DISSECTION OF THE TELOMERE COMPLEX CST IN *ARABIDOPSIS THALIANA*

A Dissertation

by

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ABSTRACT

Telomeres are the ends of linear chromosomes tasked with preventing their

recognition by the DNA damage machinery and providing a mechanism to solve the end

replication problem. The telomeric DNA is mostly double-stranded, but it terminates in a

3' protrusion termed the G-overhang. Telomeres utilize telomerase, a reverse

transcriptase, to elongate the telomere, and thus, solve the end replication problem. Both

the double strand region and the G-overhang are bound by specific proteins to facilitate

the objectives of the telomere. First discovered in budding yeast, the CST

(Cdc13(CTC1)/Stn1/Ten1) complex binds to the G-overhang and is important for both

chromosome end protection and telomere replication. Work reported in this dissertation

provided the first evidence that CST was present outside of yeast, which led to its

subsequent identification in a number of vertebrates.

Here I present the identification and characterization of the three components of

CST in *Arabidopsis thaliana*. Similar to yeast, Arabidopsis CST is required for telomere

length maintenance, for preventing telomere recombination and chromosome end-to-end

fusions. Mutations in the CST complex result in severe genomic instability and stem

cells defects. My research also shows that CST and telomerase act synergistically to

maintain telomere length. Together these data provide evidence for an essential role for

CST in maintaining telomere integrity.

Unexpectedly, I discovered that the TEN1 component of CST may have a more

complex role than other members of the heterotrimer. The majority of telomere-related

functions we can assay using molecular and cytological approaches are shared by CTC1, STN1 and TEN1, though TEN1 has additional roles in maintaining genome stability, modulating telomerase activity and possibly non-telomeric functions in the chloroplast.

I also present genetic evidence that TEN1 and STN1 act in the same pathway for the maintenance of telomere length and chromosome end protection. Interestingly, however, disrupting the STN1/TEN1 interaction reveals a separation of STN1 function for chromosome end protection versus telomere length maintenance.

Finally, I describe the design and creation of a library of STN1 and TEN1 mutants that will be used to further characterize their functions and their interaction partners. By disrupting such interactions, it will be possible to elucidate the functional significance of these interactions, and thus, provide new insight into how CST functions in Arabidopsis.

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CHAPTER I

INTRODUCTION

In the 1930s, both Barbara McClintock and Hermann Müller independently recognized that the ends of chromosomes are unique. While looking at the effects of X-rays on Drosophila chromosomes, Müller noticed that the loss of the end of the chromosome was detrimental (Müller, 1938). He named these chromosome ends telomeres (telos-end, mere-part) and hypothesized that they must encode some gene essential for survival (Müller, 1938).

McClintock's work with maize using genetics to examine the effects of x-ray-induced translocations brought her to the conclusion that the ends of the chromosome were protective and that disruption of those ends led to entrance into the chromosome breakage-fusion-bridge (BFB) cycle (McClintock, 1938) (Figure 1-1). Chromosome end-to-end fusions result in the formation of di-centric chromosomes, which have two centromeres. During the anaphase stage of mitosis the two centromeres are pulled in opposite directions and chromosome bridges appear. Tension from the separation of the centromeres causes chromosome breakage. In subsequent phases of the cell cycle, the DNA damage response (DDR) machinery will recognize the break and attempt to repair the damaged chromosome end by fusing it to another unprotected chromosome end. Thus, with each new cell division the DNA will continue to be damaged through the BFB cycle, leading to massive genomic instability and ultimately cause cell senescence or death.

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McClintock also discovered "chromosome healing," a process now known to occur via telomerase-mediated addition of telomeric sequence to the broken chromosome end. This process of de novo telomere formation (DNTF) causes the broken chromosome to lose its propensity to fuse to other chromosomes and allows it to exit the BFB cycle (McClintock, 1941). DNTF is detrimental to the cell, as the genetic material distal to the break site will be lost during cell division.

A large number of genetic and biochemical studies have subsequently shown that telomeres are specialized nucleoprotein complexes at the ends of chromosomes (Figure 1-2). They serve two important functions. The first function is to protect the chromosome end from being recognized as a double-stranded DNA break by cellular repair machinery, and feeding into the BFB cycle. Chromosome end protection is mediated in part by proteins associated with the telomere as well as the formation of a telomere loop (t-loop) on the extreme terminus, which helps to sequester the single-strand region of the telomere, masking it from the DDR machinery. Telomere dysfunction leads to telomere shortening/loss, chromosome end-to-end fusions, and genomic instability.
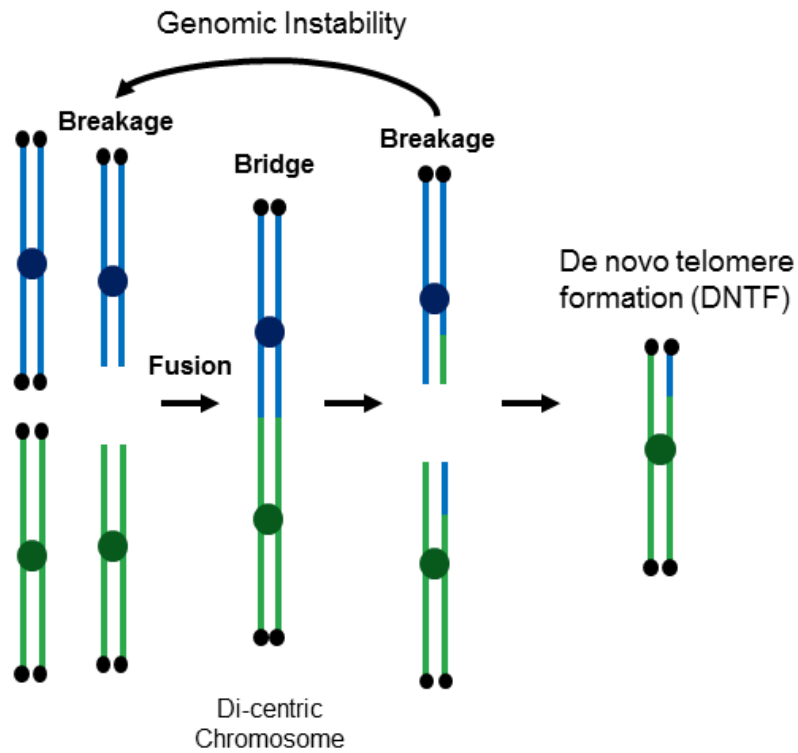
Figure 1-1. Model of the Breakage-Fusion-Bridge (BFB) cycle.
Uncapped chromosomes formed by telomere dysfunction or ds DNA breaks can fuse and form a dicentric chromosome. During anaphase the centromeres are pulled to opposite poles and the bridges can be directly observed. The chromosomes will be pulled apart resulting in the formation of newly uncapped telomeres, with genome rearrangements possible at the original break sites. These uncapped chromosomes can then continue in the BFB cycle, or exit the cycle by de novo telomere formation at the broken ends.
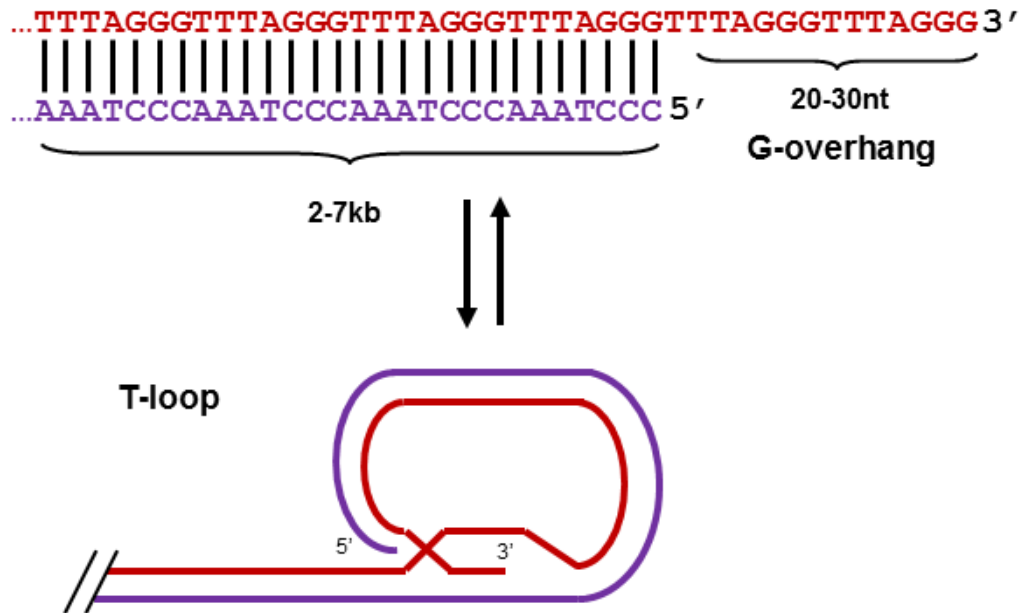
Figure 1-2. Telomere structure.
Telomeres consist of both double-stranded and single-stranded regions comprised of short G-C rich repeats. In Arabidopsis, the telomere sequence is $(TTTAGGG)_n$. The ds region is 2-7 kb, while the ss 3' overhang (G-overhang) is 20-30nts in length. The ss telomeric DNA can invade the ds portion causing strand displacement and formation of the t-loop to protect telomeres from the DDR.

The second function of the telomere is to provide a mechanism to solve the end replication problem. The discovery of the semi-conservative nature of DNA replication led to the proposition of the end replication problem by James Watson and Alexy Olovnikov in the 1970s. The end replication problem results in the shortening of linear chromosomes with each cell division due to the mechanism of conventional DNA replication (Olovnikov, 1971; Watson, 1972). The replication of DNA requires an RNA primer to start the process which is then extended by DNA polymerase (Figure 1-3A). Ultimately, the RNA primer must be removed from the DNA and the unsynthesized portion of the strand is filled in, and the nick ligated. This mechanism will result in complete replication of the 3' leading strand (Figure 1-3A). However, at the 5' end of the lagging strand, the 10-12 nts, corresponding to the size of the removed RNA primer, will not be replicated. With each cell division the chromosomes shorten, creating a dilemma concerning the complete transfer of genetic material over multiple generations. The problem is solved by the enzyme telomerase which adds telomere repeats to the chromosome ends, allowing the cell to continually divide and avoid senescence or cell death due to compromised telomeres (Figure 1-3B).
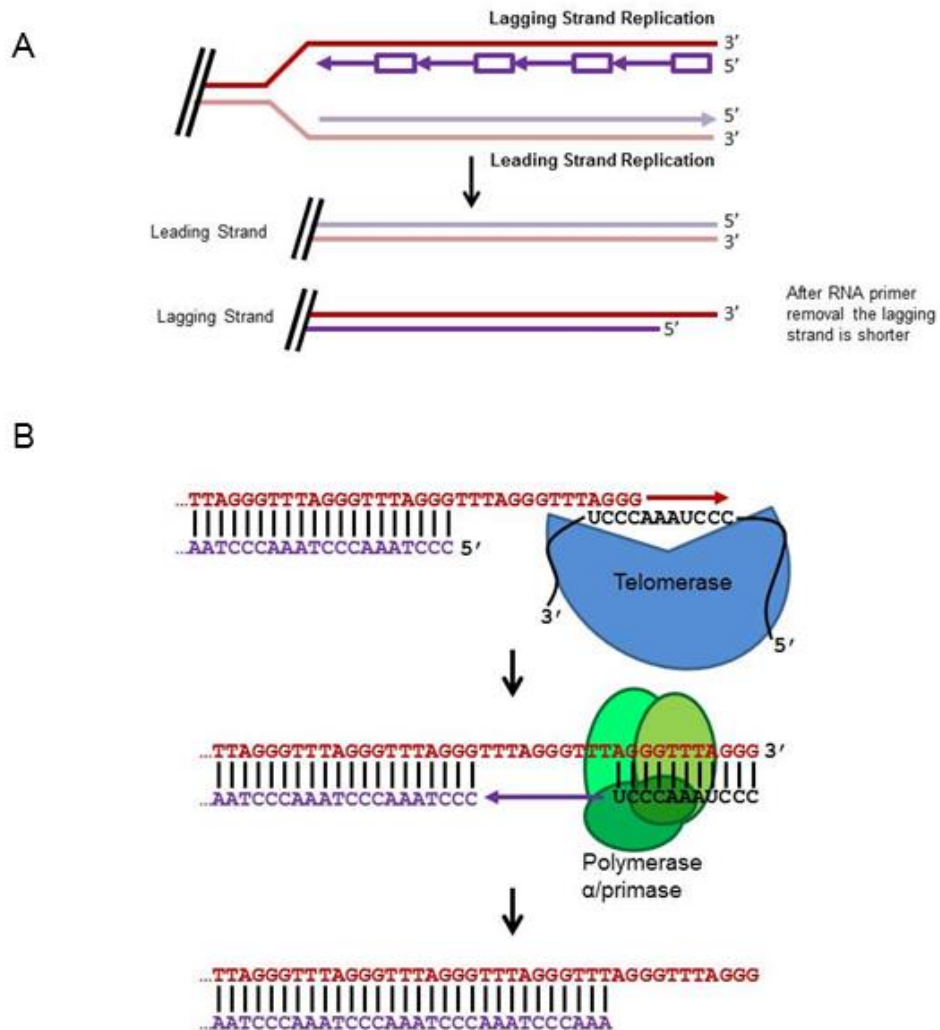
Figure 1-3. The end replication problem and its solution by telomerase.
(A) During DNA replication the leading strand is fully replicated, as the DNA polymerase moves in the opposite direction of fork progression. The lagging strand is replicated discontinuously to move in the same direction as fork progression. This requires synthesis of many RNA primers. When the final RNA primer is removed from the 5' end of the lagging strand a ss overhang is created. This incomplete replication causes the shortening of telomeres with each cell replication. **(B)** Telomerase solves the end replication problem by accessing the 3' overhang and using its RNA template and reverse transcriptase activity to add telomere repeats to the G-overhang. Following this extension polymerase α will then use a primer to replicate the C-strand. Thus, telomerase can allow for net lengthening of the chromosome ends, not shortening.

Though the first hints that telomeres were special came in the 1940s, it was not until the 1970s when Elizabeth Blackburn used new DNA sequencing technology to sequence telomeres. Sequencing of the ends of minichromosomes in *Tetrahymena thermophila* revealed that the telomeres consist of tandem repeats of the sequence GGGGTT (Blackburn & Gall, 1978). Jack Szostak later demonstrated that a foreign piece of linear DNA from *Tetrahymena* could be maintained in yeast by the addition of yeast telomere repeats to the ends (Szostak & Blackburn, 1982). This observation suggested that telomere sequence addition was a conserved mechanism in eukaryotes. The discovery of the enzymatic activity of telomerase by Blackburn and her graduate student, Carol Grieder, revolutionized the way that scientists thought about telomeres and DNA replication (Greider & Blackburn, 1985), and led to Blackburn, Greider and Szostak winning the Nobel Prize in Physiology or Medicine in 2009.

Telomeres and human health

We know now that to compensate for the end replication problem, telomerase continually adds DNA repeats to chromosome ends. In the somatic cells of vertebrates where telomerase is not active, telomeres shorten progressively with each cell division (Harley et al, 1990). Telomere attrition limits the number of divisions that a cell can undergo, and therefore the cumulative amount of DNA damage or mutations that can accrue before cellular senescence. Thus, telomere shortening is proposed to be a barrier to cancer (Kipling, 1995). Consistent with this hypothesis, in approximately 90% cancer cases, telomerase is active and telomeres are maintained, allowing cells to overcome a

major limitation in the quest to divide indefinitely (Kim et al, 1994; Shay & Wright, 2011).

In addition to well-established roles in cancer and cellular aging, telomeres are increasingly being studied in correlation with general human health. Mutations in telomere-associated genes are linked to diseases characterized by shorter telomeres. Such patients generally have stem cell dysfunction and other aging-related phenotypes (Anderson et al, 2012; Keller et al, 2012; Mitchell et al, 1999; Walne et al, 2012). Recent studies show that short telomeres also correlate with rheumatoid arthritis, stroke, coronary artery disease and hypertension (Fitzpatrick et al, 2007; Samani et al, 2001; Schonland et al, 2003; Yang et al, 2009). Shorter telomeres are associated with a poor prognosis for some diseases including renal and cardiovascular disease (Farzaneh-Far et al, 2008; Fitzpatrick et al, 2007). These studies demonstrate the importance of telomeres for human health, underscoring the need to elucidate the factors and mechanism involved in telomere maintenance and stability.

Telomeres and telomeric DNA

Telomeric DNA consists of tandem repeats of G-rich sequences (Figure 1-2). These sequences vary from species to species. Vertebrate telomeres contain T_2AG_3 repeat (Moyzis et al, 1988), while telomeres in plants are comprised of T_3AG_3 (Bae & Baumann, 2007; Richards & Ausubel, 1988; Zellinger & Riha, 2007). Most of the telomeric DNA tract is double-stranded (ds), but the very end has a 3' protrusion that is single-stranded (ss). This ss 3' extension is termed the G-overhang, due to its high G

content. Length of the telomere duplex can vary between species and even within different populations or cell types. Human telomeres range from 2-15 kb (Palm & de Lange, 2008), Arabidopsis telomeres from 2- 7 kb (Richards & Ausubel, 1988; Shakirov & Shippen, 2004) and *Nicotiana tabacum* (tobacco) telomeres from 40-160 kb (Fajkus et al, 1995). Therefore, although the telomere sequence is highly conserved, the length of the telomere repeat array varies in different organisms. Factors that influence length of the telomere tracts are discussed below. The maintenance of telomere length is critical for preventing the activation of the DDR and preventing cellular senescence.

Telomeres and DNA damage machinery

Because a chromosome end resembles a ds break, one of the main purposes of the telomere is to block activation of the DDR. Many of the telomere-associated factors function to modulate DNA repair activities that would otherwise negatively affect genome integrity. Interestingly, many of the factors important for the DDR are associated with normal telomeres and important for their maintenance. Two of these important proteins are ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3-related protein). ATM and ATR are multifaceted protein kinases that are central to the DNA damage response and telomere maintenance. Loss of ATR but not ATM is lethal in yeast (Zhao et al, 2001), as well as vertebrates (Zhao et al, 2001). The yeast homologs Tel1 (ATM) and Mec1/Rad3 (ATR) are upstream regulators of telomere length, and mutations in either causes telomere shortening due to loss of telomerase recruitment (Naito et al, 1998; Tseng et al, 2006). ATM plays an important

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role in the recognition of dsDNA damage (Khanna et al, 2001). The ds telomere binding protein TRF2 blocks activation of the ATM-mediated DNA damage response (Denchi & de Lange, 2007). Alternatively, ATR is activated in response to ss DNA by stalled replication and DNA repair processes (Nam & Cortez, 2011). ATR is also activated by loss of ss telomere binding proteins including POT1 in humans (Denchi & de Lange, 2007) and Cdc13 in yeast (Garvik et al, 1995). Notably, ATM and ATR are not essential genes in Arabidopsis (Vespa et al, 2005). Plants deleted for either gene do not exhibit telomere length perturbations (Vespa et al, 2005). However, ATM and ATR act synergistically with telomerase in the maintenance of Arabidopsis telomeres (Vespa et al, 2005; Vespa et al, 2007). Genetic interactions between ATM, ATR and the major telomere protection complex in Arabidopsis will be discussed in Appendix II.

G-overhang and the T-loop

The G-overhang provides a substrate for telomerase to bind and extend, as well as the platform for the assembly of telomere capping complexes. The length of the G-overhang at the extreme chromosome terminus can vary depending on both the species and the point of the cell cycle. The G-overhang ranges from 12-16nts in budding yeast and ciliates (Klobutcher et al, 1981; Pluta et al, 1982), and from 20-30nts in Arabidopsis (Riha et al, 2000). It is longer still in humans at 50-160 nts (Chai et al, 2006). Initially, it was believed that the G-overhang formed due to lack of complete replication of the lagging strand after removal of the RNA primer, but the origin of the G-overhang structure is in fact more complicated.

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The formation of the G-overhang occurs by different mechanisms on the leading

(Figure 1-4A) and lagging strand telomeres (Figure 1-4B) (Chow et al, 2012; Dai et al,

2010). In human cells, after replication of the leading strand in late S phase, the C-strand

is resected and the G-strand is extended by telomerase (Zhao et al, 2009). The G-

overhang on the leading strand is further lengthened in G2 due to additional C-strand

resection (Dai et al, 2010). At the lagging strand, removal of the final RNA primer after

replication results in a short 3' overhang that is further processed by C-strand resection

(Chow et al, 2012). Then, telomerase adds repeats to extend it (Zhao et al, 2009). C-

strand fill-in mediated by the recruitment of DNA polymerase α/primase follows, and

finally, removal of the last primer leaves a 3' G-overhang (Zhao et al, 2009).

After the G-overhang is generated, telomeres can form higher order secondary

structure with the help of associated proteins. Such structures provide additional

protection for the chromosome end by preventing the ss DNA region from activating an

ATR dependent DDR. In budding yeast, telomeres assume a simple fold-back structure

in which the G-overhang associates with the subtelomeric region in a Rap1-dependent

manner (de Bruin et al, 2000). Multicellular eukaryotes use another mechanism to

sequester the ss region of the telomere through the formation of a t-loop (Figure 1-2).

The t-loop was first visualized by Jack Griffith in human and mouse cells (Griffith et al,

1999). T-loops have since been detected in plants, ciliates, *Caenorhabditis elegans*, and

Kluyveromyces lactis, suggesting that this is a conserved telomere protection method

(Cesare et al, 2008; Cesare et al, 2003; Griffith et al, 1999; Murti & Prescott, 1999;

Raices et al, 2008). The t-loop is hypothesized to form when the ss region of the

11
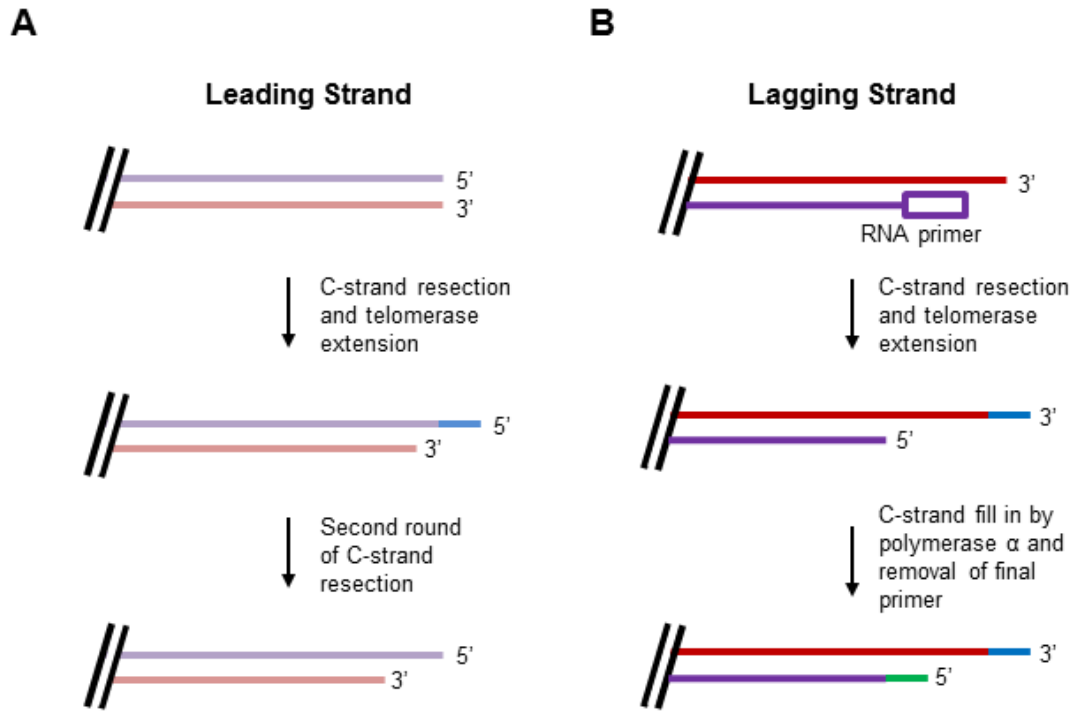
Figure 1-4. Model for G-overhang generation in human cells.

telomere invades the duplex region causing localized displacement of the original duplex strand and resulting in the formation of a conventional displacement loop (D-loop) (Figure 1-2). The D-loop is typically observed as an intermediate in homologous recombination. The t-loop hypothesis is based on the observation that t-loop formation requires that telomeres have G-overhangs (Griffith et al, 1999). The t-loop structure is stabilized by a number of telomere-related proteins that will be discussed later in detail.

Telomere length homeostasis

Telomeres that are too long or short present a problem for the cell (Stewart et al, 2012a). Therefore, telomere length is tightly regulated. Factors that contribute to telomere length regulation include telomerase, telomere bound proteins, telomere replication, homologous recombination, and exonucleolytic attack (Stewart et al, 2012a). During S phase telomerase preferentially engages the shortest telomeres in the cell, and may not act on all telomeres in a single cell cycle (Hemann et al, 2001; Shakirov & Shippen, 2004; Teixeira et al, 2004). Telomeres can exist in a non-extendible state when they are long or an extendible state when they are shorter (Teixeira et al, 2004). This interaction is mediated by the telomere conformation as well as the non-nucleosomal telomere proteins that associate with the chromosome terminus. Telomere-bound proteins can act as either positive or negative regulators of telomere length via a protein counting mechanism or recruitment/blocking telomerase (discussed below). As described above, telomere attrition will occur at a steady state in cells that are not expressing telomerase (Harley et al, 1990).

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Unlike the steady, progressive telomere shortening associated with the absence of telomerase, sudden telomere shortening can occur from homologous recombination at the t-loop. Specifically, if the t-loop is recognized as a Holliday junction it can be resolved, leading to a process known as telomere rapid deletion (TRD) (Figure 1-5) (Lustig, 2003). Resolution of t-loops creates extra chromosomal telomere circles (ECTC) as a by-product (Figure 1-5). TRD typically occurs on elongated telomeres, restoring them to wild type length. However, inappropriate TRD on wild type length telomeres can cause catastrophic consequences as the telomeres will be radically shortened. These extremely short telomeres can be recognized as dsDNA damage and then enter the BFB cycle, resulting in genomic instability. TRD was first discovered in yeast (Li & Lustig, 1996), but it is also been reported in plants (Zellinger et al, 2007) and humans (Wang et al, 2004). ECTC are present at low levels in wild type cells. It has been proposed that these ECTC can also be utilized for alternative lengthening of telomeres (ALT) with the circle serving as a template for telomere addition (Figure 1-6) (Muntoni & Reddel, 2005; Verdun & Karlseder, 2007). ECTC-mediated ALT is believed to be a major mechanism of telomere maintenance in cancer cells that do not express telomerase (Muntoni & Reddel, 2005).
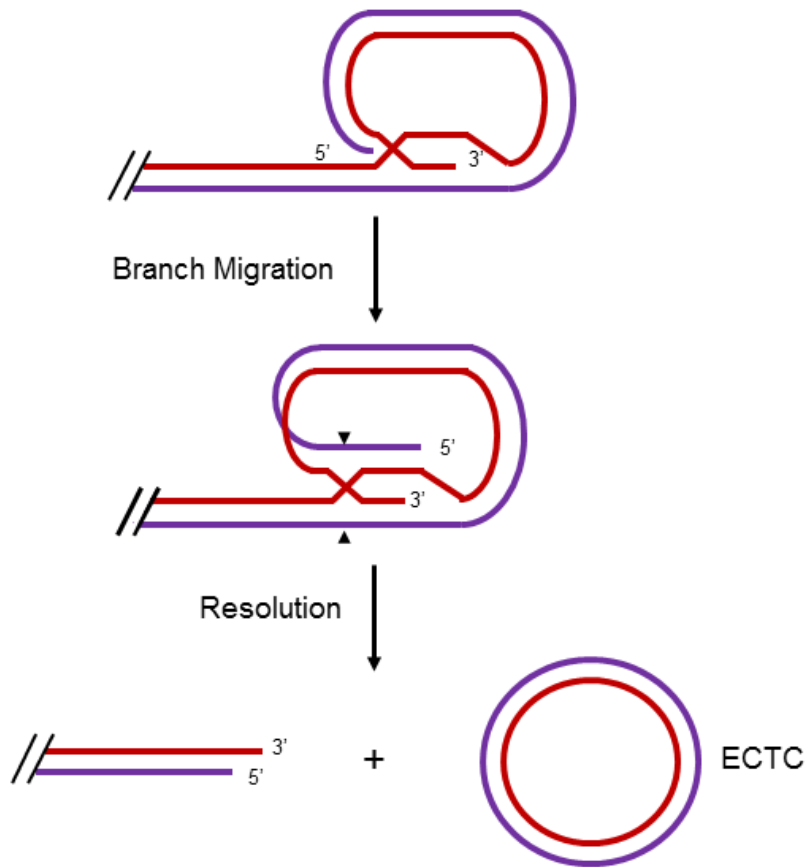
Figure 1-5. Extra-chromosomal telomere circle formation.
Branch migration in the t-loop can cause the formation of structures that resemble a
Holliday junction. Cellular machinery can recognize this secondary structure and resolve
it by cleaving at the junction site. This will cause the loss of a large portion of DNA
from the telomere that can circularize to form an extra chromosomal telomere circle.
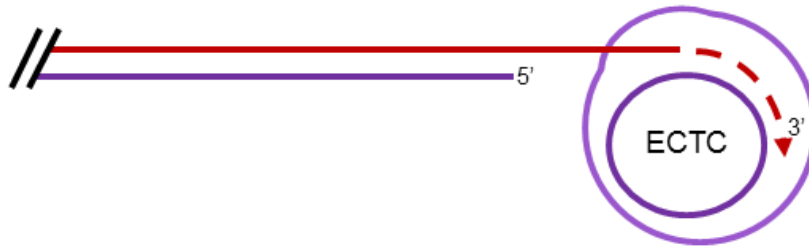
Figure 1-6. Alternative lengthening of telomeres.
Extra chromosomal telomere circles can be used as a template for telomere extension.
The ss G-overhang can invade the ds ECTC and use rolling circle amplification to
elongate the telomere.

Telomere length varies widely for different eukaryotes from a few hundred base pairs in yeast to over one hundred kb in mice. Originally, it was proposed that telomere length in multicellular eukaryotes correlates with organism lifespan. Empirical data support this model in humans and some other mammals (Gomes et al, 2011). The theory is that the longer an organism lives, the more cell divisions it requires. The number of cell divisions that are possible prior to cellular senescence is determined by both telomere length and the rate of telomere attrition due to incomplete replication, recombination and degradation (Harley et al, 1990; Shay & Wright, 2010). Therefore, in long-lived species, a large reservoir of telomere repeats will increase the number of divisions without altering replication machinery. However, the situation is clearly more complex than this simple model since the correlation between telomere length and organismal lifespan does not hold in *Mus musculus*. Mouse telomeres are up to ten times longer than human telomeres (5-15kb), and yet their life span is much shorter (Gomes et al, 2011).

Telomerase

During their initial characterization of telomerase from *Tetrahymena*, Blackburn and Greider found an RNA moiety (TER) that was a critical component of the enzyme (Greider & Blackburn, 1985). Biochemical purification of telomerase from a related ciliate, *Euplotes* (Lingner & Cech, 1996) and a screen in yeast (Lendvay et al, 1996) lead to the discovery of the telomerase reverse transcriptase subunit (TERT). Arabidopsis TERT was discovered by its homology to human TERT (Fitzgerald et al,

1996). TERT assembles into a ribonucleoprotein (RNP) complex with telomerase RNA (TER) as well as a number of accessory proteins that function in RNP biogenesis and enzyme recruitment to the chromosome end. TERT utilizes TER as a template for the addition of telomere repeats. Base pairing with the template domain aligns the G-overhang in the enzyme active site with TER. The 1.5-2x copies of the telomere repeat within TER allow for alignment and base pairing with the G-overhang to produce uniform repeats (Chen et al, 2000; Feng et al, 1995; Romero & Blackburn, 1991; Shippen-Lentz & Blackburn, 1990).

Telomerase plays a crucial role in telomere length maintenance, and mutations in the core enzyme or its accessory proteins result in an ever shorter telomere (est) phenotype (Lingner et al, 1997). Telomere shortening is a hallmark of loss of telomerase function in all organisms, including Arabidopsis, mice and humans (Feng et al, 1995; Fitzgerald et al, 1999; Lee et al, 1998; Mitchell et al, 1999). Loss of telomerase in mice causes telomere dysfunction and eventually sterility after six generations (Blasco et al, 1997). In Arabidopsis, plants lacking TERT can survive for up to ten generations. However, by the sixth generation of the *tert* mutant telomere-telomere fusions can be detected, ultimately resulting in severe genomic instability (Riha et al, 2001). This genomic instability progressively worsens, leading to developmental arrest starting in the eight generation and complete sterility by the ten generation (Riha et al, 2001).

Telomerase reverse transcriptase

TERT is a large protein of ~130 kDa (Fitzgerald et al, 1999; Nakamura et al, 1997). It is composed of several discrete domains needed for enzyme activity as well as RNA, DNA and protein interactions. The reverse transcriptase (RT) domain of TERT is well conserved among eukaryotes, and evolutionarily related to penelope-like retrotransposon RT domains (Gladyshev & Arkhipova, 2007). In addition to the RT domain, TERT contains a long variable N-terminal extension and a shorter C-terminal extension. These domains are important for protein-protein and protein-RNA interactions (Autexier & Lue, 2006). The TEN domain is a telomerase-specific domain that allows TERT to bind telomeric DNA and remain associated for the addition of multiple repeats (Robart & Collins, 2011). The TERT RNA binding domain (TRBD) near the end of the TERT N-terminal extension facilitates TER interaction (Lai et al, 2001). Finally, the TERT C-terminal extension is thought to enhance RNA/DNA interactions and contribute to the catalytic function of TERT (Wyatt et al, 2010).

Telomerase RNA

Unlike TERT, there is only limited sequence or size conservation of the TERs, even among related species. As a non-coding RNA, TER does not need to adhere to the strict codon sequence requirements that protein coding genes must, which may explain its rapid sequence divergence. Most organisms harbor a single TER (Chen et al, 2000; Feng et al, 1995; Leonardi et al, 2008; Romero & Blackburn, 1991; Singer & Gottschling, 1994). However, Arabidopsis possesses three distinct TER molecules, as

discussed in detail below. TERs range in size from 150 nts in ciliates to over 1000 nts in different species of yeast (Greider & Blackburn, 1989; Singer & Gottschling, 1994).

Conservation of TER lies within its secondary structure. Potential pseudoknots have been associated with all TERs characterized to date. These domains are important for telomerase activity and TERT binding (Autexier & Greider, 1996; Mitchell & Collins, 2000; Qiao & Cech, 2008). The CR4/5 domain in human TER is responsible for high affinity binding to TERT (Chen et al, 2002). As expected, the telomere template is the ss region and resides within the active site of telomerase. A so-called template boundary region lies at the 5' end of the template domain and stipulates the length of the nucleotide repeat that is added (Richards et al, 2006). Finally, in vertebrates and plants a conserved H/ACA box is important for binding the dyskerin complex, which is required for TER maturation (discussed below) (Chen & Greider, 2004; Fu & Collins, 2003).

