).

Telomere fusion PCR was performed as previously described (Heacock et al. 2004). PCR products were purified, cloned into pDrive vector (Quiagen), and sequenced. Quantitative TRAP assay was performed as described (Kannan et al. 2008). G-overhangs were analyzed by in-gel hybridization as previously

described for *Arabidopsis* and human telomeres (Churikov and Price 2008; Song et al. 2008). Genomic DNA was separated in native agarose gels, dried gels were then hybridized with ^{32}P 5' end-labeled telomeric C-strand probe $(C_3TA_3)_4$ for plant DNA and $(TA_2C_3)_4$ for human DNA). For quantification of *Arabidopsis* G-overhang signal, the hybridization signal from the native gel was normalized with the signal from the ethidium bromide-stained gel. The G-overhang signal obtained from mutant samples was compared to wild type signal, which was set to one. To quantify the G-overhang signal from human telomeres, the native gel was denatured and reprobed with the C-strand oligonucleotide. The signal from the denatured gel was used to normalize for gel loading.

Telomeric circle assays

For TCA and bubble trapping, DNA was digested with Alu1. TCA was performed using 50 µg of DNA as described (Zellinger et al. 2007). For the bubble trapping technique (Mesner et al. 2006), 100 µg of DNA was used. Equal volumes of DNA and 1% low-melt agarose were equilibrated at 45°C, mixed, and loaded on 0.6% agarose gel. The gel was run at 20 V at 4°C for 16 hrs. DNA was then transferred to the nylon membrane and hybridized with a G-rich telomeric probe.

Cytology, immunofluorescence and FISH

For cytological analysis of *Arabidopsis* chromosomes, spreads were prepared from pistils as described (Riha et al. 2001). Chromosomes were stained with DAPI (4',6'-diamidino-2-phenylindole) and analyzed with epifluorescence microscope (Zeiss). Immunolocalization and FISH were performed on CFP-CTC1 7-days old seedlings as discussed (Song et al. 2008). The BACs used were those described in (Surovtseva et al. 2007).

Human cells were fixed and stained for indirect immunofluorescence as described using monoclonal or polyclonal antibody to γ-H2AX, Ser139 and monoclonal to TRF2. Interphase bridges were visualized with DAPI. Colocalization of γ-H2AX and TRF2 foci was monitored using a colocalization plug-in written for Image J by Pierre Bourdoncle (Institut Jacques Monod, Service Imagerie, Paris). Two foci were considered colocalized if their respective intensities were higher than the set threshold of their channels, and if their intensity ratio was higher than the set value. Metaphase spreads were prepared and telomere FISH performed as described. FISH signals were scored using Image J using the Cell counter plug-in.

Results

Identification of CTC1

In an effort to identify mutations in *AtPOT1c*, we examined lines within a TILLING collection of EMS-mutagenized *Arabidopsis* plants. A mutant was uncovered that showed a profound telomere uncapping phenotype (described

below). However, this phenotype did not segregate with nucleotide changes in *AtPOT1c* and therefore map-based cloning was employed to identify the lesion responsible for the phenotype. A single-nucleotide transition (G to A) was found in At4g09680, which co-segregated with telomere uncapping. At4g09680 lies on chromosome 4, while *AtPOT1c* resides on chromosome 2. At4g09680 was designated *CTC1* (conserved telomere maintenance component 1) and the point mutant was termed *ctc1-1*. *CTC1* is a single copy gene and sequence analysis of *CTC1* cDNA from wild type plants revealed a large ORF with 16 exons that encodes a novel 142 kDa protein (Fig. A-1A). RT-PCR demonstrated that *CTC1* is widely expressed in both vegetative and reproductive organs (Fig. A-2A). Further analysis of the CTC1 protein sequence is discussed below.

CTC1 associates with telomeres in vitro

To determine whether CTC1 associates with telomeres *in vitro,* an N-terminal CFP-tagged version of CTC1 protein was expressed in transgenic *Arabidopsis* and immunolocalization experiments were performed on different tissues. Nuclear CFP signal was detected in plants expressing CFP-CTC1, but not in untransformed controls (Fig. A-1B, Fig. A-2B and data not shown). Telomere distribution was analyzed by fluorescence in-situ hybridization (FISH) using a telomere probe. In *Arabidopsis,* telomeres lie at the nucleolar periphery and, as expected, telomeric FISH signals were positioned in this location. Similarly, CFP-CTC1 was distributed in a punctate pattern surrounding the

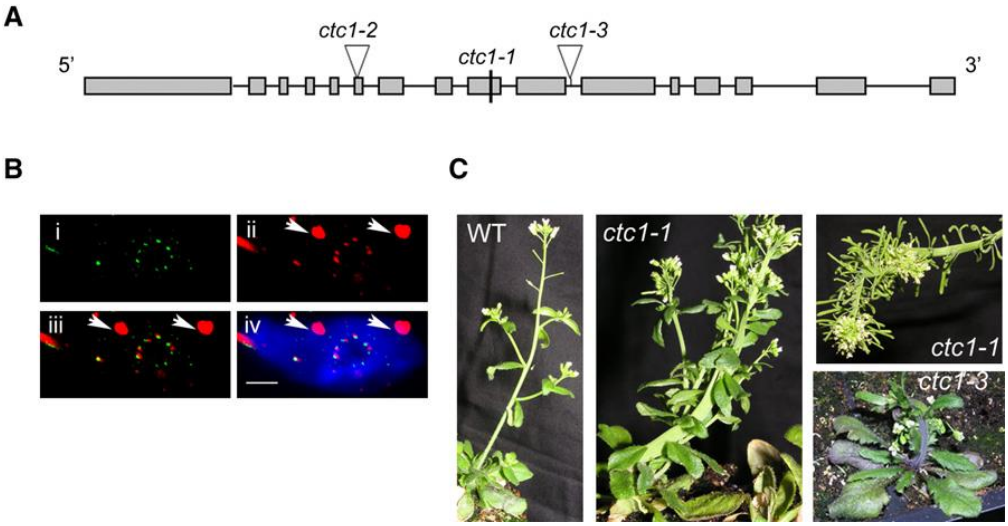


Figure A-1. Identification of CTC1 in *Arabidopsis thaliana*. (A) Schematic of the AtCTC1 gene locus. Rectangles represent exons; horizontal black lines are introns. The positions of the point mutation (*ctc1-1*) and T-DNA insertions (*ctc1-2* and *ctc1-3*) are shown. (B) Colocalization of AtCTC1 and telomeres at the nucleolus periphery of leaf nuclei from seedlings. (i) CFP-AtCTC1 localization detected with anti-GFP antibody; (ii) telomere FISH using probe made from DIG-UTP-labeled T_3AG_3-C_3TA_3; (iii) CFP-AtCTC1-telomere merge; (iv) image from (iii) is combined with DAPI-stained nucleus. The nucleolus appears as a ring where DAPI staining is excluded, arrows in (i)–(iv) indicate internal stretches of telomeric DNA sequence (Armstrong et al. 2001). Scale bar, 2.5 mm. (C) Morphological defects in *ctc1* mutants. Left panel, wild-type; middle and right panels, first generation *ctc1-1* and *ctc1-3* mutants of similar age. Fasciated stems and fused organs in *ctc1* mutants are shown. The severity of morphological defects varies among *ctc1* mutants.

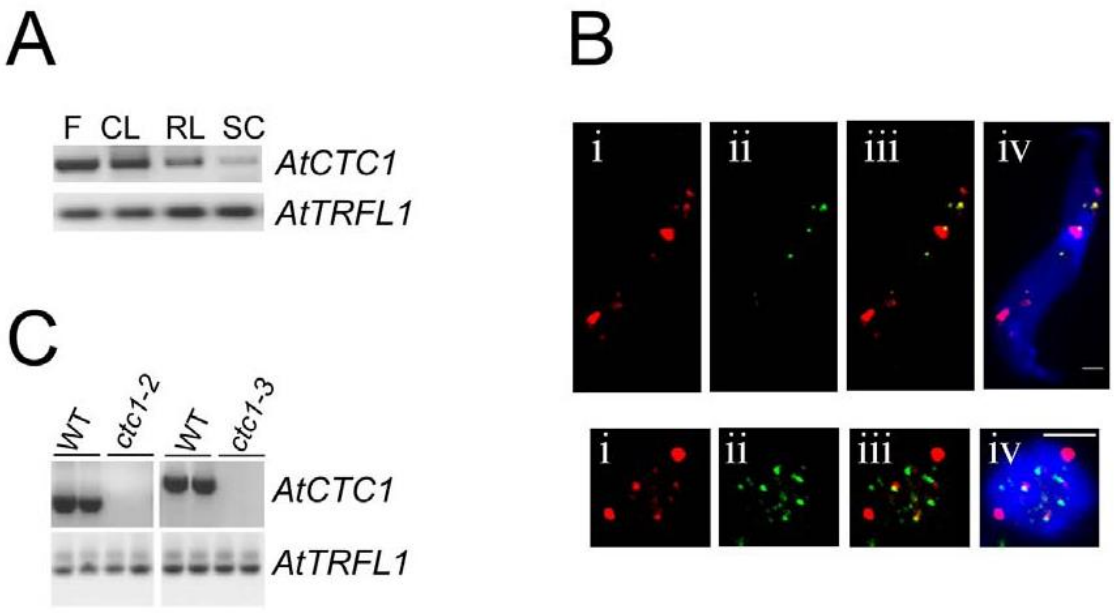


Figure A-2. *AtCTC1* gene expression in wild type and in T-DNA insertion mutants. (A) RT-PCR analysis of the *AtCTC1* gene expression in different plant tissues. F, flowers; CL, cauline leaves; RL, rosette leaves; SC, suspension culture. (B) Co-localization of AtCTC1 and telomeres. Seedling root nucleus and flower nucleus are shown in top and bottom panels, respectively. (i) CFP-AtCTC1 localization detected with anti-GFP antibody; (ii) telomere FISH; (iii) CFP-AtCTC1 – telomere merge; (iv) image from panel (iii) is combined with DAPI stained nucleus. Scale bar = 2.5 μm. (C) RT-PCR analysis of AtCTC1 gene expression in *ctc1-2* and *ctc1-3* mutants. Primers flanking the insertion were used in both cases. TRFL1, a constitutively expressed gene, was used as a loading control.

nucleolus. A merge of these images showed that much of the CFP-CTC1 co-localized with *Arabidopsis* telomeres (Fig. A-1B and Fig. A-2B). CTC1 association with telomeres was quantitated in flowers and seedlings, which contain cycling cells. On average, 51% (n = 38, SD = ±26%) of the telomere signals overlapped with CFP-CTC1. To determine if CTC1 colocalization with telomeres was retained in noncycling cells, we examined the apical half of rosette leaves that were at least 2 weeks old and arrested in G1 (Donnelly et al. 1999). In these cells, 44.1% (n = 28, standard deviation = ±24.5%) of the telomeres displayed an overlapping signal with CFP-CTC1. These data argue that CTC1 associates with telomeres throughout the cell cycle.

Severe growth defects and sterility in first-generation ctc1 mutants

We next examined the impact of CTC1 inactivation on plant morphology. Sequence analysis of *CTC1* cDNA from *ctc1-1* mutants revealed that the G(1935)A point mutation resulted in a nonsense codon within the ninth exon (Fig A-1A). Two additional *CTC1* alleles, *ctc1-2* and *ctc1-3*, bearing T-DNA insertions in the sixth exon or tenth intron, respectively, were identified within the SALK database (Fig. A-1A). RT-PCR analysis showed that no *CTC1* full length mRNA was produced in either *ctc1-2* or *ctc1-3*, indicating that these lines are null alleles of *AtCTC1* (Fig. A-2C).

All three *ctc1* mutants displayed a rapid onset of severe morphological defects in the first generation (Fig. A-1C), confirming that *CTC1* lesions are

responsible for telomere uncapping. The large majority of *ctc1* plants had grossly distorted floral phyllotaxy with an irregular branching pattern and fasciated (thick and broad) main and lateral stems and siliques (Fig. A-1C). Although most mutants produced an influorescence bolt, this structure was highly variable in size, ranging from very short to wild type (Fig. A-1C, compare middle and bottom right panels). Flowers and siliques were often fused, and seed yield was typically reduced to ~ 10% of wild type. The germination efficiency of the few seeds that could be recovered was extremely low, making propagation to the next generation almost impossible.

Telomere shortening and increased length heterogeneity in ctc1 *mutants*

Terminal restriction fragment (TRF) analysis was performed to examine bulk telomere length in *ctc1* plants derived from a single self-pollinated heterozygous parent. In contrast to the telomeres of their wild type and heterozygous siblings, this spanned 2-5 kb in length (Fig. A-3A, lanes 1 to 4), telomeres in homozygous *ctc1-1* mutants were severely deregulated (Fig. A-3A, lanes 5 and 6). The longest *ctc1-1* telomeres were in the wild type range, but a new population of shorter telomeres emerged, the shortest of which trailed to 0.5 kb. Homozygous *ctc1-2* and *ctc1-3* mutants showed a similar aberrant telomere length phenotype (Fig. A-4A).

We investigated how individual telomeres were affected by CTC1 loss using subtelomeric TRF analysis with probes directed at specific chromosome

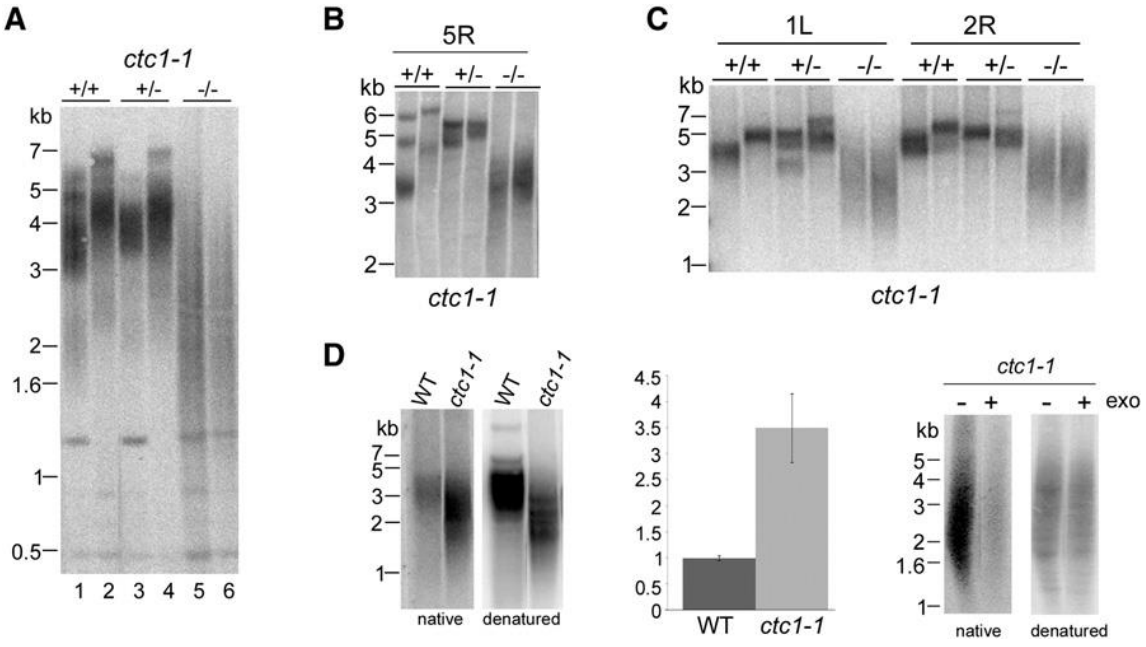


Figure A-3. Telomere length deregulation and increased G-overhangs in *AtCTC1* mutants. (A) TRF analysis of *ctc1-1*. Results are shown for progeny segregated from a parent heterozygous for *ctc1*. (B) Subtelomeric TRF analysis of DNA from *ctc1-1* mutant. DNA blots were hybridized with a probe corresponding to subtelomeric regions on the right arm of chromosome 5 (5R). (C) PETRA analysis of DNA from *ctc1-1* mutants. Results for the 1L and 2R telomeres are shown. (D) In-gel hybridization of $(C_3TA_3)_4$ probe to telomeric restriction fragments under native and denaturing conditions (left). Quantification of *ctc1-1* signal relative to wild-type is shown in the middle panel. Data are the average of eight independent experiments ± SD (p = 1.3E-5 Student's t test). Right panel, in-gel hybridization of *ctc1-1* DNA in the absence (-) or presence (+) of 3'- 5' exonuclease (T4 DNA polymerase). In (A) and (C), blots were hybridized with a radiolabeled telomeric DNA probe $(T_3AG_3)_4$. Molecular weight markers are indicated.