Regulation of telomerase activity

Telomerase activity must be tightly regulated to balance the need for telomere length maintenance in cells that continually divide, and yet allow progressive telomere shortening to drive a molecular clock that prevent cancerous cells from unlimited division. To control this TERT expression and activity are tightly regulated in multicellular organisms. Human TERT is expressed in highly proliferating cells, including gametes and stem cells, but not in most normal somatic cells (Wright et al, 1996). A similar pattern of expression is seen for in Arabidopsis, as TERT transcription is repressed in vegetative tissues, and activated in germ line and stem cells (Fitzgerald et

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al, 1999). Telomerase activity is also regulated post-translationally by phosphorylation, ubiqutination and localization (Cifuentes-Rojas & Shippen, 2012). These mechanisms are critical because inappropriate expression of TERT and telomerase activity in normal somatic cells is a hallmark of cancer, with 90-95% of cancers having active telomerase (Kim et al, 1994).

Telomerase processivity is another step that is regulated. Once bound to the DNA, telomerase will add individual nucleotides to the G-overhang utilizing the TER template domain until a full repeat is added; this ability is denoted as nucleotide addition processivity (Figure 1-7). Telomerase must then reposition the RNA template at the end of the newly extended G-overhang so that it can add another repeat. This repositioning or primer translocation step reflects telomerase repeat addition processivity (RAP) (Figure 1-7) (Greider & Blackburn, 1985). While nucleotide addition processivity is a common function that is conserved in all RTs, RAP is unique to telomerase. Regions within TERT affecting nucleotide addition processivity lie in the conserved RT domains, while RAP is modulated in the telomerase specific domains (Huard et al, 2003; Lue et al, 2003; Moriarty et al, 2004). Both the template sequence and the secondary structure of TER also are important for processivity (Bachand & Autexier, 2001; Chen & Greider, 2003; Lai et al, 2003; Moriarty et al, 2004; Tzfati et al, 2000).
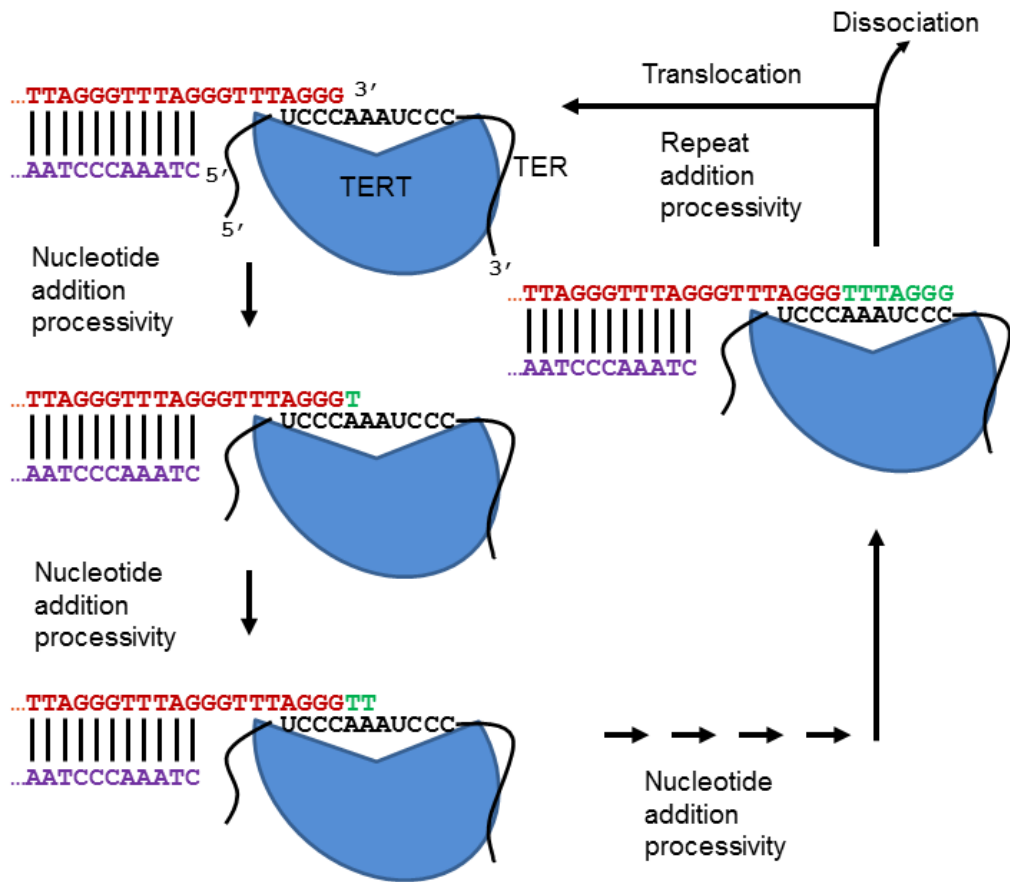
Figure 1-7. Telomerase processivity.
Telomerase uses the RNA template in TER to align and anneal with the G-overhang. The reverse transcriptase adds individual nucleotides until it reaches the template boundary region, resulting in the addition of a full telomere repeat. This process is referred to as nucleotide repeat addition processivity. After the addition of the full telomere repeat telomerase can either dissociate completely from the DNA or reposition at the newly formed end back at the beginning of the template for another round of repeat synthesis. This translocation reflects repeat addition processivity.

Telomerase processivity factors

The processivity and fidelity of telomere repeat synthesis by telomerase varies depending on the organism and growth conditions (Chang et al. 2007; Collins 2009; Zhao et al. 2011). Processivity is governed by a number of factors in vitro, including temperature, Mg^{2+} concentration, and dGTP (Shippen-Lentz & Blackburn, 1990). Telomere and telomerase-associated proteins can also modulate telomerase processivity; these include POT1-TPP1 in humans, Teb1 in *Tetrahymena* and Est1 and Est3 in *Saccharomyces cerevisiae* (discussed below).

Teb1 is a paralog of the replication protein A (RPA) subunit RPA70 in *Tetrahymena thermophila* (Min & Collins, 2009). Teb1 is similar to RPA in that it contains multiple oligonucleotide/oligosaccharide binding (OB)-folds. However, contrary to RPA, Teb1 binds with sequence-specificity to ss telomere repeats using two N-terminal OB folds (Zeng et al, 2011). An OB-fold, which consists of five beta strands that form a barrel and an alpha helix, is a common domain for protein-protein interactions and binding to ss nucleic acids, including rRNA, tRNA, ssDNA and the telomeric G-overhang (Flynn & Zou, 2010). Teb1 faciliates increased RAP by binding to both the telomere and the telomerase holoenzyme (Min & Collins, 2009; Min & Collins, 2010). Teb1 utilizes two OB–folds to bind to telomeric DNA with high affinity; it uses an additional OB-fold to modulate RAP (Zeng et al, 2011). Teb1, therefore, both recruits telomerase and modulates RAP in *Tetrahymena*.

The heterodimer POT1-TPP1 serves as a processivity factor for mammalian telomerase, in addition to its protective role at telomeres (Wang et al, 2007). TPP1-

POT1 increases RAP by slowing primer dissociation and increasing translocation efficiency (Latrick & Cech, 2010; Wang & Lei, 2011). The TEL patch in the OB-fold of TPP1 modulates both telomerase recruitment and processivity, indicating these two functions may be related (Nandakumar et al, 2012).

Telomerase-associated proteins

The core components of telomerase (TERT and TER) are sufficient to reconstitute telomerase enzyme activity in vitro, but in vivo a number of other accessory proteins are required for telomerase function. TERT and non-catalytic telomerase-associated proteins were first discovered in *S. cerevisiae*, and their loss-of-function mutants lead to the *est* phenotype. Est2 is the catalytic subunit of telomerase, and is referred to as TERT in this text. Est1 and Est3 are proteins dispensable for telomerase enzyme activity in vitro, but they are required for telomere maintenance in vivo (Cohn & Blackburn, 1995; Lendvay et al, 1996; Lingner et al, 1997; Lundblad & Szostak, 1989).

Est1 binds to ss telomeric DNA, as well as the bulged stem loop of *S. cerevisiae* TER, TLC1 in vitro (Seto et al, 2002). However, Est1 cannot bind to these two nucleic acids simultaneously (DeZwaan & Freeman, 2010) which provides a potential point of enzyme regulation. Est1 preferentially binds to TLC1 (DeZwaan & Freeman, 2010), creating a protein bridge between telomerase and Cdc13, a core subunit of the G-overhang binding complex termed CST (Cdc13/Stn1/Ten1) (Qi & Zakian, 2000). The association of Est1 with Cdc13 is needed for recruitment of telomerase to the telomere

end and facilitates the extension of the telomere (Evans & Lundblad, 1999; Pennock et al, 2001; Qi & Zakian, 2000; Wu & Zakian, 2011).

The function of Est3 is less understood, but the current model suggests that it functions in activation of telomerase and as a possible secondary recruitment mechanism. Est3 contains a single OB-fold that contacts the TEN domain of TERT (Song et al, 2008; Talley et al, 2011). The peak binding of Est3 to telomeres occurs in late S2/G phase, the same time that Est2 is bound to the telomere (Taggart et al, 2002).

Other telomerase accessory proteins that are needed for RNP maturation include dyskerin, TCAB1, GAR1, NOP10 and NHP2. TCAB1 is important for telomerase subcellular localization to Cajal bodies and in its absence telomerase association with the telomere is decreased (Venteicher et al, 2009). Dyskerin converts specific uridines into pseudouridines during the maturation of ribosomal RNAs and spliceosomal snRNAs (Meier & Thoma, 2005). Although the enzymatic function of dyskerin does not seem to be important for TER, the protein associates with telomerase RNA via the H/ACA motif within the RNA 3' terminus. Dyskerin drives telomerase RNA maturation and stability (Fu & Collins, 2003). Hence, dyskerin is required for telomerase activity in vivo (Chen & Greider, 2004; Kannan et al, 2008). Mutations in the human dyskerin cause the stem cell-related disease Dyskeratosis Congenita (DC) (Mitchell et al, 1999). Patients with DC exhibit signs of premature aging including, graying of the hair, dystrophy of the nails, anemia and increased incidence of cancer. Increasingly, more mutations in telomere-related genes are being implicated in patients with DC like symptoms (Armanios, 2012; Choudhary et al, 2012).

An unlikely accessory factor for telomerase is the Ku 70/80 heterodimer (Ku).

Ku is a multifunctional protein complex important for both the protection of

chromosome ends and DSB repair (Riha et al, 2006). Ku is a core component of the

nonhomologous end-joining (NHEJ) machinery, which is the most common dsDNA

repair process in mammals. Ku binds to dsDNA by encircling the double helix, allowing

for the stabilization of the DNA break during repair (Walker et al, 2001). In addition to

DNA repair roles, Ku is also important for telomere maintenance. Ku is required for

telomere protection, telomerase recruitment and nuclear positioning of telomeres (Fisher

& Zakian, 2005). In yeast, Ku recruits telomerase to the telomere via a direct interaction

with TLC1 in G1 where the inactive complex acts to cap telomeres and prevent C-strand

resection (Fisher et al, 2004; Hsu et al, 2007; Stellwagen et al, 2003). Ku is also

important for localization of telomeres the nuclear periphery during S-phase (Schober et

al, 2009). Due to the multifaceted roles for Ku in general chromosome maintenance, it

has been difficult to identify the telomere-specific roles. This continues to be an active

area of investigation.

Telomere binding proteins

The chromosome end associates with a variety of telomere-specific proteins.

These proteins function in two important ways. First, they regulate access of telomerase

and other DNA replication complexes to the telomere to control telomere length.

Second, they prevent the telomere from being recognized as DNA damage, thereby

preventing end resection and telomere-telomere fusions. Telomere proteins can be

classified as either dsDNA binding, ss DNA binding, or proteins that bridge these two regions of the telomere through protein-protein interactions. Proteins that directly contact the ds and ss regions of the telomere employ two major types of distinct binding domains: the Myb (myeloblastosis) domain and OB-folds, respectively. Myb domains contain three alpha helices in a helix-turn-helix domain and utilize a positively charged DNA binding domain to bind to ds telomeres (Bilaud et al, 1996). Ds telomere proteins that contain Myb domains include TRF1/TRF2 (vertebrates), Rap1 (budding yeast), and Taz1 (fission yeast). Some of the well-characterized ss binding proteins with OB-fold domains are POT1 (mammals/fission yeast) (Baumann & Cech, 2001), TEBP (*Oxytricha nova*), Cdc13 (budding yeast) (Garvik et al, 1995; Lin & Zakian, 1996; Nugent et al, 1996). In *C. elegans*, CeOB1 and CeOB2 bind to the G and C-overhangs. C-rich 5' overhangs have thus far only been identified in *C. elegans* (Raices et al, 2008). Additionally, some telomere-associated proteins interact via protein-protein interactions. These include Rif1 and Rif2 (budding yeast) and Rap1, TIN2, and TPP1 (mammals).

Shelterin is a six member protein complex that binds to telomeres in vertebrates (Figure 1-8) (Palm & de Lange, 2008). Notably, only a subset of these proteins are found in yeast and plants (Karamysheva et al, 2004; Miyoshi et al, 2008; Moser et al, 2011). Shelterin components are expressed in all cells, and bind along the length of the telomere during the entire cell cycle (Palm & de Lange, 2008). TRF1 and TRF2 bind to the ds region. Rap1 binds to TRF2. TIN2 and TPP1 provide a protein bridge from the ds binding proteins to POT1, which binds to the ss G-overhang (Figure 1-8).

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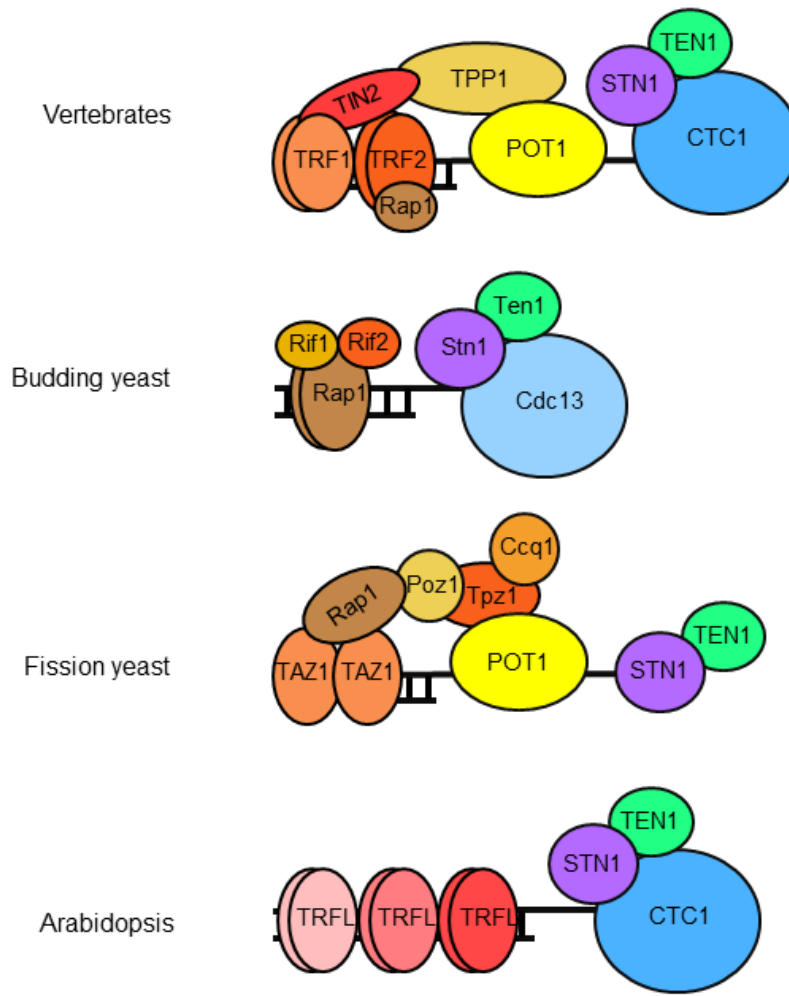
Figure 1-8. Telomere-associated proteins in different eukaryotes.
A model of the telomere-associated proteins in vertebrates, *S. cerevisiae, S. pombe* and
A. thaliana.

TFR1 and TRF2 both form homodimers and utilize their Myb domains to bind to ds telomeric DNA (Bianchi et al, 1997; Bilaud et al, 1997; Bilaud et al, 1996). TRF1 and TRF2 are negative regulators of telomere length (Smogorzewska et al, 2000; van Steensel & de Lange, 1997). They also modify the secondary structure of DNA, causing bending of the DNA and facilitating formation of the t-loop (Palm & de Lange, 2008). TRF2 plays an important role in the prevention of homologous recombination at the telomere, as loss of TRF2 results in recognition of the t-loop as a Holliday junction causing ECTC formation in human cells (Palm & de Lange, 2008; Wang et al, 2004). The TRF1 and TRF2 are critical for telomere protection. Mice lacking TRF1 or TRF2 die early in development due to DNA damage activation via p53 (Karlseder 2003, Iwano 2004, Celli and deLange 2005).

POT1 utilizes two OB-fold domains to bind ss telomeric DNA (ssDNA), though telomere binding is mediated by recruitment of POT1 by TPP1 (Lei et al, 2004; Liu et al, 2004). Loss of POT1 results in telomere elongation, as well as a telomere deprotection phenotype (Baumann & Cech, 2001). As mentioned previously, POT1-TPP1 is also a mediator of telomerase processivity (Wang et al, 2007). Loss of TPP1 results in telomere elongation, similar to POT1 mutants (Liu et al, 2004).

Like TPP1, other components of shelterin associate with the telomere via protein-protein interactions. Rap1 binds to TRF2, and is important for telomere length regulation (Li & de Lange, 2003). TIN2 is a central linch-pin of the shelterin complex, and it connects the ds binding proteins (TRF1/2) to the ss binding protein, POT1, via

TPP1 (Palm & de Lange, 2008). TIN2 binds TRF1 and TRF2 simultaneously and is required for shelterin stability (O'Connor et al, 2006; Ye & de Lange, 2004).

The CST complex

This dissertation will focus mainly on CST (Cdc13(CTC1)/Stn1/Ten1), a heterotrimeric complex that binds to the ss G-overhang (Figure 1-8). The CST complex is conserved across eukaryotes. In yeast, CST functions as a telomere capping complex that protects chromosome ends and regulates telomere replication (Chandra et al, 2001; Pennock et al, 2001). In contrast, the function of vertebrate CST complex is confined to telomere replication (Stewart et al, 2012a). Shelterin is thought to perform the telomere protective function. CST has sequence and structural similarities to the heterotrimeric RPA (replication protein A) complex (Gao et al, 2007; Sun et al, 2009). RPA coats ss DNA without sequence specificity so that ss DNA can remain unwound during replication (Wold, 1997). Like RPA, all three of the CST proteins contain OB-folds, which are important for their protein-protein interactions, as well as ss DNA binding (Price et al, 2010). However, unlike RPA, CST binds ss telomeric DNA with sequence specificity (Nugent et al, 1996).

The first CST component identified was Cdc13 (cell division control 13) from (Garvik et al, 1995). ScCdc13 has a molecular weight of 105 kDa, and is the largest subunit of the complex (Garvik et al, 1995). Biochemical analysis of Cdc13 showed that it specifically binds ss telomeric DNA with a K_d of 310 pM using a small DNA binding domain that has an even higher affinity with a K_d of 3 pM, this interaction gives the CST

complex ss telomeric DNA binding specificity (Anderson et al, 2002; Hughes et al, 2000; Nugent et al, 1996). Cdc13 is an essential gene (Garvik et al, 1995). *cdc13* ts mutants display reduced growth, longer telomeres and extended G-overhangs (Garvik et al, 1995). Further analysis of *cdc13* ts mutants showed that Cdc13 both positively and negatively regulates telomerase activity (Chandra et al, 2001; Evans & Lundblad, 1999; Grandin et al, 2000). Loss of Cdc13 binding to the telomere causes a deprotection phenotype resulting in massive nucleolytic degradation and a loss of telomerase recruitment (Garvik et al, 1995; Nugent et al, 1996).

Following the discovery of Cdc13, the Stn1 (suppressor of cdc thirteen) component of CST was identified in a suppressor screen for *cdc13* mutants (Grandin et al, 1997). Like Cdc13, Stn1 is essential in yeast (Grandin et al, 1997). Stn1 is a small protein with a molecular weight of approximately 16 kDa containing a single OB-fold that mediates its interaction with Cdc13 (Grandin et al, 1997). Cdc13-Stn1 binding is believed to block the association of the telomerase accessory factor Est1 to Cdc13, therefore preventing the recruitment of telomerase to the telomere (Puglisi et al, 2008). Thus, Stn1 is a negative regulator of telomerase activity (Grandin et al, 2000; Iyer et al, 2005; Petreaca et al, 2007). Stn1 is also an essential component for telomere end-protection as a member of the CST complex (Grandin et al, 1997; Petreaca et al, 2007). At non-permissive temperatures, *stn1* ts mutants exhibit a telomere lengthening phenotype, as well as elongated G-overhangs (Grandin et al, 1997).

The last member of the CST complex to be identified was Ten1 (Telomeric pathways in association with stn1) (Grandin et al, 2001). Ten1 was found as a suppressor

of the *stn1* mutant phenotype. Similar to Cdc13 and Stn1, Ten1 is essential in yeast. Ten1 is a 14 kDa protein that is predicted to contain a single OB-fold and it interacts strongly with Stn1 (Grandin et al, 2001; Martin et al, 2007). Additionally, Ten1 may also play a role in mediating telomerase activity via interaction with Stn1 (Grandin et al, 2001). Ten1 is important for telomere protection as ts mutants have longer, more heterogeneous telomeres with extended G-overhangs (Grandin et al, 2001; Petreaca et al, 2006). Ten1 has a weak interaction with Cdc13, but this interaction increases the telomeric DNA binding ability of Cdc13 (Grandin et al, 2001; Qian et al, 2009; Xu et al, 2009). Over-expression of Ten1 and Stn1 can rescue the telomere phenotype of a *cdc13* ts mutant (Petreaca et al, 2006) suggesting that this heterodimer can protect the chromosome end in the absence of Cdc13.

In fission yeast, there is no clear Cdc13 ortholog. However, Stn1 and Ten1 form a heterodimer that functions as part of a telomere capping complex, separate from the shelterin component Pot1 (Martin et al, 2007). The crystal structures of the *S. pombe* and *Candida tropicalis* Stn1/Ten1 heterodimers reveals the interaction interface involves the C-terminal alpha helices on both proteins (Sun et al, 2009). In contrast to the situation in *S. cerevisiae*, loss of either Stn1 or Ten1 is not lethal in *Candida albicans* or *S. pombe* (Martin et al, 2007; Sun et al, 2009). This is not to say that Stn1 and Ten1 do not play a role in stability of the genome in these organisms. In *S. pombe,* loss of Stn1 or Ten1 causes chromosome circularization, similar to *pot1* null mutants, but there is no increase in ss telomeric DNA (Martin et al, 2007). In *C. albicans,* loss of Stn1 or Ten1 causes telomere lengthening and increased telomere length heterogeneity (Martin et al, 2007;

Sun et al, 2009). Moreover, like *S. pombe*, there is no increase in the G-overhang length in C. albicans *stn1* and *ten1* mutants (Sun et al, 2009) which may help to explain the viability of these null mutants. Taken together, these data suggest that Stn1 and Ten1 are not critical for C-strand fill-in or protection in *C. albicans* and *S. pombe*. Remarkably, mutations in Ten1 that disrupt binding with Stn1 cause telomere defects similar to null mutants (Sun et al, 2009). Ten1 is no longer localized to the telomere in this genetic background (Sun et al, 2009). Thus, Stn1 binding is required for Ten1 recruitment to the telomere and for Stn1/Ten1 function in *C. albicans*, but unlike *S. cerevisiae* they are not important for C-strand maintenance.

The CST complex is required for polymerase alpha/primase (pol α) recruitment to the telomere (Qi & Zakian, 2000). Pol α is a general DNA replication polymerase that facilitates C-strand fill-in at the telomere. During S phase pol α is recruited to the telomere via interactions with CST (Qi & Zakian, 2000), where it stimulates the priming reaction at the end of the 3' overhang to facilitate the fill-in of the C-strand thereby creating ds DNA (Sun et al, 2011).

It was initially believed that CST was a telomere complex specific to yeast. However, work in the Shippen lab in 2008 showed that a STN1 ortholog was present at Arabidopsis telomeres and was critical for telomere maintenance (Chapter II). Further studies in the Shippen, Price and Ishikawa labs subsequently identified CTC1 (Conserved Telomere maintenance Component 1), an ortholog of Cdc13, in Arabidopsis, chicken and humans (Appendix I) (Miyake et al, 2009). Finally, TEN1 was identified in higher eukaryotes in human cells, as part of a STN1-interacting complex and was first

characterized in detail in Arabidopsis (Chapter III) (Miyake et al, 2009). Notably, human CTC1 and STN1 were originally identified as pol α accessory factors, not telomere specific factors (Casteel et al, 2009). Subsequent work showed that CST is at the telomere throughout the cell cycle with its association peaking during late S/G2, after telomerase extends G-overhangs (Chen et al, 2012).

Studies of CST in higher eukaryotes have yielded confounding and sometimes controversial results. siRNA knock-down of CTC1 in chicken cells causes telomere shortening, as well as the appearance of telomere dysfunction-induced foci (TIFs) (Surovtseva et al, 2009). Knock-down of CTC1 in mice likewise causes telomere loss and stem cell death, but not the formation of TIFs (Gu et al, 2012). However, unlike the results in chicken cells, depletion of individual members of the CST complex in human cancer cells causes telomere lengthening, not shortening, suggesting CST may modulate telomerase activity (Chen et al, 2012). This result is confounded by recent studies from other labs showing that in other human cell cancer lines telomere lengthening is not associated with CST knock-down (Stewart et al, 2012b; Wang et al, 2012). These data are contradictory and may reflect species or cell type specific roles for CST in vertebrate cells. Thus, the contribution of CST is unclear.

CTC1 was identified in Arabidopsis by the Shippen lab during characterization of mutants that cause telomere deprotection (Appendix I). CTC1 does not display sequence homology to Cdc13, but further analysis suggests that it is a functional ortholog. In contrast to *S. cerevisaie*, human CTC1 displays little specificity for ss telomeric DNA, opening up the possibility that CST may have a non-telomeric function

mammals (Miyake et al, 2009). This lack of DNA binding specificity would allow the CST complex to bind to ss DNA throughout the genome, similar to RPA. Studies in *Xenopus laevis* have demonstrated that STN1 is required for pol α priming on a ss DNA template (Nakaoka et al, 2012). Studies using human cells not only confirmed a role for the CST complex, especially STN1 and CTC1, in telomere replication, but also suggest that the complex plays a more general role in resolving stalled replication forks (Huang et al, 2012; Stewart et al, 2012b; Wang et al, 2012). As in yeast, STN1 interacts with both TEN1 and CTC1 in vertebrates, but no interaction between TEN1 and CTC1 has been reported (Miyake et al, 2009; Nakaoka et al, 2012). The role of TEN1 has not been examined in detail as most studies focus on cells depleted for CTC1 or STN1. Nonetheless, the current data indicate that mammalian CST evolved to play a primary role in DNA replication, while shelterin functions as the principal telomere capping complex.

Recently, mutations in CTC1 were found to be the cause of human stem cell disease. Due to the variable clinical presentation of these diseases, patients with mutations in CTC1 have been characterized as having Coats Plus, Cerebroretinal Microangiopathy with Calcifications and Cysts, and Dyskeratosis Congenita (Anderson et al, 2012; Armanios, 2012; Keller et al, 2012; Mangino et al, 2012; Polvi et al, 2012; Walne et al, 2012). Patients with these disorders present with a wide variety of phenotypes, including shorter telomeres, stem cell exhaustion, and premature aging phenotypes in rapidly dividing cells (aka skin, intestinal cells). There are conflicting reports about the effect of CTC1 mutations on telomere length in patients. Two studies

found shorter telomeres in these patients, while a third study did not find significantly shorter telomeres (Anderson et al, 2012; Mangino et al, 2012; Walne et al, 2012). The patients have compound heterozygous mutations in CTC1, suggesting that some amount of protein function in necessary for survival. This observation underscores the importance of the CST complex in human health and the need to more fully understand the functions of the CST complex.

Arabidopsis as a model for telomere biology

Arabidopsis thaliana is the preeminent plant model system and serves as the reference species for plant biology. Arabidopsis has a compact, sequenced genome that allows for comparative analysis with other plant genomes. The growth period of this multicellular eukaryote is short at approximately six weeks allowing for rapid genetic analysis. A wide variety of Arabidopsis ecotypes, as well as EMS and T-DNA insertion mutants, are available from stock centers and labs worldwide. These huge mutant collects cover most of the Arabidopsis genome, and give both knock-outs and point mutations to screen. Relevant to this study, the most important characteristic of Arabidopsis is its incredible tolerance to genomic instability. Plants survive and reproduce with mutations in genes that would cause death in the other major model organism (human cells, mice and even yeast).

Arabidopsis was developed in the Shippen lab as a model system for telomere studies in the 1990s. Arabidopsis telomeres are within a size range (2-7kB) that allows for easy monitoring of telomere length using standard molecular biology approaches.

Additionally, 8 of the 10 chromosome arms contain unique sequences, making it possible to examine the length and status of telomere tracts on individual chromosome arms. Telomerase is regulated in a manner that is similar to humans, with activity limited to rapidly dividing cells of seedlings, flowers and cell culture (Fitzgerald et al, 1999). Null mutations in many telomere related genes (ATR, CST, POT1a) are viable in Arabidopsis, providing an opportunity to study these core components of telomeres (Watson & Riha, 2010). Finally, there have been three genome duplications in the evolution of Arabidopsis (Arabidopsis Genome, 2000). These duplications have resulted in fascinating divergence of the paralogs of telomere-related genes and allow for interesting evolutionary comparisons.

Overall, telomere architecture is similar in plants and humans. However, it was recently discovered that only half of Arabidopsis telomeres harbor a G-overhang, with the remaining chromosome ends being blunt, or very nearly blunt (Kazda et al, 2012). Intriguingly, this phenomenon is not limited to Arabidopsis, but has been observed throughout the plant lineage, including the earliest diverging land plant, *Physcomitrella paten(Kazda et al, 2012)s*. These blunt telomeres have different protection mechanisms than mammals and yeast (addressed in detail below). It is hypothesized that the blunt end arises from incomplete processing of the newly created leading strand during DNA replication. At mammalian and yeast telomeres the C-strand is resected following leading strand synthesis giving rise to the 3' G-overhang (Figure 1-4A). In accordance with their different structures, the G-overhang and blunt-ends of plant telomeres have a unique suite of capping proteins (Kazda et al, 2012). It is hypothesized that asymmetry

of plant telomeres provides telomere length stability by limiting the access of telomerase and exonucleases to half of the telomeres in each cell cycle, and thus, diminishes the fluctuations in telomere length over the course of plant development (Nelson & Shippen, 2012).

Arabidopsis telomerase RNPs

Genome duplication allows genes to gain, lose, or alter their function, and as a result the organism can evolve new pathways and mechanisms for a variety of biological functions. This process is exemplified in the Arabidopsis RNPs. *Arabidopsis thaliana* harbors three distinct telomerase RNA subunits, two that are derived from gene duplication, TER1 and TER2 (Cifuentes-Rojas et al, 2011; Cifuentes-Rojas et al, 2012). TER2, which is encoded on a separate chromosome from TER1, can be processed to form TER2s by the removal of an intron and loss of the 3' tail. TERT has a higher affinity for TER2 than TER1 in vitro, and consequently, even though TER1 expression is much higher than TER2, TERT preferentially associates with TER2 in vivo (Cifuentes-Rojas et al, 2011; Cifuentes-Rojas et al, 2012). TER2s binding to TERT is not detectable in vitro and is barely perceptible in vivo (Cifuentes-Rojas et al, 2011).

TER1 and TER2 form two distinct telomerase RNPs with unique accessory proteins (below). TER1 is the canonical telomerase RNA and is required for telomere maintenance in vivo (Cifuentes-Rojas et al, 2011). Although TER2 can reconstitute telomerase activity with TERT in vitro, it is not able to maintain telomeres in vivo (Cifuentes-Rojas et al, 2011; Cifuentes-Rojas et al, 2012). Instead TER2 acts as a

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negative regulator of the TER1 RNP in vivo (Cifuentes-Rojas et al, 2012). Moreover, expression of TER2 is upregulated in response to ds DNA breaks (Cifuentes-Rojas et al, 2012). Upregulation of TER2 triggers a decrease in telomerase activity (Cifuentes-Rojas et al, 2012). It is hypothesized that the TER2 regulatory pathway helps to protect broken chromosome ends from DNTF by telomerase, thereby promoting genome stability. The function of TER2s is unknown.

Gene duplication also gave rise to three sequence homologs of the shelterin component POT1: POT1a, POT1b and POT1c. Interestingly, none of these POT1 proteins bind to ss telomeric DNA in vitro (Shakirov et al, 2009), but instead bind either TER1 or TER2 (Cifuentes-Rojas et al, 2011; Surovtseva et al, 2007). Evidence is emerging that POT1a is a functionally orthologous to Est1, the telomerase recruitment factor in budding yeast. POT1a binds to TER1 (Cifuentes-Rojas, 2011 #1481) as well as CTC1 and STN1 (X. Song, unpublished data). In addition, telomere shortening in *pot1a* mutants is reminiscent of a progressive decline in telomere length over subsequent generations seen in *tert* mutants (Fitzgerald et al, 1999; Shakirov et al, 2005; Surovtseva et al, 2007). Finally, *pot1a* mutants have decreased telomerase activity (K. Renfrew, unpublished data), a property associated with yeast Est1 (Singh & Lue, 2003). These data indicate that POT1a is part of the canonical telomerase RNP and may play a role in telomerase recruitment and activity stimulation (Figure 1-9).
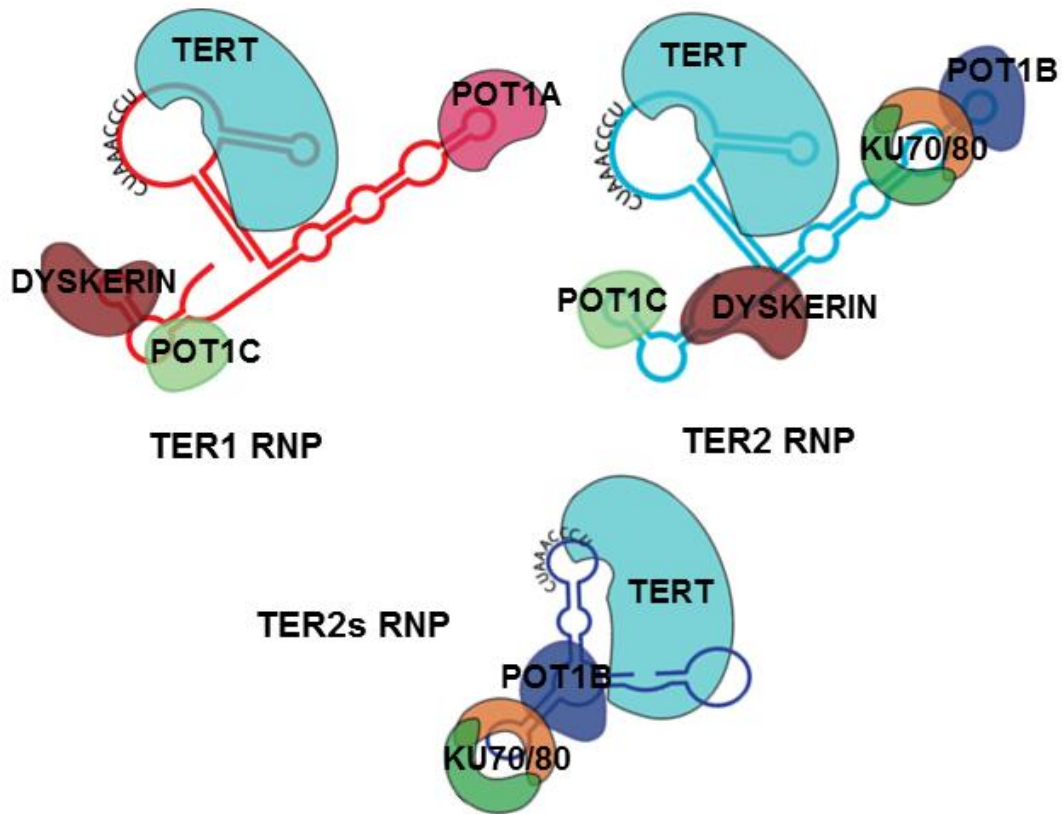
Figure 1-9. *A. thaliana* **telomerase RNP complexes.**
The TER1, TER2 and TER2s RNP complexes in *A. thaliana* associate with different protein subunits. The TER1 RNP is comprised of TERT, POT1a, POT1c, and dyskerin. It is responsible for telomere maintenance in vivo. The TER2 RNP is comprised of TERT, POT1b, POT1c, dyskerin and Ku70/K80. TER2 RNP is a negative regulator of the TER1 RNP telomerase activity. The TER2s complex is less well defined but it contains TERT, POT1b and Ku70/Ku80. It may play a role in telomere protection (Cifuentes-Rojas et al, 2011; Cifuentes-Rojas et al, 2012).

The TER2 RNP is associated with two unique accessory factors POT1b and POT1c, as well as the Ku heterodimer (Figure 1-9) (Cifuentes-Rojas et al, 2011; Cifuentes-Rojas et al, 2012). Loss of POT1b results in increased telomerase activity though telomeres are wild type length (Dr. A. Nelson, unpublished data). POT1c associates with both TER1 and TER2 in vitro (Dr. A. Nelson, unpublished data). Knockdown of POT1c by RNA interference causes decreased telomerase activity, shorter and more heterogeneous telomeres, loss of the G-overhang and increased ECTC (Dr. A. Nelson, unpublished data). These data suggest POT1c may play a primary role in telomere capping, and hence its relativity to telomerase binding is unclear.

The Ku70/Ku80 heterodimer (Ku) in Arabidopsis is important for the maintenance of the telomere architecture (Riha & Shippen, 2003b). In addition to ds DNA binding, Ku can bind TER2 and TER2s (Cifuentes-Rojas et al, 2012). Ku prevents C-strand degradation and homologous recombination resulting in formation of ECTC (Riha & Shippen, 2003a; Zellinger et al, 2007). Telomeres are grossly elongated in the null background, reaching 15-20kb in length, due to ALT (Riha & Shippen, 2003a). Interestingly, Ku also seems to be important for the binding and protection of the blunt telomeres in Arabidopsis, potentially through its interaction with TER2 and TER2s (Nelson & Shippen, 2012). The increase in G-overhang signal in *ku* mutants may be a reflection of processing of the blunt telomeres to generate G-overhangs (Kazda et al, 2012). It is therefore predicted that in *ku* mutants all of the telomeres may have G-overhangs instead of only 50% of the telomeres. In this background telomeres may be symmetrical with all of ends bound by CST.

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Arabidopsis CST and telomere-associated proteins

Arabidopsis contains six punitive homologs to the shelterin TRF proteins, called TRF-like (TFRL). The TRFLs utilize Myb domains for ds telomeric DNA binding. Likely due to functional redundancy, individually the knock-outs do not have a phenotype. TPP1, RAP1, and TIN2 homologs have not been detected by sequence homology in any plant genome. Therefore, a major question in the field is how CST, the TRFL proteins and POT1c work together to promote telomere protection and maintenance in Arabidopsis.

Dissertation overview

Characterization of the *Arabidopsis thaliana* CST complex is the main focus of this dissertation. Initial characterizations of STN1 and CTC1 are provided in Chapter II. Analysis of TEN1 is covered in Chapter III. Genetic interactions of STN, TEN1 and TERT are examined in Chapter IV. Finally, studies to examine the function of the TEN1/STN1 interaction interface are discussed in Chapter V.

The work described in Chapter II concerns the identification and characterization of STN1 in Arabidopsis. This study provides the first identification of a CST complex member in a higher eukaryote. Here we showed that a null mutation in STN1 causes an immediate onset of gross morphological abnormalities, massive telomere shortening and chromosome fusions. These data suggest a critical role for STN1 in chromosome end protection.

Chapter III describes the identification and characterization of TEN1 in Arabidopsis. TEN1 was identified based on its sequence homology to the human TEN1. A variety of mutants were analyzed to elucidate the function of TEN1 including T-DNA insertion in the 5' UTR of TEN1, two separate anti-sense constructs and the generation of two TALENs targeting TEN1. During the course of this study, a paper was published describing the identification of Meristem Disorganization 1 (MDO1). This gene mapped to the predicted TEN1 locus, At1g56260. A point mutation at amino acid 77 in MDO1/TEN1 (mod1-1/ten1-3) causes stem cell death and a DNA damage phenotype similar to *stn1* and *ctc1* null mutants (Appendix I) (Hashimura & Ueguchi, 2011). My analysis of *ten1-3* mutants revealed telomere defects similar to *stn1* and *ctc1*. However, I found a significant increase in genomic instability in the *ten1-3* mutant compared to *ctc1* and *stn1* mutants. In addition, *ten1-3* mutants show an increase in telomerase RAP, while there was change in telomerase activity in *stn1* or *ctc1* mutants. Finally, cytological data indicate that TEN1 co-localized at far fewer telomeres than STN1 or CTC1. Together these data suggest that TEN1 functions within context of the CST complex, but also has some unique functions.

Chapter IV reports the results from genetic analysis of *TEN1*, *STN1* and *TERT*. Loss of both *STN1* and *TEN1* results in a more severe morphological and telomere phenotype than either single mutant, indicating that while some of the functions are redundant there are unique contributions from these proteins (likely TEN1). Data are also provided showing that CST components interact synergistically with TERT to maintain chromosome ends. Finally, data are presented indicating that the role of TEN1

is more critical than CTC1 or STN1 in plants lacking telomerase, supporting the conclusion that TEN1 functions extend beyond its role in the CST complex.

Chapter V explores the importance of the STN1/TEN1 interface for telomere protection. By threading Arabidopsis STN1 and TEN1 protein sequence onto the *S. pombe* crystal structure, our collaborator Ming Lei (University of Michigan) determined a number of amino acids on each protein that would be potentially important for STN1/TEN1 binding. Site-directed mutagenesis was used to mutate these residues and in vitro binding assays and genetic complementation were used to assess STN1/TEN1 interaction and its function in vivo. Preliminary data in suggests that loss of STN1/TEN1 binding results in telomere fusions, but does not decrease the length of the telomeres. These apparent separation of function mutants indicate that the STN1/TEN1 interaction is important for the prevention of the activation of the DDR, but it is not critical for telomere maintenance.

The appendices present work published in collaboration with other members of the Shippen lab. Appendix I describes the initial characterization of the CTC1 in Arabidopsis and vertebrates. This paper reported the discovery of CTC1 in higher eukaryotes and its role in telomere biology. Appendix II describes the role of STN1 and CTC1 in the prevention of the DNA damage response. Data presented in this section indicate that STN1 and CTC1 block activation of the ATR-mediated DNA damage response at the telomere.

CHAPTER II

STN1 PROTECTS CHROMOSOME ENDS IN *ARABIDOPSIS THALIANA**

Summary

Telomeres shield the natural ends of chromosomes from nucleolytic attack,

recognition as double-strand breaks, and inappropriate processing by DNA repair

machinery. The trimeric Stn1/Ten1/Cdc13 complex is critical for chromosome end

protection in *Saccharomyces cerevisiae*, while vertebrate telomeres are protected by

Shelterin, a complex of six proteins that does not include STN1 or TEN1. Recent studies

demonstrate that Stn1 and Ten1 orthologs in *Schizosaccharomyces pombe* contribute to

telomere integrity in a complex that is distinct from the Shelterin components, Pot1 and

Tpp1. Thus, chromosome end protection may be mediated by distinct subcomplexes of

telomere proteins. Here we report the identification of a *STN1* gene in Arabidopsis that is

essential for chromosome end protection. AtSTN1 encodes an 18 kDa protein bearing a

single oligonucleotide/oligosaccharide binding fold (OB-fold) that localizes to telomeres

in vivo. Plants null for AtSTN1 display an immediate onset of growth and developmental

defects and reduced fertility. These outward phenotypes are accompanied by

catastrophic loss of telomeric and subtelomeric DNA, high levels of end-to-end

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chromosome fusions, increased G-overhang signals and elevated telomere recombination. Thus, AtSTN1 is a crucial component of the protective telomere cap in Arabidopsis, and likely in other multicellular eukaryotes.

Introduction

Telomeres distinguish the natural ends of chromosomes from double-strand breaks by virtue of their unusual architecture and protein composition. Vertebrate telomeres are bound by a core complex of six proteins, termed Shelterin, which regulates the length of the telomeric DNA tract, suppresses the activation of a DNA damage response at the terminus, and protects the ends from inappropriate recombination, nuclease attack and end-to-end fusion (de Lange, 2005; Palm & de Lange, 2008). Shelterin is composed of two double-strand telomere binding proteins, TRF1 and TRF2, a single-strand telomere binding protein, POT1, and three bridging proteins TIN2, RAP1 and TPP1 (de Lange, 2005; Palm & de Lange, 2008). TRF2 and the OB-fold containing protein POT1 are critical for chromosome end protection (Hockemeyer et al, 2006; van Steensel et al, 1998; Wu et al, 2006; Yang et al, 2005). Studies in *S. pombe* confirm the presence of several Shelterin homologs, including Taz1 (a TRF1/TRF2 homolog), Rap1, Pot1 and Tpz1 (a TPP1 homolog) (Baumann & Cech, 2001; Chikashige & Hiraoka, 2001; Ferreira & Cooper, 2001; Kanoh & Ishikawa, 2001; Miyoshi et al, 2008).

In contrast, budding yeast telomeres are protected by a trimeric complex of three OB-fold proteins, Stn1/Ten1/Cdc13 (Lustig, 2001; Pennock et al, 2001). Recent studies demonstrate that Stn1 and Ten1 orthologs in *S. pombe* also contribute to telomere capping (Martin et al, 2007). Notably, SpStn1 and SpTen1 interact with each other, but

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thus far evidence is lacking for a physical interaction between these proteins and SpPot1 (Martin et al, 2007). Furthermore, Tpz1, but not Stn1/Ten1, was identified by mass spectrometry of Pot1-associated proteins in *S. pombe* (Miyoshi et al, 2008), indicating that in *S. pombe* chromosome ends are protected by two distinct telomere protein subcomplexes.

To note, several candidate orthologs of the SpStn1 protein can be found in the genomes of multicellular eukaryotes, including humans, by position-specific iterative BLAST (PSI-BLAST) (Gao et al, 2007; Martin et al, 2007). Here we use a genetic approach to demonstrate that the *STN1* gene in the flowering plant Arabidopsis is essential for chromosome end protection. In striking contrast to plants lacking telomerase, which display a progressive but gradual loss of telomeric DNA that ultimately leads to end-to-end chromosome fusions and worsening growth and developmental defects beginning in the sixth generation (G6) (Riha et al, 2001), telomeres are immediately and catastrophically compromised in Arabidopsis mutants null for STN1. Telomeric as well as subtelomeric DNA is extensively eroded and mutants exhibit increased G-overhang signals, elevated telomere recombination and massive telomere fusion, resulting in severe growth defects and sterility. These findings not only indicate that AtSTN1 is required for telomere capping in Arabidopsis, but further suggest that additional key components of the telomere complex remain to be elucidated in metazoa.

Materials and methods

Plant materials and plasmids

The *stn1* mutants were obtained from the Arabidopsis Biological Resource Center (ABRC). The T-DNA insertion lines, *stn1-1* (CS023504) and *stn1-2* (CS846727), were genotyped by PCR using primers 5'-ATGGATCGATCCCTCCAAAG-3' and 5'-TTGAATACGAACACGATAACAAC-3'. Plants were grown according to the conditions described (Surovtseva et al, 2007). Siliques from wild type and *stn1-1* mutants were dissected ~ 10 days after fertilization and photographed using a Zeiss Axiocam digital camera coupled to a Zeiss microscope. A transgenic construct of STN1 was prepared by inserting a C-terminal YFP tag using an Ala (Gly)$_5$ Ala linker sequence. Tagged STN1 was cloned into a Gateway entry vector pENTR (Invitrogen) and then subcloned into a binary vector pB7WG2 (Invitrogen) according to manufacturer instructions. The resultant binary vector was used to transform plants as described (Surovtseva et al, 2007).

RT-PCR

Total RNA was extracted from plant tissues using an RNA purification kit (Fisher Scientifics). Reverse transcription was performed using Superscript III reverse transcriptase (Invitrogen) per manufacturer instructions. PCR of *STN1* cDNA was performed using the above primers, with the following program: 95 °C 3 min; 25 cycles of 94 °C 20 sec, 55 °C 30 sec, 72 °C 1 min 30 sec; 72 °C 7 min.

Cytology, immunofluorescence and FISH

To monitor anaphase bridge formation, cells were prepared from pistils, stained with DAPI Vectashield (Vector Laboratories), and then analyzed with an epifluorescence microscope (Zeiss) as described (Riha et al, 2001). Anaphase bridges were scored as a percentage of total anaphase cells. For combined immunolocalization and FISH, second generation transformants (T2) expressing a C-terminal YFP tagged version of STN1 were grown to seedlings (~ 7-day old) and fixed with 4% formaldehyde for 30 min on ice. Root nuclei from the seedlings were extracted and dried onto polylysine coated slides, and immunolocalization was performed as described (Onodera et al, 2005). A rabbit anti-GFP antibody (Abcam) was used as the primary antibody and a FITC-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch) was used as the secondary antibody. After immunolocalization, the nuclei were postfixed with 4% formaldehyde and 0.1% glutaraldehyde for 30 min prior to FISH. Nuclei were washed with $1 \times$ PBS, passed through an ethanol series (70%, 80%, 90%, 100%) at -20 °C and then dried. Digoxygenin-dUTP labeled telomere probe was prepared as described (Armstrong et al, 2001). FISH was performed as described (Kato et al, 2004). Detection of digoxygenin labeled probes was with a rhodamine conjugated anti-digoxygenin antibody (Roche). Nuclei were counterstained with DAPI Vectashield and analyzed with an epifluorescence microscope (Zeiss).

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TRF, PETRA and telomere fusion PCR

DNA from individual whole plants was extracted as described (Cocciolone & Cone, 1993). TRF analysis was performed using 50 μg of DNA digested with $Tru1$I (Fermentas) and hybridized with a ^{32}P 5' end–labeled $(T_3AG_3)_4$ oligonucleotide probe (Fitzgerald et al, 1999). The average length of bulk telomeres was determined by Telometric 1.2 (Grant et al, 2001); the range of telomere length was obtained using ImageQuant software. Subtelomeric TRF analysis was performed using 100 μg of DNA digested with SpeI and PvuII (New England Biolabs) and hybridized with a 5R probe (Shakirov & Shippen, 2004). Telomere fusion PCR and PETRA were performed as described (Heacock et al, 2004).

In-gel hybridization and telomeric circle amplification (TCA)

In-gel hybridization was performed as described (Heacock et al, 2007). The relative amount of single-strand G-overhang was calculated by quantifying the hybridization signal obtained from the native gel and then normalizing this value with the loading control of either interstitial telomere signal from the denaturing blot or ethidium bromide staining of the agarose gel. The single-strand G-overhang signal obtained from wild type DNA was set to one and each sample was normalized to this value. Exonuclease treatment was performed by incubating DNA samples with T4 DNA polymerase (New England Biolabs) prior to in-gel hybridization at 12 °C for 30 min. The telomeric circle amplification (TCA) was performed as described (Zellinger et al, 2007).

Results

Identification of AtSTN1

To search for a STN1 protein in the plant kingdom, PSI-BLAST was employed using the protein sequence of SpStn1 as the query. In the second iteration, a previously uncharacterized protein, NP_563781, from *Arabidopsis thaliana* was uncovered with an E-value of $2e^{-06}$, well above the program threshold (0.005). The corresponding single-copy gene, At1g07130, was designated *AtSTN1*. A combination of EST database searches and 3' RACE was used to verify the boundaries of the *AtSTN1* coding region. *AtSTN1* lacks introns and is predicted to encode a small protein of 160 aa that can assume a single OB-fold (Fig 2-1A).

Database searches revealed potential STN1 homologs from other sequenced plant genomes including rice and a single-celled green algae (Figure 2-1A). As expected (Gao et al, 2007; Martin et al, 2007), putative STN1 homologs were also uncovered in a wide variety of other eukaryotes, including fishes, amphibians, birds, rodents and primates (Figure 2-1A and data not shown). In contrast to STN1 orthologs from yeast, the plant STN1 proteins lack a C-terminal extension (Figure 2-1A).
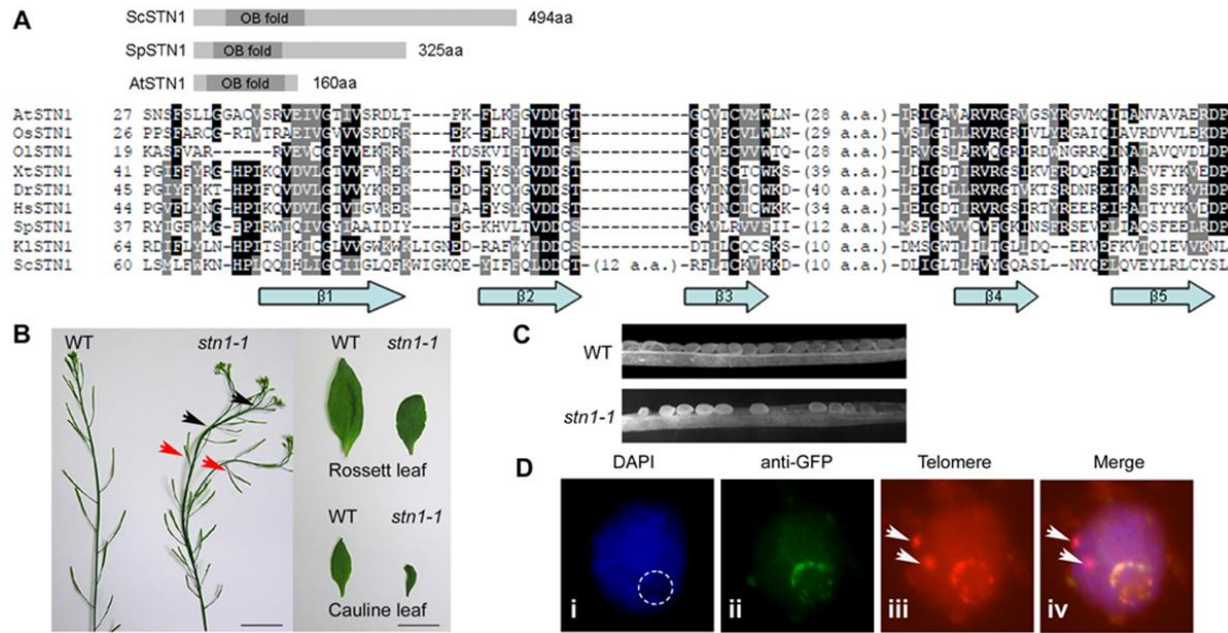
Figure 2-1. Identification of AtSTN1 and severe morphological defects in *STN1* deficient plants.
(A) Top, Diagram showing the OB-fold domain structure of STN1 homologs from S. cerevisiae (Sc), S. pombe (Sp) and A. thaliana (At). Bottom, Alignment of putative STN1 orthologs from plants and other organisms generated by Macvector and Boxshade software. The secondary structure was predicted by PSIPRED (McGuffin et al, 2000). At, *Arabidopsis thaliana* (NP_563781); Dr, *Danio rerio* (NP_956683); Hs, *Homo sapiens* (NP_079204); KL, *Kluyveromyces lactis*, (XP_452728); Ol, *Ostreococcus lucimarinus* (green algae, XP_001417183); Os, *Oryza sativa* (Rice, NP_001050181); Sc, *Saccharomyces cerevisiae* (CAA98902); Sp, *Schizosaccharomyces pombe* (XP_001713126); Xt, *Xenopus tropicalis* (NP_001004908). **(B)** Morphological defects in *stn1-1* mutants. Stems (left panel), rosette leaves (top right) and cauline leaves (bottom right) are shown for wild type plants (WT) and stn1-1 mutants. Fused stems (black arrows) and altered phyllotaxy (red arrows) are indicated. Bars, 1 cm. **(C)** Aborted seed development in stn1-1 mutants. Siliques from wild type plants and *stn1-1* mutants were visualized by microscopy. **(D)** STN1 colocalizes with telomeres. Isolated nuclei from STN1-YFP transformants were stained with DAPI (i), STN1-YFP was detected with an anti-GFP antibody (ii), and the telomeres were labeled by FISH with a telomere probe (iii) (see Materials and methods for details). Panels (i) to (iii) were superimposed to produce panel (iv). Arrows in (iii) and (iv) indicate internal stretches of telomere signals as described in (Armstrong et al, 2001). AtSTN1 mRNA is expressed in all plant tissues examined (Figure 2-2), unlike the mRNA for TERT, the catalytic subunit of telomerase, which accumulates only in highly proliferative organs (Fitzgerald et al, 1999).
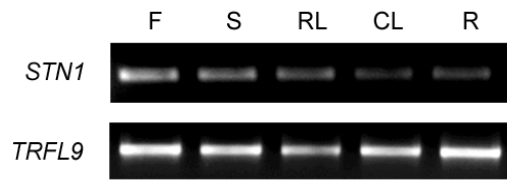
Figure 2-2. Ubiquitous gene expression of *STN1* in Arabidopsis.
RT-PCR of *STN1* mRNA was performed in the indicated Arabidopsis tissues. CL,
cauline leaf; F, flower; R, root; RL, rosette leaf; S, stem. RT-PCR of *TRFL9* is shown as
a loading control.

Protein sequence alignment indicated that AtSTN1 displays limited sequence

similarity to SpStn1 (Figure 2-1A), but this similarity is statistically significant within

the predicted OB-fold domain. Positions 7-143 of AtSTN1 align to positions 16-136 of

SpStn1 with 23% identity/ 40% similarity. Secondary structure prediction by PSIPRED

(McGuffin et al, 2000) indicated that residues within four of the five essential beta

strands of the OB-fold (β1, β2, β3 and β4) in AtSTN1 share significant similarity to that

of functionally verified STN1 protein from yeasts as well as the putative STN1 proteins

from other multicellular eukaryotes (Figure 2-1A). In β5, sequence conservation is

reduced in the Stn1 sequences from multicellular eukaryotes relative to their

counterparts in yeasts. PFAM analysis confirmed that both AtSTN1 and SpStn1 proteins

contain a "tRNA_anti" OB-fold nucleic acid binding domain, arguing that the OB-fold

domain of the two proteins belongs to the same family. Results of PFAM analysis can be

retrieved for AtSTN1 (http://pfam.sanger.ac.uk/protein?entry=Q9LMK5) and for SpStn1

(http://pfam.sanger.ac.uk/protein?entry=Q0E7J7).

Severe morphological defects in Arabidopsis **stn1** *mutants*

We examined the *in vivo* function of *AtSTN1* by studying two T-DNA insertion

lines, designated *stn1-1* and *stn1-2*, which were obtained from the Arabidopsis

Biological Resource Center. RT-PCR analysis of homozygous mutants confirmed that

full-length *AtSTN1* mRNA was disrupted in both lines (Figure 2-3). Both mutant lines

displayed a fasciated phenotype with severe morphological abnormalities in G1,

although the severity of the defects varied somewhat in different individuals. In nearly

all mutants, apical dominance was completely abolished, leading to multiple

inflorescence bolts that were often fused (Figure 2-1B, black arrows). In addition, floral

phyllotaxy was perturbed and siliques developed at irregular positions on the

inflorescence bolt (Figure 2-1B, red arrows). Similar to what has been observed in late

generation (G8-G9) *tert* mutants (Riha et al, 2001), leaf size was substantially reduced in

stn1 mutants, likely reflecting defects in cell proliferation (Figure 2-1B, right). *stn1*

mutants produced numerous undeveloped ovules (Figure 2-1C) and the germination rate

declined dramatically through successive generations. Only 17% (n=144) of the seeds

from G1 mutants germinated to produce G2 plants. G2 progeny (G3) arrested early in

vegetative development without producing a germline (data not shown). Many of these

phenotypes are reminiscent of late generation *tert* mutants (Riha et al, 2001).
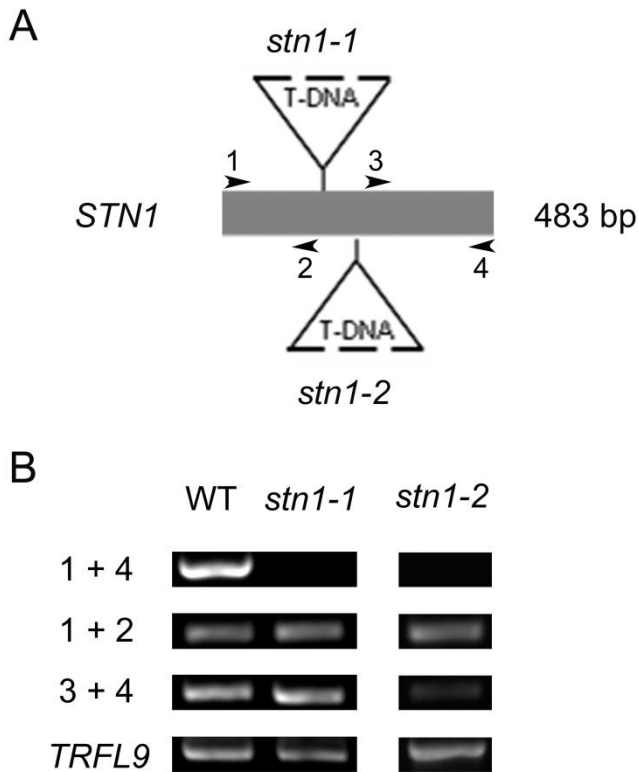
Figure 2-3. Identification of two *stn1* mutant alleles.
Top, The relative positions of *stn1-1* and *stn1-2* T-DNA insertions are shown. Bottom, RT-PCR analysis of *STN1* mRNA in wild type, *stn1-1 and stn1-2* mutants is shown. Primer positions are denoted by arrows. RT-PCR with primers flanking the T-DNA insertion suggests the full length mRNA of *STN1* was disrupted in both *stn1-1* and *stn1-2* mutants. RNA transcripts can be detected both upstream and downstream of the T-DNA insertion site. The upstream transcripts in *stn1-1* and *stn1-2* mutants encode small polypeptides (64 aa and 77 aa, respectively) and are likely to be non-functional. Additional analysis revealed that the downstream transcripts are likely derived from a cryptic promoter in the T-DNA construct and contained part of the T-DNA and an in-frame stop codon prior to the exon (data not shown). RT-PCR of *TRFL9* was used as a loading control.

AtSTN1 localizes to Arabidopsis telomeres

To monitor the subcellular localization of AtSTN1, we generated a *stn1-1* line

expressing a C-terminal YFP tagged version of *AtSTN1* under the control of the CaMV

35S promoter. The transgene fully complemented the telomere defects in *stn1-1* mutants

(see below). In root tip meristems, distinct spots of YFP signal formed a ring around the

periphery of the nucleolus (data not shown). The arrangement of Arabidopsis telomeres

at the nucleolar periphery has previously been noted in meiotic interphase (Armstrong et

al, 2001). Fluorescence in situ hybridization (FISH) with a telomere probe also produced

signals at the nucleolar periphery in somatic cells from roots and immature pistils (e.g.,

Figure 2-1D, panel iii). Immunolocalization using an anti-GFP antibody (Figure 2-1D,

panel ii) combined with telomere FISH on the same nuclei produced co-localizing

signals (Figure 2-1D, panel iv). This localization was specific to terminal telomeric

DNA sequences as the STN1-YFP signal did not overlap with internal stretches of

telomeric DNA sequence on chromosome 1 (Armstrong et al, 2001) (shown by the

arrows in Figure 2-1D, panel iv). We conclude that AtSTN1 colocalizes with telomeres

in Arabidopsis.

Extensive telomere erosion in plants lacking **AtSTN1**

In *S. pombe*, the absence of Stn1 leads to an immediate and profound loss of

terminal DNA sequences (Martin et al, 2007). To determine if AtSTN1 protects

chromosome ends in Arabidopsis, Terminal Restriction Fragment (TRF) analysis was

performed to examine bulk telomere length. In both *stn1-1* and *stn1-2* mutants, telomere

tracts appeared as a broad, heterogeneous smear (Figure 2-4A). Although the average

length of bulk telomeres was only slightly shorter than in wild type siblings (2.4 kb

versus 2.7 kb, respectively), the shortest telomere tracts in *stn1-1* mutants were

significantly shorter than in wild type, trailing down to ~ 600 bp (1.4 kb shorter than the

shortest wild type telomeres) (Figure 2-4A). In contrast, telomeres in *tert* mutants

decline much more gradually, reaching 600 bp in G6 or G7 (Riha et al, 2001).

Next we monitored telomere length dynamics on individual chromosome arms

using subtelomeric TRF and Primer Extension Telomere Repeat Amplification

(PETRA). For subtelomeric TRF, we used a probe corresponding to the right arm of

chromosome 5 (5R) (Figure 2-4B). For PETRA, the left arms of chromosomes 1 and 3

(1L and 3L) were assessed (Figure 2-4C). Consistent with conventional TRF analysis,

both assays revealed dramatic telomere erosion in plants lacking *AtSTN1*. Moreover,

individual telomere tracts in *stn1* mutants spanned a broader size range than those in

wild type (Figure 2-4B and C). By contrast, telomere tracts on homologous

chromosomes in *tert* mutants are even more homogenous in size than in wild type,

typically forming a single sharp band that spans 100-200 bp on an agarose gel (Heacock

et al, 2004). We confirmed that the telomere defect in *stn1-1* mutants was due to the T-

DNA insertion in the *AtSTN1* gene by complementation. Bulk telomere analysis (data

not shown) and PETRA demonstrated that the profile of telomere tracts in *stn1-1* plants

expressing an *AtSTN1* transgene, expressed from a CaMV 35S promoter ,was restored to

wild type (Figure 2-4D).
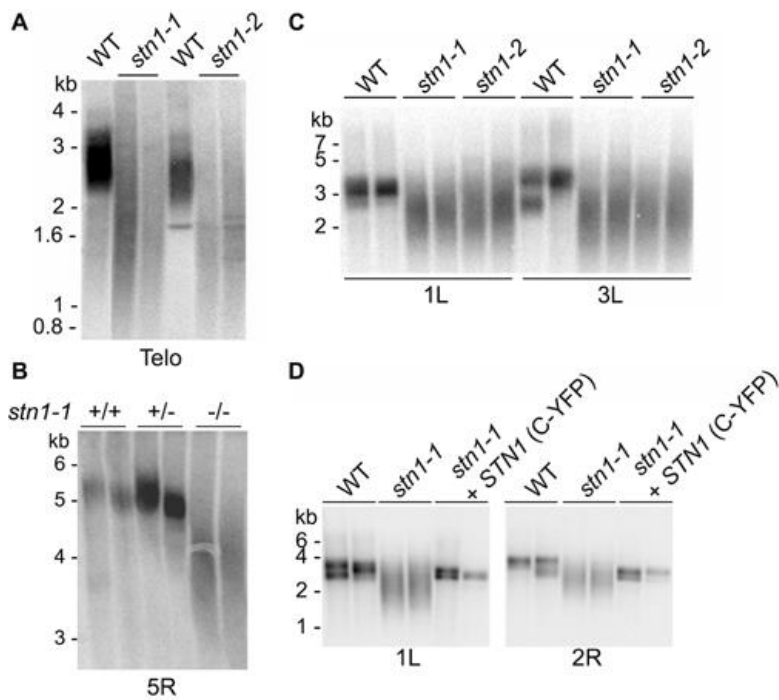
Figure 2-4. Extensive telomere erosion in *stn1* mutants.
(**A**) TRF analysis of wild type, *stn1-1* and *stn1-2* mutants. For each genotype, data from two individual sibling plants are shown. The blot was hybridized with a radiolabelled G-rich telomeric probe. Molecular weight makers are indicated. (**B**) Subtelomeric TRF analysis of wild type, heterozygous and homozygous *stn1-1* mutants. The blot was hybridized with a probe specific for the right arm of chromosome 5 (5R). (**C**) PETRA analysis of wild type, *stn1-1* and *stn1-2* mutants. The blot was hybridized with a telomeric probe. Telomere length on the left arm of chromosomes 1 and 3 (1L and 3L) was measured. (**D**) PETRA analysis of *stn1* mutants expressing a C-terminal YFP tagged wild type *STN1* transgene. Telomere length was examined on the chromosome arms indicated.

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Finally, we asked whether telomerase activity was diminished in *stn1* mutants using a real-time Telomere Repeat Amplification Protocol (Herbert et al, 2006; Kannan et al, 2008). In vitro telomerase activity levels in *stn1-1* mutants were approximately the same as in wild type plants (Figure 2-5). Thus, the loss of telomeric DNA observed in *stn1* mutants is not due to telomerase enzyme deficiency, but we cannot rule out the possibility that telomerase access to the telomere is impeded in the absence of AtSTN1.

AtSTN1 is required to prevent telomere fusions

Extensive loss of telomeric DNA can trigger end-to-end chromosome fusions. To determine whether telomeres in *stn1* mutants engage in end-joining reactions, we monitored the frequency of anaphase bridges in the pistils of these plants. As expected, no bridged chromosomes were observed in wild type plants (Figure 2-6A, Table 2-1). However, up to 29% of the anaphases in *stn1-1* mutants showed evidence of fused chromosomes (Figure 2-6B-D, Table 2-1). This degree of genome instability is not observed in *tert* mutants until G8 or G9 (Riha et al, 2001). The immediate and catastrophic onset of genome instability in *stn1* mutants reinforces the conclusion that AtSTN1 plays a critical role in chromosome end protection in Arabidopsis.