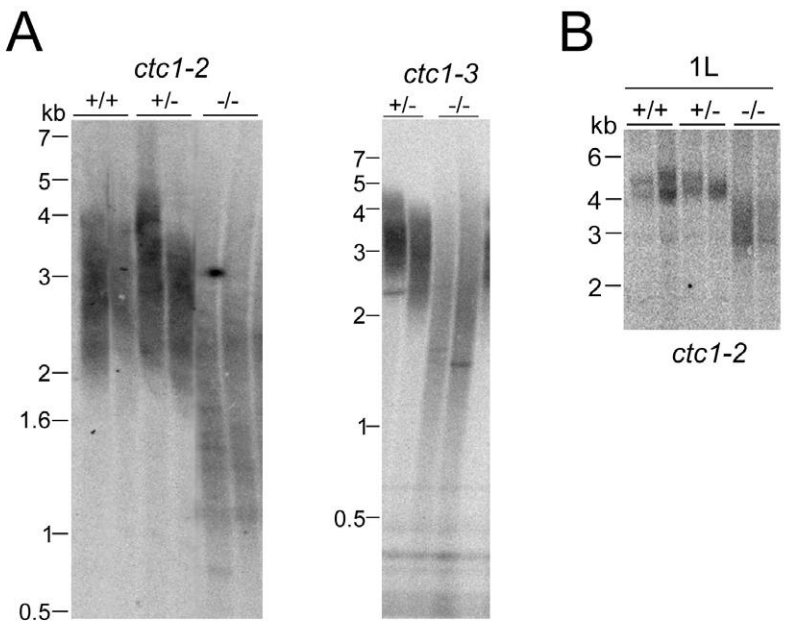


Figure A-4. Telomere length deregulation in *AtCTC1* deficient mutants. (A) TRF analysis of *ctc1-2 and ctc1-3* mutants. Results are shown for progeny segregated from a parent heterozygous for *ctc1*. DNA blots were hybridized with a radiolabeled telomeric probe. (B) Subtelomeric TRF analysis of DNA from *ctc1-2* mutant. Blots were hybridized with a probe corresponding to subtelomeric region on the left arm of chromosome 1 (1L). In both panels, molecular weight markers are indicated.

termini. As expected (Shakirov and Shippen 2004), sharp bands were produced from wild type telomeres (Fig. A-3B and A-4B). In contrast, telomeres in *ctc1* mutants gave rise to a broad heterogeneous hybridization signal spanning 1.5 kb (Fig. A-3B and A-4B). Primer extension telomere repeat amplification (PETRA) also generated broad smears in *ctc1* mutants, confirming that the length of individual telomere tracts was grossly deregulated (Fig. A-3C). Telomere shortening and increased heterogeneity at individual telomere tracts in *ctc1* mutants is not due to a reduction in telomerase activity. Quantitative Telomere Repeat Amplification (Q-TRAP) revealed no significant difference in the *in vitro* telomerase activity levels in *ctc1* mutants relative to wild type (Fig. A-5).

Increased G-overhang signals and telomere recombination in ctc1 *mutants*

Next we studied the G-overhang status in *ctc1* mutants using non-denaturing in-gel hybridization. Strikingly, the G-overhang signal was ~three times greater in *ctc1* mutants relative to wild type (3.5 ± 0.7) (Fig. A-3D). A similar increase in G-overhang signal is observed in *Arabidopsis stn1* mutants (Song et al. 2008). Exonuclease treatment reduced the G-overhang signal by approximately 95%, indicating that the majority of ss telomeric DNA is associated with the chromosome terminus (Fig. A-3D, right panel).

To investigate whether telomeres in *ctc1* mutants are subjected to increased recombination, we used t-circle amplification (TCA)

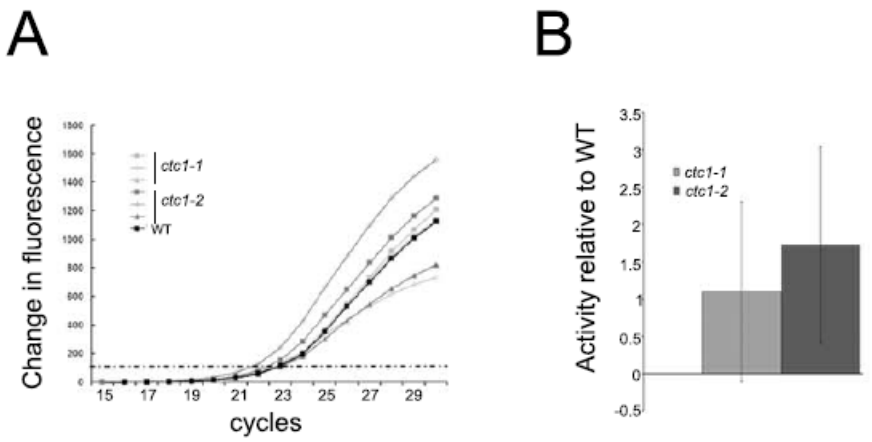
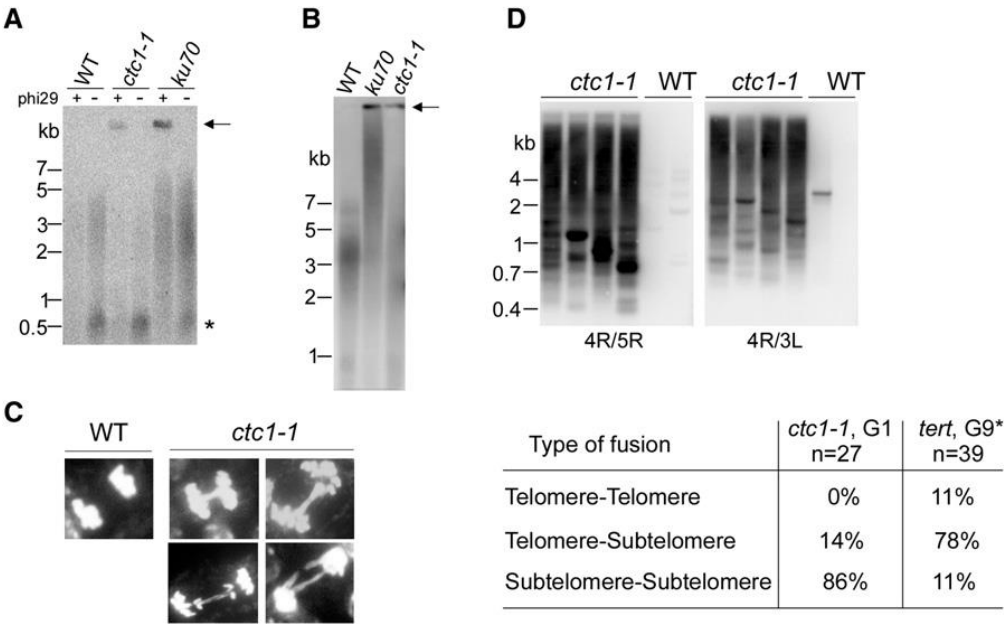


Figure A-5. Results of real time TRAP on *ctc1-1* and *ctc1-2* mutants. Left panel shows raw data. Dashed line represents the threshold change in fluorescence. Right panel shows quantification of the telomerase activity levels in *ctc1* mutants relative to wild type.

(Zellinger et al. 2007) to look for evidence of ECTCs, a by-product of t-loop resolution. In this procedure, telomere sequences are amplified by phi29, a polymerase with strand displacement activity that generates high molecular weight ssDNA products from a circular template. As a positive control, TCA was performed on DNA from *ku70* mutants previously shown to accumulate ECTCs (Zellinger et al. 2007). A high molecular weight DNA band was detected in both *ku70* and *ctc1* DNA samples, but not in wild type (Fig. A-6A). To verify the presence of ECTCs in *ctc1* mutants, we employed the bubble trapping technique (Mesner et al. 2006), which relies on the ability of linear DNA fragments to enter the gel, while circular DNA cannot. A telomeric signal was detected in the well with DNA from *ctc1* and *ku70* mutants, but not with wild type (Fig. A-6B). These data confirm that ECTCs accumulate in the *ctc1* background and argue that loss of CTC1 results in elevated rates of homologous recombination at telomeres. Altogether, these results indicate that the architecture of the chromosome terminus is perturbed in the absence of CTC1.

End-to-end chromosome fusions in ctc1 *mutants*

In *Arabidopsis,* telomeres shorter than 1 kb are prone to end-to-end chromosome fusions (Heacock et al. 2007). Since a substantial fraction of *ctc1* telomeres dropped below this critical threshold, we looked for evidence of mitotic abnormalities. Anaphase bridges were scored in four individual *ctc1-1* mutants and in their wild type siblings. As expected, there was no evidence of genome



Type of fusion	*ctc1-1*, G1 n=27	*tert*, G9* n=39
Telomere-Telomere	0%	11%
Telomere-Subtelomere	14%	78%
Subtelomere-Subtelomere	86%	11%

Figure A-6. *ctc1-1* mutants display elevated telomere recombination and end-to-end fusions. (A) TCA with *ctc1-1* DNA. Reactions were performed in the presence or absence of phi29 polymerase. *ku70* DNA was used as a positive control. (B) Bubble-trapping results for *ctc1-1* and *ku70* mutants. All blots were hybridized with a radiolabeled telomeric probe. In (A) and (B), the probe hybridized to both circular and linear telomeric DNA products. Arrows mark TCA product/circles, smears correspond to TRFs, and the asterisk indicates an interstitial telomeric repeat signal. (C) Cytogenetic analysis of *ctc1-1* mutants showing DAPI-stained chromosome spreads with anaphase figures. (D) Telomere fusion PCR analysis of *ctc1-1* mutants. Primers were specific for 4R and 5R (left) or 4R and 3L (right). The table shows types of fusion junctions found after sequencing PCR products.

instability in wild type plants, but in all four *ctc1-1* mutants a high fraction of mitotic cells (up to 39%) exhibited anaphase bridges (Fig. A-6C and Table A-1). Many anaphases contained multiple bridged chromosomes as well as instances of unequal chromosome segregation (Fig. A-6C). FISH using a mixture of probes from nine subtelomeric regions produced signals in 20/23 anaphase bridges, indicating that the bridges represent end-to-end fusions (Table A-2). FISH probes from eight chromosome ends were individually applied to chromosome preparations from a single *ctc1-1* flower cluster. Signals from each probe were observed in anaphase bridges suggesting that all chromosome arms participated in chromosome fusions (Table A-1).

Telomere fusion PCR confirmed end-to-end chromosome fusion. Abundant telomere fusion products were generated from *ctc1-1* homozygous plants, but not from heterozygous or wild type siblings (Fig. A-6D and data not shown). Sequence analysis of 27 cloned fusion junctions failed to detect joining events involving direct fusion of telomere repeats. Instead, telomere-subtelomere fusions (14%) and subtelomere-subtelomere fusions (86%) were recovered (Fig. A-6D), which were characterized by extensive loss of subtelomere sequences (792 bp average loss). In contrast, in G9 *tert* mutants, telomere-subtelomere fusions are the most prevalent (78%), and the average loss of subtelomeric DNA sequences is only 290 bp (Heacock et al. 2004). Thus, chromosome ends are subjected to dramatic DNA loss prior to fusion in *ctc1* mutants.

Table A-1. Frequency of anaphase bridges in *ctc1-1* mutants.

Genotype	# of analyzed pistils	# of anaphases		% anaphase bridges
		with bridges	total scored	
ctc1-1 #1	4	50	127	39
ctc1-1 #2	6	95	395	24
ctc1-1 #3	3	80	278	29
ctc1-1 #4	4	54	190	28
WT	4	1	140	0
tert, G6				6*
tert, G9				~40*

* Data reported in (Heacock et al 2004).

Table A-2. FISH labeling to identify chromosome ends present in anaphase bridges from *ctc1-1* mutants.

Chromosome Arms	Probe (BAC)	Bridges with Signal	Bridges Observed
All but 4R	9 BAC mix	20[a]	23
1L	F6F3	6	21
1R	F516	5	22
2R	F11L15	3	10
3R	F16M2	5	29
4R	F6N15	6	32
5L	F7J8	7	29
5R	K919	1	8
4R, 2L	25S rDNA	1	7

[a] All but four signals were doublet. Cases in which the signal was a doublet are counted as one signal.

CTC1 and STN1 act in the same genetic pathway for chromosome end protection

Since the rapid telomere-uncapping phenotype associated with loss of AtCTC1 is remarkably similar to AtSTN1 deficiency (Song et al. 2008), we asked whether the two proteins act in the same genetic pathway for chromosome end protection. Plants heterozygous for *ctc1-1* were crossed to *stn1-1* heterozygotes and F1 progeny were self-pollinated to generate homozygous *ctc1-1 stn1-1* mutants, and their *ctc1-1* and *stn1-1* single mutant siblings. Double *ctc1 stn1* mutants were viable, and the severity of morphological defects was similar to the single mutants (Fig. A-7A).

TRF analysis and PETRA revealed the same heterogeneous, shortened telomere profile in double mutants as in the *ctc1* or *stn1* single mutants (Fig. A-8A and Fig. A-7B). Similarly, G-overhang signal intensity and the level of ECTC were comparable, implying that double *ctc1-1 stn1-1* mutants did not undergo additional telomeric DNA depletion or increased telomere recombination (Fig. A-8B and Fig. A-7C). Finally, the frequency of anaphase bridges was similar in double mutants and their *ctc1* and *stn1* siblings (Table A-3). Altogether these findings indicate that *AtCTC1* and *AtSTN1* act in the same pathway for chromosome end protection.

We looked for evidence of a physical association between AtCTC1 and AtSTN1 proteins. Full length AtSTN1 and truncation fragments of AtCTC1 were expressed in rabbit reticulocyte lysate as T7-tagged proteins or radiolabeled with

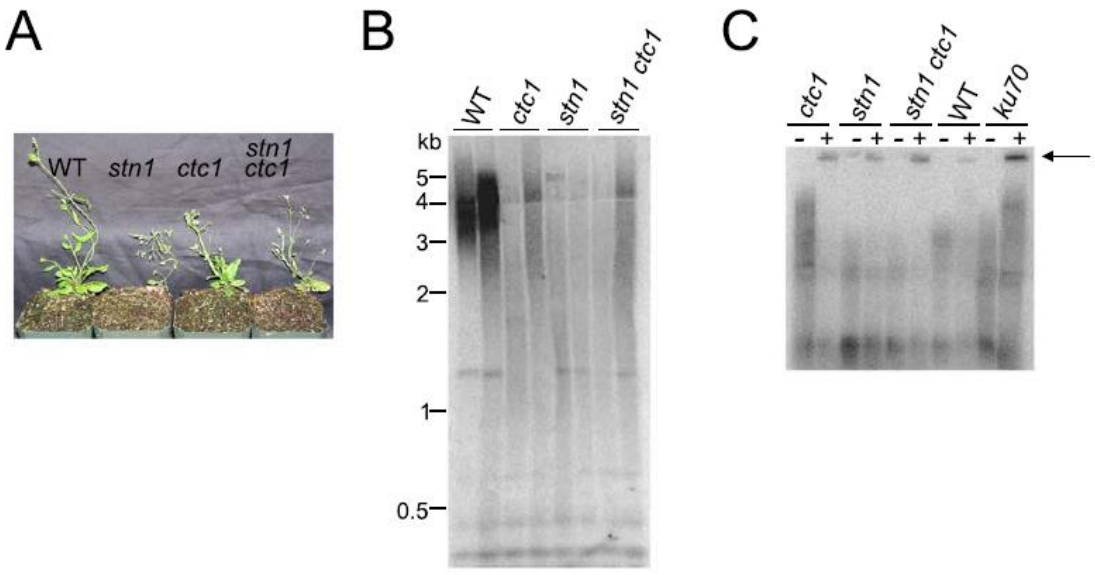


Figure A-7. Morphological and telomere phenotypes in *ctc1-1 stn1-1* double mutants. (A) Morphological and developmental defects in *ctc1-1 stn1-1* double mutants and their *ctc1-1* and *stn1-1* siblings. (B) TRF analysis of *ctc1-1 stn1-1*, *ctc1-1* and *stn1-1* siblings. (C) T-circle amplification of DNA extracted from *ctc1-1 stn1-1*, *ctc1-1* and *stn1-1* siblings. All panels show progeny of a single parent heterozygous for both *ctc1-1* and *stn1-1.*