To further characterize the architecture of chromosome fusion junctions in *stn1* mutants, we employed telomere fusion PCR using primers directed at unique subtelomeric sequences on different chromosome arms (Heacock et al, 2004). Abundant telomere fusion PCR products were generated with G1 *stn1-1* DNA, which appeared as an intense, heterogeneous smear (Figure 2-6E).
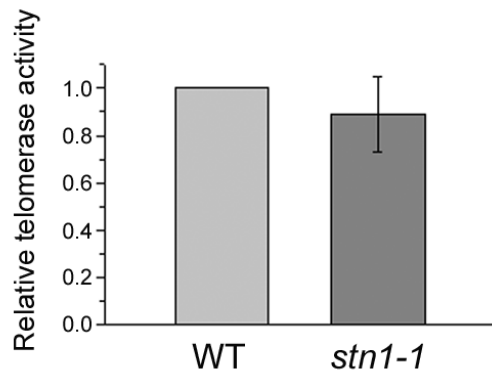
Figure 2-5. In vitro telomerase activity levels are approximately the same in *stn1* mutants as in wild type plants.
Real-time telomere repeat amplification protocol was performed with protein extracts from wild type (n=3) and *stn1-1* mutants (n=3). The telomerase activity obtained from wild type extracts was set to one and each sample was normalized to this value. Data are represented as mean ± SEM.
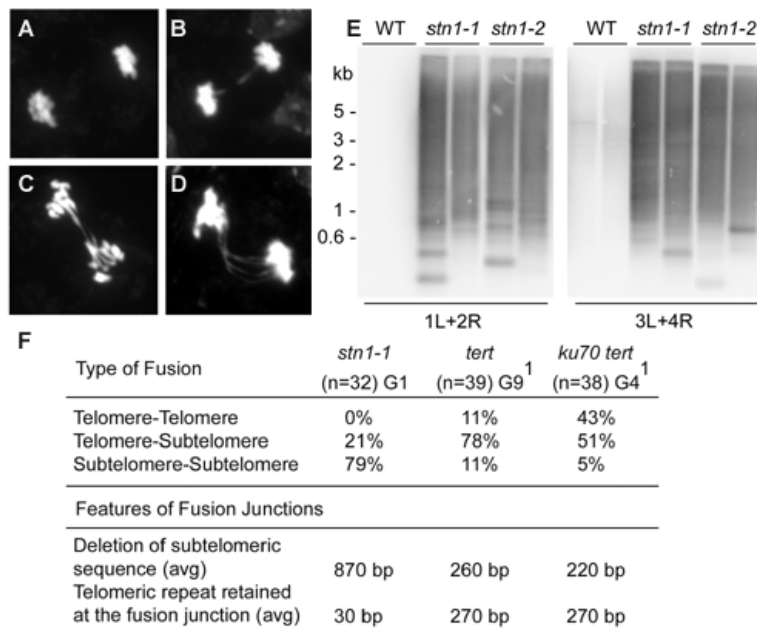
Figure 2-6. STN1 is required to prevent telomere fusions.
(A-D) Cytology of mitotic chromosomes in wild type **(A)** and *stn1-1* mutants **(B-D)** is shown. DAPI-stained chromosome spreads were prepared from pistils. Examples of *stn1-1* anaphases with one **(B)**, two **(C)** or four **(D)** bridges are shown. **(E)** Telomere fusion PCR products obtained from wild type, *stn1-1* and *stn1-2* mutants were hybridized using a telomeric probe. Primer pairs used to amplify specific subtelomeric regions are indicated. **(F)** Summary of DNA sequence analysis of cloned telomere fusion junctions in *stn1-1* (G1) mutants. Data for *tert* (G9) and *tert ku70* (G4) were taken from a previous study (Heacock et al, 2004).

	No. of pistils analyzed	total anaphase cells analyzed	anaphase cells with fusions	percentage anaphase cells with fusions
WT	4	203	0	0%
stn1-1 #54	5	241	41	17%
stn1-1 #55	3	222	54	24%
stn1-1 #70	4	229	66	29%

Table 2-1. Analysis of anaphase bridges in *stn1* mutants.
DAPI-stained chromosome spreads were prepared from pistils. Anaphase cells from three individual *stn1-1* mutants were analyzed. Anaphase bridges were scored as a percentage of total anaphase cell s.

This observation is consistent with our previous studies showing that telomere fusion is initiated when telomeres shorten below 1kb (Heacock et al, 2004). Sequence analysis of cloned PCR products showed that the majority (79%) of end-joining events in *stn1-1* mutants involved subtelomere-to-subtelomere fusion (Figure 2-6F). In contrast, chromosome fusion junctions primarily reflect telomere-to-subtelomere joining in late generation *tert* mutants (78%), and telomere-to-telomere (43%) or telomere-to-subtelomere (51%) fusions in *ku70 tert* mutants (Figure 2-6F) (Heacock et al, 2004). Notably, the average deletion of subtelomeric DNA was four-fold greater in *stn1-1* mutants (~ 870 bp) (Figure 2-6F) than in *tert* (G9, ~ 260 bp) or *ku70 tert* mutants (G4, ~ 220 bp) (Heacock et al, 2004). Because bulk telomere length is much shorter in *tert* (G9) and in *ku70 tert* (G4) mutants where an equivalent level of genome instability is observed, our G1 *stn1-1* results indicate that at least a subset of telomeres in these mutants suffer extensive nucleolytic attack prior to being recruited into end-to-end chromosome fusions.

AtSTN1 is required to maintain proper telomere architecture and to block formation of extra-chromosomal telomeric circles

Mutations in Stn1, Ten1 or Cdc13 in *S. cerevisiae* (Grandin et al, 2001; Grandin et al, 1997; Nugent et al, 1996) and Stn1 in *K. lactis* (Iyer et al, 2005) lead to gross elongation of the G-overhang. These data are interpreted to mean that the Stn1 complex protects the telomeric C-strand from degradation. In-gel hybridization was used to determine if AtSTN1 contributes to the maintenance of telomere end structure in

Arabidopsis. Relative to wild type, the G-overhang signal was increased by approximately four-fold in *stn1-1* mutants (Figure 2-7A, left panel and Figure 2-7B). Exonuclease treatment indicated that the hybridization signal detected in the native gel correlated with terminal G-overhangs (Figure 2-7A, right panel). Thus, AtSTN1 is required to maintain the proper architecture of the chromosome terminus.

The frequency of telomere recombination is dramatically increased in *K. lactis stn1* mutants (Iyer et al, 2005). To determine whether this is also true in plants lacking *AtSTN1*, we looked for evidence of Telomere Rapid Deletion (TRD). TRD results in large, stochastic deletions of telomere tracts and is thought to occur when the t-loop on the chromosome terminus undergoes branch migration, giving rise to a Holliday junction intermediate that is subsequently resolved to produce a truncated telomere and an extrachromosomal telomeric circle (Lustig, 2003). We monitored TRD using telomeric circle amplification (TCA), which detects the telomeric circle by-products of TRD (Zellinger et al, 2007). In this procedure, phi29 polymerase is used to amplify telomeric DNA circles into extremely long ssDNA, which is distinguished from endogenous linear telomere fragments based on its slower migration on a denaturing agarose gel. As expected, telomeric circles were enriched in our *ku70* mutant control reaction (Zellinger et al, 2007) (Figure 2-7C). A similar high molecular weight product was generated in *stn1-1* mutants, but not in the wild type control. We conclude that STN1 suppresses telomere recombination in Arabidopsis.
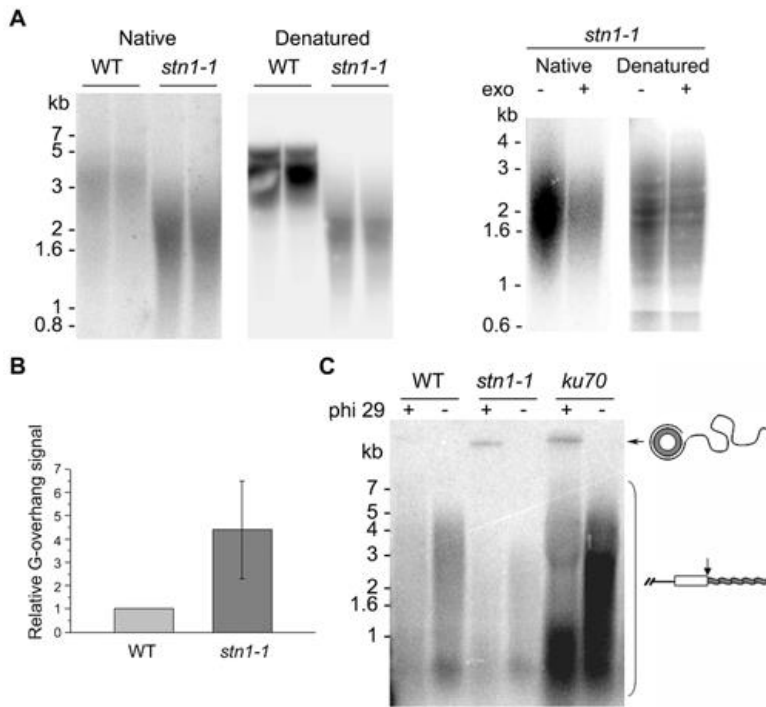
Figure 2-7. Loss of *STN1* leads to increased G-overhang signals and increased telomeric circle formation.
(**A**) In-gel hybridization analysis of DNA isolated from wild type and *stn1-1* mutants using a C-strand telomeric probe under native and denaturing conditions (left panel). The hybridization signal in the native gel was strongly reduced by exonuclease treatment, demonstrating that the signal was dependent on G-overhangs (right panel). (**B**) Quantification of the G-overhang signal. The relative G-overhang signal was determined from five independent experiments as described in Materials and methods. Data are represented as mean ± SEM. (**C**) Telomeric circle amplification (TCA) was performed with wild type, *stn1-1* and *ku70* mutant DNA in the presence or absence of phi 29 polymerase. DNA from *ku70* mutants served as a positive control. The hybridization signal for linear telomere tracts is indicated by the bracket.

Taken together, our data indicate that AtSTN1 is an essential component of the protective telomere cap in Arabidopsis that prevents nucleolytic attack, end-to-end chromosome fusions and telomere recombination.

Discussion

Although Barbara McClintock described the protective "capping" function of maize telomeres nearly 70 years ago (McClintock, 1939), we still know relatively little about why natural chromosome ends are recalcitrant to nuclease attack and end-joining reactions, especially in multicellular eukaryotes. In part, our understanding has been stymied by the rapid evolution of the telomere protein complex. Here we provide evidence that STN1 is conserved in metazoa and plays an essential role in chromosome end protection.

AtSTN1 was identified in the second iteration of PSI-BLAST as a protein bearing sequence similarity to the OB-fold domain of *S. pombe* Stn1. Subsequent analysis revealed putative STN1 orthologs in a variety of plants and vertebrates (this study; [Gao et al, 2007; Martin et al, 2007]). Structure-based alignment shows significant sequence similarity within four of the five essential beta strands of the core of the OB-fold. While the overall similarity among the Stn1 orthologs is not high, minimal sequence similarity among telomere proteins from different taxa is not without precedent. For example, Pot1 from *S. pombe* shows only 19% identity/ 40% similarity to the TEBP α subunit in ciliates, and yet the two proteins are functional and structural homologs (Baumann & Cech, 2001; Lei et al, 2003).

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One notable distinction between the STN1 proteins from plants and yeasts is the absence of a C-terminal extension in the former. Recent studies indicate that the N- and C-terminus of ScStn1 encode independent and separable functions at the telomere (Petreaca et al, 2007; Puglisi et al, 2008). The N-terminal OB-fold of ScStn1 is required for cell viability and mutation of this domain leads to an increase of single-strand DNA at the chromosome terminus (Puglisi et al, 2008), arguing that the N-terminal OB-fold is essential for chromosome end protection. In contrast, the C-terminal domain of ScStn1 is required for telomere length control and plays no detectable role in telomere capping (Puglisi et al, 2008). Like Arabidopsis *stn1* mutants, a null mutation in the *S. pombe* Stn1 leads to severe telomere deprotection phenotype, suggesting the major role of Stn1 in *S. pombe* and Arabidopsis may be in chromosome end protection. Notably, *S. pombe* Stn1 protein is significantly truncated relative to *S. cerevisiae* Stn1 (325 aa versus 494 aa), consistent with rapid evolution of the C-terminal domain. We hypothesize that the C-terminal domain of STN1 is not crucial for its telomere capping function in plants and hence was lost in the 1.5 billion years since plants and yeasts shared a common ancestor.

The strongest evidence that AtSTN1 is a functional homolog of the yeast Stn1 proteins is based on the genetic data. Plants lacking STN1 display phenotypes that strongly parallel the *S. pombe stn1* null mutants (Martin et al, 2007). In both cases, *stn1* mutants exhibit an immediate and profound telomere deprotection phenotype. In Arabidopsis mutants, both telomeric and subtelomeric tracts are subjected to extensive nuclease attack. Telomeric C-strands are particularly vulnerable to digestion, creating extended G-overhangs. As a likely consequence, *stn1* mutants exhibit increased

intrachromosomal telomere recombination as evidenced by an accumulation of telomere circles. TRD may further fuel the erosion of terminal DNA sequences in this setting. The degraded telomeres engage in end-joining reactions, triggering genome-wide instability and the cell proliferation arrest typical of plants experiencing severe telomere dysfunction (Riha et al, 2001). Thus, STN1 is a crucial component of the telomere complex in Arabidopsis that is essential for chromosome end protection.

Shelterin homologs have not been clearly defined in plants. Arabidopsis harbors at least six myb-related proteins that bind double-strand telomeric DNA in vitro in a manner similar to vertebrate TRF1 and TRF2 (Karamysheva et al, 2004), as well as three putative POT1 paralogs. Although the functions of AtPOT1b and AtPOT1c are still under investigation (Shakirov et al, 2005) (Dr. A. Nelson and D.E. Shippen, unpublished work), AtPOT1a is a physical component of the telomerase RNP that is required for telomerase action *in vivo* (Surovtseva et al, 2007). Strikingly, homologs for RAP1, TPP1 and TIN2 cannot be discerned in the Arabidopsis genome with the current search algorithms, underscoring the conclusion that telomere proteins are evolving at a rapid pace.

Besides STN1, the only other plant protein directly implicated in chromosome end protection is from rice. Like mammalian TRF2, rice telomere binding protein 1 (RTBP1) bears a myb-like DNA binding domain (Hong et al, 2007). However, in contrast to TRF2-depleted mammalian telomeres, which activate a strong DNA damage response and massive end-to-end chromosome fusions (Denchi & de Lange, 2007; Karlseder et al, 1999; van Steensel et al, 1998), plants lacking RTBP1 display very

gradual telomere lengthening over successive plant generations and only in G2 do

telomere fusions become evident (Hong et al, 2007). This mild phenotype may reflect

functional redundancy of myb-bearing telomere proteins in plants (Shippen, 2006).

STN1, by contrast, is a single-copy gene in all of the sequenced plant genomes we

surveyed.

The conserved function of Stn1 in yeasts (Grandin et al, 1997; Martin et al, 2007;

Pennock et al, 2001) and STN1 in flowering plants (this study), and the existence of

putative homologs in primates, rodents, amphibians, birds and fishes (Gao et al, 2007;

Martin et al, 2007) argues that this family of proteins may contribute to chromosome end

protection in a broad range of eukaryotes. Notably, STN1 was not identified as a

component of the Shelterin complex (Liu et al, 2004; O'Connor et al, 2004; Ye & de

Lange, 2004) in mammals. It is conceivable that STN1 interacts only transiently with

telomeres, e.g. during a specific period of the cell cycle. Alternatively, STN1 may be

part of an end protection complex distinct from Shelterin. In support of this hypothesis, a

TPP1 homolog, Tpz1, but not Stn1/Ten1, was recently identified by mass spectrometry

of Pot1-associated proteins in *S. pombe* (Miyoshi et al, 2008). Interestingly, SpPot1 does

not interact with Stn1/Ten1 in a yeast two-hybrid assay (Martin et al, 2007), implying

that *S. pombe* telomeres are composed of two distinct capping complexes, one bearing

Pot1 and Tpz1 (from Shelterin) and a second containing Stn1 and Ten1. Given that

mammalian Shelterin contains orthologs only from the former complex, POT1/TPP1,

and that STN1 is a key component of the telomere cap in plants, the data suggest that

higher eukaryotic telomeres are protected by a network of telomere protein

subcomplexes, the full constituency of which is yet to be elucidated.

CHAPTER III

MERISTEM DISORGANIZATION 1 ENCODES TEN1, AN ESSENTIAL

TELOMERE PROTEIN THAT MODULATES TELOMERASE PROCESSIVITY IN

ARABIDOPSIS*

Summary

Telomeres protect chromosome ends from being recognized as DNA damage, and they facilitate the complete replication of linear chromosomes. CST (CTC1(Cdc13)/STN1/TEN1) is a trimeric chromosome end binding complex implicated in both aspects of telomere function. Here we characterize TEN1 in the flowering plant *Arabidopsis thaliana*. We report that *TEN1* is encoded by a previously characterized gene, *Meristem Disorganization 1 (MDO1). A* point mutation in *MDO1*, *mdo1-1/ten1-3* (G77E), triggers stem cell differentiation and death, and a constitutive DNA damage response. We provide biochemical and genetic evidence that *ten1-3* is likely to be a null mutation. As with *ctc1* and *stn1* null mutants, telomere tracts in *ten1-3* are shorter and more heterogeneous than wild type. Mutants also exhibit frequent telomere fusions, increased single-strand telomeric DNA and telomeric circles. However, unlike *stn1* or *ctc1* mutants, telomerase enzyme activity is elevated in *ten1-3* mutants due to an

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increase in repeat addition processivity. In addition, TEN1 is detected at a significantly smaller fraction of telomeres than CTC1. These data indicate that TEN1 is critical for telomere stability and also has a novel role in modulating telomerase enzyme activity.

Introduction

Telomeres are essential for chromosome integrity and consist of tandem arrays simple G-rich repeats terminating in a single-strand 3' extension termed the G-overhang. The telomere tract is bound by proteins that protect the terminus from nucleolytic degradation, inappropriate recombination and activation of a DNA damage response. Telomeres also promote replication of the chromosome terminus through recruitment of telomerase and lagging strand replication machinery. Mammalian telomeres are capped by a six member protein complex called shelterin (de Lange, 2005), while the telomere ends in budding yeast are bound by the trimeric RPA-like CST (Cdc13;Stn1;Ten1) complex (Giraud-Panis et al, 2010).

All three of the yeast CST genes are essential (Garvik et al, 1995; Grandin et al, 2001; Grandin et al, 1997; Nugent et al, 1996). CST binds the G-overhang primarily through interactions with Cdc13 (Nugent et al, 1996). Cdc13 plays a central role in coordinating telomeric DNA replication by promoting G-strand synthesis via an interaction with the Est1 component of the telomerase RNP, and C-strand synthesis through its association with DNA polymerase α/primase (Chandra et al, 2001; Evans & Lundblad, 1999; Qi & Zakian, 2000; Wu & Zakian, 2011). Mutation of any of the CST components triggers nucleolytic degradation of the telomeric C-strand, leading to gross

extension of the G-overhang (Garvik et al, 1995; Grandin et al, 2001; Grandin et al, 1997).

In budding yeast Ten1 and Stn1 are proposed to regulate telomerase. Telomeres in temperature-sensitive *ten1* and *stn1* mutants elongate in a telomerase-dependent manner (Grandin et al, 2001; Grandin et al, 1997). Furthermore, Stn1 appears to block the binding of Est1 to Cdc13, preventing telomerase action on the telomere (Chandra et al, 2001). Ten1 may act in concert with Stn1 since a Stn1-Ten1 fusion protein rescues the telomere lengthening phenotype of *stn1* (Grandin et al, 2001). Ten1 could also regulate telomerase via a separate mechanism as over-expression of *TEN1* partially rescues telomere defects in *stn1* mutants, hence its discovery as a partial suppressor of *stn1* (Grandin et al, 2001).

The consequences of CST depletion are much less severe in *Candida albicans.* Null mutations in *STN1 or TEN1* are not lethal, and cells do not accumulate single-stranded G-rich telomere DNA (Sun et al, 2009), implying that the essential contributions of CST components are not universally conserved. CST orthologs (CTC1/STN1/TEN1) have been reported in vertebrates and plants (Miyake et al, 2009; Nakaoka et al, 2012; Song et al, 2008; Surovtseva et al, 2009). Although CTC1 shares no sequence similarity with Cdc13 from budding yeast, CST complexes appear to function in similar capacities across eukaryotes.

As in yeast, vertebrate and plant CST interact with pol α (pol α) (Casteel et al, 2009; Huang et al, 2012; Nakaoka et al, 2012; Price et al, 2010). Indeed, mammalian CTC1 and STN1 were first identified as pol α accessory factors (AAF) (Casteel et al,

2009). Biochemical studies reveal that vertebrate CST stimulates pol α/primase activity on telomeric substrates (Dai et al, 2010; Huang et al, 2012; Nakaoka et al, 2012), and promotes new origin firing at non-telomeric sites (Stewart et al, 2012b). CST also interacts with the shelterin components TPP1 in human cells (Wan et al, 2009) and POT1b in mice (Wu et al, 2012). Moreover, recent data suggest that human CST modulates telomerase enzyme activity through primer sequestration (Chen et al, 2012). These findings argue that CST coordinates replication of telomeric C and G-strands via dynamic interactions with shelterin, pol α and telomerase.

Despite these biochemical findings, the *in vivo* function of vertebrate CST remains poorly understood. A conditional CTC1 knock-out in mice triggers telomere loss, increased G-overhangs and ultimately activation of an ATR-dependent DNA damage response, primarily in highly proliferating tissues (Gu et al, 2012). Knock-down of CTC1 in HeLa and MCF7 human cell lines leads to similar phenotypes (Surovtseva et al 2009). More recent studies of CTC1 knock-down in other human cancer lines revealed telomere elongation in one case (Chen et al, 2012), and no significant change in telomere length in another (Wu et al, 2012).

The first reports of CTC1 and STN1 in multicellular eukaryotes came from studies in *Arabidopsis thaliana*. A null mutation in either *AtCTC1* or *AtSTN1* profoundly affects telomere integrity and stem cell proliferation. Although mutant plants are viable, they exhibit dramatic morphological phenotypes, including abnormally small leaves, irregular phyllotaxy, fasciated stems and reduced fertility (Song et al, 2008; Surovtseva et al, 2009). In addition, telomere tracts are drastically shorter and plants display

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abundant end-to-end chromosome fusions, enhanced G-overhang signals and telomeric circles (Song et al, 2008; Surovtseva et al, 2009).

Recently, CST was shown to work in concert with the Ku70/80 heterodimer to promote telomere integrity in Arabidopsis (Kazda et al, 2012). Unlike other model organisms, half of the chromosome ends in Arabidopsis, presumably those replicated by the leading strand machinery, are blunt-ended and protected by Ku. The remaining telomeres, replicated by the lagging strand mechanism, possess a canonical G-overhang bound by CST (Kazda et al, 2012). Plants encode only a subset of the vertebrate shelterin components. This observation coupled with the unusual architecture of plant telomeres suggests that CST evolved a more pivotal role than its vertebrate counterparts in protecting chromosome ends throughout the cell cycle (Nelson & Shippen, 2012).

In this study, we examine the contribution of TEN1 in Arabidopsis. We show that *AtTEN1* is encoded by *Meristem Disorganization 1*, a gene recently discovered by Hashimura and Ueguchi (2011) that is crucial for stem cell viability. A point mutation in *MDO1* (*mdo1-1/ten1-3*) causes severe shoot apical meristem aberrations including stem cell death or differentiation, developmental defects and a constitutive DNA damage response. Here we demonstrate that the defects associated with *mdo1-1/ten1-3* result from severe telomere dysfunction. Although most of the mutant phenotypes closely parallel those in plants lacking CTC1 or STN1, an unexpected role for TEN1 in the negative regulation of telomerase repeat addition processivity was uncovered. Thus, in conjunction with its essential function in telomere protection/replication as a component of CST, TEN1 plays an additional novel role in modulating telomerase activity.

Materials and methods

Plant materials and plasmids

The *ten1-1* mutant was obtained from ABRC. Plants were genotyped using TEN1-1 F and TEN1-1 R (Table 3-1). The *ten1-2$_{AS}$* lines were created utilizing the Gateway vector pB7WG2 with a 35S promoter (Karimi et al, 2002); two separate constructs were created targeting two separate regions of *TEN1* (Figureure 3-1A, Table 3-1). Antisense constructs were introduced using *Agrobacterium*-mediated transformation (Zhang et al, 2006). Transformed plants were selected on MS (Murashige and Skoog) + Basta plates. The *ten1-3* mutant and the complementation line were obtained from the Ueguchi lab at the Bioscience and Biotechnology Center, Nagoya University, Chikusa-ku, Nagoya 464-8601, Japan. Plants were genotyped as previously described (Hashimura & Ueguchi, 2011). Plants were grown in the conditions described (Surovtseva et al, 2007).

Cytology, immunofluorescence and FISH

To examine anaphase bridge formations flower pistils were prepared and analyzed as described (Riha et al, 2001; Song et al, 2008). Immunolocalization and FISH were performed on Arabidopsis suspension cells, MM2d (Menges & Murray, 2002), that were grown under continuous darkness at 130 rpm and a temperature of 25°C. Nuclei were extracted from one week old cells (Song et al, 2008). The TEN1 was detected with Rabbit anti-TEN1 (1:200) antibody and the signal was amplified using FITC Donkey

anti-Rabbit antibody (1:200, Jackson ImmunoResearch). FISH was performed as described (Armstrong et al, 2009).

Antibody preparation, protein extraction and western blot analysis

200 ug *E. coli* expressed AtTEN1 and adjuvant were mixed and injected into rabbits. Blood was collected from the central ear artery and clotted blood was clarified by centrifugation at 2,500 rpm for 20 min. Protein A purification was performed to purify anti-TEN1 antibody. To determine a suitable dilution of the antibody, immunoblotting was conducted with serial dilutions of antigen and stored at -20°C.

To analyze the expression of TEN1, protein was extracted from wild type and mutant seedlings using CelLytic P protein extraction buffer (Sigma). 45 μg of each protein was used for SDS-PAGE, followed by western blotting. The PVDF membrane was blocked in 6% (w/v) non-fat dry milk in 1X TBST buffer for 2h at RT. The membrane was incubated with anti-TEN1 antibody (1:7,500) in 6% (w/v) non-fat dry milk in 1X TBST buffer for 2h at RT. Anti-rabbit-HRP secondary antibody (1:6,667 of 0.4mg/ml in 50% glycerol, Jackson ImmunoResearch) in the same conditions using ECL prime western blotting detection kit (GE Healthcare).

Transient expression of Flag-HA-TEN1 was performed as described (Zhu et al, 2011) with the following modifications. Leaves were collected 20h after agroinfiltration, ground in liquid nitrogen and resuspended in 1XSDS loading dye (1 ml/g of tissue). 25ul of *N. benthamiana* and 45ug of Arabidopsis total protein were run on an SDS-PAGE gel. Western blotting with anti-HA was performed with anti-HA

antibody (1:3,000; Sigma) and anti-mouse-HRP secondary (1:3,000; GE). Membrane was stripped and probed with anti-TEN1 antibody.

Protein interaction assays

STN1, TEN1, Ku70 and Ku80 cDNA were cloned into pET28a (T7-tag fusion) and pCITE4a vectors (Novagen). Proteins were expressed in RRL (Promega) according to manufacturer's instructions with [^{35}S]-methionine (Perkin Elmer) to label the protein expressed from pCITE4a, and in some cases pET28a. Co-immunoprecipitation was performed as described (Karamysheva et al, 2004). Quantification was preformed by calculating the ratio of TEN1: STN1 signal and comparing, with the wild type interaction set to 100%. For yeast two-hyrbid assays, GAL4-AD or GAL4-BD constructs of TEN1 and STN1 cDNA were transformed and expressed in yeast strain PJ69-4A. To eliminate false positives, the yeast two-hybrid assay was conducted under stringent media conditions consisting of synthetic drop-out (SD)/-Leu/-Trp/-His/-Ade selection medium with 50 mM 3-aminotriazole (3-AT). To confirm positive interactions, we switched inserts from the GAL4-AD vector to the GAL4-BD vector and repeated the assay.

RNA analysis

Total RNA was extracted from plant tissue using a plant RNA extraction kit (OMEGA). Reverse transcription was performed with cDNA Supermix (Quanta) per manufacturer's instructions. TEN1 mRNA levels were measured by Q RT-PCR with

Primers TEN1 Q RT-PCR 1F and TEN1 Q RT-PCR 1R (Table 3-1) using Sso Fast Eva

Green Supermix (BioRad) in accordance with manufacturer's specifications. mRNA

levels were normalized to GAPDH and TIP41L mRNA levels in corresponding samples.

TRF, PETRA, and TF-PCR

DNA from individual plants was extracted as described (Cocciolone & Cone,

1993). TRF analysis was performed with 50 μg of DNA as previously described

(Fitzgerald et al, 1999). For Bal31 assay 200ug of DNA was incubated with 65 units of

*Bal*31 (New England Biolabs) in 1x *Bal*31 reaction buffer. Equal amounts of sample

were taken out at 15 or 30 minute intervals for 60 or 90 minutes. Phenol:choloroform

extraction was performed followed by isopropanol precipitation. Resuspended DNA was

digested by *Tru1I* following the TRF protocol. PETRA and TF-PCR were performed as

described (Heacock et al, 2004).

In-gel hybridization and telomere circle amplification

In-gel hybridization was performed as described with the following

modifications (Heacock et al, 2007). Exonuclease treatment was performed with T4

DNA polymerase (New England Biolabs) at 37°C for 10 min. Prior to drying the gel the

lower portion containing the interstitial telomere repeats was removed and a Southern

blot was performed using a $[^{32}P]$ 5' end labeled $(T_3AG_3)_4$ oligonucleotide probe. The

relative amount of single-strand G-overhang was calculated by quantifying the

hybridization signal obtained from the native gel and then normalizing this value with

the loading control of either interstitial telomere signal from the Southern blot or ethidium bromide staining. The value for wild type was set to one. TCA was performed as previously described (Zellinger et al, 2007).

Telomerase activity assays

Protein for TRAP assays was extracted from flowers and reactions were conducted as described (Fitzgerald et al, 1996). Q-TRAP was performed as discussed in (Kannan et al, 2008). pTRAP protocol was adapted from (Szatmari & Aradi, 2001). Telomerase extension was performed with reaction mix containing 1xGo Taq MasterMix, colorless (Promega), 50 ng protein extract, 0.1μM TRAP forward (Table 3-1), ~5uCi [α-^{32}P]dGTP (PerkinElmer). After telomerase extension, 0.04μM 1RPgg (Table 3-1) was added to the reaction followed by two PCR cycles (95°C for 30s, 60°C for 1m, and 72°C for 45s). After addition of 0.1μM 2RP (Table 3-1) 33 PCR cycles were conducted (95°C for 30s, 64°C for 30s, and 72°C for 1m). Products were ethanol precipitated and resolved on 6% denaturing PAGE, followed by autoradiography.

Primer	Sequence 5'-3'
MDO1-GF	GCG AAG CAC GAT TCA AAC CCT TTT CGT
MDO1-GR	GGT TCG ACA CCA AAC ATC GAG TCC T
MDO1-IF	GTG TTA TTG AAG ATG GAG GCA GAA GTC TC
MDO1-IR	CTA AGA TGC TGA ACC TAC ATC GTC TTG AG
TEN1Q RT-PCR IL	CCG TCC ACA TTT CTT CCT GT
TEN1Q RT-PCR IR	TGG AGG CAG AAG TCT CAA AA
TEN1G77E	GGC TCT ATT TAT CAG TTT ATT GAA GAG CTT CAC ATT GAA CAA CC
TEN1G77E_antisense	GGT TGT TCA ATG TGA AGC TCT TCA ATA AAC TGA TAA ATA GAG CC
TEN1Antisense Const. 1F	CAC CCG CCT CAT TAT TGG GTT GTT
TEN1Antisense Const. 1R	TTG AAC CTG GTG TTC CCA TT
TEN1Antisense Const. 2	CAC CGA CCA AAA CAT ATC CAC CAT CC
TEN1Antisense Const. 2	GGC TCG AAC AGG AAG AAA TG
TEN1-1F	CAC CCA AAA CTG TCA TCA TTG CTT CA
TEN1-1R	GCC ATG GCG GCG GTG CAG TTT TTG TAG TTC CAA CAA AG
LBa1	TGG TTC ACG TAG TGG GCC ATC G
TRAP Forward	CAC TAT CGA CTA CGC GAT G
IRP gg	TAG AGC ACA GCC TGT CCG TGC TAA ACC CTA AAC CCT AAA CCC TAA ACC GG
2RP	TAG AGC ACA GCC TGT CCG TG
PT3	AGC ATC CGT CGA GCA GAG TTA GGG TTT AGG GTT TAG GGT TTA G
PT6	AGC ATC CGT CGA GCA GAG TTA GGG TTT AGG GTT TAG GGT TTA GGG TTT AGG GTT TAG GGT TTA G

Table 3-1. Primers

Results

Identification of Arabidopsis TEN1

In the second iteration of PSI-BLAST using human TEN1 (Miyake et al, 2009) as a query we retrieved a single hit: NP_176022.2 (E-value=2e-07). This gene, At1g56260, corresponds to *MDO1* (Hashimura & Ueguchi, 2011), and hereafter is termed *AtTEN1*. *AtTEN1* is a single copy gene with one open reading frame interrupted by two introns (Figure 3-1A). The ORF encodes a 127 amino acid protein with 23% identity/48% similarity to human TEN1. Secondary structure prediction by PSIPRED (McGuffin et al, 2000) revealed a single OB-fold that shares significant similarity TEN1 in other plants, as well as in *S. pombe* and humans (Figure 3-1B). RT-PCR amplified a single *AtTEN1* mRNA species that was expressed widely in Arabidopsis tissues (Figure 3-2A)

A hallmark of TEN1 is its ability to interact with STN1 (Grandin et al, 2001; Martin et al, 2007; Miyake et al, 2009; Nakaoka et al, 2012). To assay for TEN1-STN1 interaction, recombinant proteins were expressed in rabbit reticulocyte lysate (RRL) and labeled with [^{35}S] methionine. One of the proteins was expressed as T7-tagged fusion. Reciprocal co-immunoprecipitation (co-IP) experiments with T7 antibody showed a direct interaction between AtSTN1 and AtTEN1 (Figure 3-1C). Yeast-two-hybrid analysis confirmed this association (Figure 3-1D).

Figure 3-1. Arabidopsis TEN1 is a member of a CST complex

(A) Schematic of AtTEN1 gene structure. The T-DNA insertion in *ten1-1* is illustrated, along with the position of two anti-sense constructs and the point mutation responsible for the G77E mutation in ten1-3. **(B)** Alignment of TEN1 proteins from different eukaryotes. At, *Arabidopsis thaliana*; Os, *Oryza sativa* (rice); Pt, *Populus trichocarpa* (poplar); Mm, *Mus musculus*; Hs, *Homo sapiens*; Sp, *Schizosaccharomyces pombe*. The positions of beta-strands of the OB-fold are indicated below the alignment. **(C)** TEN1 interacts with STN1 in vitro. Results of co-immunoprecipitation performed with recombinant proteins. One protein is [35S] methionine-labeled (asterisk) and the other is T7-tagged and unlabeled. S, supernatant; P, pellet. Results for the positive (KU70/KU80) and negative (KU70/KU70) controls are shown. **(D)** Yeast-two hybrid assay results for AtSTN1 and AtTEN1. The two proteins fused to GAL4-AD and GAL4-BD were co-expressed and grown on selection plates for His auxotrophy (left) or assayed to detect β-galactosidase activity of positive transformants (right). (-) indicates empty vector. **(E)** Nuclear localization of TEN1 in purified nuclei. TEN1 was detected by anti-TEN1 antibody in hexaploid Arabidopsis suspension cell culture, Telomeres were labeled by FISH using a rhodamine-labeled telomere probe. DAPI stained nuclei are shown. In the merge, filled white arrows denote sub-centromeric stretches of telomeric DNA on chromosome 1. TEN1 co-localization with telomeres is indicated by the open white arrow.
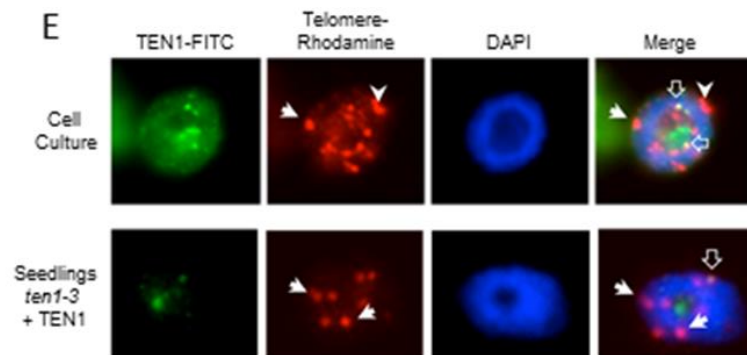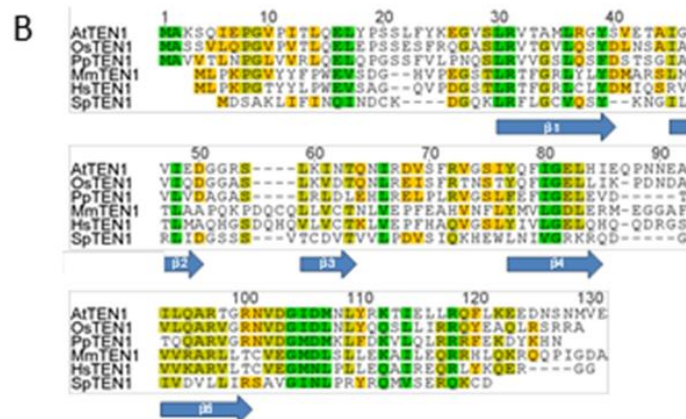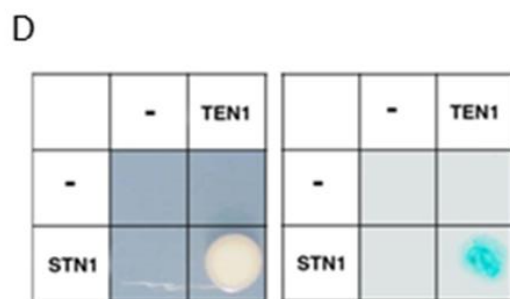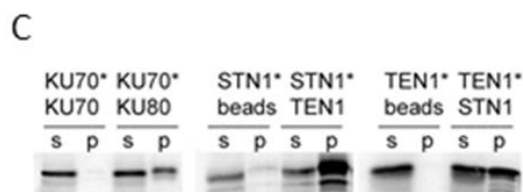
We next asked whether AtTEN1 co-localizes with telomeres using a polyclonal antibody raised specifically against recombinant AtTEN1 (see methods). Immunolocalization was performed with the TEN1 antibody on the nuclei of asynchronously dividing Arabidopsis suspension cell culture. Fluorescence in situ hybridization (FISH) using a rhodamine-labeled telomere probe was used to identify telomeres. AtTEN1 appeared as punctate spots in the nucleus (Figure 3-1E). A merged image of the TEN1 and telomere signals revealed that AtTEN1 co-localized with 13% (15/114) of the telomeres examined (Figure 3-1E, top). A similar value, 12.1% (18/149) was obtained with Arabidopsis seedlings. These observations were unexpected, as previous experiments with transgenic plants expressing a tagged version of CTC1 found that it associated with approximately 50% of the chromosome ends in cycling cells and cells arrested in G1 (Surovtseva et al, 2009). Thus, our data suggest that TEN1 association with telomeres may be more transient than STN1 or CTC1. Moreover, because some of the punctate spots recognized by the TEN1 antibody do not co-localize with telomeres, TEN1 may have extra chromosomal functions.

To test whether increased TEN1 expression would drive telomere localization, immunolocalization was conducted on seedling nuclei from a genetic complementation line in which *TEN1* was expressed from its native promoter in a *ten1-3* background (Hashimura & Ueguchi, 2011). In this line the level of TEN1 mRNA was three to ten-fold higher than in wild type plants (Figure 3-2C). The number of telomeres bound by TEN1 increased slightly to 26/127 (20.45%), implying that the low frequency of TEN1-telomere association is not due to coincident overlap or inaccessibility of telomere-
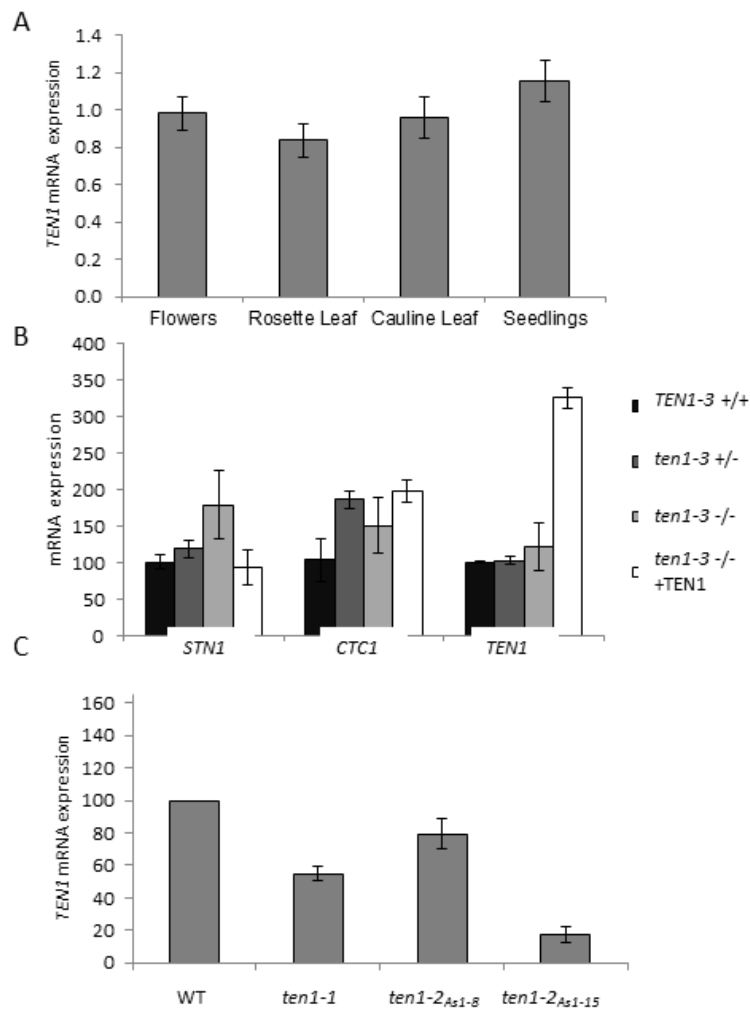
Figure 3-2. *TEN1* mRNA expression levels monitored by quantitative real time PCR.

(**A**) TEN1 mRNA expression in different plant tissues. RNA was analyzed from four week-old wild type (WT) Arabidopsis leaves and flowers and from two week-old seedlings. QRT-PCR was performed with TEN1 specific primers, as well as TIP41L and GAPDH for normalization of cDNA levels. Experiments were performed in triplicate. (**B**) CST mRNA expression in the *ten1-3* line for whole plant tissue. Results are shown for sibling offspring of *ten1-3* heterozygotes as indicated. Also shown are data for the Native::TEN1 complementation line. Expression data were normalized to GAPDH and compared to expression in wild type siblings. Each bars represent standard deviation for 4 biological individuals, each sample done in triplcate. (**C**) TEN1 mRNA levels in anti-sense knockdown lines. mRNA was isolated from flowers, data were normalized for GAPDH expression, and values for each experiment compared to wild type. Experiments were performed in quadruplicate.

bound TEN1 to antibody. Instead, the data indicate that TEN1 is associated with a substantially smaller fraction of telomeres than CTC1.

The ten1-3 *mutation causes profound defects in plant development and fertility*

To examine the function of AtTEN1 *in vivo* we initially characterized a T-DNA insertion line (*ten1-1*) that contains a disruption in the 5' UTR of *AtTEN1*, 180bp upstream of the start codon (Figure 3-1A). Quantitative RT-PCR showed ~50% reduction in TEN1 mRNA in homozygous *ten1-1* mutants (Figure 3-2B). In an attempt to achieve a greater TEN1 knockdown, we targeted two regions of *AtTEN1* with anti-sense RNA (TEN1-2$_{AS1}$ and TEN1-2$_{AS2}$)(Figure 3-1A). Quantitative RT-PCR revealed a wide range of TEN1 mRNA depletion. For example, *ten1-2$_{AS1-15}$* showed an 82% reduction in TEN1 mRNA, while TEN1 mRNA levels were reduced by only 10% in *ten1-2$_{AS1-8}$* (Figure 3-2B). Similar results were obtained for *ten1-2$_{AS2}$* knock-down lines. Strikingly, despite the significant reduction of TEN1 mRNA in *ten1-2$_{AS1-15}$*, mutant plants were morphologically wild type, indicating that only a small amount of TEN1 is required for wild type function.

In marked contrast to *ten1-1* and *ten1-2$_{AS}$* lines, plants harboring the *mdo1-1* mutation, exhibited severe growth and developmental defects (Hashimura & Ueguchi, 2011) (Figure 3-3), much like those described for *stn1 and ctc1* null mutants (Song et al, 2008; Surovtseva et al, 2009). Hereafter we term the *mdo1-1* mutation, *ten1-3*. Homozygous *ten1-3* mutants showed a lack of apical dominance. Many plants had fused stems, smaller leaves and irregular siliques (Figure 3-3A, middle). Seeds collected from

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first generation (G1) *ten1-3* mutants displayed remarkably variable germination rates spanning 30-90%. Compared to their parents, second generation (G2) *ten1-3* mutants developed even more slowly and the majority had more severe morphological defects, including the complete absence of apical meristems and exceptionally short roots (Figure 3-3C). As reported earlier (Hashimura & Ueguchi, 2011), over-expression of wild type *AtTEN1* rescued these morphological phenotypes (Figure 3-3A, right), confirming that the *ten1-3* mutation is responsible for the developmental abnormalities.

The *ten1-3* mutation is caused by a glycine to glutamic acid amino acid substitution at position 77 (Figure 3-1A) (Hashimura & Ueguchi, 2011). As expected, the level of *TEN1* mRNA was essentially wild type in *ten1-3* plants (Figure 3-2C). Western blotting was performed to assess TEN1 protein expression. To confirm the specificity of the TEN1 antibody, FLAG-HA-tagged TEN1 was transiently expressed in tobacco leaves and the extracted proteins were used for Western blot analysis with anti-HA and anti-AtTEN1 antibodies. A single band corresponding to the fusion protein was detected with anti-HA antibody (Figure 3-4A). The same size product was detected with the anti-AtTEN1 antibody, along with another band of 17 kDa that may represent partial loss of the tags (Figure 3-4A). As expected, a single band corresponding to the predicted size of TEN1 (16 kDa) was observed in wild type Arabidopsis (Figure 3-4A). We found a slight increase in TEN1 abundance in the TEN1 complementation line, and decreased TEN1 in *ten1-3* plants (Figure 3-5A). Notably, attempts to express recombinant TEN1$_{G77E}$ in *E. coli* resulted in significantly lower protein yields than wild type TEN1, arguing that the mutation reduced TEN1 stability.
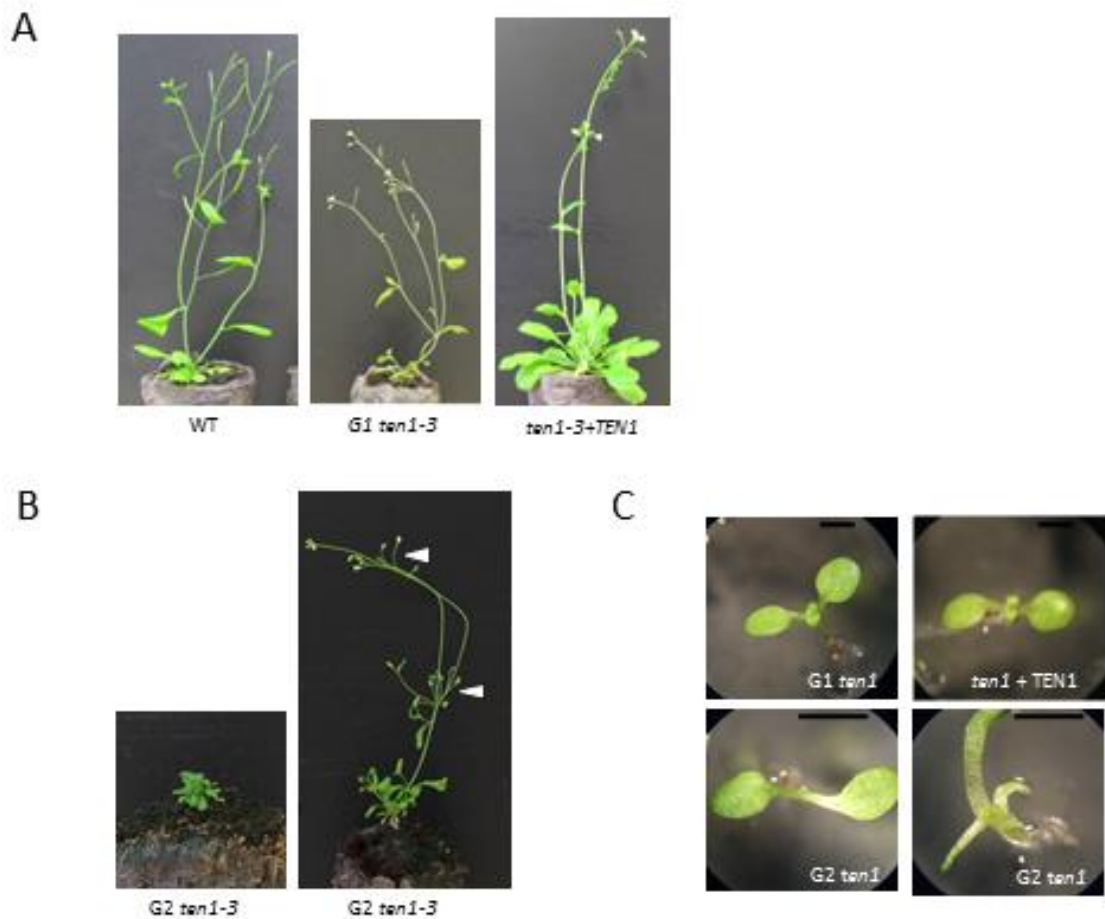
Figure 3-3. The *ten1-3* mutation causes severe morphological defects.
(A) First generation (G1) *ten1-3* mutants are smaller in stature than wild type (WT) and harbor smaller leaves, fused stems and irregular phyllotaxy (middle panel). These phenotypes are rescued by expression of a wild type copy of TEN1 (right panel). **(B)** Second generation (G2) *ten1-3* mutants display more severe growth phenotype than G1 mutants, and are infertile. Arrowhead denotes aborted siliques. **(C)** Two week-old seedlings of the genotypes indicated were grown on MS without selection. G2 *ten1-3* mutants exhibit shoot apical meristem abnormalities and fail to produce true leaves. G2 mutants are shown in a 2x zoom to show abnormal apical meristem.
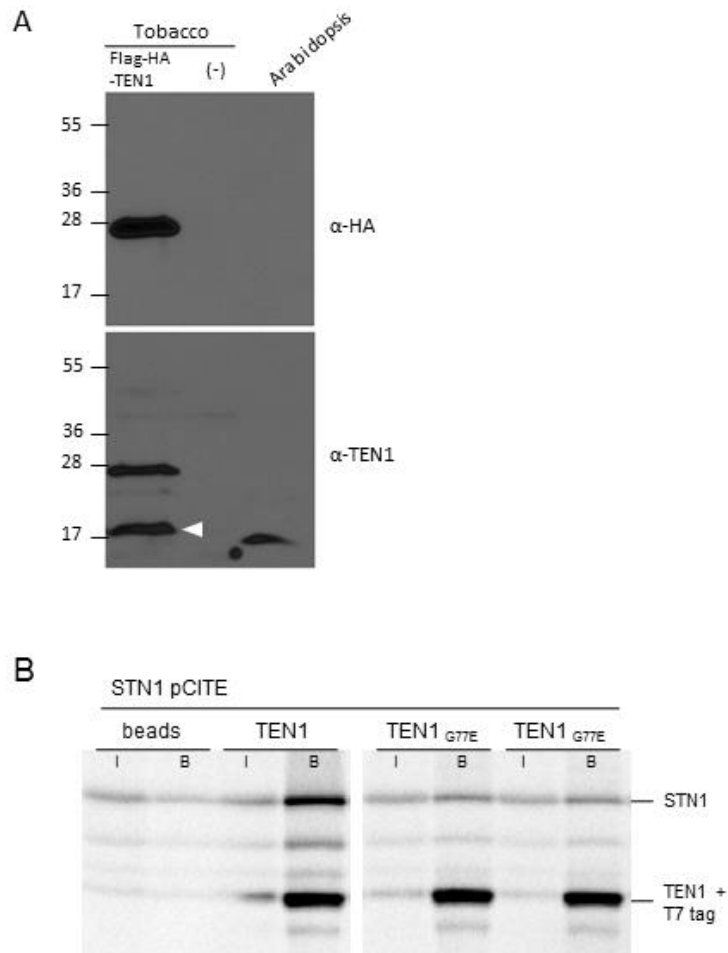
Figure 3-4. G77E mutation alters TEN1 expression *in vivo* and ability to bind STN1 in vitro.
(A) TEN1 antibody recognizes transiently expressed Flag-HA-TEN1 in tobacco and endogenous TEN1 in Arabidopsis. The western blot was probed with anti-HA, then stripped and re-probed with polyclonal anti-body raised against Arabidopsis TEN1. White arrow indicates lost of tag from transiently expressed TEN1 protein. **(B)** Pull – down of STN1 by a T7 tagged TEN1 in vitro from RRL expressed proteins by T7 conjugated agarose beads. Proteins are labeled with [^{35}S]-methionine to visualize and run on a 15% gel. This is an example of the data used to calculate the binding in figure 3C. (I) Input, (B) Beads
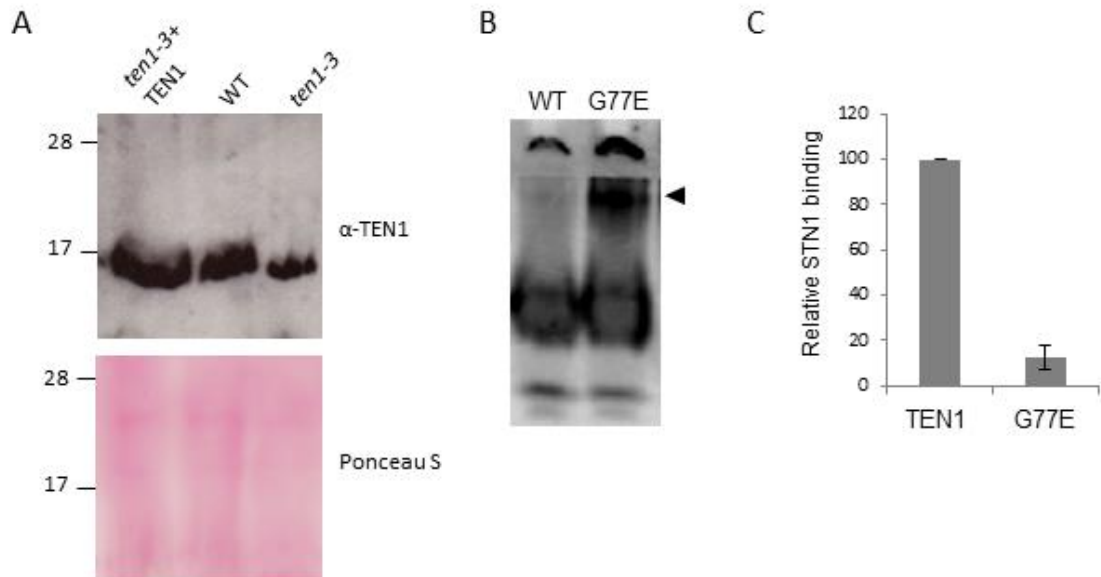
Figure 3-5. The TEN1_{G77E} mutant protein is unstable and does not interact with STN1 in vitro.

(A) Western blot results for wild type (WT), *ten1-3* and *the TEN1 complementation line (TEN1-3 + TEN1) are shown*. Ponceau S stain loading controls included. Molecular weight size markers in kD are on the left. The blot was probed with a polyclonal antibody raised against Arabidopsis TEN1. **(B)** Native PAGE results for recombinant WT TEN1 or TEN1_{G77E} protein expressed in rabbit reticulocyte lysate. Arrow indicates a higher molecular weight polypeptide in the TEN1_{G77E} protein sample. **(C)** Quantification of recombinant TEN1 protein binding to STN1. Shown are results of co-immunoprecipitation experiments with recombinant WT TEN1 and TEN1_{G77E}. The interaction for WT TEN1-STN1 was set to 100%. An example of raw data is shown in Figure 4B.

Although recombinant TEN1$_{G77E}$ expressed in rabbit reticulocyte lysate was soluble, a large fraction of the protein migrated much more slowly than wild type TEN1 on a non-denaturing gel (Figure3-5B), consistent with significant structural perturbation. In addition, co-IP experiments showed that the G77E mutation abolished the interaction of TEN1 with STN1 in vitro (Figure3-5C, Figure3-4B). Since the G77 residue on TEN1 is not predicted to lie within the AtSTN1 binding interface based on the Stn1-Ten1 crystal structure from *S. pombe* (Sun et al, 2009), we conclude that the *ten1-3* mutation profoundly disrupts TEN1 structure and stability. Thus, *ten1-3* mutants can be classified as null or nearly null for TEN1.

AtTEN1 is required for telomere length maintenance

Terminal restriction fragment (TRF) analysis was performed to analyze bulk telomere length in *ten1* mutants. Although there was no obvious change in telomere length in *ten1-1* or in the *ten1-2$_{AS1-8}$* antisense line relative to wild type (Figure3-6A and B), the telomere profile was altered in *ten1-2$_{AS1-15}$* mutants (Figure 3-6B), where TEN1 mRNA is decreased by 82%. The size range of telomeres contracted and longer telomeres were absent. Stronger evidence that TEN1 is important for telomere length maintenance came from analysis of *ten1-3* mutants. Telomere tracts were more heterogeneous, and significantly shorter overall than wild type or any of the *ten1-2$_{AS}$* lines, and closely resembled *ctc1* and *stn1* mutants (Figure 3-6C). Primer Extension Telomere Repeat Assay (PETRA), which measures telomere length on individual chromosome arms, confirmed telomere shortening on all arms tested (Figure3-7A). As

expected, telomeres in the TEN1 complementation line were wild type (Figure 3-6C), verifying that the *ten1-3* mutation is responsible for the defects in telomere length maintenance.

Unlike *tert* mutants that suffer progressive telomere shortening in subsequent plant generations (Riha et al, 2001), bulk telomeres did not shorten further in second generation (G2) *ten1-3* mutants (Figure 3-6D). However, TRF analysis revealed a new profile of products, consisting of heterogeneous telomere tracts punctuated by multiple discrete bands (Figure 3-6D). G2 *ten1-3* DNA was digested with BAL31 exonuclease prior to TRF analysis to determine whether the bands correspond to terminal DNA sequences (Figure 3-6E, Figure 3-7B). Although bulk telomeric DNA was completely degraded within 30 minutes, the sharp bands were insensitive to exonuclease treatment. Because this banding profile was not observed in G1 *ctc1* or G1 *stn1* mutants (Song et al, 2008; Surovtseva et al, 2009), we asked whether it represented a general response to the prolonged absence of CST. TRF analysis performed with DNA from G2 *stn1* mutants also revealed sharp bands (Figure 3-6F). We hypothesize that these products reflect gross rearrangements of telomeric DNA, resulting from chromosome end de-protection and multiple rounds of the breakage-fusion-bridge cycle (see below).
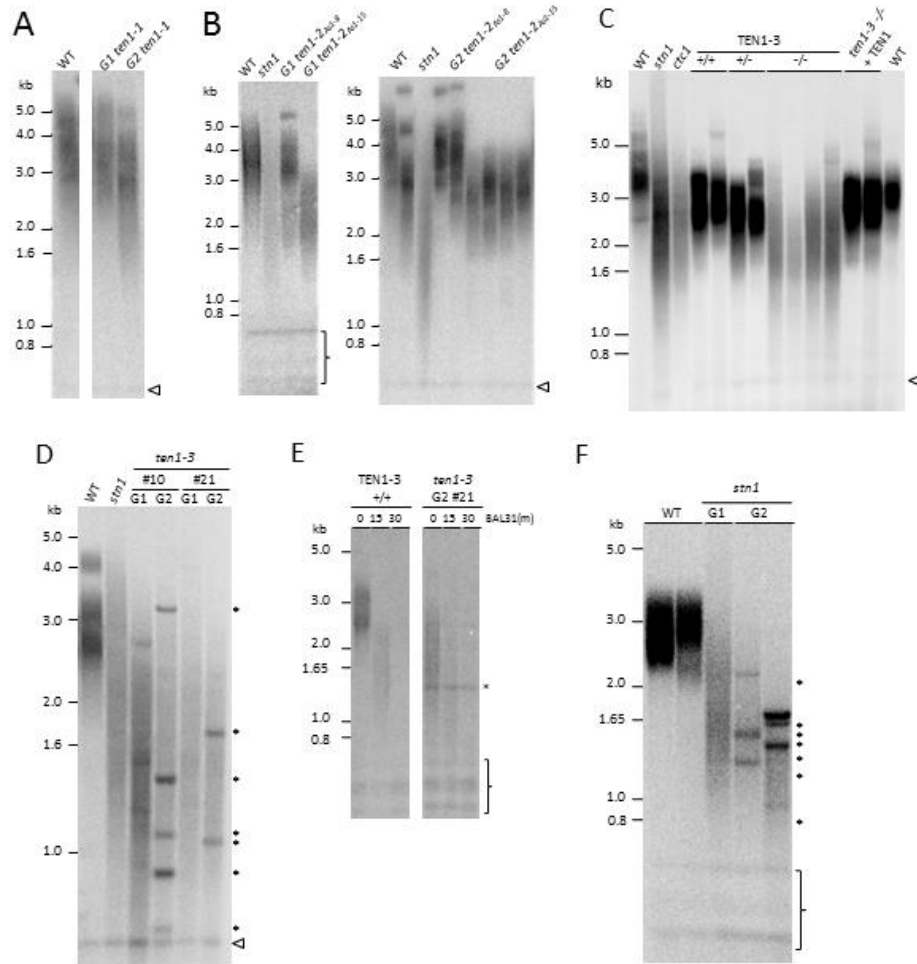
Figure 3-6. TEN1 is important for telomere length regulation and genome maintenance.
TRF analysis of *ten1* mutants. Blots were hybridized with a radiolabeled G-rich telomeric probe.**(A)** Results for first (G1) and second (G2) generation *ten1-1* are shown relative to wild type (WT). **(B)** Telomere length in first (left) and second (right) generations of two anti-sense knock-down lines of TEN1. For comparison results are shown with first generation *stn1-1* mutants. **(C)** TRF analysis of *ten1-3* mutants. Results for offspring of *ten1-3* heterozygous plants are analyzed. **(D)** Parent-progeny analysis for two different *ten1-3* mutants. **(E)** BAL31 time course of DNA with WT and a G2 *ten1-3* mutant. **(F)** Telomere profile of G1 and G2 *stn1-1* mutants. Asterisks indicated abnormally sharp TRF bands. Interstitial telomeric DNA repeats are denoted by the bracket, or arrowhead.
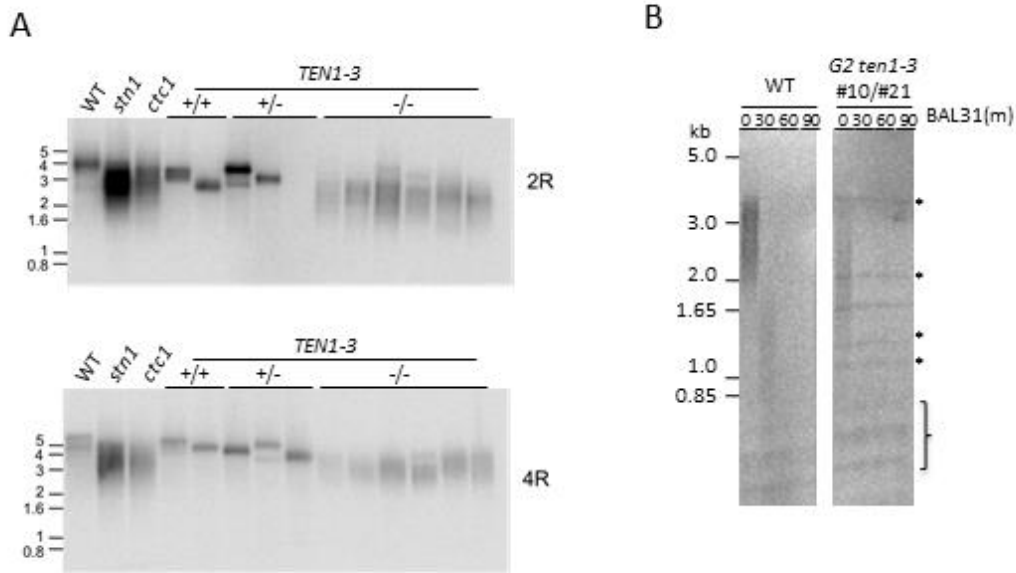
Figure 3-7. Telomere shortening and length deregulation in *ten1-3* mutants.
(A) Results of PETRA using subtelomeric primers for 2R and 4R are shown. **(B)** BAL 31 nuclease time course experiment. DNA from WT and two G2 *ten1-3* mutant plants were treated with BAL 31 for the times indicated followed by digestion with Tru1I nuclease. Asterisk indicates BAL31 resistant digestion products. Previously characterized interstitial telomeric bands are indicated by the bracket. Blots were hybridized with a labeled G-rich telomeric probe. Molecular weight makers are shown.

AtTEN1 promotes telomere integrity

Telomere Fusion PCR (TF-PCR) was employed to ask if TEN1 is needed to prevent end-to-end chromosome fusions. As for *ctc1* and *stn1* mutants*,* abundant TF-PCR products were generated in reactions with *ten1-3*, but not wild type DNA samples (Figure 3-8A). Cloning and sequence analysis showed evidence of extensive nucleolytic resection prior to chromosome end-joining (Table 3-2). To obtain a quantitative estimate of telomere fusion events, mitotic chromosomes were examined for evidence of anaphase bridges (Figure 3-8B). 30-50% of anaphases examined in *ten1-3* mutants contained bridged chromosomes, with several involving multiple chromosomes. Notably, this value is significantly higher than number of bridges found in *ctc1* or *stn1* mutants (18-30%).

In-gel hybridization was performed to determine if TEN1 modulates G-overhang architecture. Compared to wild type siblings, *ten1-3* mutants displayed a two-fold increase in G-overhang signal, similar to *stn1* and *ctc1* mutants (Figure 3-8C and D) (Song et al, 2008; Surovtseva et al, 2009). Exonuclease controls confirmed that the hybridization signal was derived from terminal single-stranded telomeric DNA (Figure 3-9A). As expected, the enhanced G-overhang signal was absent in the TEN1 complementation line (Figure 3-8C).
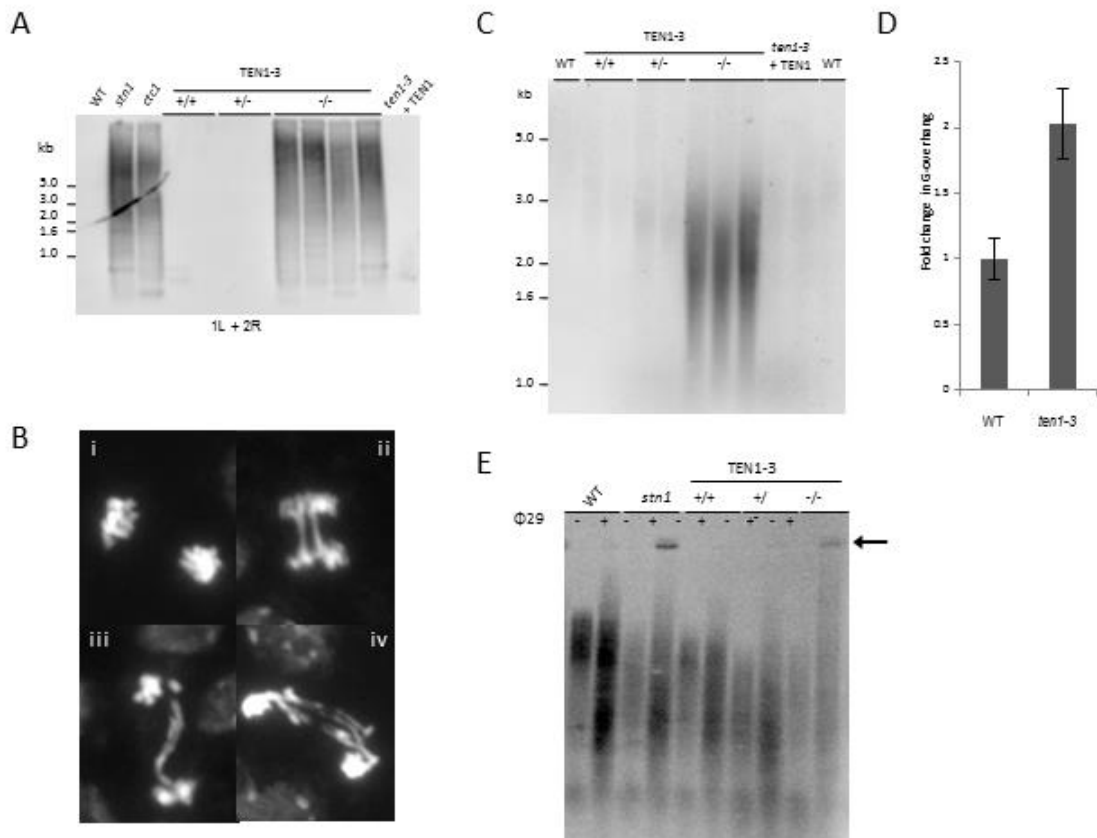
Figure 3-8. TEN1 prevents end-to-end chromosome fusions and promotes proper telomere architecture.
(**A**) Telomere fusion PCR products obtained from wild type (WT) and *ten1*-3 mutants are shown. Primer pairs used to amplify specific subtelomeric regions are indicated. (**B**) Cytology of mitotic chromosomes in WT (i) *and ten1-3 mutants* (ii-iv) are shown. DAPI-stained chromosome spreads were prepared from pistils. (**C**) In-gel hybridization analysis of DNA isolated from WT and *ten1-3* mutants using a C-strand telomeric probe under native conditions. (**D**) Quantification of the G-overhang signal for *ten1-3* mutants. A Southern blot of interstitial telomere DNA or ethidium bromide staining of DNA was used as a DNA loading control for quantification of G-overhang signal. Data represent 7 individual biological replicates of *ten1-3*. (**E**) Telomeric circle amplification (TCA) was performed with WT, *ten1-3 +/-* offspring, and *stn1-1* DNA in the presence (+) or absence (-) of phi (φ) 29 polymerase. Arrow indicates extra-chromosomal telomere circles.