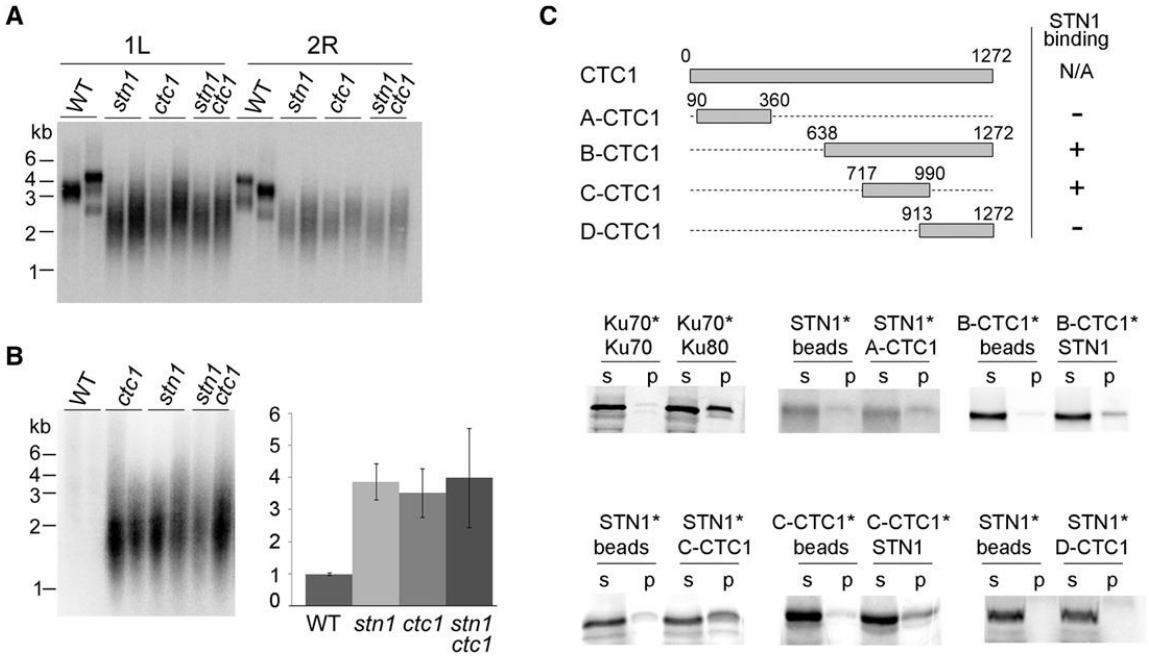


Figure A-8. *AtCTC1* and *AtSTN1* function in the same genetic pathway for chromosome end protection and physically interact *in vitro*. (A) PETRA analysis of telomere length with DNA from *ctc1-1 stn1-1* double mutants, and their *ctc1-1*, *stn1-1*, and wild-type siblings. (B) G-overhang analysis using in-gel hybridization. Native gel and quantification results (the average of six independent experiments ± SD) are shown. $p \leq 0.005$ for all mutant samples compared to wild-type, and $p \geq 0.4$ for mutant samples compared to each other. In (A) and (B), all progeny were segregated from a double heterozygous *ctc1-1 stn1-1* parent. Blots were hybridized with a radiolabeled telomeric DNA probe. (C) Top, schematic of the full-length AtCTC1 protein and its truncation derivatives. AtCTC1 fragments that bind AtSTN1 are indicated. Bottom, coimmunoprecipitation experiments conducted with recombinant full-length AtSTN1 and truncated AtCTC1 fragments A-D. Asterisks indicate ^{35}S-methionine-labeled protein; the unlabeled protein was T7 tagged. S, supernatant; P, pellet. KU70-KU80 interaction was the positive control.

Table 3-3. Frequency of anaphase bridges in *ctc-1- stn1-1* double mutants and their wild type, *ctc1-1* and *stn1-1* siblings.

Genotype	# of analyzed pistils	# of anaphases		% anaphase bridges
		with bridges	total scored	
WT	1	2	207	1
ctc1-1	1	39	184	21
	2	74	273	27
stn1-1	1	42	213	20
	2	30	202	15
ctc1-1 stn1-1	1	28	234	12
	2	51	287	18

^{35}S methionine. Immunoprecipitation experiments showed no interaction between AtSTN1 and fragments A-CTC1 or D-CTC1. However, AtSTN1 bound the B-CTC1 and C-CTC1 fragments in reciprocal immunoprecipitation asays (Fig. A-8C). These data indicate that AtSTN1 and AtCTC1 directly interact *in vitro* and hence may also associate with each other *in vitro*.

Genome instability in human cells depleted of CTC1

TBLASTN and EST database searches revealed CTC1 homologs in a wide range of plant species, while searches using PSI-BLAST and HHpred uncovered putative CTC1 orthologs in many vertebrates. Although the putative plant and animal orthologs exhibited considerable sequence divergence, a global profile-profile alignment indicated that the secondary structures had similarity throughout the length of the protein. Further analysis indicated that the C-terminal domain of human and *Arabidopsis* CTC1 shows homology to OB-fold regions from RPA orthologs, while the N-terminal domain may contain an OB-fold that is distantly related to OB2 from POT1 (Fig. A-9A and Fig. A-10). Interestingly, the mammalian ortholog of CTC1 is identical to one subunit of Alpha Accessory Factor (AAF-132) while the second subunit of AAF (AAF-44, also known as OBFC1) corresponds to the mammalian ortholog of Stn1. AAF is a heterodimeric protein that was originally identified as a factor that stimulates Pol α-primase. It was subsequently shown to enhance Pol α-primase association with ssDNA allowing the enzyme to prime and extend DNA in a reiterative

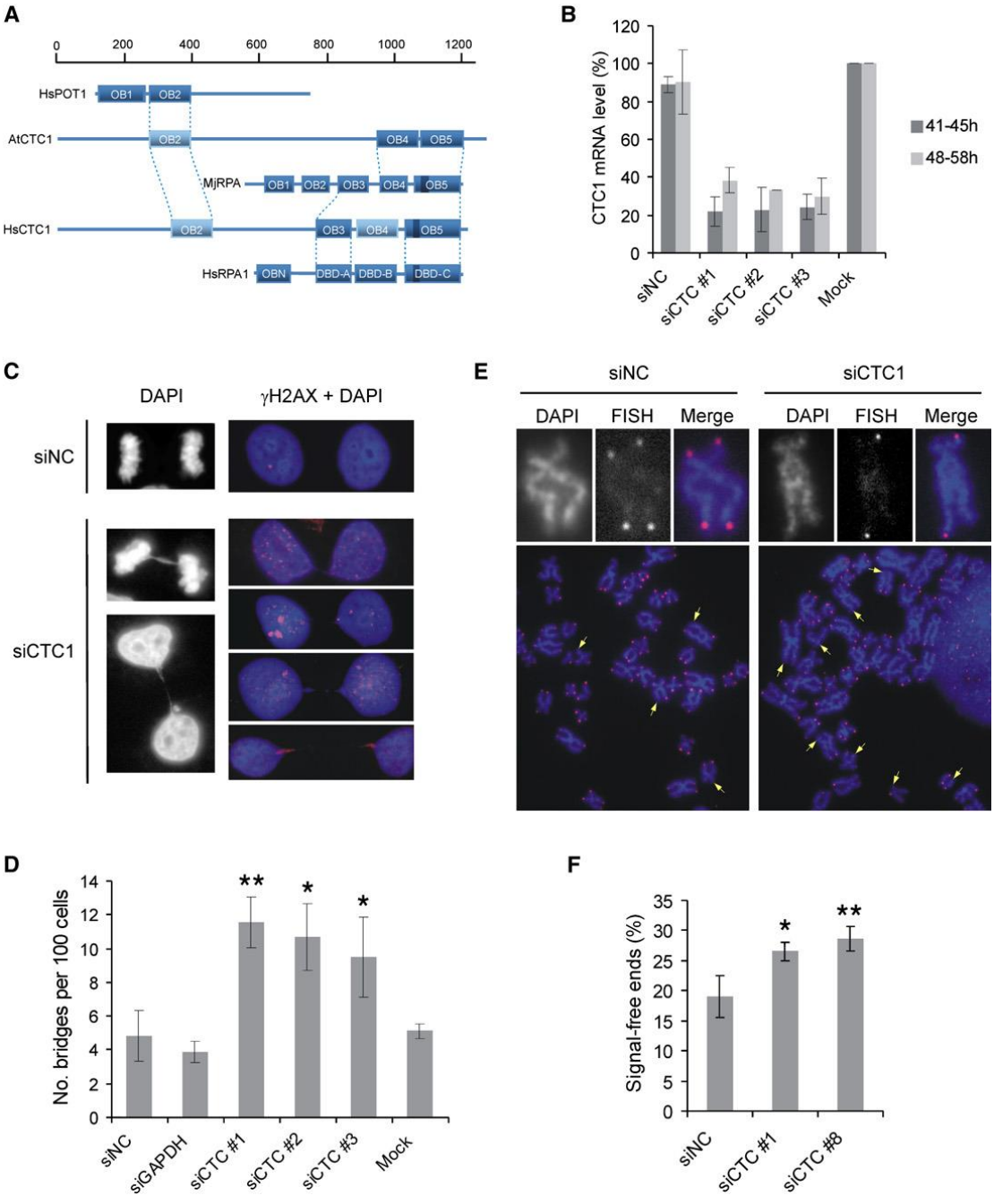


Figure A-9. Depletion of human CTC1 causes genomic instability and sudden telomere loss. (A) Alignment of potential OB folds in *Arabidopsis* and human CTC1 with OB-fold domains from POT1 and RPA.. MjRPA, archeal RPA from *Methanococcus jannaschii*; HsRPA1, human RPA70. (B) Knockdown of CTC1 mRNA in HeLa cells at indicated times after the second transfection. Values are the mean of five independent experiments ± SEM. The percent knockdown is relative to the mock transfection, which was set at 100%. (C and D) Chromatin bridges and γH2AX staining after CTC1 knockdown in HeLa cells. (C) DAPI staining (blue) shows bridges between interphase cells, γH2AX (red) shows DNA damage foci. (D) Frequency of chromatin bridges. (E and F) Telomere FISH showing signal-free ends 48 hr after CTC1 knockdown in HeLa cells. (E) Representative metaphase spreads hybridized with Cy3-OO-$(TTAGGG)_3$ PNA probe. The top panels show magnified view of selected chromosomes. (F) Percent of chromosome ends that lack a telomeric DNA signal after treatment with nonsilencing control or CTC1 siRNA. Asterisks indicate significance of the increase in signal-free ends.

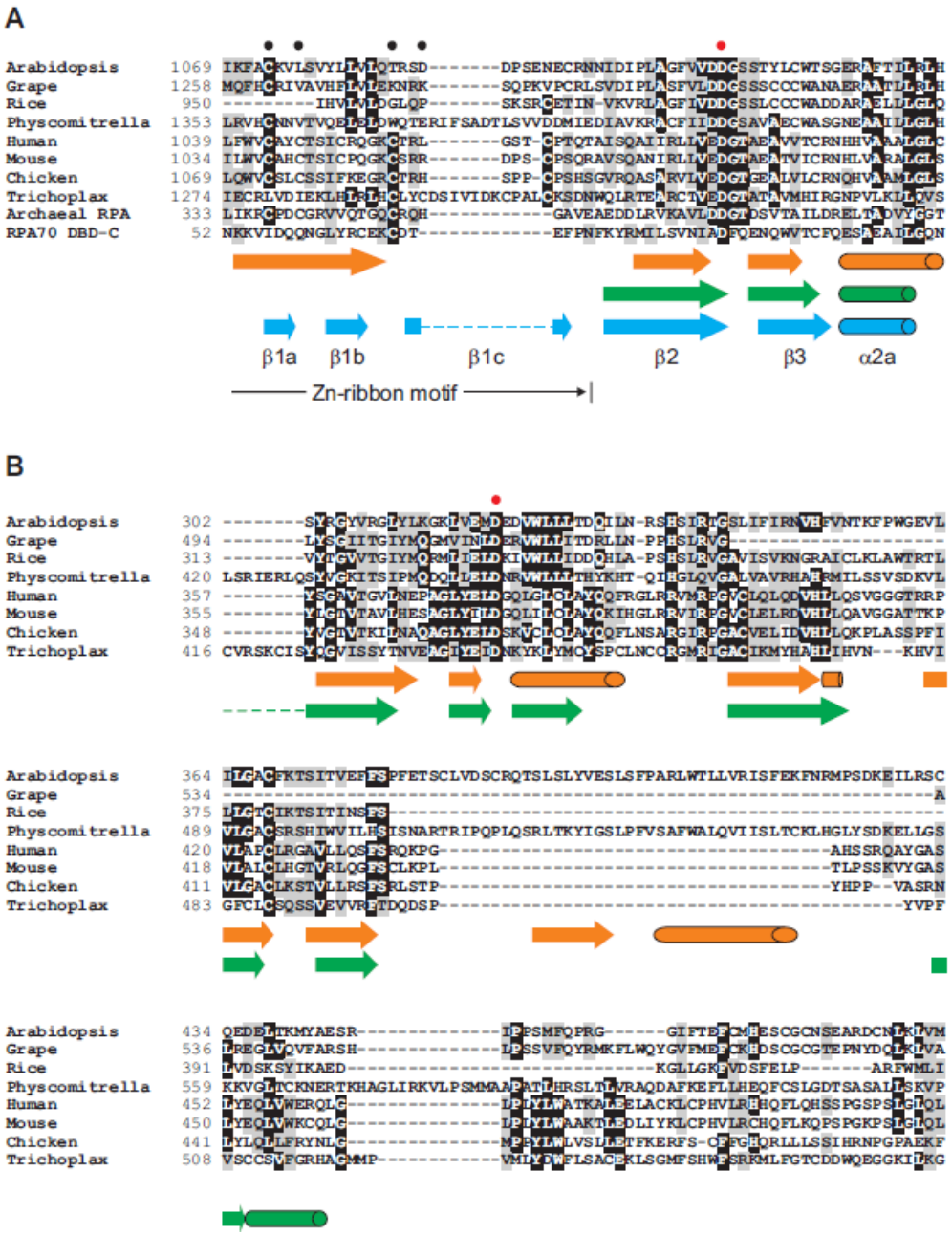


Figure A-10. Sequence alignments showing conservation between CTC1 homologs. (A) Sequence alignment of the C-terminal region of CTC1 homologs with the homologous region of archeal (*Methanocaldococcus jannaschii*) RPA and human RPA70 (DBDC). Secondary structure elements were taken from the crystal structure for HsRPA (shown in blue) and were predicted with PSIPRED for AtCTC1 (orange) and HsCTC1 (green). Arrows and cylinders represent β-sheets and α-helices. Red dot indicates aspartic acid that is conserved in the second β-sheet of OB-folds. Black dots indicate conserved residues in the $CX_2CX_8CX_2H$ Zn finger motif present in archael RPAs and chicken CTC1. (B) Alignment of the N-terminal region that is best conserved between CTC1 homologs. Secondary structure predictions (Orange; AtCTC1, green, HsCTC1) suggest the presence of an OB fold that is distantly related to POT1 OB2.

fashion without falling off the DNA template (Goulian and Heard 1990). Genes encoding the two subunits of AAF were identified recently and AAF-44 was predicted to contain OB-folds resembling those from RPA32 (Casteel et al. 2009).

To investigate whether the human CTC1 protein is important for telomere integrity, we examined the effect of knocking down CTC1 expression in human cells. HeLa and MCF7 cells were subject to two rounds of transfection with individual siRNAs and the level of CTC1 transcript was analyzed by quantitative real-time RT-PCR. Out of eight siRNAs tested, six routinely gave a 60-80% knockdown (Fig. A-9B and A-11C, data not shown). The effect of CTC1 knockdown was monitored after the cells had recovered from the dual transfection.