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Type of Fusion	G2 *ten1-1* (n=15)	G1 *ten1-3* (n=12)	G1 *stn1*[1] (n=32)	G1 *ctc1*[2] (n=27)
Telomere-Telomere	13.0%	41.7%	0.0%	0.0%
Telomere-Subtelomere	87.0%	41.7%	21.0%	14.0%
Subtelomere-Subtelomere	0.0%	16.6%	79.0%	86.0%
Features of Fusion Junctions				
Deletion of subtelomereic sequence (avg)	130 bp	213 bp	870 bp	N.A.
Telomeric repeat retained at the fusion junction (avg)	280 bp	103 bp	30 bp	N.A.

[1]Data reported in Song *et al* 2008

[2]Data reported in Surovtseva *et al* 2009

Table 3-2. Sequence analysis of *ten1* mutant telomere fusion PCR products.
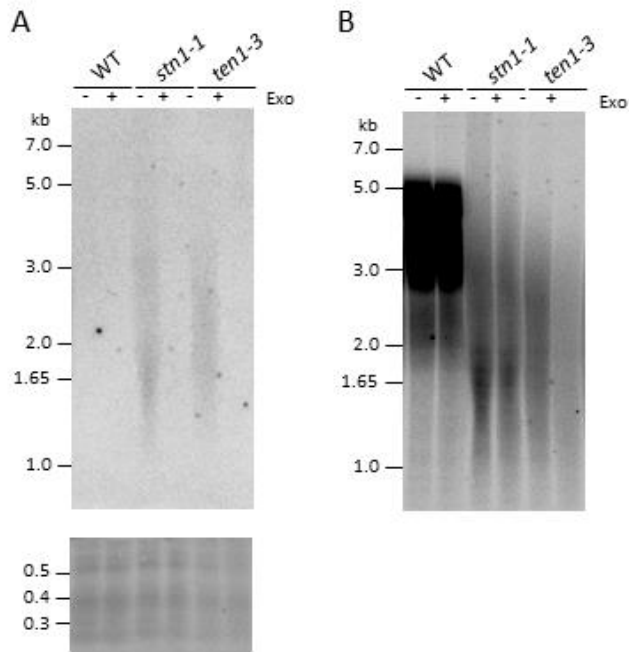
Figure 3-9. Increased G-overhang signals in *ten1-3* mutants in exonuclease sensitive.
In gel hybridization results are shown for *ten1-3* and *stn1-1* mutants. Samples were treated with exonuclease as indicated. **(A)** Products from native in-gel hybridization of G-overhangs (top), Southern blot of interstitial telomeric DNA (bottom). (Right) Denatured gel showing telomere signal.

One other hallmark of telomere instability is an increase in the frequency of telomere recombination. The telomere circle assay (TCA) (Zellinger et al, 2007) was used to monitor telomere recombination events. As in *stn1* and *ctc1* mutants, *ten1-3* plants displayed increased production of extra-chromosomal telomere circles (Figure 3-8E). We conclude that TEN1, like the other components of CST, promotes telomere integrity by maintaining telomere length and proper architecture of the chromosome terminus.

TEN1 negatively regulates telomerase repeat addition processivity

Plants lacking STN1 or CTC1 exhibit no change in telomerase activity levels (Song et al, 2008; Surovtseva et al, 2009). Therefore, we were surprised to find that telomerase activity was elevated in *ten1-3* mutants (Figure 3-10A). Quantitative telomere repeat amplification protocol (Q-TRAP) revealed, on average, a two-fold increase in enzyme activity in *ten1-3* mutants compared with wild type siblings. The alteration in enzyme activity was somewhat variable with some plants showing only slightly increased activity, while others showed a three-fold increase (Figure 3-11A). When products of the TRAP reaction were resolved by denaturing PAGE, it was evident that *ten1-3* mutants generated substantially more of the longer telomere repeat arrays than wild type (Figure 3-10B). Importantly, the ratio of shorter products generated with *ten1-3* versus wild type was slightly less than 1:1, but increased to more than 10:1 for longer products. This skewed ratio indicates that the *ten1-3* extracts exhibit qualitatively

different telomerase activity. Specifically, the findings indicate that telomere repeat addition processivity (RAP) was increased in the absence of TEN1.

A direct, non-PCR based telomerase activity assay is not available for Arabidopsis. Therefore, we modified the processivity TRAP (pTRAP) assay devised for human telomerase (Szatmari & Aradi, 2001) to assess RAP in extracts from *ten1-3* mutants. In this assay telomerase extension products are tagged with a unique sequence that is used for amplification in conventional PCR (Figure 3-12A). Control reactions with synthetic oligonucleotide substrates bearing either three or six telomere repeats yielded the expected products (Figure 3-12A). Examination of pTRAP products showed that the abundance of short products was essentially the same for wild type, *ten1-3* and *stn1-1* mutants. However, the longer products were strongly over-represented in reactions with *ten1-3* (Figure 3-12B). Although the TEN1 complementation line had a slightly elevated processivity, the product profile more closely resembled that of wild type than the mutant (Figure 3-12B). We conclude that TEN1 negatively regulates telomerase enzyme activity by decreasing RAP.
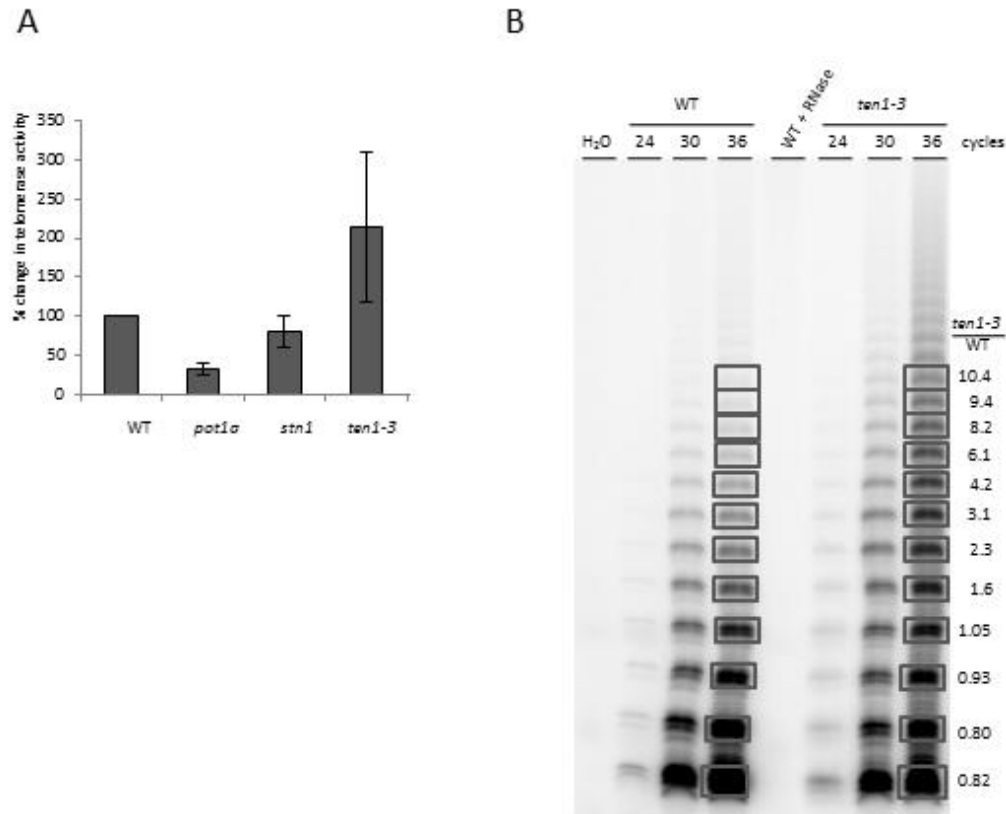
Figure 3-10. TEN1 is a negative regulator of telomerase activity.
(A) Telomerase activity in flowers measured by Q-TRAP. Data are normalized to wild type (WT); each data point represents two or three biological replicates, with three technical replicates. Standard deviation represents the deviation between biological replicates. **(B)** TRAP products from WT and *ten1-3* mutants at 24, 30 and 36 cycles of PCR resolved by PAGE. Quantification (right) represents the signal for the corresponding bands of *ten1-3* divided by WT for the 36 cycle PCR reaction.
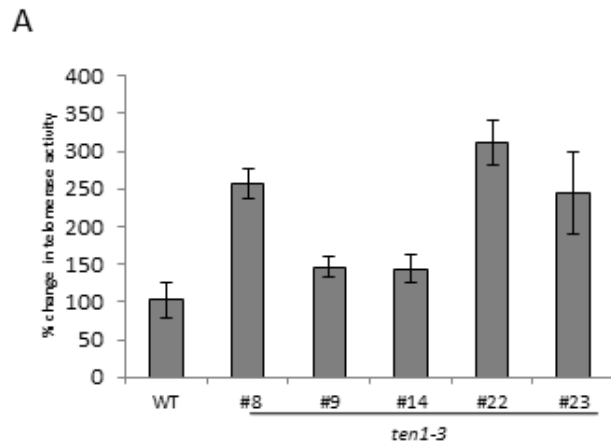
Figure 3-11. Analysis of telomerase enzyme activity in *ten1-3* mutants.
(A) Q-TRAP results for flowers from individual *ten1-3* plants. Each data point represents three technical replicates. Error bars represent the standard deviation in technical replicates.
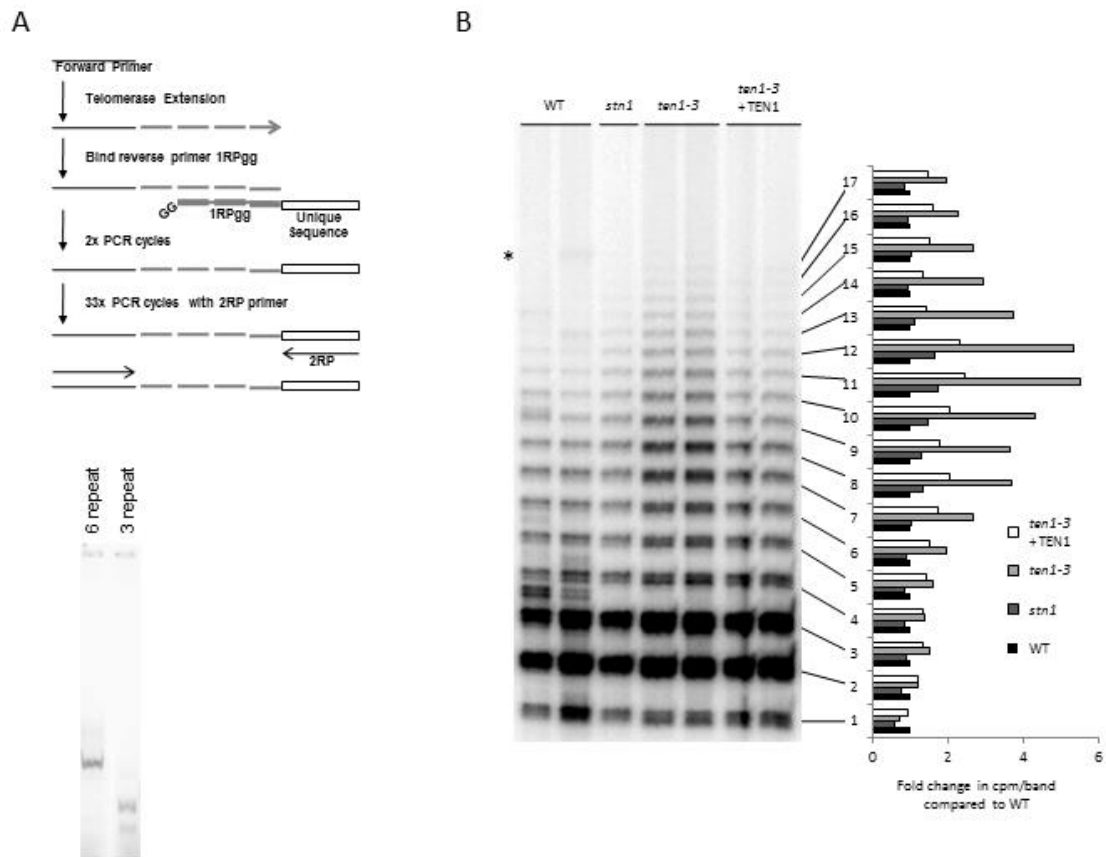
Figure 3-12. TEN1 decreases telomerase repeat addition processivity.
(A) (Left) Schematic of processivity TRAP (Szatmari & Aradi, 2001). (1) Telomerase
extends a forward primer substrate. (2-3) Binding of reverse primer 1RPgg, which
contains a unique sequence tag on the 5' end, and terminates in two 3' non-
complementary G nucleotides that precisely position the primer at the terminus of the
extension product. Two cycles of PCR are used to tag the telomerase product with the
unique sequence tag. (4) 33 cycles of PCR using the forward primer and 2RP, a reverse
primer complementary to the unique sequence tag. Right, Results for control reactions
with oligonucleotides containing three (PT3) or six (PT6) telomere repeats (Table 3-1)
subjected to steps 2-4 of processivity TRAP. **(B)** Results of processivity TRAP for floral
extracts from wild type (WT), *stn1* and *ten1-3* mutants. Left, telomerase extension
products displayed by PAGE. Asterisk denotes non-specific PCR amplification products.
Right, quantification of processivity TRAP. Signal was quantified for the individual
bands indicated. The average signal was calculated for each genotype (except *stn1*) and
that average was compared to the average for WT.

Discussion

Telomere integrity is critical for genome stability and the long-term proliferative capacity of stem cell pools. The *MDO1/TEN1* gene was originally identified in a forward genetic screen for defects in meristem maintenance (Hashimura & Ueguchi, 2011). Here we demonstrate that the molecular basis for stem cell failure is telomere dysfunction. Plants lacking TEN1 harbor short, highly heterogeneous telomere tracts with aberrant G-overhangs that are subjected to inappropriate recombination including massive end-to-end chromosome fusions. These phenotypes are strikingly similar to those of *ctc1* and *stn1* null mutants (Song et al, 2008; Surovtseva et al, 2009), and together with biochemical data showing that AtTEN1 physically interacts with AtSTN1, our findings argue that TEN1 is a key component of the Arabidopsis CST complex required for genome integrity.

A critical role for CST in cell proliferation in humans is highlighted by a spate of genetic studies demonstrating that compound heterozygous mutations in CTC1 underlie the stem cell disorders Coats plus and Dyskeratosis congenita (Anderson et al, 2012; Keller et al, 2012; Mangino et al, 2012; Polvi et al, 2012). Interestingly, only a subset of patients with CTC1 mutations exhibit telomere shortening (Walne et al, 2012), consistent with the prevailing model that mammalian CTC1 is a multifunctional protein that contributes to different facets of DNA metabolism. We found that only a small amount of TEN1 is sufficient in Arabidopsis, suggesting that conflicting reports pertaining to CST deficiency in mammalian cell lines (Chen et al, 2012; Gu et al, 2012; Surovtseva et al, 2009; Wu et al, 2012) could reflect different levels of depletion. Studies

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to elucidate how CST contributes to human disease are in their infancy, and thus far no mutations in TEN1 or STN1 have been reported. Relative to CTC1, these genes are much smaller targets for mutation, and given the essential role of STN1 and TEN1 in plants (Hashimura & Ueguchi, 2011; Song et al, 2008); this study), disease-related mutations in their human counterparts may ultimately be recovered.

Several lines of evidence indicate that TEN1 does not always function in concert with CTC1 and STN1. Purification of AAF from human cells revealed the presence of CTC1 and STN1, but not TEN1 (Casteel et al, 2009). In addition, we found that TEN1 associates with a smaller fraction of Arabidopsis telomeres than CTC1. The current model for telomere protection in Arabidopsis proposes that CST is bound to half of the chromosome ends (Kazda et al, 2012). Consistent with this model, CTC1 co-localizes with approximately 50% of the telomeres (Surovtseva et al, 2009). In contrast, we found only 13-20% of telomeres were bound by TEN1. These results are particularly striking, given that the same relatively low fraction of mammalian telomeres are bound by CST (13-20%) (Miyake et al, 2009). Unlike Arabidopsis CST, mammalian CST plays no significant role in chromosome end protection. Therefore an intriguing possibility is that Arabidopsis TEN1, unlike CTC1 and perhaps STN1, promotes telomere integrity through transient interactions with the chromosome terminus.

The most compelling argument that TEN1 makes a unique contribution outside the context of the trimeric CST complex comes from the unexpected observation that telomerase enzyme activity is elevated in plants lacking TEN1, but not CTC1 or STN1 (Song et al, 2008; Surovtseva et al, 2009); this study). In both conventional TRAP and

pTRAP assays significantly longer telomere repeat arrays were generated in *ten1-3*

reactions than with *stn1* or wild type extracts, indicating that TEN1 negatively regulates

telomerase activity by controlling RAP. Studies in other model systems have uncovered

telomerase-associated OB-fold proteins that stimulate RAP. These include p82 (Teb1), a

stable component of the *Tetrahymena* telomerase RNP (Min & Collins, 2009), and the

mammalian shelterin components TPP1/POT1 (de Lange, 2009). TPP1 stimulates the

DNA binding ability of POT1 and the heterodimer then provides a bridge linking

telomerase to the telomere (Wang et al, 2007; Xin et al, 2007). We were unable to detect

telomerase activity in TEN1 immunoprecipitates (Dr. J.R. Lee and D. Shippen,

unpublished data). This result may reflect the transient nature of the TEN1-telomerase

interaction, or the association of TEN1 with telomerase may result in non-processive

elongation (e.g. the addition of less than one full telomere repeat). Such products would

not be readily detected by TRAP.

It is noteworthy that studies in budding yeast (Grandin et al, 2001) and more

recently in human cancer cells (Chen et al, 2012) suggest that CST contributes to the

negative regulation of telomerase at chromosome ends. Although we did not observe

telomere elongation in *ten1-3* mutants, enhanced telomerase action at chromosome ends

would be masked by profound telomere de-protection in this background. Our data are

thus consistent with yeast and mammalian CST studies, and go a step further by directly

implicating TEN1 in telomerase regulation.

CHAPTER IV

TELOMERASE ACTS SYNERGISTICALLY WITH STN1 AND TEN1 FOR

TELOMERE MAINTENANCE

Summary

Telomeres are important for protecting the chromosome end and solving the end replication problem. These functions are mediated by telomerase and a number of telomere-associated proteins. CST (CTC1/STN1/TEN1) is a single-strand telomere binding complex that is required for telomere integrity in *Arabidopsis thaliana*. Here we describe genetic evidence that TEN1 and STN1 act in the same pathway for the maintenance of telomere length and end protection. Loss of both STN1 and TEN1 causes defects in telomere length maintenance and telomere fusions. These phenotypes mimic those associated with loss of STN1 or TEN1 alone. We further show that CST and telomerase act synergistically to maintain telomere length. Telomeres in *stn1 tert* double mutants are shorter that *stn1* null mutants. Analysis of the *ten1 tert* double was initially hampered, but identification of double mutants using a different strategy showed that they were very severely affected and their growth arrested at the seedling stage. Telomeres in the *ten1 tert* mutant were more heterogeneous with some telomeres elongated relative to their *tert* siblings. This finding suggests that in the absence of TEN1 and TERT a recombination event like alternative lengthening of telomeres occurred. Altogether, these data demonstrate that CST and TERT are both important for the maintenance of telomere length. The study also reveals a more critical role for TEN1 than STN1 in maintaining genomic integrity.

Introduction

Telomeres are the ends of linear chromosomes, and they are comprised of DNA and associated proteins. Telomeric DNA consists of tandem G-rich repeats, with the majority of the sequence being double-stranded. The very end of the chromosome has a 3' single-strand protrusion called the G-overhang. Telomere repeats are added to the G-overhang by telomerase. Telomerase is minimally composed of the telomerase reverse transcriptase (TERT) and the telomerase RNA (TER) (Greider & Blackburn, 1989; Shippen-Lentz & Blackburn, 1990). Accessory proteins associated with the telomerase RNP promote telomerase activity in vivo (Lingner et al, 1997). Telomerase has two types of activity: nucleotide addition processivity and repeat addition processivity (RAP) (Greider & Blackburn, 1985). RAP is a unique property of telomerase that allows the enzyme to add multiple repeats to the telomere prior to dissociation.

The flowering plant *Arabidopsis thaliana* is unusual as it harbors at least two telomerase RNP complexes with two distinct RNA subunits, TER1 and TER2, and different cohorts of accessory proteins (Cifuentes-Rojas et al, 2011; Cifuentes-Rojas et al, 2012). The TER1 RNP is the canonical RNP; it adds telomere repeats to the chromosome end to solve the end replication problem (Cifuentes-Rojas et al, 2011). POT1a is an accessory component of the TER1 RNP that binds TER1 and is important for telomerase activity (Cifuentes-Rojas et al, 2011; Surovtseva et al, 2007). The TER2 RNP, in contrast, is a novel negative regulator of TER1-mediated telomerase activity (Cifuentes-Rojas et al, 2012). TER2 RNA is induced in response to DNA double-strand breaks (Cifuentes-Rojas et al, 2012).

Prolonged inactivation of telomerase through a null mutation in the catalytic subunit TERT results in gradual telomere shortening leading to genomic instability and eventually to developmental arrest after successive generations of telomerase deficiency (Fitzgerald et al, 1999; Riha et al, 2001). Similar to *tert* mutants, *pot1a* mutants also exhibit gradual loss of telomere length over progressive generations (Surovtseva et al, 2007). Telomerase activity is reduced ten-fold in the *pot1a* mutant due to decreased telomerase RAP (Surovtseva et al, 2007)(K. Renfrew unpublished data). Telomeres in these mutants appear as distinct bands when DNA is resolved on a denaturing gel (Fitzgerald et al, 1999; Surovtseva et al, 2007). The homogeneity of the telomere tracts reflects the loss of telomerase extension at G-overhangs, and the continued protection of the ends from nucleolytic attack (Riha et al, 2001).

In addition to its contacts with telomerase, the G-overhang is bound by an RPA-like complex termed CST (Cdc13/Stn1/Ten1). CST was initially identified in yeast, where it is important for both telomere protection and telomere replication (Evans & Lundblad, 1999; Garvik et al, 1995; Grandin et al, 2001; Grandin et al, 1997; Nugent et al, 1996). Cdc13 can positively and negatively regulate telomerase activity through interactions with Est1, a telomerase accessory factor, and Stn1, respectively (Chandra et al, 2001; Evans & Lundblad, 1999; Grandin et al, 1997; Lustig, 2001; Nugent et al, 1996; Pennock et al, 2001; Qi & Zakian, 2000). Competition for the same binding interface on Cdc13 by Stn1 and Est1 is believed to regulate telomere length (Chandra et al, 2001; Grandin et al, 2001; Pennock et al, 2001).

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Stn1 is a negative regulator of telomerase activity and Ten1 may assist Stn1 in this role, though the mechanism is unclear (Grandin et al, 2001; Grandin et al, 1997). Additionally, the CST complex facilitates complete replication of the chromosome end by recruiting polymerase α/primase to the telomere during G-overhang extension to facilitate C-strand fill-in (Qi & Zakian, 2000). Therefore, the CST complex coordinates the replication of the telomere by coupling G and C-strand synthesis (Qi & Zakian, 2000).

Interestingly, the function of CST at mammalian telomeres seems to be confined to telomere replication and C-strand fill-in and not chromosome end protection (Stewart et al, 2012b; Wang et al, 2012). It was previously believed that CST was confined to yeast, as telomere protection and telomerase regulation are controlled by shelterin in vertebrates (Palm & de Lange, 2008). The discovery of CST components in Arabidopsis led the way for their discovery in other higher eukaryotes. STN1, the first CST protein to be identified, is a small 18 kDa protein with a single OB-fold (Song et al, 2008). The next to be discovered was CTC1, a much larger 142 kDa protein with multiple OB-folds and the capacity to specifically contact ss telomeric DNA (Surovtseva et al, 2009) (Dr. J.R. Lee, unpublished data). CTC1 is a functional but not sequence homolog to Cdc13, and was identified in a screen for telomere capping proteins. The last Arabidopsis CST subunit to be identified was TEN1, which was found based on homology to the human TEN1 protein (Leehy et al, 2013). TEN1 is the smallest of the three at only 16 kDa. STN1 interacts strongly with both TEN1 and CTC1, but the interaction between TEN1 and CTC1 is very weak (Leehy et al, 2013; Surovtseva et al, 2009). STN1 and CTC1 co-

localize with ~50% of the telomere ends in plant cells regardless of cell cycle stage (Song et al, 2008; Surovtseva et al, 2009). In contrast, TEN1 is found at only 11% of telomeres (Leehy et al, 2013).

Loss of CST complex members in Arabidopsis leads to telomere shortening, extended G-overhangs, rampant telomere fusions and increased telomere recombination (Leehy et al, 2013; Song et al, 2008; Surovtseva et al, 2009). However, *ten1-3* mutants exhibit some phenotypes that are distinct from those of *stn1* or *ctc1* mutants. These phenotypes include decreased co-localization of TEN1 with telomeres, as mentioned above, as well as a dramatic increase in anaphase bridges consistent with much more severe telomere dysfunction (Leehy et al, 2013). Interestingly, unlike STN1 and CTC1, TEN1 plays a role in regulating telomerase activity. It does so by negatively regulating telomerase RAP (Leehy et al, 2013; Song et al, 2008; Surovtseva et al, 2009). Thus, TEN1 not only contributes to the core functions of CST, but also has additional roles in the cell.

Here we use a genetic strategy to more precisely define the roles of STN1 and TEN1 in telomere protection and length maintenance. First, we examine plants bearing null mutations in both *STN1* and *TEN1*. We show that similar to a *stn1-1 ctc1-2* double mutant, the telomere length defect in *stn1-1 ten1-3* mutants is generally not more severe than the single mutants (Surovtseva et al, 2009). This observation indicates that chromosome end protection afforded by CST requires all three members of the complex. We also examined the plants lacking STN1 or TEN1 in conjunction with a loss of TERT. We find a more severe phenotype in *stn1 tert* than either single mutant, but

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surprisingly the *ten1 tert* mutant was even more compromised than *stn1 tert*. Taken together our data indicate that telomerase acts on telomeres in the absence of STN1 and TEN1. The data also support the conclusion that TEN1 has a more complex role in telomere maintenance than STN1.

Materials and methods

Plant materials

The *stn1-1* and *tert* mutant lines were obtained from the ABRC stock center. The *ten1-3* mutant and the complementation line were a gift from the Ueguchi lab at Nagoya University, Japan. Plants were grown in the conditions described (Surovtseva et al, 2007). Plants were genotyped (Fitzgerald et al, 1999; Hashimura & Ueguchi, 2011; Song et al, 2008) and genetic crosses were performed as previously described (Surovtseva et al, 2009). In brief, heterozygous mutants were crossed both male to female and female to male. The seeds from these crosses were planted on soil and genotyped. Offspring from double heterozygous plants were planted on soil, genotyped and used for experiments. To obtain *ten1-3 tert* double mutants, the offspring from a *ten1-3 +/- tert -/-* plant were grown on soil, genotyped and used for experiments.

TRF, PETRA, and TF-PCR

DNA from individual plants was extracted as described (Cocciolone & Cone, 1993). TRF analysis was performed with 50 μg of DNA (Fitzgerald et al, 1999). PETRA and TF-PCR were performed as described (Heacock et al, 2004).

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Results

TEN1 and STN1 act in the same genetic pathway

Since TEN1 seems to have functions in addition to those of CTC1 and STN1, analysis of *ten1-3 stn1-1* double heterozygous mutant offspring was performed to further characterize STN1 and TEN1 and to assess their individual contributions. The *stn1-1* allele has a T-DNA insertion in the *STN1* coding region, causing a complete loss of *STN1* mRNA (Song et al, 2008). The *ten1-3* mutant is the result of an amino acid substitution (G77E) in the *TEN1* coding sequence (Hashimura & Ueguchi, 2011). Although TEN1$_{G77E}$ protein can be detected in vivo by western blotting, the mutant protein is unable to associate with STN1 in vitro (Leehy et al, 2013). Moreover, *ten1-3* mutants display severe morphological defects consistent with a null mutation in TEN1 (Leehy et al, 2013).

To obtain a *stn1-1 ten1-3* mutant, plants heterozygous for *stn1-1* were crossed to a *ten1-3* heterozygote. Heterozygous plants must be used for this cross as homozygous *stn1-1* and *ten1-3* mutants have significantly reduced fertility (Leehy et al, 2013; Song et al, 2008). Seeds from the cross were grown and genotyped to identify a double heterozygous mutant. Large numbers of seeds from these plants were germinated to obtain a *stn1-1 ten1-3* double mutant, and single mutant siblings for controls. We found that double homozygous mutants were morphologically similar to single mutants (Figure 4-1A). Shared phenotypes included faciated stems, smaller leaves, and irregular phyllotaxy of the siliques, indicative of increased stem cell disorganization within the shoot apical meristem (Hashimura & Ueguchi, 2011; Song et al, 2008). Notably, there

were some properties of double mutants that were more compromised that in the single mutants. The double mutant displayed smaller leaves that were fewer in number and a delay in the generation of siliques, resulting in most of them being present at the top of the stem (Figure 4-1A).

To understand how loss of both STN1 and TEN1 affects telomere maintenance and chromosome end protection, we examined telomere length and looked for the presence of telomere-telomere fusions. Bulk telomere length was assessed using terminal restriction fragment (TRF) analysis. This experiment revealed that telomere length in the double mutant was no more affected than in the single mutants. The telomeres in both the single and double mutants were significantly shorter and more heterogeneous (Figure 4-2A). To determine if telomere length was similar for all telomere arms, we performed primer extension telomere repeat assay (PETRA). This assay utilizes PCR primers for unique subtelomeric arms and a G-overhang adaptor primer to amplify the telomere from a single chromosome end. Again, analysis of several chromosome arms confirmed that telomere length was similar for all tested in both *stn1-1 ten1-3* double mutants and their single mutant siblings (Figure 4-2B).
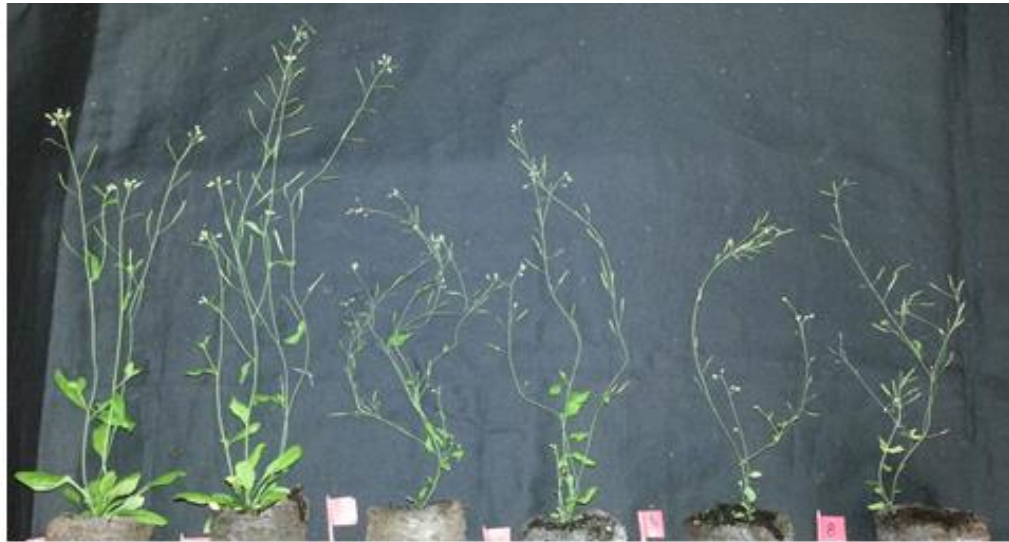
Figure 4-1. Morphological defects in plants bearing *stn1-1* and *ten1-3* mutations.
stn1 and *ten1* single null mutants have irregular silique placement and fasciated stems.
stn1 ten1 double mutants exhibit these phenotypes as well as additional abnormalities
like very small leaves and delayed formation of siliques.

Both *ten1-3* and *stn1-1* single mutants have chromosome end-to-end fusions as a result of telomere deprotection (Leehy et al, 2013; Song et al, 2008). Telomere fusion PCR (TF-PCR) amplifies telomere-telomere fusions using primers for two different subtelomere sequences that will only produce a PCR product in the presence of a chromosome end-to-end fusion. These PCR products can then be visualized by Southern blotting with a telomere specific probe. Using this approach, we found an abundance of fusions in *stn1-1 ten1-3* double mutants (Figure 4-2C). However, because TF-PCR is not quantitative, we could not assess whether there were more or less end-joining events in the double mutants.