FACS analysis of DNA content revealed that CTC1 knockdown affected cell cycle progression. MCF7 cultures showed an accumulation of cells in G1 and a decrease in the S/G2 fraction (Fig. A-12A). Microscopy of DAPI stained cells revealed that CTC1 knockdown perturbed chromosome segregation. For HeLa cells, we observed an ~ 2-fold increase in the frequency with which interphase cells remained connected by chromatin bridges (Fig. A-9C, A-9D and A-12B). Although the incidence of chromatin bridges was lower in MCF7 cells, there was an increase in the number of cells with micronuclei (Fig. A-12C). These micronuclei probably reflect anaphase or interphase bridges that were later resolved (Hoffelder et al. 2004). We were unable to determine whether

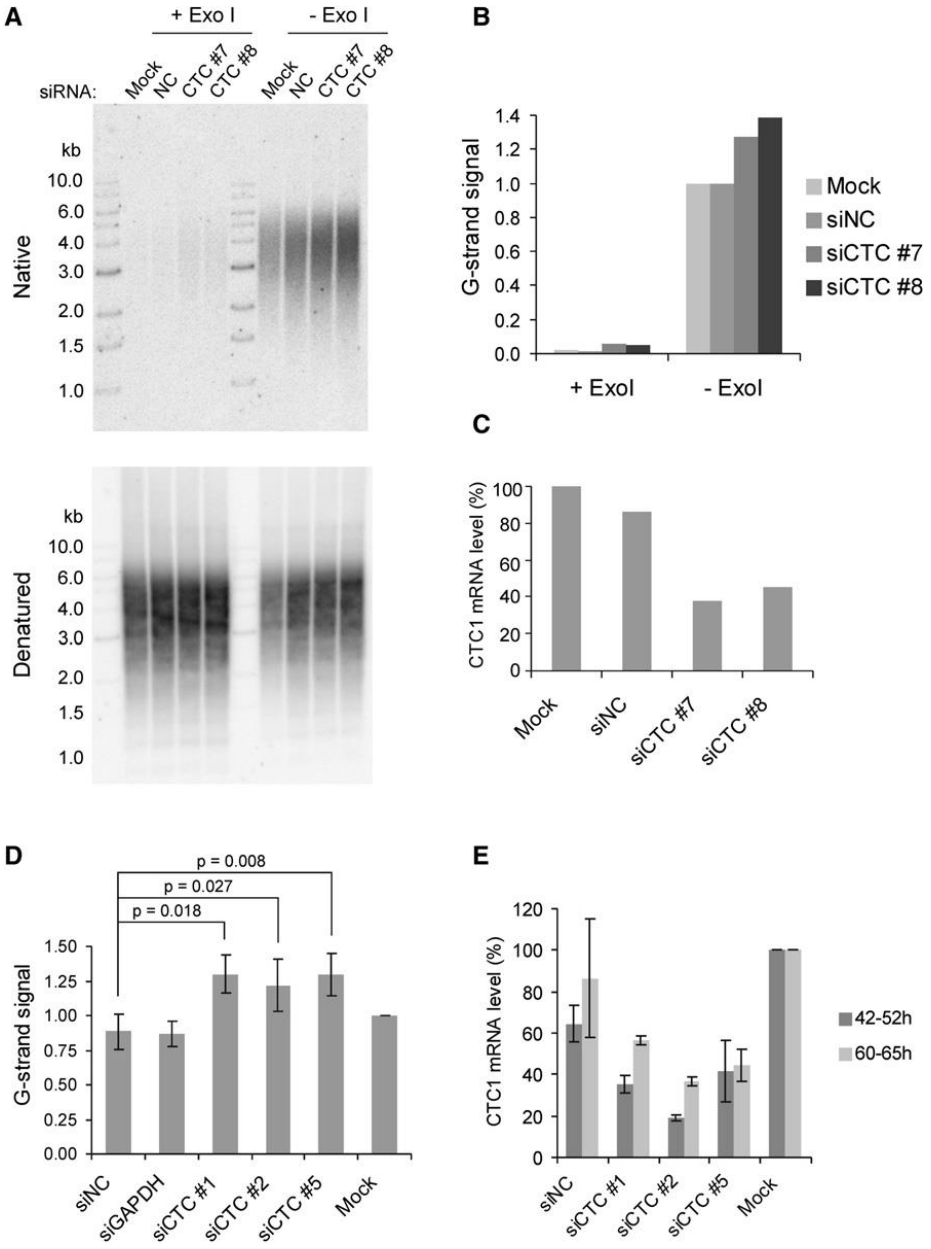
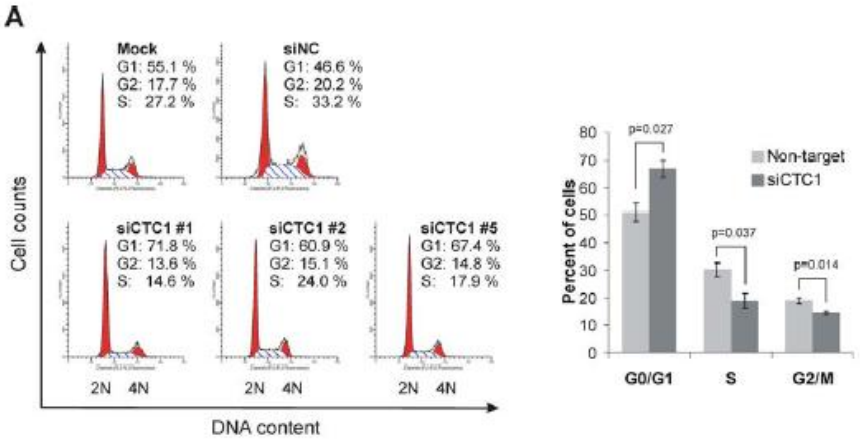


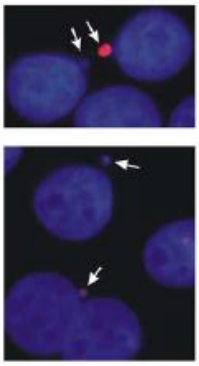
Figure A-11. Deregulation of the G-strand overhang after CTC1 knockdown in MCF7 cells. (A) In-gel hybridization of $(CCCTAA)_4$ probe to telomeric restriction fragments under native (upper panel) or denaturing (lower panel) conditions. +ExoI, DNA samples were treated with Exonuclease I prior to restriction digestion. (B and C) Quantification of G-strand signal (B) or CTC1 mRNA depletion (C) for experiment shown in (A). Change in G-strand signal or CTC1 mRNA level is shown relative to the mock transfection. (D) Mean change in G-strand signal after CTC1 knockdown. Data are from three independent experiments ± SEM; p values are from one-tailed Student's t test. (E) Mean change in CTC1 mRNA level for experiments shown in (D)



B

siRNA	Mean no. of bridges	SEM	Total cells analyzed
siNC	4.80	1.49	232
siGAPDH	3.87	0.63	245
siCTC #1	11.56	1.52	339
siCTC #2	10.67	2.01	222
siCTC #3	9.50	2.34	246
Mock	5.11	0.44	185

C



D

	siRNA	CTC1 mRNA	Total ends	FISH signals	Signal-free	Fraction	SEM	t-test
HeLa	siNC	100%	1151	932	219	0.190	0.035	
exp.1	siCTC #1	32%	1084	796	288	0.266	0.016	p=0.033
	siCTC #7	25%	1400	1000	400	0.286	0.021	p=0.005
HeLa	siNC	100%	1736	1368	368	0.212	0.017	
exp.2	siCTC #1	49%	1794	1313	481	0.268	0.016	p=0.184
	siCTC #7	33%	1857	1291	566	0.305	0.010	p=0.001
293T	siNC	100%	1115	967	148	0.133	0.019	
	siCTC1 #7	50%	1033	850	183	0.177	0.025	p=0.147
	siCTC1 #8	51%	1122	889	233	0.208	0.011	p=0.003

Figure A-12. Effects of CTC1 knockdown in human cells. (**A**) FACS analysis showing accumulation of MCF7 cells in G1 at 64 hrs after treatment with CTC1 siRNA. NC, non-silencing control RNA; Mock, transfection reagent alone. The percent of cells in each phase of the cell cycle was determined using ModFit LT (Verity Software). The graph on the right shows the mean percentage of cells at each stage ± SEM. (**B**) Mean number (± SEM) of interphase chromatin bridges in HeLa cells after treatment with CTC1 siRNA. GAPDH, siRNA to GAPDH. (**C**) Micronuclei in MCF7 cells 60 hrs after treatment with CTC1 siRNA. Nuclei are stained with DAPI (blue) and antibody to γH2AX (red). (**D**) Data from 3 separate telomere FISH experiments showing the number of chromosome ends with or without FISH signals and percent of residual CTC1 mRNA.

CTC1 knockdown causes an increase in anaphase bridges as the frequency of mitotic cells was too low. However, the cut-like phenotype with interphase bridges is similar to what was observed after POT1 knockdown in HeLa cells (Veldman et al. 2004), suggesting that like *Arabidopsis* CTC1, human CTC1 is needed to prevent chromosome fusions.

To determine whether the defects in chromosome segregation led to a DNA damage response, we looked for the appearance of γH2AX foci. Treatment with CTC1 siRNA caused an increase in foci in both HeLa and MCF7 cells. These foci were fewer in number and larger than the foci observed after UV irradiation. Moreover, they persisted for the duration of the knockdown whereas UV-induced foci were resolved after a few hours (data not shown). We looked for co-localization of γH2AX and TRF2 staining but this was not readily apparent (data not shown) suggesting that either the DNA damage was not telomeric or that disruption of CTC1 results in complete loss of the telomeric tract from a subset of telomeres. Overall our results indicate that loss of human CTC1 causes a DNA damage response and genome instability.

Depletion of human CTC1 alters G-overhang structure and results in the accumulation of signal-free ends

To determine whether CTC1 knockdown has a direct effect on telomere structure, we used non-denaturing in-gel hybridization to examine the status of the G-overhang. CTC1 depletion caused a modest but consistent increase in ss

G-strand DNA in both HeLa and MCF7 cells (Fig. A-11 and data not shown). In MCF7 cells, the G-strand signal increased by 33%-41% relative to the non-silencing control siRNA (Fig. A-11). This increase was statistically significant. Treatment with Exo1 removed essentially all the G-strand signal from the control DNAs, but a small amount remained in the samples from CTC1 depleted cells (Fig. A-11A). Thus, removal of CTC1 causes an increase in G-overhang length and may also result in internal regions of ss G-strand DNA.

Given the failure of the γH2AX foci to co-localize with TRF2 after CTC1 knockdown, we analyzed metaphase spreads to determine whether depletion of CTC1 lead to sporadic telomere loss. Metaphase spreads were prepared from siRNA-treated HeLa and 293T cells and hybridized with Cy3-labeled $(TTAGGG)_3$ PNA probe. Subsequent analysis of individual chromosomes revealed an increase in signal free ends (Fig. A-9E and A-9F). This increase was statistically significant in four out of six trials, with the greatest frequency of signal free ends correlating with the deepest CTC1 knockdown (Fig. A-12D). We therefore conclude that like *Arabidopsis* CTC1, human CTC1 is required to maintain telomere integrity.

Discussion

Although overall telomere architecture and the general mechanism of telomere replication are well conserved, telomere protein sequence and composition have evolved rapidly (Bianchi and Shore 2008; Linger and Price

2009). The resulting divergence has complicated telomere protein identification so it is still unclear whether the full complement of dedicated telomere proteins is known for any organism. It is also unclear whether additional telomere-specific factors are required to address the unique problems associated with replicating the DNA terminus. In this study we employed a genetic approach to uncover CTC1, a new telomere protein that is required for genome integrity in multicellular eukaryotes. The *CTC1* gene is predicted to encode a large protein (142 kDa in *Arabidopsis* and 134.5 kDa in humans) that has orthologs dispersed widely throughout the plant and animal kingdoms. Both *Arabidopsis* and human CTC1 interact with STN1, an ortholog of *S. cerevisiae* Stn1 that was recently found at *Arabidopsis* and human telomeres (this study; [Casteel et al, 2009; Dejardin & Kingston, 2009; Song et al, 2008]). Moreover, we discovered that the mammalian CTC1/STN1 complex corresponds to the recently identified DNA polymerase AAF, previously shown to stimulate Pol α-primase (Casteel et al. 2009). Thus, CTC1 appears to be a novel protein that is required for telomere end protection and/or telomere replication.

In *Arabidopsis*, the phenotype of a *ctc1* null mutant reflects rapid and catastrophic deprotection of all chromosome ends. Telomere tracts are grossly deregulated in both length and terminal architecture and are subjected to increased recombination and extensive loss of both telomeric and subtelomeric sequences prior to end-to-end fusion. The dramatic effect of CTC1 depletion contrasts with the gradual loss of telomeric DNA in *tert* mutants and the

correspondingly later onset of developmental defects (Fitzgerald et al. 1999; Riha et al. 2001). It is striking that plants null for CTC1 are viable, because in other model organisms, loss of telomere-capping proteins activates an ATM or ATR-mediated DNA damage checkpoint and is a lethal event (e.g. loss of *CDC13*, *STN1* or *TEN1* in budding yeast, *STN1*, *TEN1* or *POT1* in fission yeast, and *TRF2* or *POT1* in vertebrates (Grandin et al. 1997; Churikov and Price 2008; Palm and de Lange 2008). The extraordinary tolerance of plants to telomere uncapping may reflect a difference in pathways used to monitor genome integrity (Gutierrez 2005), the partial duplication of the *Arabidopsis* genome, which permits some degree of aneuploidy. In addition, developmental plasticity may mitigate the consequences of genome instability by allowing healthy cells to assume the function of their more severely compromised neighbors.

Depletion of the human CTC1 mRNA revealed a more modest, but significant role for this protein in chromosome end protection. Several cell lines exhibited hallmarks of genome instability such as chromatin bridges, micronuclei and γH2AX staining. Moreover, telomere architecture was perturbed with cells showing an increase in G-overhang signal and sporadic telomere loss. The milder phenotypes associated with HsCTC1 depletion relative to *Arabidopsis* may reflect the partial knockdown. Plants that are heterozygous for *CTC1* show no deleterious phenotypes, thus only low levels of protein may be needed for telomere protection. This is the case for vertebrate POT1 as the knockdown

causes a less severe phenotype than the full gene knockout (Churikov et al. 2006). It is also possible that the function of HsCTC1 only partially overlaps that of AtCTC1. In *Arabidopsis*, POT1 variants seem to be telomerase subunits rather than stable components of the telomere (C. Cifuentes-Rojas, K. Kannan, J. Levy, A.D.L. Nelson, L. Tseng and D.E. Shippen, unpublished data) (Surovtseva et al. 2007). Thus, plant CTC1 may have evolved to function both in telomere end protection and telomere replication. In contrast, mammalian CTC1 may function only in telomere replication.

How CTC1 promotes telomere integrity in multicellular eukaryotes is unknown, but important clues come from recent studies of AAF (HsCTC1/STN1) (Casteel et al. 2009). AAF-44 (HsSTN1) contains an OB-fold that is required for AAF to bind ssDNA and stimulate Pol α-primase activity. Thus, as in the budding yeast Cdc13/Stn1/Ten1 (CST) complex, the mammalian CTC1/STN1 complex binds ssDNA and provides a link to the lagging strand replication machinery. This connection also appears to be conserved in plants, as AtCTC1 physically interacts with both AtSTN1 (this study) and the DNA pol α catalytic subunit (X. Song and D.E. Shippen, unpublished data). These findings raise the possibility that plant and mammalian CTC1 and STN1 are part of a CST complex that, like budding yeast CST, functions in telomere capping and/or coordination of G- and C-strand synthesis during telomere replication. If CTC1 functions in a CST-like complex, we would expect multicellular eukaryotes to possess a Ten1-like protein. Indeed, a putative TEN1 ortholog has been identified in humans (Miyake

et al. 2009) and *Arabidopsis* (X. Song, K. Leehy and D.E. Shippen, unpublished data). Like its counterpart in budding yeast, the *Arabidopsis* TEN1 protein exhibits strong affinity for AtSTN1 *in vitro*.

The observation that both *S. cerevisiae* CST and mammalian CTC1/STN1 (AAF) modulate DNA pol α-primase is particularly striking. In yeast, both Cdc13 and Stn1 interact with Pol α subunits and are proposed to couple telomeric G- and C-strand synthesis (Qi and Zakian 2000; Grossi et al. 2004; Puglisi et al. 2008). This coupling prevents accumulation of long G-strand overhangs following G-strand extension by telomerase or C-strand resection by nuclease. Previous studies of mammalian CTC1/STN1 (AAF) only explored Pol α-primase stimulation *in vitro* and did not investigate *in vitro* telomeric function or interactions with telomeric DNA (Goulian et al. 1990; Casteel et al. 2009). Thus, this work did not indicate whether CTC1/STN1 promotes general DNA replication or telomere replication. Our results reveal a clear role for CTC1/STN1 in telomere maintenance. However, we cannot rule out additional non-telomeric functions. Indeed, the non-telomeric γH2AX staining after CTC1 knockdown suggests a role in DNA replication or repair. One possibility is that mammalian CST acts as a specialized replication/repair factor that is needed to reinitiate DNA synthesis by DNA Pol α if a replication block causes uncoupling of polymerase and helicase activity at the replication fork (Heller and Marians 2006; Yao and O'Donnell 2009). Such a function might explain the residual exonuclease-resistant G-strand signal after CTC1 depletion.

Many of the telomere defects observed after CTC1 depletion could be explained by defects in lagging strand replication either at the chromosome terminus or within the telomeric tract. For example, failure to fill in the C-strand following telomerase action or C-strand resection would lead to long G-overhangs. Damage to the G-strand might, in turn, result in telomere loss and/or telomere fusions. Likewise, failure to reinitiate lagging-strand synthesis after replication fork stalling could lead to loss of large stretches of telomeric DNA and signal-free ends.

Given the role played by the *S. cerevisiae* CST complex, one attractive model for CTC1/STN1 function is that it serves to recruit Pol α-primase to the telomeric G-strand after telomerase action and/or C-strand processing. Pol α appears to be recruited to replication forks by Mcm10, which may in turn interact with the Cdc45/Mcm2-7/GINS replicative helicase (Warren et al. 2008). However, since the G-strand overhang cannot support a conventional replication fork, telomeres appear to require a specialized mechanism to recruit Pol α-primase for C-strand fill in. Further studies will be needed to test this model for CTC1/Stn1 function. Additional work will also be required to determine the extent to which the telomeric function of CTC1/STN1 stems from its role in telomere replication versus a more passive function in G-overhang protection. Perhaps the balance between these activities will differ between organisms. For example, the *Arabidopsis* and *S. cerevisiae* complexes may function in both capacities, while the mammalian complex is specialized for telomere replication.

APPENDIX B

EVOLUTION OF CST FUNCTION IN TELOMERE MAINTENANCE*

Summary

Telomeres consist of an elaborate, higher-order DNA architecture, and a suite of proteins that provide protection for the chromosome terminus by blocking inappropriate recombination and nucleolytic attack, and facilitate telomeric DNA replication by physical interactions with telomerase and the lagging strand replication machinery. The prevailing view has been that two distinct telomere capping complexes evolved, shelterin in vertebrates and a trimeric complex comprised of Cdc13, Stn1 and Ten1 (CST) in yeast. The recent discovery of a CST-like complex in plants and humans raises new questions about the composition of telomeres and their regulatory mechanisms in multicellular eukaryotes. In this review we discuss the evolving functions and interactions of CST components and their contributions to chromosome end protection and DNA replication.

Telomere protein complexes: Shelterin versus CST

Vertebrate telomeres are bound by six telomere-specific proteins that assemble into a complex termed shelterin (Palm and de Lange 2008) (Fig. B-

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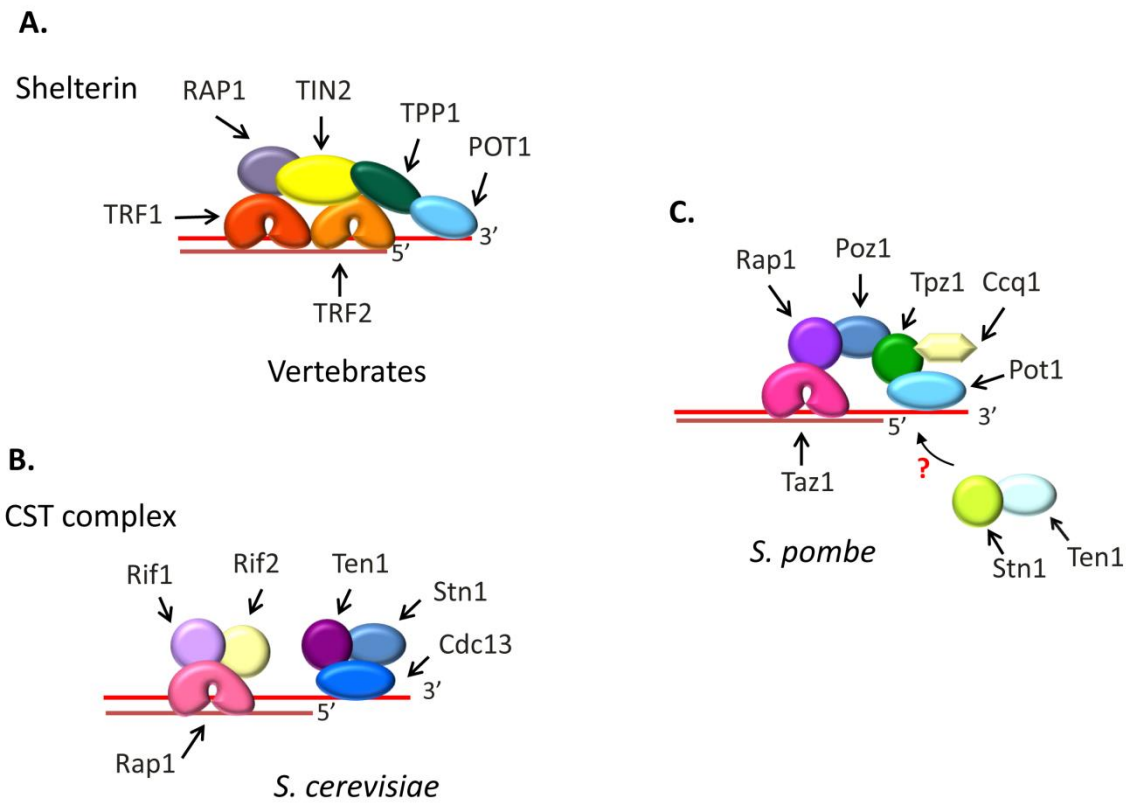

Figure B-1. Telomere capping complexes in vertebrates and yeast. A) The six-member shelterin complex associates with both single- and double-strand regions of the vertebrate telomeric DNA. B) Budding yeast telomeres are protected by the trimeric Cdc13 Stn1 Ten1 (CST) complex, which assembles on the G-overhang. The duplex region of the telomere is bound by a separate complex containing Rap1, Rif1 and Rif2. C) Fission yeast telomeres associate with a six member shelterin-like complex. In addition, Stn1 and Ten1 contribute to chromosome end protection, but it is not known how they interact with other telomere proteins.

1A). The individual components, TRF1, TRF2, TIN2, Rap1, TPP1 and POT1, each play defined roles in telomere protection. These include limiting DNA degradation, preventing ATM and ATR-activation and inhibiting DNA repair activities such as non-homologous end joining or homology directed repair (O'Sullivan and Karlseder 2010). TRF1 and TRF2 bind to the duplex region of the telomere while POT1 binds to the 3' overhang on the G-rich strand. TIN2 and TPP1 form a bridge between TRF1/2 and POT1 linking the telomere duplex and the G-overhang (Palm and de Lange 2008). Pot1 binds to the overhang via two adjacent oligonucleotide-oligosaccharide binding folds (OB-folds) (Lei et al. 2004; Linger and Price 2009). Fission yeast telomeres also assemble with a shelterin-like complex that contains obvious orthologs of vertebrate TRF1/2 (Taz1), Rap1 and Pot1 (Miyoshi et al. 2008). Although the vertebrate and fission yeast complexes differ in subunit arrangement, the overall structure seems quite similar as the *S. pombe* duplex binding protein Taz1 is linked to Pot1 and the G-overhang via a series of bridging proteins which include Rap1, Poz1, Tpz1 and Ccq1 (Fig. B-1B). Tpz1 appears to be the functional homolog of TPP1.

Intriguingly, budding yeast telomeres are not protected by a shelterin-like complex. Although the double-strand region of the telomere is bound Rap1 and two associated factors, these proteins are not involved in chromosome end protection. Instead this function is fulfilled by a trimeric complex, CST, comprised of Cdc13, Stn1 and Ten1, which associates with the G-overhang (Fig. B-1C) (Lundblad 2006). None of the CST components show obvious sequence

identity to Pot1, TPP1/Tpz1 or other shelterin constituents (Linger and Price 2009). *S. cerevisiae* CST plays a dual role in telomere protection and modulation of telomere replication (Bianchi and Shore 2008). Although Cdc13 is the main DNA-binding subunit, all three proteins function in end-protection and removal of any subunit results in degradation of the telomeric C-strand, accumulation of long G-overhangs, activation of a DNA-damage response and a late S/G2 cell cycle arrest. Cdc13 and Stn1 play key roles in telomere replication (Garvik et al. 1995; Grandin et al. 1997; Grandin et al. 2001). During late S/G2, phosphorylation of Cdc13 promotes a direct interaction between Cdc13 and the Est1 subunit of telomerase (Li et al. 2009). This interaction enhances telomerase extension of the chromosome terminus (Chan et al. 2008; DeZwaan and Freeman 2009). Subsequent dephosphorylation of Cdc13 limits telomerase action by reducing Est1 binding and increasing Stn1 binding (Puglisi et al. 2008; Li et al. 2009). Cdc13 and Stn1 then appear to coordinate fill-in of the complementary C-strand by recruiting DNA Polα/primase through direct interactions with the Pol1 and Pol12 subunits of DNA Polα (Qi and Zakian 2000; Grossi et al. 2004). Despite the lack of sequence similarity to Pot1, the DNA binding domain of Cdc13 consists of an OB-fold that is structurally similar to the OB-folds in the DNA-binding domain of Pot1 (Mitton-Fry et al. 2002; Lei et al. 2004; Linger and Price 2009). This discovery led to the idea that Cdc13 is the functional homolog of Pot1 and further suggested that shelterin had replaced CST in vertebrate cells. This impression was reinforced when POT1 or TPP1

depletion was shown to cause a severe telomere uncapping phenotype analogous to that observed after removal of the *S. cerevisiae* CST complex (Churikov et al. 2006; Hockemeyer et al. 2006; Kibe et al. 2010). However, recent genetic and structural studies reveal that budding yeast CST is more closely related to Replication Protein A (RPA) than to POT1-TPP1 (Gao et al. 2007; Sun et al. 2009)

RPA is a heterotrimer that binds ssDNA through a series of OB-folds (Fanning et al. 2006) (Fig. B-2). RPA70 contains four OB-folds, three of which contact DNA. RPA32 contains one OB-fold that also contacts DNA and a C-terminal winged helix (WH) protein interaction domain. RPA14 is comprised of a single OB-fold that is needed for complex formation. Protein structure prediction first suggested that Stn1 and Ten1 might contain OB-folds resembling those of RPA32 and RPA14 (Gao et al. 2007). X-ray crystallography has since confirmed that budding yeast (*C. tropicalis*) Stn1-Ten1 and Rpa2-Rpa3 (RPA32-RPA14) complexes have substantial structural similarity in their OB-fold motifs, subunit interaction surfaces and in the Stn1 N- and C-terminal extension regions (Sun et al. 2009) (Fig. B-2). However, there are significant differences in the relative orientation of the subunits and in the structure of most of the connecting loop regions. Also, the C-terminal extension of Stn1 contains two WH motifs instead of the single motif found in Rpa2 (Gelinas et al. 2009; Sun et al. 2009). Thus, while CST shares significant structural features with RPA, it is tailored to perform a different biological function.

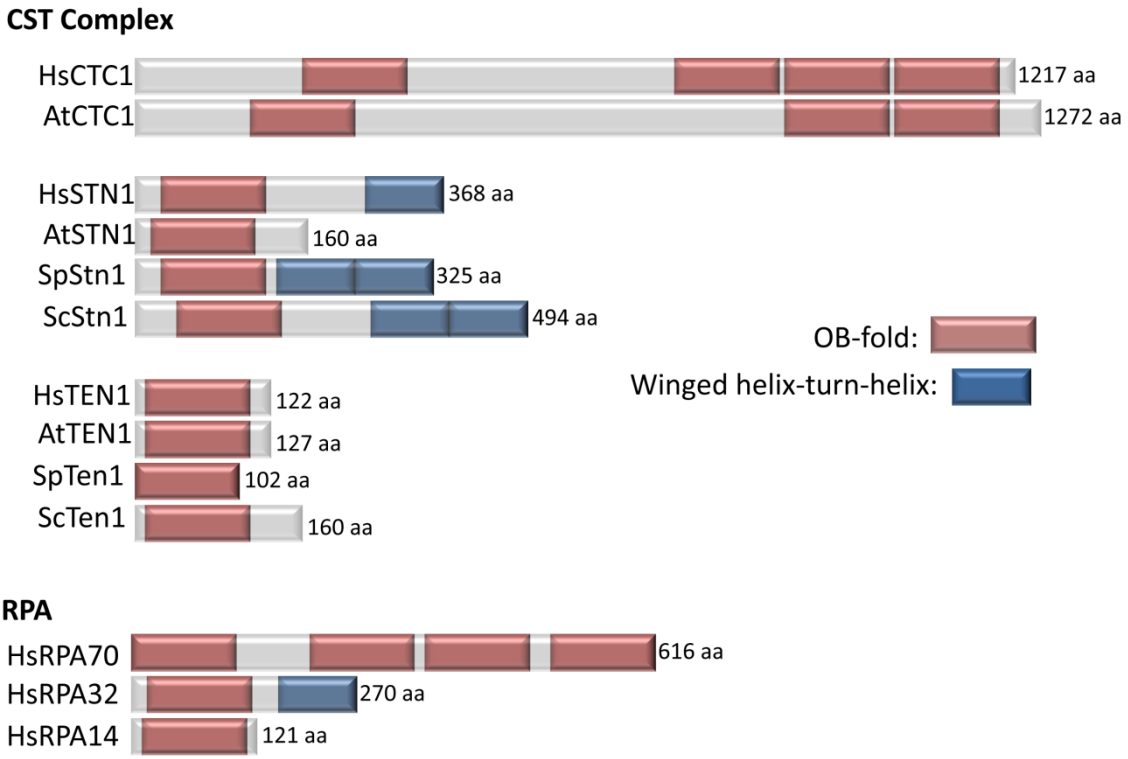

Figure B-2. Similar domain structure in CST and RPA. The predicted human and Arabidopsis CTC1 domain structure is illustrated along with STN1 and TEN1 from humans, Arabidopsis, *S. pombe* and *S. cerevisiae*. For comparison, human RPA subunit structure is shown.

Conservation of the CST complex.

The rapid evolution and resulting sequence divergence of telomere proteins makes it difficult to identify orthologs from other species using purely bioinformatics approaches (Linger and Price 2009). When database searches revealed potential Stn1 orthologs in a wide range of organisms (Gao et al. 2007; Martín et al. 2007), it was unclear whether they were bona-fide telomere proteins. The same cross-species database searches failed to identify orthologs of Ten1 and Cdc13. The potential Stn1 orthologs showed low sequence identity with budding yeast Stn1 (17.7% identity and 54.4% similarity for ScStn1 vs. Arabidopsis STN1; 21.5% identity and 59.5% similarity for ScStn1 vs. human STN1), but structure prediction programs revealed OB-fold domains similar to that found in RPA32 (Fig. B-2). Subsequent disruption of the *S. pombe STN1* gene demonstrated a role in telomere protection as the cells exhibited rapid telomere loss and end-to-end fusion of chromosomes (Martin et al. 2007). A tentative Ten1 ortholog with a putative OB-fold was then identified by more sensitive bioinformatic analyses. *TEN1* gene disruption gave the same telomere loss and end fusion phenotype as the *STN1* disruption. The Ten1 protein was also shown to interact with Stn1 and to co-localize with Stn1 and Pot1 at telomeres (Martin et al. 2007). Interestingly, the Stn1-Ten1 complex does not appear to interact with Pot1, suggesting that fission yeast contain separate Stn1-Ten1 and Pot1/shelterin-like complexes that are both required for telomere

protection. Thus far an ortholog of Cdc13 or the plant and vertebrate CTC1 remains to be identified in *S. pombe*.

Arabidopsis CST was uncovered through a combination of bioinformatic and genetic approaches (Song et al. 2008; Surovtseva et al. 2009). A putative *STN1* ortholog was identified in the plant genome and the *in vivo* function was determined by analyzing the phenotype of STN1 null plants (Song et al. 2008). Mutants showed profound defects in chromosome end protection and telomere maintenance (see below). Bioinformatics was also used to reveal a putative TEN1 ortholog based on similarity to human TEN1. As in yeast, AtSTN1 and AtTEN1 interact *in vitro* (K. Leehy and D. Shippen, unpublished data). CTC1 (Conserved Telomere maintenance Component 1) was identified using a genetic screen for mutations that cause telomere capping defects (Surovtseva et al. 2009). CTC1 lacks sequence identity to any known gene but structure prediction programs (HHpred and Metaserver) indicate that the encoded protein contains multiple OB-folds with homology to the OB-folds from RPA70 (Fig. B-2). CTC1 and STN1 interact *in vitro* and the phenotype of a CTC1 null plant is similar to that of a STN1 mutant or the CTC1/STN1 double mutant. Thus, CTC1 and STN1 appear to function in the same pathway for chromosome end protection and telomere maintenance in Arabidopsis.

The plant CTC1 sequence was employed in database searches using PSI-BLAST and HHpred to identify vertebrate CTC1(Surovtseva et al. 2009). While the overall level of sequence identity was low (14% identity, 26% similarity

between the human and Arabidopsis CTC1), the predicted secondary structure was similar throughout the length of the protein and the potential OB-folds again resembled those of RPA70 (Fig. B-2). Subsequent siRNA knockdown of human CTC1 resulted in various telomere defects and genomic instability (see below). Mammalian CTC1 and the CST complex were identified independently through analysis of the putative STN1 ortholog (Miyake et al. 2009). Mass spectrometry of STN1-interacting proteins uncovered CTC1 and TEN1. Subsequent analysis demonstrated that these proteins form a trimeric complex.

Although *S. pombe* Stn1-Ten1 and Arabidopsis and mammalian CST clearly localize to telomeres and play a role in telomere maintenance, the level of sequence conservation between proteins identified as being homologous to Stn1, Ten1 or CTC1 is extremely low. Thus, one has to ask whether these proteins are true orthologs of *S. cerevisiae* Cdc13, Stn1 and Ten1. For Cdc13 and CTC1, this is still an open question as structural information is available for only a single OB-fold from Cdc13. Since Cdc13 and CTC1 are both predicted to contain multiple OB-folds, it is possible that the two proteins will turn out to resemble each other and RPA70.