The data obtained so far suggest that STN1 and TEN1 largely function in the same genetic pathway to maintain telomere length and prevent chromosome fusions. Further research is needed to determine how loss of both of these proteins will affect the G-overhang status, the level of chromosome instability and telomerase activity.
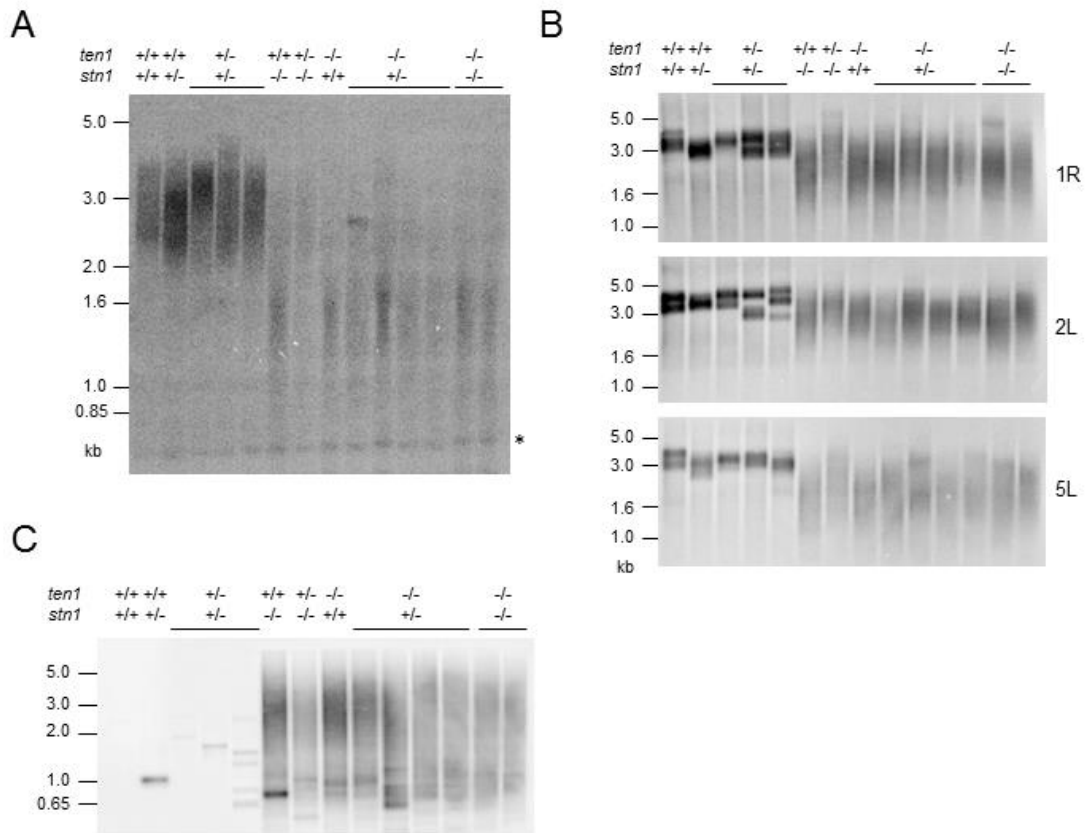
Figure 4-2. STN1 and TEN1 act in the same genetic pathway to maintain telomere length.
Analysis of the telomere length phenotype in offspring from a *stn1+/- ten1+/-* parent.
(A) TRF results for *stn1 ten1* and their single mutant siblings. Asterisk denotes interstitial telomeric DNA. **(B)** PETRA results for telomere length on chromosomes arms 1R, 2L and 5L for wild type, and different *stn1*, *ten1* and *stn1 ten1* mutants. **(C)** TF-PCR results using primers directed at 1R/5L for wild type, and different stn1, ten1 and stn1 ten1 mutants. Abundant fusions are detected in both single and double mutants.

Telomerase contributes to telomere maintenance in the absence of STN1

To investigate how telomerase contributes to the maintenance of short telomeres in CST mutants, we created a *stn1 tert* double mutant. A *stn1-1* heterozygote was crossed to a *tert* heterozygote to obtain a *stn1 tert* double heterozygote. Offspring were genotyped to obtain double mutants, as well as single null mutant siblings. The growth and development of the *stn1 tert* double mutant was much more severely affected than *stn1-1*. Double mutants were very small, consisting only of vegetative tissue and were completely sterile (Figure 4-3A). Morphologically, the *stn1 tert* double mutants resembled third generation *stn1-1* mutants (Song et al, 2008).

TRF was performed to examine the telomere length profile in the *stn1 tert* double mutants. These plants had substantially shorter, more deregulated telomeres than *stn1-1* (Figure 4-3B). Unlike *tert* mutants, the telomeres in *stn1 tert* double mutants were heterogeneous, similar to the *stn1-1* mutants. This observation suggests that at least part of the heterogeneity of telomeres in the *stn1-1* mutants may be a result of nuclease attack. The telomere length pattern was the same for all chromosome arms tested (Figure 4-3C). As expected, telomere fusions were abundant in these double mutants (X. Song unpublished data). These data suggest that TERT is required to maintain telomere length in *stn1-1* mutants, by either physically protecting the chromosome end or more likely, by telomerase-mediated extension of the telomere.
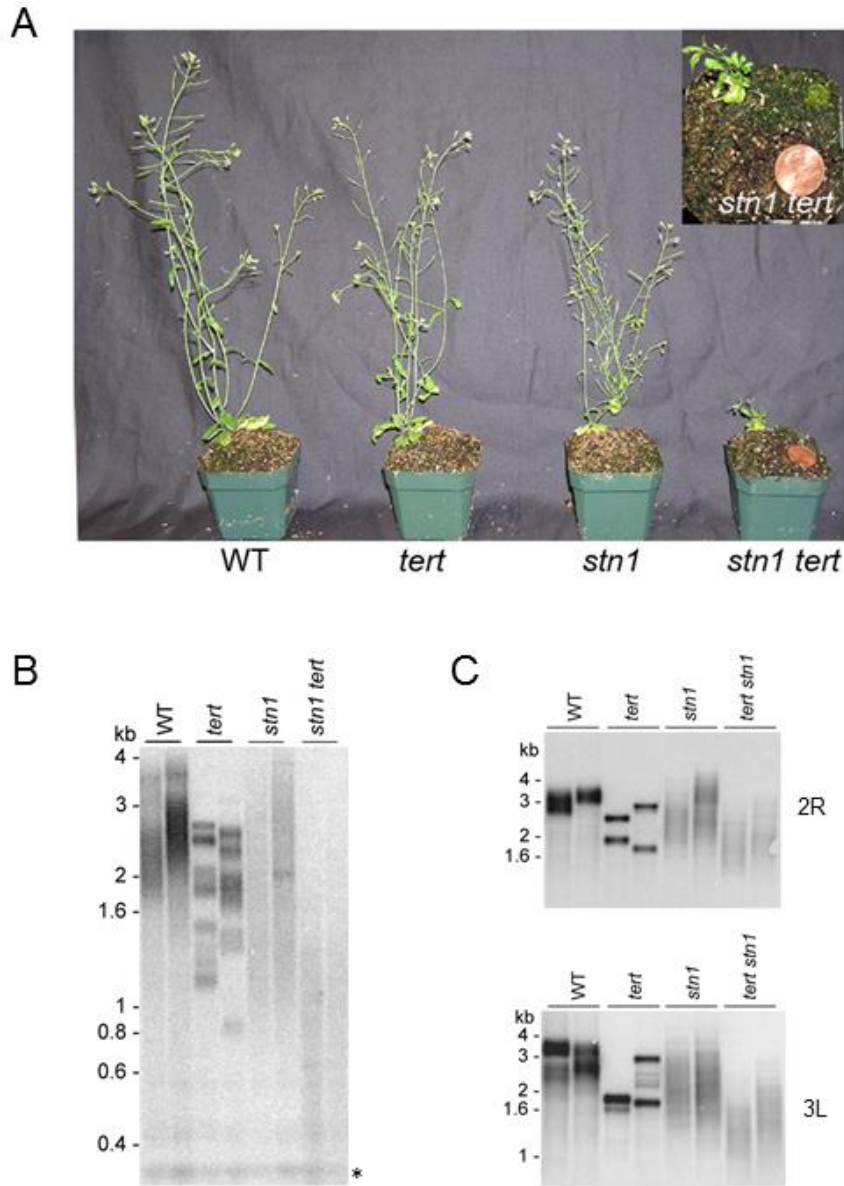
Figure 4-3. STN1 and TERT act synergistically to maintain telomere length.
(A) *stn1 tert* double mutants have a more severe morphological phenotype compared to their single mutant siblings. An enlarged picture of *stn1 tert* is shown in the upper right corner. **(B)** TRF analysis of *stn1 tert* double mutants and their wild type (WT), *stn1* and *tert* mutant siblings. **(C)** PETRA analysis of *stn1 tert* double mutants and their WT, *stn1* and *tert* mutant siblings.

Evidence for increased telomere recombination in plants lacking TEN1 and TERT

Previous analysis of the *ten1-3* mutant indicates that TEN1 may have unique roles in telomere protection and the regulation of telomerase processivity (Leehy et al, 2013). To investigate how TEN1 impacts telomerase-mediated telomere maintenance, we created a *ten1-3 tert* double mutant by crossing *ten1-3* heterozygote and *tert* heterzygote. Seeds from double heterozygous offspring were planted to identify a mutant that was a *ten1-3-/- tert-/-*. In striking contrast to *stn1 tert*, no double mutant was identified from this cross even after extensive genotyping. *TEN1* and *TERT* are unlinked, therefore Mendelian inheritance predicts that 1 in 16 offspring would have a double mutant genotype. Analysis of more than 250 plants failed to identify such a mutant. As an alternative strategy, we planted the offspring of a *tert* null, *ten1-3* heterozygous plant to increase the odds of obtaining a double mutant. Analysis of these offspring led to the recovery of a few *ten1 tert* double mutants. These plants had an extremely severe morphological phenotype (Figure4-4A). Upon reaching the two cotyledon stage, *ten1 tert* mutant growth arrested, the seedlings turned white, and died shortly thereafter. The white color of the *ten1 tert* mutants is especially interesting as TEN1 is primarily localized in the chloroplast with only a small fraction of the protein in the nuclear compartment where it functions in telomere biology (Dr. J.R. Lee, unpublished data). Although the function of TEN1 in the chloroplast in unknown, no other obvious chloroplast-related phenotypes were observed in *ten1-3* single mutants.
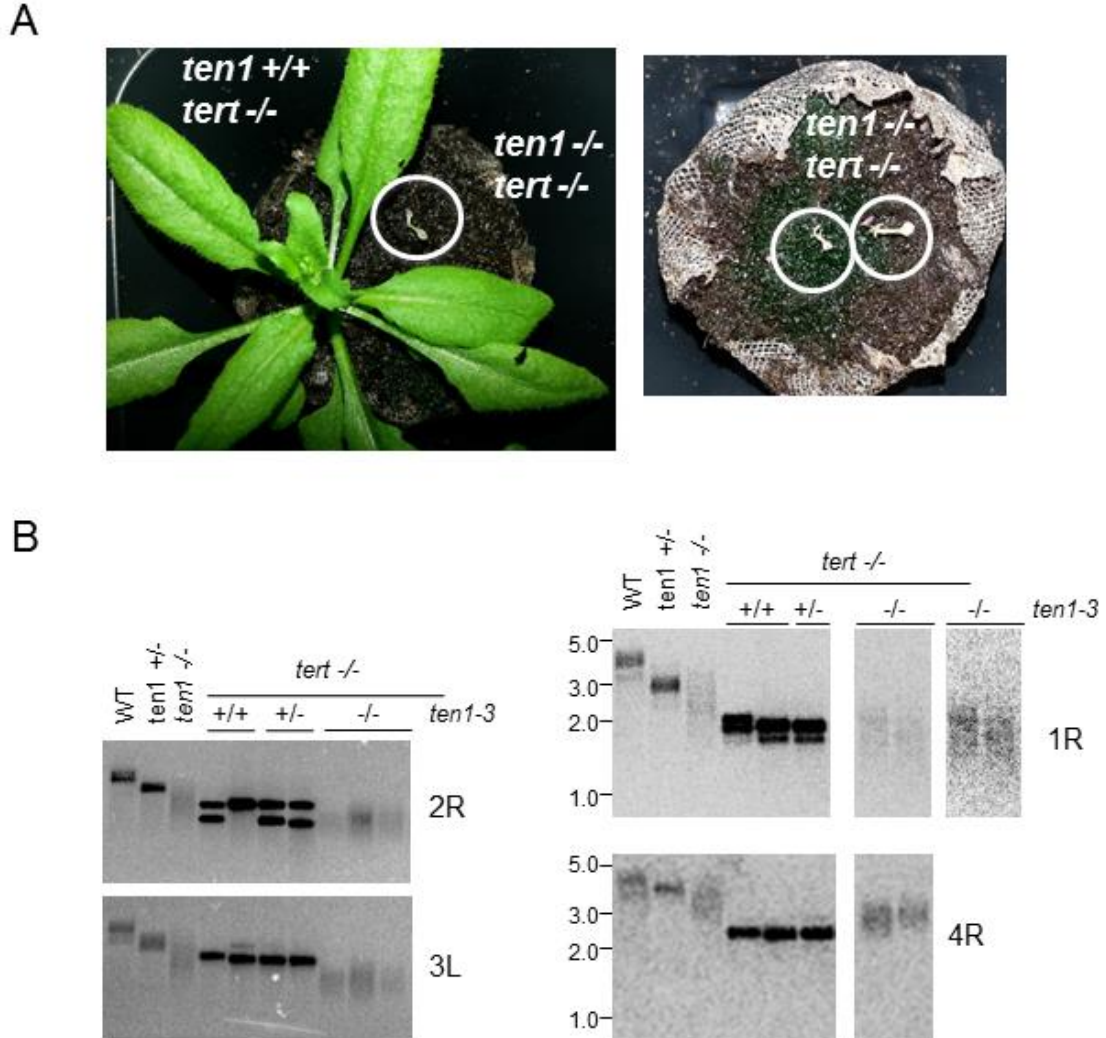
Figure 4-4. Combined loss of TEN1 and TERT leads to severe growth defects and telomere recombination.
(**A**) *ten1 tert* double mutants are indicated by white circles and a *ten1+/+ tert -/-* sibling is shown for comparison. Double mutants arrested growth at the cotyledon stage and turned white. (**B**) PETRA analysis of the offspring of a *ten1+/- tert -/-* plant to measure telomere length on chromosomes arms 2R, 3L, 1R and 4R. A longer exposure the PETRA with primers for chromosome 1R for *ten1 tert* is show to the right of the matched data. Elongated telomeres were observed with some PETRA experiments.

Due to the very small size of *ten1 tert* plants, insufficient DNA was available for bulk telomere length analysis. Therefore, we analyzed telomere length using PETRA. As expected, telomeres in *tert* mutant siblings consisted of discrete homogenous populations shorter than wild type, while telomeres in *ten1-3* mutants were shorter, but more heterogeneous (Figure 4-4B). In *ten1 tert* double mutants some of the chromosome arms (2R and 3L) showed a decrease in telomere length relative to the *ten1-3* siblings, but telomeres on other chromosome arms (1R and 4R) were substantially longer. Because the lengthening of the telomeres occurred in a telomerase-independent manner, the data indicate telomeres in the *ten1 tert* double are subject to increased recombination. Altogether, these data support the conclusion that TEN1 not only plays a role in chromosome end protection as part of the CST complex, but also functions outside the context of CST.

Discussion

The CST complex is required for telomere length maintenance and chromosome end protection in Arabidopsis, but the contribution of individual constituents is not clear. Moreover, it is also unknown how CST interfaces with the telomerase enzyme. In this study we used a genetics approach to more rigorously define the roles of STN1 and TEN1 in telomere length maintenance and chromosome end protection. Our data indicate that these roles are similar, as was previously found for STN1 and CTC1. We also employed a genetic approach to understand how telomerase and CST coordinate telomere maintenance. The results suggest that CST and telomerase independently

contribute to telomere length maintenance. Furthermore, these data supplement the model that TEN1 has additional roles in stabilizing the Arabidopsis genome.

STN1 and TEN1 act in the same genetic pathway to maintain telomeres

The data presented here indicate that STN1 and TEN1 act in the same genetic pathway and are both required for telomere integrity. We found that telomeres in the *stn1 ten1* double mutant have the same length as either single mutant. Moreover, single and double mutants display end-to-end telomere fusions. To further ascertain the roles of STN1 and TEN1, it will be important to more carefully evaluate telomeres in the double mutant, specifically counting the number of anaphase bridges. This will give us a quantitative understanding of the genomic instability resulting from simultaneous loss of STN1 and TEN1 and delineate if they maintain genome stability thorugh the same mechanism. Additionally, we can look for changes in G-overhang signal in *stn1 ten1*. Changes in G-overhang signal would suggest that the telomere replication may be uncoupled or that the C-strand is more/less accessible for nucleolytic degradation (Wei & Price, 2003). Finally, we can examine level of telomere circles in the *stn1 ten1* double mutant which will indicate if telomere recombination is altered in a *stn1 ten1* double mutant. Increased recombination could result from increased G-overhang length or alterations in the telomere architecture (Lustig, 2003).

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CST and telomerase cooperate for telomere maintenance

In yeast, Stn1 negatively regulates telomerase activity, possibly in conjunction with Ten1 (Grandin et al, 2000; Grandin et al, 2001; Grandin et al, 1997; Iyer et al, 2005). Stn1 blocks recruitment of telomerase to the telomere by binding to Cdc13 and preventing the association of the telomerase accessory protein Est1 (Chandra et al, 2001; Lustig, 2001; Qi & Zakian, 2000). In the absence of Stn1 or Ten1 telomeres increasingly elongate in a telomerase-dependent manner. Notably, Arabidopsis *ten1* mutants exhibit increased telomerase RAP (Leehy et al, 2013; Song et al, 2008), thus, it is possible that telomerase may still be recruited to telomeres in the *ten1* mutant but cannot compensate for the massive nuclease attack on the unprotected telomeres. It is unclear if or how CST modulates telomerase functions at the telomere in multicellular eukaryotes. Although telomeres are dramatically shorter in the absence of CST components in plants this effect is so severe that it may mask the failure to recruit telomerase. To understand the interplay between CST and telomerase in Arabidopsis we examined the effects of loss of both telomerase and STN1 or TEN1 on telomere length maintenance.

The telomeres in the *stn1 tert* mutant were shorter than the *stn1* single mutant, a result similar to the telomere shortening seen in *ctc1 tert* double mutants (K. Boltz, unpublished data). These shorter telomeres could be the result of loss of telomerase-mediated telomere extension or loss of chromosome end protection by TERT. Studies in vertebrates indicate that in addition to extension of the chromosome end, TERT is also important for chromosome protection (Kondo et al, 1998; Sharma et al, 2003; Zhu et al, 1999). TERT's protective function is independent of its ability to add telomere repeats

(Kondo et al, 1998; Sharma et al, 2003; Zhu et al, 1999). Parallel genetics experiments in the *stn1 pot1a* double null showed that telomere shortening was similar to *stn1 tert* (X. Song, unpublished data). Because TERT is intact in the *stn1 pot1a* mutants, these data suggest that the accelerated loss of telomeric DNA in plants lacking STN1 and telomerase reflects a failure of telomerase to extend the chromosome end, and not the loss of chromosome end protection by TERT.

Surprisingly, we have been unable to identify a *ten1 tert* double mutant from a *ten1 tert* double heterozygote parent. The mechanism of this potential lethality is unclear. The morphological defects of the *ten1 tert* double were significantly worse than in the *stn1 tert* mutant. The growth of the *ten1 tert* double mutants we obtained was arrested early in development, resulting in a small seedling that quickly turned white. This chlorotic phenotype indicates that there is also chloroplast dysfunction in these double mutants. Notably, the majority of TEN1 is localized in the chloroplast, while only a small fraction is localized in the nucleus for telomere functions (Dr. J.R. Lee, unpublished data). The functional significance of TEN1 in the chloroplast is unclear.

The range of telomere length was greater in *ten1 tert* double mutants, than in *stn1 tert*. Most notably, telomere length was increased on some of the chromosome arms in the *ten1 tert* double mutants. This finding indicates that telomere recombination may be more active in this background leading to elongation of telomeres by a telomerase-independent manner such as alternative lengthening of telomeres (Lustig, 2003). Unfortunately, due to insufficient DNA, we cannot investigate the level recombination in *ten1 tert* double mutants. Telomere recombination can cause elongation or shortening of

the telomere tract (Nabetani & Ishikawa, 2011). Resolution of the t-loop as a Holliday

junction intermediate results in formation of extra-chromosomal telomere circles

(ECTC) (Nabetani & Ishikawa, 2011). ECTCs can be used as a template for alternative

lengthening of telomeres. Loss of TEN1 may encourage recombination due to a change

in the telomere architecture, resulting in branch migration. It is possible that TEN1 may

associate with the single-stranded displacement loop or stabilize the single-strand

invasion site at the base of the t-loop.

Together these data suggest that STN1 and TEN1 act synergistically with TERT

to maintain telomere length, but that TEN1 has additional roles outside of its function in

the CST complex. Our findings are similar to recent reports for human TEN1, which

indicate that loss of TEN1 causes increased telomere dysfunction relative to STN1

(C.Price, personal communication). Historically, CST has been thought to work as a

cohesive trimeric telomere complex, but there is increasing evidence that in addition to

core functions members of CST may make individual contributions to telomere integrity.

CHAPTER V

THE STN1/TEN1 INTERACTION IS REQUIRED FOR CHROMOSOME END PROTECTION

Summary

Telomeres are the ends of linear chromosomes that are required for preventing the DNA damage response (DDR) and providing a mechanism to solve the end replication problem. The majority of the telomeric DNA is double-stranded, the extreme terminus contain a single-stranded 3' extension called the G-overhang. Both the double strand region and the G-overhang are bound by proteins that are important for the functions of the telomere. In *Arabidopsis thaliana*, the CST complex binds to the G-overhang and promotes telomere stability. Mutations in STN1 and TEN1 lead to telomere shortening, increased telomere recombination, telomere-telomere fusions and an increase in the G-overhang signal. TEN1 also serves as a negative regulator of telomerase activity and plays a more important role in the protecting genome stability. The STN1/TEN1 interaction is conserved in all organisms with CST. To understand how that interaction mediates the functions of TEN1 and STN1 in Arabidopsis, we sought to disrupt that interaction and examine the effects on telomeres in vivo using genetic complementation. Preliminary data presented here with $STN1_{\Delta TEN1}$ mutants indicate a separation of function for STN1 in which wild type telomere length is restored, but plants are unable to prevent telomere fusions. Additionally, the design and creation of a library of mutations in STN1 and TEN1 is described. These mutants will be valuable tools to study interactions of STN1 and TEN1 with their other binding partners.

Introduction

Telomeres protect the chromosome terminus from being recognized as DNA damage and facilitate the complete replication of the genome. Telomeres contain tandem repeats of G-rich sequence with the 3' end culminating in a single-strand (ss) 3' protrusion termed the G-overhang. Telomeres disguise the ss region to prevent activation of the DNA damage response (DDR) by binding specialized proteins. In addition, the terminus forms an unusual secondary structure called the t-loop, which secludes the G-overhang by tucking it back into the duplex portion of the telomere. Telomeres that are recognized as DNA damage will be shunted into DNA repair pathways that lead to end-to-end chromosome fusions and entrance into the breakage-fusion-bridge cycle (Cheung & Deng, 2008). Chromosomes can exit this cycle if telomere repeats are added to broken chromosome ends by telomerase in a process called de novo telomere formation (DNTF) (Pennaneach et al, 2006).

Telomerase ribonucleoprotein (RNP) minimally contains a reverse transcriptase (TERT) and a telomerase RNA (TER) (Greider & Blackburn, 1989; Shippen-Lentz & Blackburn, 1990). The two subunits work together to solve the end replication problem by extending chromosomes via the addition of telomeric repeats. TER contains a template corresponding to 1.5-2 copies of the C-rich telomere repeat sequence. This segment anneals to the G-overhang and serves as a template for the addition of telomeric sequence. *Arabidopsis thaliana* is unusual as it harbors three telomerase RNA subunits: TER1, TER2 and TER2s (Cifuentes-Rojas et al, 2011; Cifuentes-Rojas et al, 2012). TER1 forms a canonical telomerase RNP that is active both in vitro and in vivo

(Cifuentes-Rojas et al, 2011). In contrast, TER2 forms an inhibitory telomerase RNP that does not extend telomeres in vivo and negatively regulates telomere addition by the TER1 RNP (Cifuentes-Rojas et al, 2012). TER2 is induced in response to DNA damage and this may help prevent DNTF (Cifuentes-Rojas et al, 2012). TER2 is spliced in vivo to form TER2s (Cifuentes-Rojas et al, 2012). The function of this smaller TER isoform is unknown.

Chromosome end protection is another important function of telomeres. In Arabidopsis, this function is fulfilled by the CST (CTC1/STN1/TEN1) complex (Leehy et al, 2013; Song et al, 2008; Surovtseva et al, 2009). CST (Cdc13/Stn1/Ten1) is a heterotrimeric G-overhang binding complex that was initially identified in *Saccharomyces cerevisiae* (Grandin et al, 2001; Grandin et al, 1997; Lin & Zakian, 1996). Recently, CST was discovered in higher eukaryotes (Miyake et al, 2009; Nakaoka et al, 2012; Song et al, 2008; Surovtseva et al, 2009). In *S. cerevisiae*, the CST complex is required to maintain telomere integrity and to facilitate telomere replication, with mutants exhibiting extensive resection of the telomeric C-strand and the activation of DDR resulting in G2/M cell cycle arrest (Garvik et al, 1995; Grandin et al, 2001; Grandin et al, 1997). CST functions appear to be more specialized in vertebrates and limited to telomeric DNA replication (Huang et al, 2012; Nakaoka et al, 2012; Stewart et al, 2012b; Wang et al, 2012). Chromosome end protection and telomerase recruitment are mediated by shelterin (Longhese et al, 2012).

The initial characterization of the CST complex in multicellular eukaryotes came from studies in Arabidopsis (Leehy et al, 2013; Song et al, 2008; Surovtseva et al, 2009).

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Genetic analysis revealed that CST is important for chromosome end protection, as well as telomere length regulation (Leehy et al, 2013; Song et al, 2008; Surovtseva et al, 2009). CST mutant plants exhibit gross morphological phenotypes indicative of stem cell disorganization, differentiation and death (Boltz et al, 2012; Hashimura & Ueguchi, 2011). The telomeres in these mutants are shorter and more deregulated, with an increase in ss G-overhang signal (Leehy et al, 2013; Song et al, 2008; Surovtseva et al, 2009). Additionally, chromosome end-to-end fusions are abundant, and there is an increase in recombination at the telomere. Interestingly, *ten1-3* mutants exhibit greater genomic instability that *stn1* or *ctc1* mutants. Moreover, they display increased telomerase activity (Leehy et al, 2013). Thus, TEN1 has additional roles beyond those seen for CTC1 and STN1.

Sub-complexes of CST have been described. In mammals, CTC1 and STN1 were independently found as polymerase α accessory proteins, but TEN1 was not a part of that complex (Casteel et al, 2009). In *S. cerevisiae*, over-expression of Stn1 together with Ten1 can prevent activation of DDR in the absence of Cdc13, but telomere length maintenance relies on an alternative mechanism for telomerase recruitment (Petreaca et al, 2006). These data argue that while Stn1 and Ten1 can function as a heterodimer to protect telomeres in the absence of Cdc13, they cannot independently recruit telomerase. Although no Cdc13 homolog has been identified in *Schizosaccharomyces pombe*, Stn1 and Ten1 are critical for telomere integrity in this organism (Martin et al, 2007). Analysis of the *S. pombe* and *Candida tropicalis* STN1/TEN1 crystal structures indicate that STN1 and TEN1 interact via their conserved C-terminal alpha helices (Sun et al,

2009). Finally, genetic experiments in *Candida albicans* indicate that the STN1/TEN1 protein interaction is required for chromosome end protection, telomerase regulation and TEN1 localization at the telomere (Sun et al, 2009).

Members of the CST complex interact with other telomere-associated factors, including the polymerase alpha/primase complex required for telomeric C-strand extension, and proteins that promote telomerase activity like Est1 (yeast) and TPP1 (humans) (Price et al, 2010; Qi & Zakian, 2000; Wan et al, 2009). Studies of the Arabidopsis CST components show that STN1 strongly interacts with both CTC1 and TEN1. STN1 also weakly interacts with POT1a, a component of the TER1 RNP (Leehy et al, 2013; Surovtseva et al, 2009) (X. Song unpublished data). Additionally, CTC1 can also interact with POT1a as well as polymerase alpha (Price et al, 2010) (X. Song unpublished data). Although CTC1 can directly bind TEN1, this association is much weaker (Leehy et al, 2013). Finally, TEN1 can form homo-oligomers and can bind TER2 in vitro (Dr. J.R. Lee, K. Leehy, Dr. C. Cifuentes-Rojas unpublished data). The biological significance of most of the CST interactions is still not clear.

In this study we examine the STN1/TEN1 interaction to better understand how this association promotes telomere stability. We describe a strategy to generate and analyze mutants that disrupt the STN1/TEN1 binding interface and we provide preliminary data that suggests that the two functions of the CST complex, chromosome end protection and length regulation, can be separated. Additionally, we generated a large collection of STN1 and TEN1 point mutants with the goal of disrupting other protein interactions mediated by STN1 and TEN1. These studies will further our

understanding of the functions of the core CST components and help to develop a model for telomere protection and replication in Arabidopsis.

Materials and methods

Protein interaction assays

STN1, TEN1, CTC1 and POT1a cDNA were cloned into pET28a (T7-tag fusion) and pCITE4a vectors (Novagen). Mutant STN1 and TEN1 constructs were created by site-directed mutagenesis of pET28a and pCITE4a vectors containing STN1 and TEN1 sequences using the primers listed in Table 5-1. Primers for site-directed mutagenesis were designed using the QuikChange Primer Design program (Agilent). Primers were designed to introduce a single amino acid change, except for a STN1 E139R/I140A double mutant. Proteins were expressed in rabbit reticulocyte lysate (RRL) (Promega) according to manufacturer's instructions with [^{35}S]-methionine (Perkin Elmer) to label the protein expressed from pCITE4a, and in some cases pET28a. Co-immunoprecipitation was performed as described (Karamysheva et al, 2004). Quantification was performed by calculating the ratio of TEN1: STN1 signal and comparing it with the wild type interaction set to 100%.

Plant materials and Arabidopsis transformation

Seeds for the *stn1-1* mutant were obtained from the ABRC. STN1 complementation was performed as described (Song et al, 2008) with some modifications. STN1 mutants were created using the same site-directed mutagenesis

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primers (Table 1) on STN1 in pENTR. The sequenced pENTR constructs were used to clone STN1 and STN1 mutants into a vector containing a cauliflower mosaic virus (CaMV) promoter and a C-terminal YFP tag with a BASTA resistance gene. The construct was transformed into *Agrobacterium tumefaciens* using electroporation. For Arabidopsis transfection the floral buds of *stn1* heterozygous plants were dipped in *A. tumefaciens* resuspended in 5% sucrose with silwet-77 for 1 m, wrapped in plastic and placed in the dark for 24 h following the previously established protocol (Zhang et al, 2006). The plants were allowed to grow to maturity and once dry seeds were collected. Seeds were selected on MS+ BASTA to confirm the insertion of the transgene. Genotyping for endogenous STN1 locus was performed using primers targeting the sequences adjacent to STN1 (Table 5-1). Confirmation of the transgene was accomplished using a primer complementary to the 5' start of the STN1 coding sequence and a YFP reverse primer (Table 5-1)

RNA analysis

Total RNA was extracted from plant tissue using a plant RNA extraction kit (OMEGA). Reverse transcription was performed with cDNA Supermix (Quanta) per manufacturer's instructions. STN1 mRNA levels were measured by Q RT-PCR with primers to STN1 (Table 5-1) using Sso Fast Eva Green Supermix (BioRad) in accordance with manufacturer's specifications. mRNA levels were normalized to GAPDH mRNA levels in corresponding samples.

TRF, PETRA, and TF-PCR

DNA from individual plants was extracted as described (Cocciolone & Cone, 1993). TRF analysis was performed with 50 μg of DNA as previously described (Fitzgerald et al, 1999). PETRA and TF-PCR were performed as described (Heacock et al, 2004).

Results

Disrupting the STN1/TEN1 interaction in vitro

To determine residues involved in the STN1/TEN1 interaction interface, the Arabidopsis STN1 and TEN1 sequences were threaded onto the *S. pombe* crystal structure of the Stn1 N-terminus bound to Ten1 using Chimera (UCSF) (Figure 5-1). This analysis was performed by Dr. Ming Lei, University of Michigan, and subsequently provided to us. Four residues in STN1 (E139, I140, W143 and L147) were predicted to be in the C-terminal alpha helix that interacts with TEN1. These amino acids were chosen based the residues in the STN1/TEN1 interface in crystal structures of *S. pombe* and *C. albicans*, that were confirmed by amino acid substitution to disrupt STN1/TEN1 binding in vivo (Sun et al, 2009). Additional STN1 residues (W85, L89 and I149) outside of this predicted STN1/TEN1interface were chosen as controls to demonstrate that similar amino acid changes would not affect STN1/TEN1 binding. The amino acid substitutions in our mutagenesis were chosen to disrupt electrostatic interactions (R to E, E to R and L to R) or to disrupt hydrophobic interactions (alanine scanning: I/L/T/W).
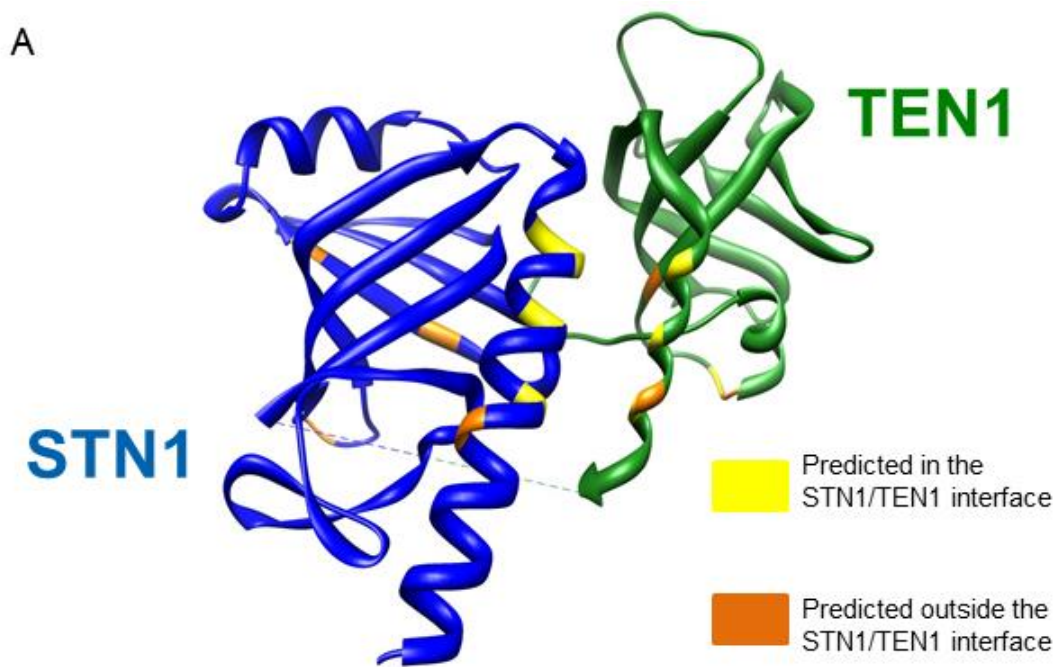
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Figure 5-1. Arabidopsis STN1 and TEN1 threaded onto the *S. pombe* crystal structure.

Chimera (UCSF) was used to thread the protein sequence of *A. thaliana* STN1 and TEN1 onto the *S. pombe* Stn1 N-terminus/Ten1 crystal structure (RCSB Protein Data Bank accession number 3KF6). Blue-STN1 amino acids, Green-TEN1 amino acids, Yellow-amino acids predicted by Dr. Ming Lei (University of Michigan) to be in the STN1/TEN1 binding interface. Orange-amino acids denote STN1/TEN1 binding controls, predicted not to be in the STN1/TEN1 interface.

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Site-directed mutagenesis was performed on STN1 and TEN1 expression

constructs. The amino acid substitutions for STN1 and TEN1 are listed in Figure 5-1.