An orthologous relationship between the budding yeast Stn1 and Ten1 and STN1 and TEN1 proteins in other organisms is supported by several lines of evidence. First, the crystal structure of the SpStn1-Ten1 complex has essentially the same architecture as the Stn1-Ten1 complex from the budding yeast *C. tropicalis* (Sun et al. 2009). Second, similar to ScStn1 and ScTen1 the OB-folds

of SpStn1 and SpTen1 resemble those of Rpa2 and Rpa3 (corresponding to HsRPA32 and HsRPA14, respectively). Finally, the C-terminal domain of SpStn1, although shorter than that of ScStn1, is also predicted to contain two WH motifs. Similarly, the C-terminal domain of hSTN1 encodes at least one predicted WH domain, and NMR analysis confirms the existence of this motif in mouse STN1 (PDB1wj5). Interestingly, AtStn1 lacks a C-terminal domain altogether (Fig. B-2). In ScStn1 the C-terminal domain is required for telomere length control, but plays no detectable role in telomere capping (Puglisi et al. 2008). Since the OB-fold domain and adjacent α-helix mediate the interaction between STN1 and TEN1 (Sun et al. 2009), it is possible that the WH motif is required for a function that was lost in the 1.5 billion years since plants, humans and yeasts shared a common ancestor.

Separate domains in multi-domain proteins often have different evolutionary histories (Koonin et al. 2000; Richards and Cavalier-Smith 2005). Phylogenetic analysis of the OB-fold domains of STN1 and RPA32 (Rpa2) from 16 different eukaryotes (Fig. B-3) indicates that STN1 and RPA32 form distinct monophyletic groups: ScStn1 and all other putative Stn1 orthologs, including AtSTN1, form a statistically well-supported clade while the putative RPA32 orthologs form a second separate clade. Thus, all identified STN1 proteins are likely orthologs; the ability to bind TEN1 is conserved and for all STN1 sequences identified using bioinformatic approaches, their OB-fold domains are related by common ancestry.

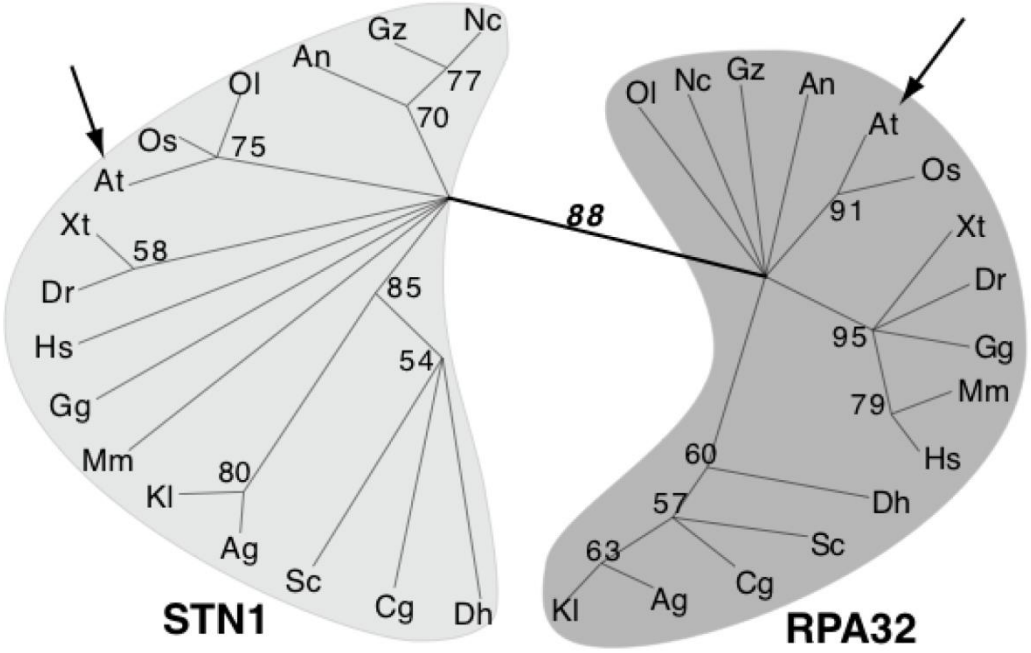

Figure B-3. Stn1 and Rpa32 cluster in distinct monophyletic groups**.** Shown is an unrooted maximum likelihood phylogeny of the OB-fold domains of STN1 and RPA32 inferred using the WAG amino-acid transition model in RAxML(Stamatakis 2006) from the alignment of Gao et al with the addition of STN1 from plants and green algae. Numbers along branches are bootstrap percentages from 500 replicates and indicate that STN1 and RPA32 form distinct monophyletic groups. Arrows indicate the placement of Arabidopsis. Other species are: Ag, *Ashbya gossypii*; An, *Aspergillus nidulans*; Cg, *Candida glabrata*; Dh, *Debaryomyces hansenii*; Dr, *Danio rerio*; Gg, *Gallus gallus*; Gz, *Gibberella zeae*; Hs, *Homo sapiens*; Kl, *Kluyveromyces lactis*; Nc, *Neurospora crassa*; Mm, *Mus musculus*; Os, *Oryza sativa*; Ol, *Ostreococcus lucimarinus*; Sc, *Saccharomyces cerevisiae*; Xt, *Xenopus tropicalis*.

From a functional standpoint, evidence is also emerging that the various activities of CST are conserved, although there is there is considerable species to species variation in the relative importance of each activity. Both *S. pombe* and Arabidopsis STN1 play an essential role in telomere protection (Martin et al. 2007; Song et al. 2008), and as discussed below, Arabidopsis CST may also contribute to telomeric DNA replication. In contrast, the vertebrate CST appears to have lost its essential function in telomere protection perhaps as shelterin components emerged, but it has retained a specialized role in telomere replication.

Plant CST

CTC1 localizes to telomeres in both cycling and non-cycling Arabidopsis cells as expected for a protein involved in chromosome end protection (Surovtseva et al. 2009). Although null mutations in telomere capping proteins are lethal events in mammalian cells and even yeast (e.g. loss of *CDC13*, *STN1* or *TEN1* in budding yeast, *STN1*, *TEN1* or *POT1* in fission yeast, and *TRF2* or *POT1* in vertebrates (Palm and de Lange 2008)), Arabidopsis mutants lacking STN1 and CTC1 are viable at least initially(Song et al. 2008; Surovtseva et al. 2009). Telomere length in null mutants is much more heterogeneous, and overall significantly shorter than in wild-type plants. 20-35% of mitotic cells from mutant pistils contain end-to-end chromosome fusions, and these are predominately subtelomere-to-subtelomere fusion events. In addition,

extrachromosomal telomeric circles, a marker for telomere recombination, are observed in null mutants, arguing that telomere stability is decreased in the absence of CST components.

As in yeast and vertebrate CST mutants, plants lacking CTC1 or STN1 display increased G-overhang signals, in this case three to four times higher than in wild-type. While this phenotype could result from loss of C-strand protection, it is also possible that the Arabidopsis CST has an additional function in telomere replication. *In vitro* co-immunoprecipitation experiments support this conclusion by revealing an interaction between the C-terminus of CTC1 and the C-terminus of the catalytic subunit of Polα, Incurvata2 (ICU2) (Barrero et al. 2007) (Fig. B-4). These data indicate that the physical association between CST and lagging strand replication machinery is conserved and further that the Arabidopsis CST complex may participate in both telomere capping and telomeric DNA replication.

Only a subset of the vertebrate shelterin components have been identified in plants; no RAP1, TPP1 or TIN2 can be discerned. Arabidopsis encodes multiple TRF-like proteins, which bind ds telomeric DNA *in vitro* and negatively regulate telomere length *in vivo* (Karamysheva et al. 2004; Hong et al. 2007) (Fig. B-5A). Notably, the two POT1 paralogs in Arabidopsis, POT1a and POT1b, do not bind single-strand telomeric DNA *in vitro* (Shakirov et al. 2009) and do not function in telomere capping (Surovtseva et al. 2007) (A. Nelson, E. Shakirov and D. Shippen, unpublished data). Rather, these proteins associate with the

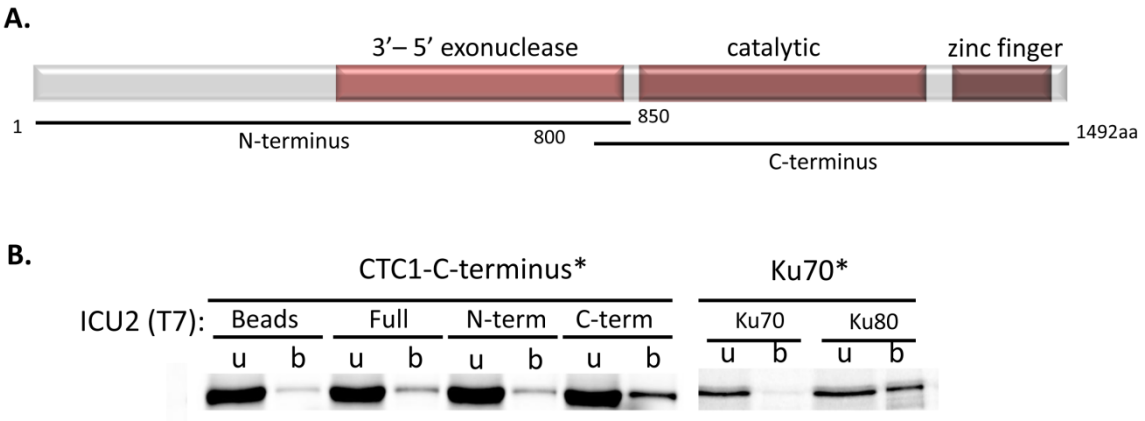

Figure B-4. *Arabidopsis* CTC1 interacts with the catalytic subunit of DNA polymerase alpha (ICU2) *in vitro*. A) Diagram of DNA polymerase alpha domain structure**.** B) Co-immunopreciptiation was conducted with ^{35}S-Met labeled (asterisk) and T7-tagged unlabeled protein expressed in rabbit reticulocyte lysate. The C-terminal half of AtCTC1 was used for binding reactions with different regions of ICU2. When bound to a tagged partner, labeled protein is precipitated on T7-beads (b) from the unbound supernatant (u) fraction. Ku70/80 interaction served as a positive control.

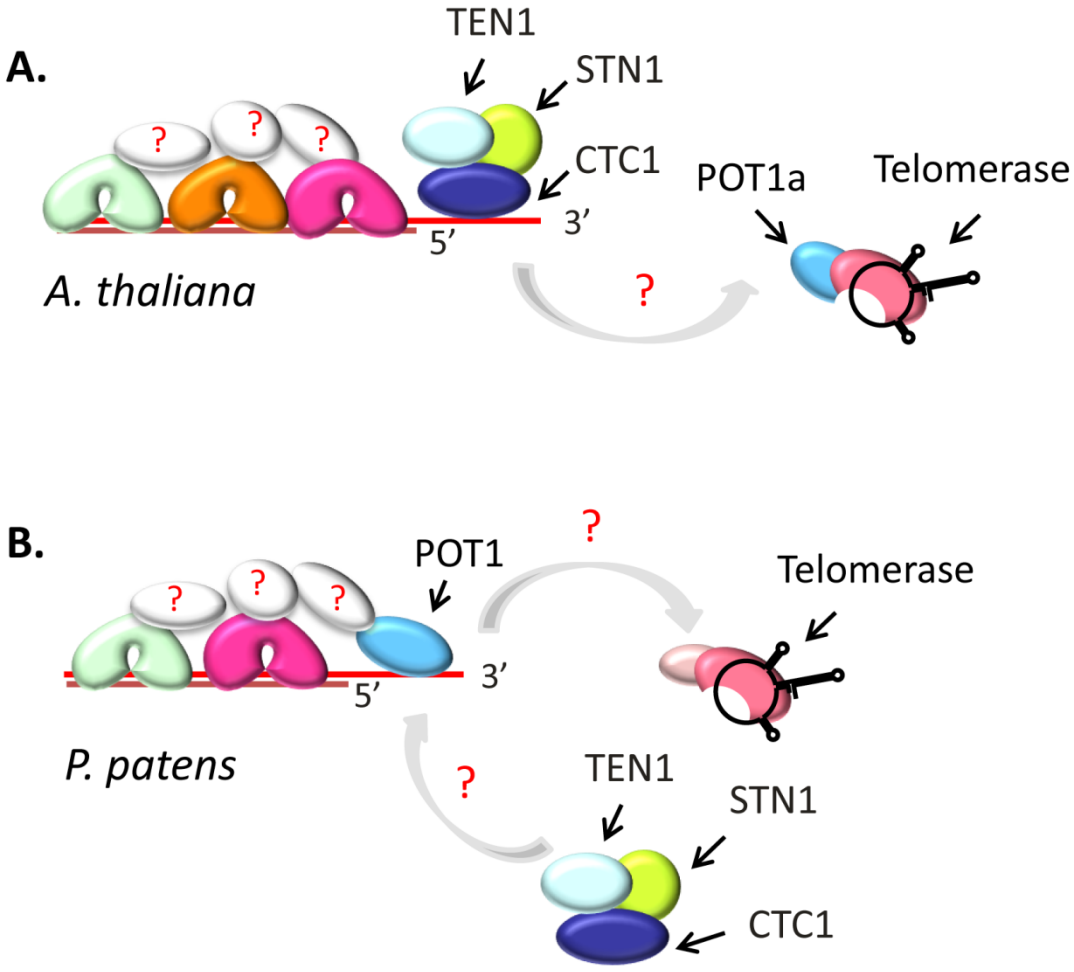

Figure B-5. Model for telomere capping complexes in the flowering plant *Arabidopsis thaliana* and the moss *Physcomitrella patens*. A) CST functions as the major telomere capping complex in Arabidopsis. Multiple TRF-like proteins have been described, but other shelterin-like components cannot be identified in plant genomes. POT1a is a telomerase accessory factor and is not required for chromosome end protection. B) P. patens encodes two TRF-like proteins and a single POT1 protein. The moss POT1 binds single-stranded G-rich telomeric DNA and functions in a manner analogous to vertebrate and yeast POT1. CST components are encoded in the *P. patens* genome, but their function is unknown.

telomerase RNP (Cifuentes-Rojas et al.) (Fig. B-5A). These findings raise the possibility that chromosome end protection proceeds by a fundamentally different mechanism in plants versus other eukaryotes. This hypothesis is refuted by recent analysis of POT1 from the moss *Physcomitrella patens* (Shakirov et al. 2010). *P. patens* POT1 binds G-rich telomeric DNA *in vitro* and is critical for chromosome end protection *in vivo*. Thus, the telomere capping function of POT1 is conserved in early diverging land plants, but appears to have been lost in Arabidopsis. *P. patens* also encodes STN1, TEN1 and CTC1 orthologs (Fig. B-5B). The extent to which these CST components contribute to chromosome end protection, telomeric DNA replication or both processes remains to be determined. Nevertheless, the current data suggest that plants represent an evolutionary bridge between *S. cerevisiae*, which has only CST as a capping complex and vertebrates, which use shelterin for end protection and CST for special replication functions.

Mammalian CST

While mouse and human CST localize to telomeres as shown by both indirect immunofluorescence and chromatin immunoprecipitation, several lines of evidence suggest that mammalian CST has both telomeric and non-telomeric functions (Miyake et al. 2009). First, only ~20% of CST-containing foci localize to telomeres. The remaining ~80% are present in the nucleus but the identity of these foci is unclear. They do not correspond to replication foci and the relative

distribution of CST between telomeric and non-telomeric locations is unaffected by cell cycle stage.

The DNA binding properties of mammalian CST also suggest non-telomeric as well as telomeric functions (Miyake et al. 2009). The complex binds specifically to ssDNA, but the interaction is not sequence specific and all three subunits are required for high affinity binding (nM Kd). Moreover, the minimum binding site size is large and binding affinity increases with increasing length of DNA (20nt<<34nt<74nt). This contrasts with the situation in budding yeast where binding of CST is sequence specific and only Cdc13 is required for high affinity binding to the well-defined 11 nt consensus sequence. The DNA-binding properties of mammalian CST more closely resemble those of RPA which also lacks sequence specificity and prefers an extended DNA binding site (Wold 1997; Fanning et al. 2006). The extended RPA binding site (28-32 nt) is thought to reflect the sequential binding of multiple OB-folds along the length of the DNA. It will be interesting to learn whether this also occurs with CST.

Depletion of either CTC1 or STN1 results in a variety of telomeric and non-telomeric defects (Miyake et al. 2009; Surovtseva et al. 2009). RNAi depletion of either protein causes an elevation in H2AX phosphorylation and an increase in anaphase bridges and chromatin bridges between newly separated daughter cells. The γH2AX foci do not localize to telomeres and telomere fusions between metaphase chromosomes are rarely observed. Thus, CTC1 or STN1 knockdown seems to cause a general increase in genome instability rather than

a loss of telomere protection. Other knockdown phenotypes indicate a role for CST in telomere replication. Telomere FISH with cells depleted of either CTC1 or STN1 shows an increase in telomeres with aberrant structure and telomeres with multiple hybridization signals are particularly apparent (Fig. B-6). Such multi-telomeric signals indicate discontinuities in the telomeric chromatin (akin to fragile sites) and are a hallmark of defects in telomere replication (Sfeir et al. 2009). Thus, the appearance of multi-telomeric signals after CTC1 or STN1 knockdown suggests mammalian CST may be important for replication of the duplex region of the telomeric tract. CTC1 or STN1 knockdown also causes an increase in single-stranded (ss) telomeric DNA (Miyake et al. 2009; Surovtseva et al. 2009). Analysis by non-denaturing hybridization reveals that a portion of this ssDNA is resistant to exonuclease 1 digestion and hence must occur within the duplex region of the telomere. Accumulation of ssDNA within the telomeric tract fits with replication defects that lead to replication fork stalling. The remainder of the ssDNA corresponds to an increase in G-overhang length. This phenotype again suggests a defect associated with telomere replication such as increased G-overhang processing, increased telomerase action or decreased C-strand fill-in (Verdun and Karlseder 2007).

Although CST does not appear to play a primary role in telomere protection, it can complement the protective function of POT1/TPP1 (Miyake et al. 2009). Knockdown of POT1 causes an increase in the number of telomeres with DNA damage signals (TIFs) and this is exacerbated if both POT1 and STN1

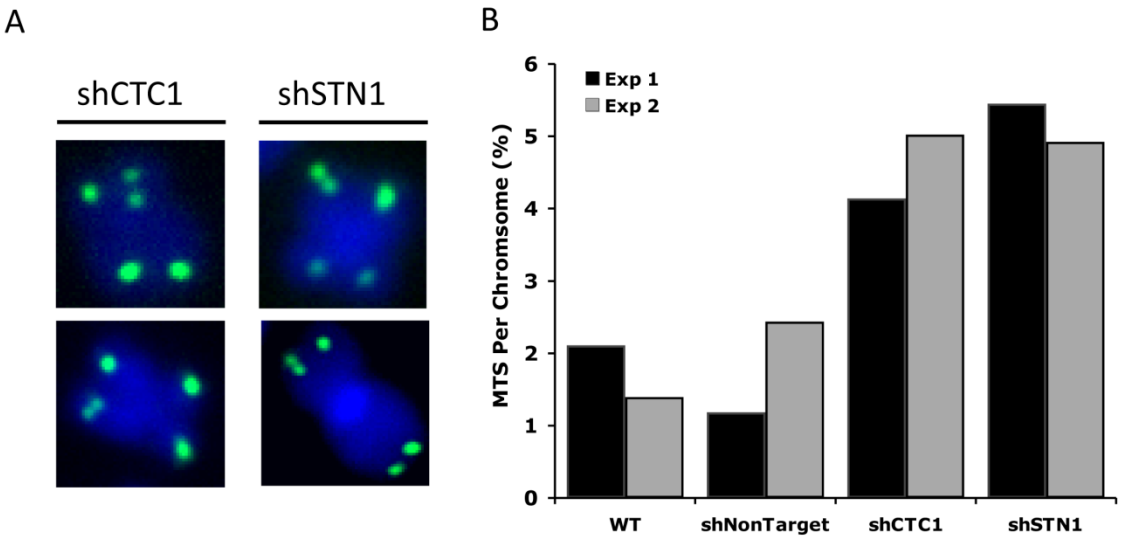

Figure B-6. Knockdown of human CST components, CTC1 or STN1, results in multi-telomeric signals (MTS). A) Examples of MTS in stable shRNA knockdown clones of either CTC1 or STN1. Telomeric PNA-FITC probe (green); DAPI (blue). B) Quantification of MTS. Black and gray bars represent independent experiments.

are depleted even though no TIFs are observed after depletion of STN1 alone. Likewise, a POT1/STN1 double knockdown causes an even greater increase in G-overhang length than the single STN1 knockdown.

Independent evidence that CST contributes to telomere maintenance in humans comes from a large genome-wide association study looking for SNPs associated with fluctuation in leukocyte telomere length. In this study involving 3,417 participants, only two new genes were identified as having association at a genome wide significance level. One of these was STN1 (otherwise known as OBFC1). Evidence for association was also obtained for regions of TERC, the telomerase RNA subunit. The association between STN1/OBFC1 and leukocyte telomere length was replicated using *de novo* genotyping and a retrospective look-up analysis of data from additional individuals.

Mechanism of Action

Clues concerning the mechanism of CST action in mammalian cells come from analysis of a DNA Polα accessory factor called AAF (alpha accessory factor) that was recently found to be composed of CTC1 and STN (Churikov et al. 2006; Casteel et al. 2009; Miyake et al. 2009; Surovtseva et al. 2009). The complex of AAF132/CTC1 and AAF44/STN1 was originally identified as a factor that co-purified with DNA Polα (Goulian et al. 1990). Subsequent biochemical studies indicated that AAF/CTC1-STN1 functions by increasing the affinity of DNA Polα/primase for template DNA, leading to the suggestion that AAF/CTC1-

STN1 might assist DNA Polα/primase in synthesis of the lagging strand of a replication fork (Goulian and Heard 1990). Given the well-established role of *S. cerevisiae* Cdc13 and Stn1 in recruiting DNA Polα/primase to fill in the complementary C-strand following telomerase action(Bianchi and Shore 2008), it is striking that both mammalian and Arabidopsis CTC1 and/or STN1 associate with DNA Polα/primase (Fig. B-4; ref (Casteel et al. 2009)) and the mammalian proteins act as a template affinity factor (Goulian and Heard 1990). These findings lead us to suggest that plant and mammalian CST may also serve to recruit DNA Polα/primase for C-strand fill-in (Fig. B-7). The need for a specialized mechanism to recruit DNA-Polα to the chromosome terminus is anticipated because telomerase acts after the replication fork reaches the chromosome terminus (Moser et al. 2009a; Zhao et al. 2009). Hence, the components of the replication progression complex that normally recruit DNA Polα/primase to the replication fork (And1 and Mcm10) (Chattopadhyay and Bielinsky 2007) are unlikely to remain at the chromosome terminus at the time of C-strand fill in (Zhao et al. 2009).

One obvious question concerns how CST is loaded at the telomere given its lack of DNA-binding specificity. A recent study indicates that human STN1 interacts with TPP1 (Wan et al. 2009), suggesting that in mammalian cells CST loading may occur through interactions with shelterin (Fig. B-7). Given that the telomeric function of mammalian CST was discovered only recently, it is also possible that CST is brought to the chromosome terminus through interactions

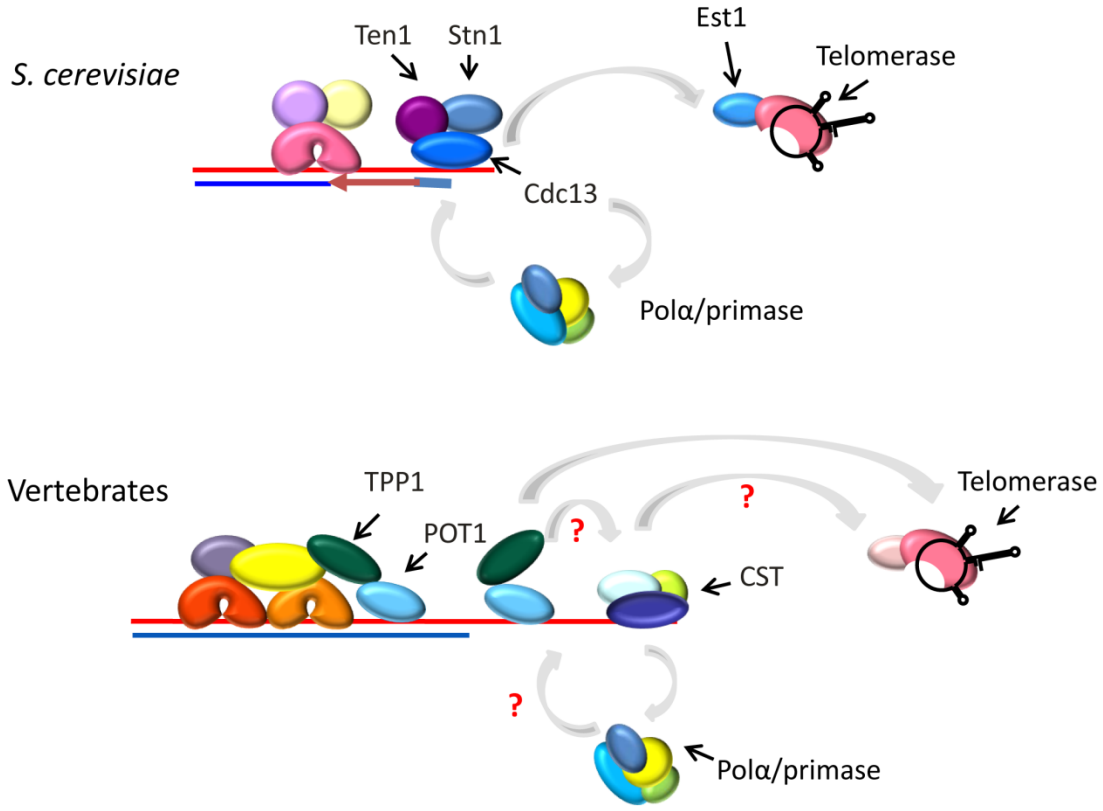

Figure B-7. Model for CST in telomeric DNA replication in budding yeast and vertebrates. *S. cerevisiae* CST interacts with the Est1 component of telomerase to promote telomeric DNA synthesis on the G-overhang, and with Polα/primase to facilitate lagging strand replication of the C-strand. Vertebrate CST associates with Polα/primase and stimulates its priming activity. The shelterin component TPP1 contacts telomerase and is postulated to recruit it to the chromosome terminus. TPP1 may also recruit CST to the telomere via interactions with STN1.

with as yet unidentified telomere proteins. TPP1 also interacts with telomerase (Xin et al. 2007; Zaug et al.) and enhances its action by increasing enzyme processivity (Latrick and Cech 2010). Thus, TPP1 could potentially function in manner similar to Cdc13 by serving to first enhance telomerase activity and then to recruit DNA Polα/primase via interactions with CST.

The current data argue that mammalian CST may have both telomeric and non-telomeric functions. Specifically, this complex may serve as a DNA Polα/primase recruitment factor elsewhere in the genome (Fig. B-8). Conditions that promote replication stress, for example replication through highly repetitive sequences or after certain types of DNA damage, will cause the polymerase to become uncoupled from the MCM1-7 replicative helicase. This scenario would lead to accumulation of ssDNA and a need to re-initiate leading or lagging strand synthesis. Recruitment of CST to stalled replication forks could stimulate synthesis by Polα/primase to restart replication and thus contribute to global genome stability.

Evolution of CST function

It is striking how CST complexes from different organisms perform similar activities in telomere protection and aspects of DNA replication, but in any one organism only a subset of these activities predominate. In terms of telomere protection, budding yeast and mammalian cells represent the two ends of the spectrum as CST is essential in *S. cerevisiae,* while in human cells it only

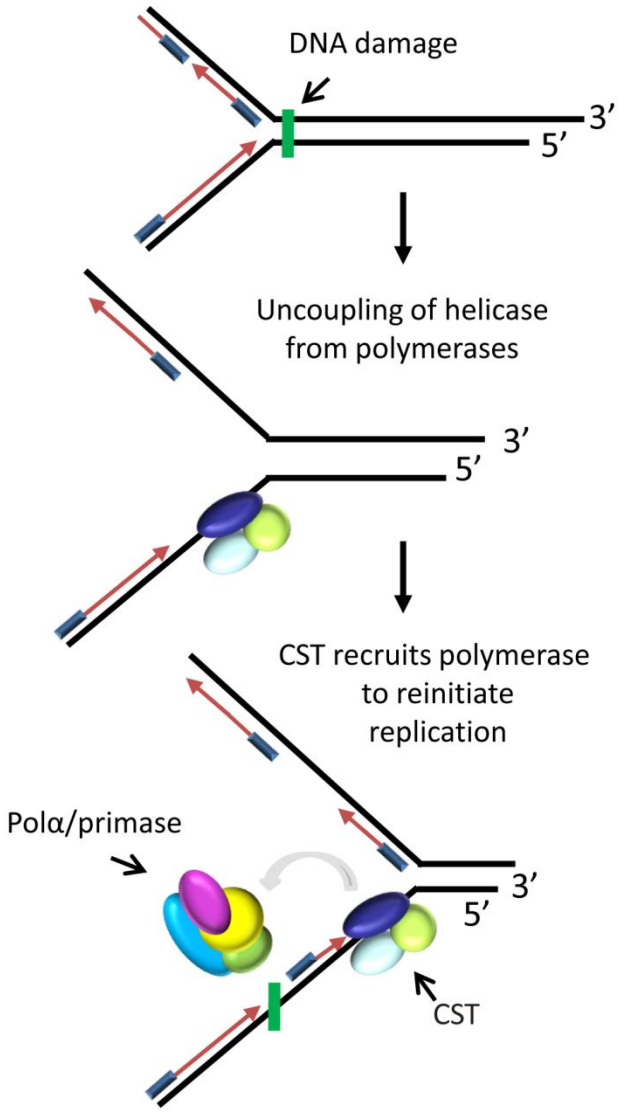

Figure B-8. Model for CST function during replication of non-telomeric DNA. Replication stress (following DNA damage or synthesis through highly repetitive sequences) results in polymerase dissociation from replicative helicases. CST may recruit and stimulate the activity of DNA polα/primase to promote lagging strand replication at such sites.

contributes when POT1/TPP1 is depleted. Plants provide a fascinating middle ground as some have evolved to use CST as their main telomere protection complex, while others have retained the capping function of POT1 (and likely other shelterin components). The current data indicate that budding yeast CST function is confined to replication of the extreme chromosome terminus. However, it is conceivable that ScCST has additional replication/repair functions resembling those of the mammalian complex. Such functions may not have been uncovered because removal of CST subunits leads to such a severe and immediate telomere uncapping phenotype. In support of this idea, when overexpressed, STN1 localizes to replication forks and interferes with the S-phase checkpoint in a DNA Polα-dependent manner (Gasparyan et al. 2009).

Finally, the structural similarity between CST and RPA is remarkable given that the two protein complexes have distinct biological roles. The similarity suggests that CST may resemble RPA in having multiple interaction partners and alternative DNA binding modes that involve a variable number of OB-folds. Such diversity in interactions might allow CST to mediate the sequential events that take place during telomere replication in much the same way that RPA mediates nucleic acid transactions during DNA replication, recombination and repair (Fanning et al. 2006; Haring et al. 2008). It is noteworthy that an RPA-like protein Teb1 is a key component of the telomerase holoenzyme from Tetrahymena (Min and Collins 2010). The OB-folds from Teb1 bind telomeric G-strand DNA thus anchoring telomerase and allowing enzyme processivity.

Again the sequential binding of the multiple OB-folds may be important as this could prevent Pot1 binding as the telomere is extended. Thus, RPA-like proteins may be much more common than originally thought and play key roles in a wide variety of chromosomal processes.

APPENDIX C

PRELIMINARY ANALYSIS OF THE MECHANISM OF ZEOCIN-INDUCED AND ATR-REGULATED TELOMERASE ACTIVITY LEVELS IN *Arabidopsis*

Introduction

Telomerase is the reverse transcriptase that adds telomeric DNA repeats to the ends of chromosomes during each cell cycle. Telomerase consists minimally of a protein reverse transcriptase (TERT) and an RNA template (TER). Loss of telomerase activity causes progressive telomere shortening with each cell division. When telomeres reach a critically short length, stability of chromosome ends is compromised and a DNA damage response (DDR) is activated (Palm and de Lange 2008). This can result in end-to-end chromosome fusions, nucleolytic attack, or inappropriate recombination. These events lead to further genome instability that ultimately results in cellular senescence or apoptosis (Palm and de Lange 2008).

When telomerase is overactive or functions at the wrong place or time, deleterious effects also occur. Telomerase is reactivated in approximately 90% of cancer cells and is an important step in establishing cellular immortality in cancers. Even in non-cancerous cells, unrestrained telomerase can be harmful. If telomerase extends chromosome ends too much, the long telomeres will be unstable and will be more likely to recombine (Palm and de Lange 2008). Finally, telomerase must be prevented from adding telomeres *de novo* to internal

regions of chromosomes at sites of DNA double-strand breaks (DSBs) (Pennaneach et al. 2006). Addition of telomeres within a chromosome will create an acentric chromosome fragment that will be lost during cell division. Telomerase therefore requires multiple layers of regulation to ensure it acts when needed and at the correct level of activity needed. More details about regulation of telomerase activity can be found in Chapter I above.

We have previously reported two novel types of telomerase regulation in *Arabidopsis thaliana* (Boltz et al. 2012; Cifuentes-Rojas et al. 2012). First, we found that plants lacking the DNA damage kinase ATR, but not the related kinase ATM, have a dramatic decrease in telomerase activity compared to wild type plants (Boltz et al. 2012). Although both ATR and ATM have been implicated in regulating telomerase access to the telomere, and Tel1 (ATM) in yeast promotes telomerase processivity at critically short telomeres, a direct modulation of telomerase activity by ATR has not been previously identified in other organisms (see Chapter I for more information about the DDR). Remarkably, the decrease in telomerase activity was not observed until the second generation (G2) of *atr* mutants. To explain this curious finding, we proposed that *atr* mutants accumulated low levels of DNA damage that did not detectably affect telomerase activity until the second generation. This hypothesis ties into our second discovery, which was that treatment of seedlings with the radiomimetic drug zeocin, which induces DSBs, caused a rapid drop in telomerase activity (Boltz et al. 2012).

Further investigation into the zeocin-mediated telomerase decrease revealed that levels of the TER2 telomerase RNA increased after zeocin treatment (Cifuentes-Rojas et al. 2012). TER2 is an alternative telomerase RNA which functions in an inhibitory telomerase complex (Cifuentes-Rojas et al. 2012). The canonical telomerase RNA TER1 was at wild type levels after zeocin treatment. TER2 shows higher binding affinity for TERT than TER1, so we hypothesized that increased TER2 levels after zeocin led to the presence of more inhibitory telomerase complexes (Cifuentes-Rojas et al. 2012). The inhibition of telomerase immediately after induction of a high number of DSBs is consistent with the need to repress formation of *de novo* telomeres at those breaks.

We also showed that the TER2-mediated response seems to be restricted to DSBs. Hydroxyurea (HU) induces stalled replication forks by inhibiting ribonucleotide reductase, which leads to depletion of dNTPs. HU treatment did not cause a change in telomerase activity (Cifuentes-Rojas et al. 2012). Additionally, plants with high levels of telomere dysfunction due to mutations in critical telomere capping proteins, such as *ctc1,* had wild type telomerase activity. Further, *atr* mutants displayed wild type TER levels, suggesting that the decrease in telomerase in these mutants occurs by a different mechanism than in zeocin-treated seedlings (Cifuentes-Rojas et al. 2012). Even if the mechanism is different in *atr* mutants, it does not preclude the

possibility that ATR (or ATM) is required for the down-regulation of telomerase after zeocin treatment.

ATR could function in telomerase activity regulation either by its kinase activity or through physical interactions with other proteins or nucleic acids. Further, the kinase activity could be directed at specific telomeric proteins, or ATR could activate a DDR signaling cascade. In *Arabidopsis* the ATR and ATM kinases act at the top of the DDR signaling pathway and lead to activation of a transcription factor, SOG1. The expression of hundreds of DDR genes require SOG1 activation (Yoshiyama et al. 2009). To investigate whether signaling through the canonical DDR pathways regulates telomerase activity, I employed *atr, atm* and *sog1* mutants. I found that ATM is not required for telomerase activity in response to zeocin treatment. SOG1 and ATR are both required for wild type telomerase activity in untreated seedlings. It is unclear whether they are required for the response to zeocin. The requirement for both ATR and SOG1 to promote telomerase activity suggests that ATR signaling through SOG1 is required for maintenance of wild type telomerase activity.

Materials and methods

Plant lines, growth conditions, and zeocin treatment

The *atr* and *atm* alleles have been previously described (Vespa et al. 2005; Boltz et al. 2012). Seeds used were for *atm* or *atr* from the third

generation (G3) of the deficiency or beyond. *sog1-1* seeds were a gift from Anne Britt (Yoshiyama et al. 2009) and the generation was unknown. Seeds were washed in 70% ethanol and then sterilized in 50% bleach/0.1% Triton-X. After washing with water, liquid MS (1/2x) was added to the seeds and tubes were stored at 4°C for two days. The seeds were then transferred to 1/2x liquid MS in 6-well petri plates. Plates were placed under constant light with gentle shaking for 7 days. On day seven, MS was removed from all wells and replaced with either new 1/2x MS or 1/2x MS containing freshly added zeocin (20µM final concentration). Plates were then placed in the dark with gentle shaking for 2 hours. Seedlings were then gently removed and blotted dry and immediately frozen in liquid nitrogen. Seedlings were frozen in pools of approximately 10-20 seedlings.

RNA and protein extractions

Protein was extracted from individual pools of seedlings that had been finely ground in liquid nitrogen using CelLytic P (Sigma) at a concentration of 100µL per 0.1 g of seedlings. Protein was quantified using a Bradford assay (Bio-Rad protein reagent) and samples were frozen in liquid nitrogen and stored at -80°C until used.

RNA was extracted using the Direct-zol RNA miniprep kit (Zymo). On column DNAseI treatment was used and samples were eluted in 30uL nuclease-free water. RNA was run on a 1% agarose to check for RNA integrity.

cDNA was made using qScript cDNA supermix (Quanta Bioscience) with 1ug of total RNA. cDNA was then diluted 1:4 in 10µg/mL of yeast tRNA in water. 4µL of cDNA was used for PCR to check for the presence of genomic DNA.

qTRAP

qTRAP was performed as previously described (Kannan et al. 2008), except that 1µL of a 1:10 dilution of each sample was used in the telomerase extension reaction. To quantify telomerase activity, raw data was run in the LinRegPCR program (Ruijter et al. 2009). The resulting N_0 values, which had been corrected for PCR efficiency, were used for calculations. To adjust for protein loading, the N_0 values were divided by the relative protein concentrations for each sample. These adjusted values were compared to wild type untreated samples by dividing by the average activity level for WT untreated. Averages for each genotype and treatment were calculated.

qRT-PCR

SsoAdvanced SYBR Green Master Mix (Bio-Rad) was used for qPCR. 4µL of diluted cDNA was used in each reaction. All samples were run in duplicate. *BRCA1* primers were described previously (Boltz et al. 2012). PDF2 was used as the reference gene to correct for loading differences (Czechowski et al. 2005). Raw data was entered into LinRegPCR using default settings and

calculating average PCR efficiency by amplicon group. The resulting N_0 values were used for further calculations. The average N_0 value for each *BRCA1* reaction was divided by the average N_0 for the corresponding PDF2 reaction. This adjusted value was then divided by the average N_0 for WT untreated seedlings. Finally, average expression for each genotype and treatment was calculated.

Results

To determine if zeocin-mediated telomerase repression depends on signaling from the DDR kinases ATR or ATM, 7-day-old WT Columbia-0, G3 *atr*, *atm*, and *sog1-1* seedlings were treated with 20µM zeocin for 2 hours. qRT-PCR of *BRCA1* was used to verify that zeocin induced DNA damage (Figure C-1A). The levels of *BRCA1* in untreated seedlings were similar in all genotypes (Figure C-1A, blue bars). Further, in WT, *atr* and *atm* samples, *BRCA1* levels were at least seven-fold higher than in WT untreated seedlings (Figure C-1A, red bars), indicating that zeocin had induced DSBs. *BRCA1* expression was not

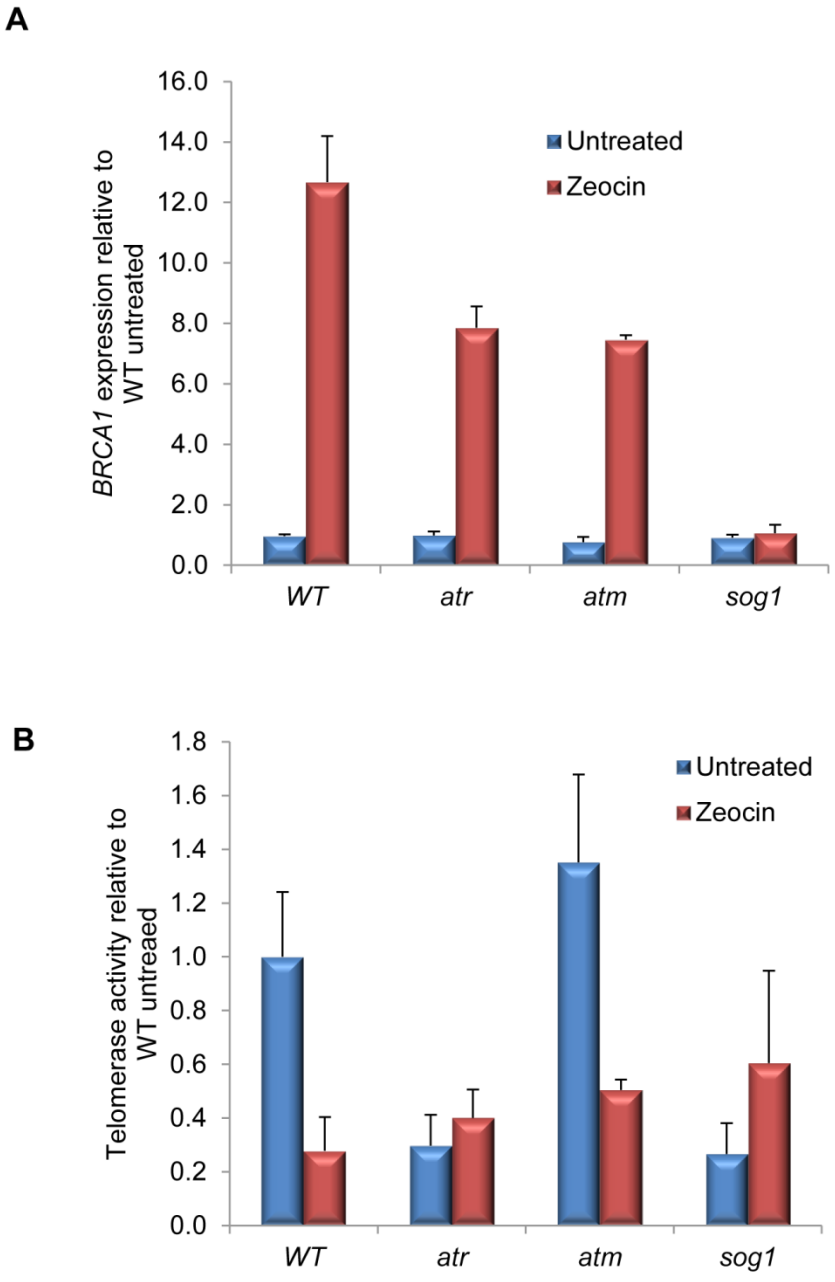

Figure C-1. Telomerase activity in seedlings after zeocin treatment in DDR mutants. (A) qRT-PCR of *BRCA1* expression relative to WT untreated following 2h zeocin treatment. (B) Quantitative TRAP for 7-day-old seedlings treated with zeocin for 2h. Telomerase activity levels are relative to WT untreated. Each value represents the average of three experiments.

induced in *sog1* mutants, which is expected because SOG1 is required for most transcriptional responses to DSBs in *Arabidopsis* (Yoshiyama et al. 2009).

Telomerase activity was measured using quantitative telomere repeat amplification protocol (qTRAP) (Figure C-1B). Telomerase activity levels in untreated *atm* and *atr* mutants were similar to our previous findings (Boltz et al. 2012). *atm* mutants that were not zeocin-treated had telomerase activity levels within the WT range, whereas *atr* mutants retained only about 30% of WT telomerase activity levels (Figure C-1B, blue bars). The basis for the decrease in telomerase activity in *atr* mutants is unknown, but could have several causes. For example, ATR could directly phosphorylate proteins involved in maintaining WT telomerase levels, or ATR could act indirectly by activating SOG1, which would then transcriptionally regulate telomerase activity. To investigate the second possibility we measured telomerase activity levels in *sog1* mutants. Similar to *atr* mutants, telomerase activity was reduced to approximately 30% of WT levels (Figure C-1B, blue bars). These results suggest that ATR and SOG1, but not ATM, are required for WT telomerase activity levels.

qTRAP was also performed on extracts from seedlings treated with zeocin (Figure C-1B, red bars). As seen before (Cifuentes-Rojas et al. 2012), zeocin treatment in WT seedlings led to an approximately 70% decrease in telomerase activity. *atm* mutants responded similarly to WT with about 50% of telomerase activity gone after zeocin, indicating that ATM is not required for the down-regulation of telomerase activity following zeocin treatment. In both *atr*

and *sog1* mutants, no further decrease in telomerase activity occurred after zeocin treatment. While this result suggests that ATR and SOG1 are required for the decrease in telomerase activity after zeocin treatment, it could reflect the fact that telomerase activity was already low in the untreated mutants.

Discussion

In this preliminary study we have several pieces of evidence that ATR signaling, and not physical interactions with other proteins, is important for wild type telomerase activity. Telomerase activity was measured in *sog1* mutants for the first time, and we found that *sog1* and *atr* mutants share a similar phenotype of decreased telomerase activity. This fact, coupled with the knowledge that ATR can activate SOG1 (Yoshiyama et al. 2009), suggests that ATR initiates a signaling cascade that activates SOG1, which in turn regulates transcription of some factors that regulate telomerase activity. An *atr sog1* double mutant should be analyzed to verify that they act in the same pathway for telomerase regulation. What is curious about this scenario is that it requires constitutive activation of ATR. Normal human telomeres have been shown to elicit a transient DDR in G2 of the cell cycle (Verdun et al. 2005), so perhaps during S-phase, ATR is activated in *Arabidopsis*. Telomerase extension of the telomeres requires that it have access to the single-stranded G-overhang. The presence of

an accessible G-overhang could activate ATR, which in turn initiates signaling to ensure that telomerase is active to extend the chromosome end.

This model also does not explain why telomerase activity is present in wild type levels in G1 *atr* mutants, but is then decreased in all subsequent generations. Previously we hypothesized that loss of ATR generated a low level of DNA damage, probably in the form of replication stress, and not enough DNA damage accumulated until the second generation to cause downregulation of telomerase activity (Boltz et al. 2012). However, we later showed that 8 hour treatment with hydroxyurea, which leads to replication fork stalling, does not cause a reduction in telomerase activity in WT seedlings (Cifuentes-Rojas et al. 2012). Perhaps in the case of replication stress, telomerase is not down-regulated until the DNA damage accumulated over a longer time period. Consistent with this hypothesis, we saw that TER2 levels were within the wild type range in *atr* mutants (Cifuentes-Rojas et al. 2012). If the increase in TER2 levels represents a mechanism for immediate inhibition of telomerase activity, then long term mechanisms for telomerase repression would not necessarily depend on TER2. Thus, we cannot eliminate the possibility that long term loss of ATR or SOG1 leads to slow accumulation of DNA damage, which triggers a TER2-independent pathway for telomerase down-regulation. A careful analysis in first generation *atr* and *sog1* mutants will be needed to compare the differences in later generation mutants.

The pathway(s) responsible for zeocin-induced inhibition of telomerase activity are not clear based on these preliminary results. ATM is not required for telomerase regulation, which is unexpected since zeocin induces DSBs and should activate ATM. In previous analysis of DSB induction by gamma radiation, most transcriptional responses required ATM and SOG1 (Yoshiyama et al. 2009). It is possible that telomerase repression in response to zeocin does not require transcriptional changes and is instead caused by post-translational protein modifications or changes in TER2 stability. Interpreting the low levels in telomerase activity in treated *atr* and *sog1* mutants is hindered by the fact that the mutants already had greatly reduced telomerase activity. To clarify if either ATR or SOG1 is important, first generation mutants, which have wild type levels of telomerase activity, must be used for zeocin treatment to see whether either protein is required.

The only signaling pathway known to increase telomerase activity levels in *Arabidopsis* is auxin-mediated (Ren et al. 2004). BT2 and TAC1 (Telomerase Activator 1) regulate *TERT* expression by negatively regulating an unknown negative regulator of telomerase activity. Further, plants can be treated with exogenous auxin to turn on *TERT* expression (Ren et al. 2004). To determine if ATR or SOG1 function within this pathway, G2 or older *atr* and *sog1* mutants could be grown with or without exogenous auxin to see if telomerase activity is restored. Further, qRT-PCR could be used to measure levels of *BT2, TAC1* and *TERT* in *atr* and *sog1* mutants. If ATR or SOG1 regulates telomerase through

that pathway, then all three transcripts would be expected to be decreased in *atr* or *sog1* mutants.

The relationship between the DDR and telomeres is paradoxical. Telomerase activity at a DSB is potentially lethal whereas it is vital at critically short telomeres. We are only beginning to understand how cells differentiate the different contexts that might activate or repress telomerase. Understanding how cells signal in response to a DSB versus the need to elongate telomeres is also important in the context of cancer where the DDR is perturbed and therefore telomerase regulation is altered.