Two expression constructs, pET28a with a T7-tag and pCITE4a, were generated to

produce only one of the proteins with a T7 tag to allow for co-immunoprecipitation (co-

IP) (Figure 5-2). Recombinant STN1 and TEN1 proteins were expressed in rabbit

reticulocyte lysate (RRL). The proteins were individually expressed, with one protein

containing a T7-tag. The other protein, which lacks a T7-tag, was labeled during

expression with radioactive methionine. After co-incubation, the sample was centrifuged

to remove aggregated proteins and an input sample was taken from the supernatant.

Immunoprecipitation was performed using T7 conjugated agarose beads. These co-IP

assays allow for semi-quantitative analysis of binding affinity for the mutants.

Pull down assays with wild type TEN1 and the STN1 variants predicted to

disrupt the interaction with TEN1 indicated that the $STN1_{E139R}$, $STN1_{I140A}$, $STN1_{W143A}$

and $STN1_{L147A}$ mutations completely disrupted the interaction with TEN1 in vitro

(Figure 5-3A,C). In contrast, $STN1_{L147E}$ maintained a low level of binding with TEN1

(Figure 5-3A bottom panel and C). To confirm that the STN1 point mutations did not

cause gross unfolding of the protein, they were tested for binding to the C-terminal half

of CTC1, previously shown to interact with wild type STN1 (Figure 5-3B, C)

(Surovtseva et al, 2009). The STN1 point mutants retained their ability to bind CTC1,

though $STN1_{L147E}$ did have reduced binding. As negative controls for interaction loss,

point mutations outside of the C-terminal alpha helix of STN1 were also tested for

interaction and found to maintain this interaction ($STN1_{W85A}$, $STN1_{T89A}$)(Figure 5-3C).

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The results of these experiments indicate that amino acids in the STN1 C-terminal alpha helix predicted to mediate associated with TEN1 are required for this interaction in vitro. The STN1 mutants that are unable to interact with TEN1 are subsequently referred to in the text as STN1$_{\Delta TEN1}$.

Based on the crystal structure, TEN1 is thought to use its C-terminal alpha helix for binding to STN1. A similar mutagenesis strategy was used to produce TEN1 variants. Three residues in the C-terminal alpha helix of TEN1 (L105, T109 and L113) and a single residue in the beta barrel (R31) were predicted to be important for the interaction with STN1 (Figure 5-1) (M. Lei, personal communication). As controls, mutations were designed to mimic similar amino acid changes in L30, R37 and alanine mutations for residues L105 and T109. As with STN1, amino acid substitutions were chosen to disrupt electrostatic interactions (L to R, R to E) or using alanine scanning to disrupt hydrophobic interactions (L to A).
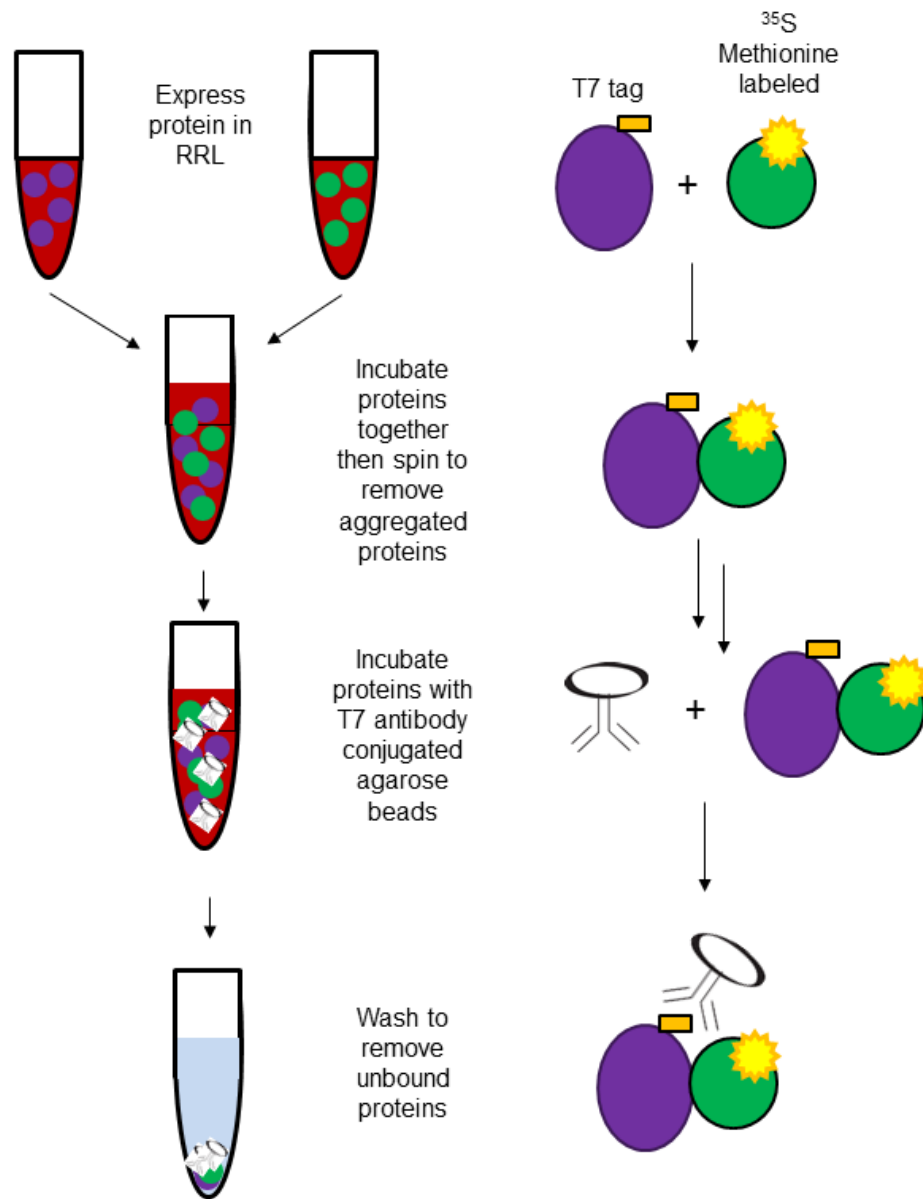
Figure 5-2. In vitro co-IP method.
For binding quantification, both proteins were labeled with radioactive methionine to confirm that there were similar amounts of the tagged protein added to each binding reaction. This was also used for binding calculations to determine the amount of tagged protein pulled down. Additionally, the input lane is a control for the expression level of the untagged protein and confirmation that that protein was not an aggregate that would have been removed prior to the binding reactions.
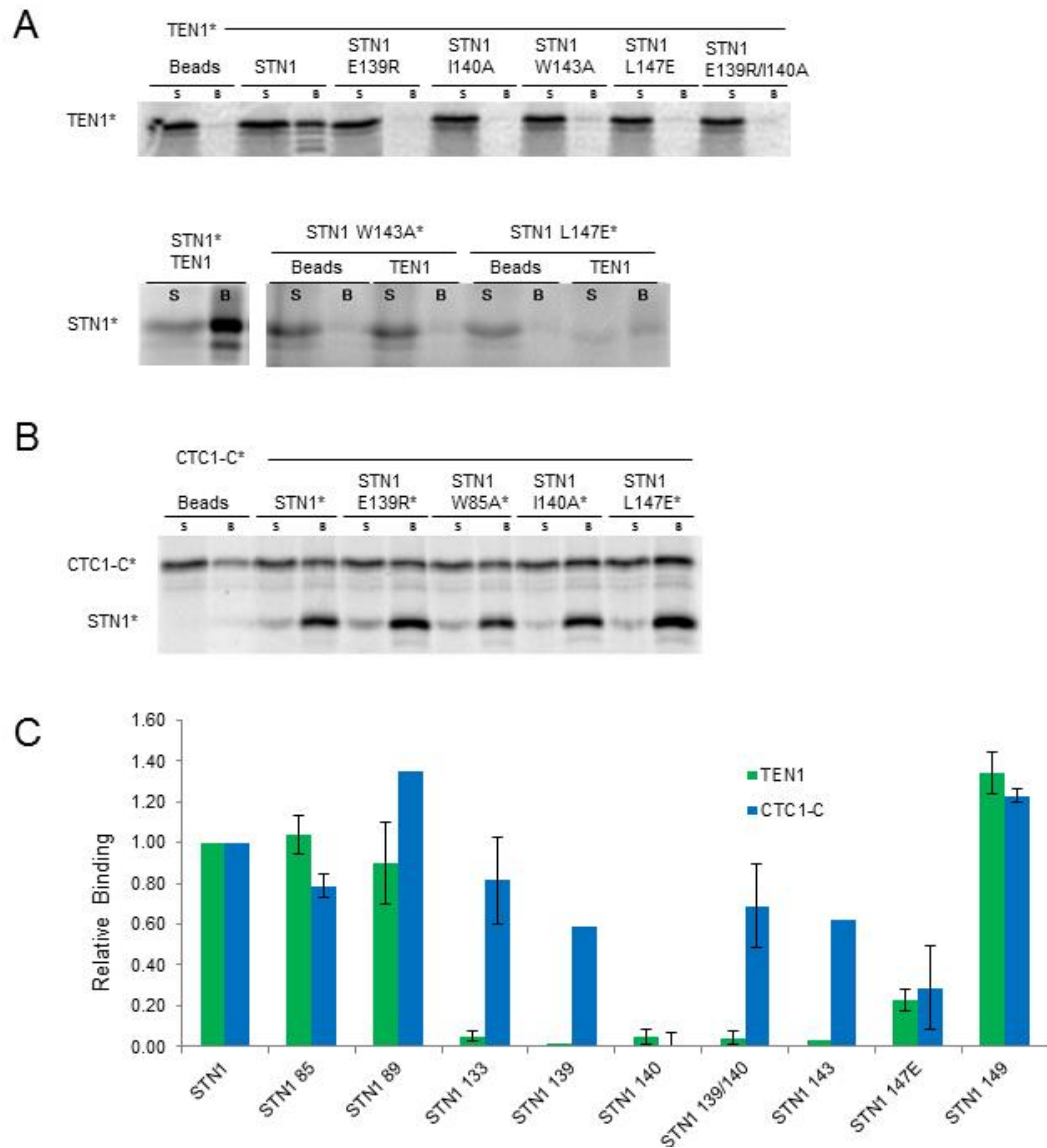
Figure 5-3. Identification of STN1$_{\Delta TEN1}$ mutants.
(A-C) Results of co-immunoprecipitation performed with recombinant proteins to determine in vitro binding for STN1 mutants. In vitro co-IP with T7-tagged STN1 mutant and ^{35}S methionine radiolabeled TEN1 **(A)**, radiolabeled STN1 and CTC1 C-piece. **(B)**. Supernatant (S) was taken after removal of aggregated protein, B denotes protein pulled down by beads. Asterisk-[^{35}S] methionine-labeled protein. **(C)** Quantification of recombinant STN1 mutant protein binding to TEN1. Shown are results of co-immunoprecipitation experiments with recombinant WT TEN1 and WT STN1 and STN1 mutants. The interaction for WT STN1/TEN1 was set to 100%. Error bars reflect standard deviation for at least three replicates, and are not shown for those interactions that have not been tested a minimum of three times.

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We found that the TEN1$_{L105R}$ and TEN1$_{T109R}$ mutations interrupted interaction with STN1, but this was not the case for alanine substitutions at the same site (Figure 5-5A/B). This difference in binding indicates that replacement of leucine at amino acids 105 and 109 with a positively charged arginine hindered the association with STN1, possibly due to a positive charge in the corresponding binding region of STN1.

Notably, the TEN1$_{R31E}$ and TEN1$_{L30A}$ mutations also disrupt the interaction with STN1, though TEN1$_{L30A}$ was not predicted to be in the interaction interface (Figure 5-5A/B). The recombinant protein is soluble, but it is possible that the TEN1$_{L30A}$ subtly alters folding of the protein. Notably, the *ten1-3* phenotype is a result of a point mutation at amino acid G77 TEN1 and the TEN1$_{G77E}$ mutant protein is still expressed and not grossly unfolded (Leehy et al, 2013). Interestingly, although this amino acid is not in the predicated STN/TEN1 interface it does cause a loss of binding to STN1 and telomere integrity is severely affected (Leehy et al, 2013). TEN1 is capable of forming high molecular weight homo-oligomers, but TEN1$_{G77E}$ is known to form higher order structures with greater frequency and of increased molecular weight, possibly facilitating the loss of STN1 binding for TEN1$_{G77E}$ (Dr. J.R. Lee, unpublished data).
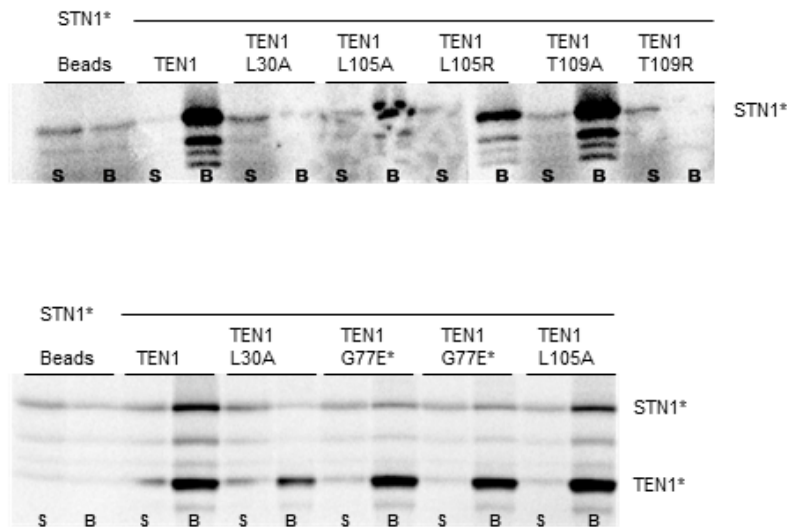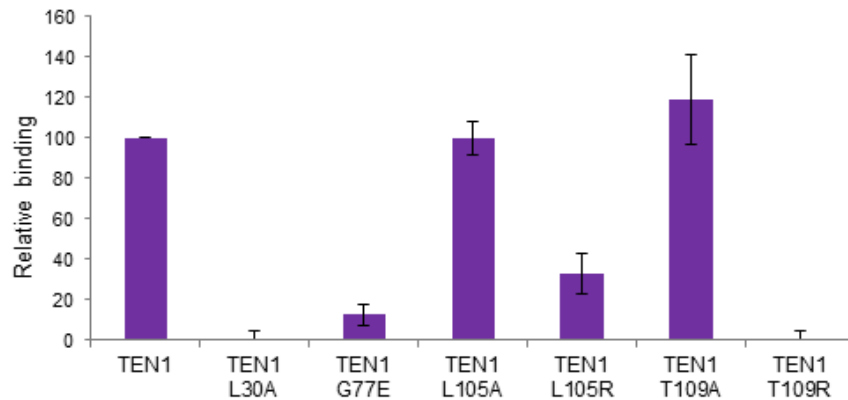
Figure 5-4. Identification of TEN1$_{\Delta STN1}$ mutants

(A-B) Results of co-IP performed with TEN1 mutants. **(A)** In vitro co-IP with T7-tagged TEN1 mutants and ^{35}S methionine radiolabeled wild type (WT) STN1 (top). TEN1 and STN1 are both radiolabeled (bottom). Supernatant (S) was taken after removal of aggregated protein, B denotes protein pulled down by beads. Asterisk-[^{35}S] methionine-labeled. **(B)** Quantification of recombinant TEN1 mutant protein binding to STN1. Shown are results of co-IP experiments with WT STN1 and WT TEN1 and TEN1 mutants. The interaction for WT STN1/TEN1 was set to 100%. Error bars reflect standard deviation for at least three replicates.

Separation of function defined by STN1/TEN1 disruption

In *C. albicans* the Stn1 and Ten1 are important for maintaining telomere length

and the prevention of recombination (Sun et al, 2009). Abrogating the Stn1/Ten1

interaction demonstrated that this association was required for the function of Stn1 and

Ten1 as the telomere phenotype resembled the null mutants (Sun et al, 2009). The

Stn1/Ten1 interaction is also required Ten1 localization at the telomere (Sun et al, 2009).

To understand the role of the STN1 interaction with TEN1 in promoting telomere

integrity and length regulation in Arabidopsis, we performed genetic complementation

using a method previously employed to complement the telomere phenotypes of *stn1*

with a *STN1* wild type transgene (Song et al, 2008). Transgene constructs that express

$STN1_{\Delta TEN1}$ point mutants were transformed into plants carrying a T-DNA insertion in

the *STN1* locus for genetic complementation. The STN1 constructs ($STN1_{E139R}$,

$STN1_{I140A}$, $STN1_{W143A}$ and $STN1_{L147E}$) contained a cauliflower mosaic virus (CaMV)

35S promoter to drive expression, a C-terminal YFP tag to monitor protein expression in

vivo and a BASTA selectable marker to identify transformants. Selected offspring from

transformants were transferred from plates to soil and later the mature plants were

genotyped to determine the state of the endogenous *STN1* locus and the transgene. Plants

null for endogenous *STN1* and expressing the *STN1* transgene were confirmed by RT-

PCR.

The $STN1_{\Delta TEN1}$ mutants were analyzed for telomere defects by primer extension

telomere repeat assay (PETRA) and telomere fusion PCR (TF-PCR). PETRA utilizes

primers specific to different subtelomere regions and a primer that anneals to the single

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strand overhang to assess telomere length on individual chromosomes arms. Wild type

Arabidopsis telomeres range from 2-7 kb (Richards & Ausubel, 1988; Shakirov &

Shippen, 2004). As expected this size was observed for wild type DNA. In contrast, the

telomeres in *stn1-1* null mutants were much more heterogeneous and significantly

shorter, with the shortest telomeres trailing down to 0.8 kb (Figure 5-5 A, left). The

$STN1_{L147E}$ transgene that was able to bind to TEN1 in vitro fully complemented the

telomere length defect of the *stn1-1* null mutant (Figure 5-5A, right). Notably, the

$STN1_{W143A}$ transgene, which failed to bind TEN1 in vitro, was also able to complement

the telomere length defect in a *stn1-1* null background. These findings indicate that a

direct physical interaction between STN1 and TEN1 is not required for telomere length

maintenance in Arabidopsis.

Next, we assessed chromosome end protection in the $STN1_{\Delta TEN1}$ mutants using

telomere fusion PCR (TF-PCR) to monitor the integrity of chromosome ends. TF-PCR

uses primers specific for two different subtelomeric regions to perform a PCR reaction.

The synthesis of a PCR product is dependent on the formation of a covalent bond

between to chromosome ends. A Southern blot is performed with a telomere specific

probe to confirm the presence of telomere repeats in the PCR product. As expected, no

TF-PCR products were generated for wild type Arabidopsis, while a large smear of

products was produced in the *stn1-1* sample (Figure 5-5B left). In *stn1-1* heterozygous

mutants or wild type plants expressing the STN1E139R and STN1I140A transgenes

there was no evidence of telomere-telomere fusions (Figure 5-5B). Furthermore, the

STN1L147E construct, which retains TEN1 binding, was able to fully complement the
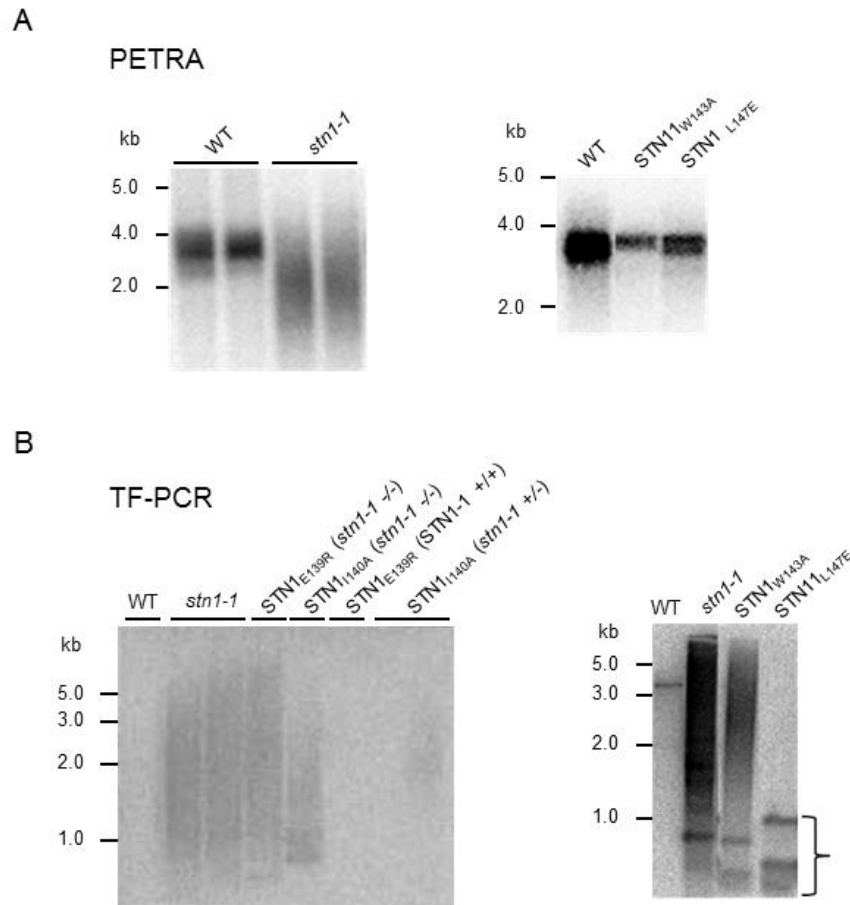
Figure 5-5. STN1$_{\Delta TEN1}$ mutants complement the telomere length defect, but not the chromosome end protection phenotype of a *stn1-1* null mutant.
(A) Results of PETRA on chromosome arm 1L for telomere length measurement are shown for a *stn1-1* null mutant and *stn1-1* null mutants complemented with STN1$_{W143A}$ and STN1$_{L147E}$. **(B)** TF-PCR results for a *stn1-1* null mutant and genetic complementation with STN1$_{\Delta TEN1}$ in wild type, *stn1-1* heterozygous and *stn1-1* null plants (Left). Additional results are shown for TF-PCR with primers for chromosome arms 1L and 2R in *stn1-1* null mutants and genetic complementation with STN1$_{W143A}$ and STN1$_{L147E}$ in *stn1-1* null plants (Right). Bracket indicates spurious PCR products. Each lane shows the results for an individual plant with transgene indicated.

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telomere fusion phenotype in *stn1-1*. In contrast, the STN1 transgenes (STN1E139R, STN1I140A, and STN1W143A), which failed to bind TEN1 by in vitro, displayed telomere-telomere fusions (Figure 5-5B). Thus, these mutants were unable to complement the chromosome end protection phenotype in the stn1 null mutant.

The STN1 $_{E139R}$ and STN1 $_{I140A}$ transgenes did not act as dominant negatives of the endogenous STN1, as there were no telomere fusions in plant expressing both the mutant and wild type STN1 (Figure 5-5B left). Finally, the STN1 $_{L147E}$ mutation that reduced, but did not abolish, the STN1/TEN1 interaction in vitro was capable of complementing both telomere length and chromosome end protection phenotypes of the *stn1-1* mutants (Figure 5-5A/B). Taken together, these data indicate that chromosome end protection is mediated by the STN1/TEN1 interaction, while telomere length maintenance afforded by STN1 and TEN1 does not require their direct binding.

Generation of STN1 and TEN1 point mutants that affect other interaction interfaces

STN1 and TEN1 both have additional protein interaction partners, but these relationships are poorly defined (Figure 5-6). The two proteins can interact with CTC1, although preliminary data suggests that STN1 cannot interact with CTC1 and TEN1 simultaneously (K. Renfrew unpublished data). The C-terminal half of *S. cerevisiae* Stn1 is responsible for Cdc13 binding, but unlike *S. cerevisiae* Stn1, Arabidopsis STN1 does not have this C-terminal extension (Petreaca et al, 2007; Song et al, 2008). Thus, the STN1/CTC1 binding interface is unknown in Arabidopsis. Additionally, Arabidopsis STN1 can interact with POT1a, a component of the TER1 RNP (X.Song unpublished

data), while TEN1 associates with the TER2 RNA subunit, which is part of the inhibitory telomerase TER2 RNP (data not shown) (Cifuentes-Rojas et al, 2012). Finally, TEN1 can form homo-oligomers in vitro, associated with protein chaperone activity (Dr. J.R. Lee unpublished data). Therefore, we set out to generate a large collection of TEN1 and STN1 mutants with the goal of dissecting their many interaction interfaces.

STN1 and TEN1 interactions with other proteins may be conserved (STN1/CTC1) or are evolving (STN1/POT1a, TEN1/TEN1 and TEN1/TER2). Since STN1 and TEN1 appear to have acquired several new interaction partners in Arabidopsis relative to yeast and vertebrates, the amino acids responsible for these interactions are entirely unknown. Alignment of STN1 and TEN1 with their homologs in plants as well as more divergent eukaryotes was used to identify conserved and variable residues in both proteins (Price et al, 2010, Mark Beilstein unpublished data). Based on sequence alignment, we identified residues that were conserved among plant STN1 and TEN1 proteins, and those that were also conserved in vertebrates STN1 and TEN1 proteins (Figure 5-7 and 8). Additionally, we found a few very highly conserved residues that were also present in the yeast Stn1 proteins. We used this information to generate mutations throughout STN1 and TEN1. These mutations are described in for STN1 in Figure 5-7 and TEN1 in Figure 5-8.
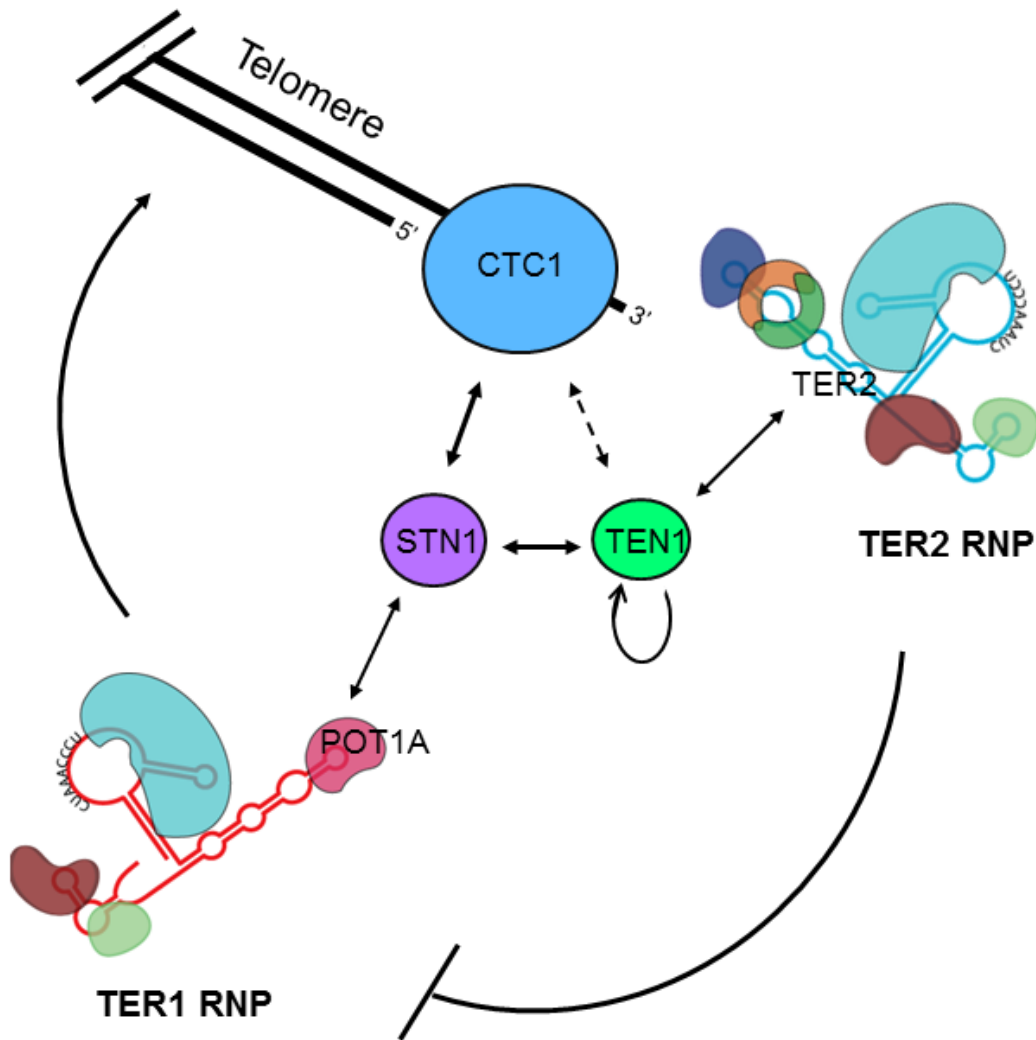
Figure 5-6. STN1 and TEN1 interaction network in *A. thaliana*.
The G-overhang is bound by CTC1. STN1 can interact with CTC1 as well as POT1a, part of the TER1 RNP. The TER1 RNP is the canonical telomerase complex required for telomere maintenance. TEN1 can interact with itself to form homo-oligomers. TEN1 can also interact with TER2, which is part of the TER2 RNP, a negative regulator of the TER1 RNP. TEN1 shows a weak interaction with CTC1.

Figure 5-7. STN1 alignment and mutations designed to disrupt STN1 interactions.
(A) Alignment of Arabidopsis STN1 with STN1 proteins from vertebrates and plants showing conserved residues. AtSTN1 amino acid numbers (top row), AtSTN1 amino acids (second row), consensus vertebrate amino acids (third row) and plant consensus amino acids (bottom row). Asterick denotes amino acids chosen for mutagenesis. Secondary structure, as predicted by PSIPRED, is shown underneath the sequence. Orange amino acids denote residues completely conserved throughout yeast, Arabidopsis, vertebrates and other plants. Yellow amino acids denote residues that are conserved in Arabidopsis, vertebrates and other plants. Green amino acids are those that are similar in Arabidopsis, vertebrates and other plants. **(B)** Amino acid substitutions in STN1 that may disrupt its binding to CTC1, POT1a or other as yet undefined binding partners are shown.

A
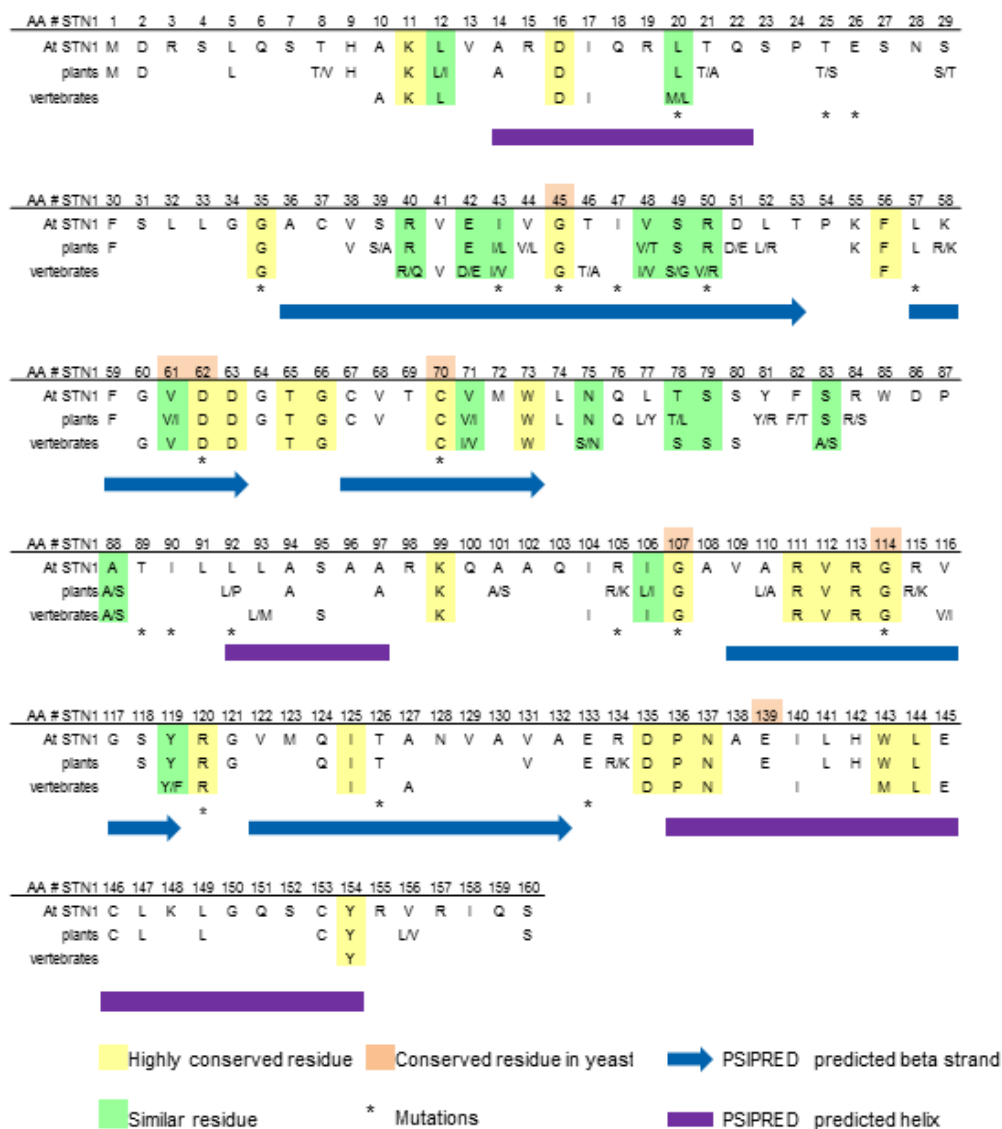
B

STN1 mutants generated

L20A	I47A	T89A	G114A
T25A	R50A	I90A	R120E
E26A	L57A	L92A	T126A
I43R	D62A	R105A	R133E
G45A	C70A	G107A	

Figure 5-8. TEN1 Alignment and mutations designed to disrupt TEN1 interactions.
(A) Alignment of Arabidopsis TEN1 with TEN1 proteins from vertebrates and plants
showing conserved residues. AtTEN11 amino acid numbers (top row), AtTEN1 amino
acids (second row), and plant consensus amino acids (bottom row). Asterick denotes
amino acids chosen for mutagenesis. Secondary structure, as predicted by PSIPRED,
show underneath the sequence. Yellow amino acids are conserved in Arabidopsis and
other plants. Green- amino acids are similar in Arabidopsis and other plants. **(B)** Amino
acid substitutions in TEN1 designed to disrupt homo-oligomerzation or other yet
undefined protein interactions (left) and RNA interactions (right) are indicated.

A total of 19 (out of 160) amino acids in STN1 were chosen for mutagenesis based on conservation, and amino acids most likely to be involved in protein-protein interactions, but were not likely to cause a disruption of the protein structure (Bogan & Thorn, 1998; Lichtarge et al, 1996; Moreira et al, 2007). With advice from Dr. Feng Qiao, now at University of California, Irvine, we made mutations in residues that are hydrophilic, charged or potential substrates for post-translational modifications.

We first chose to mutate STN1 amino acids that are highly conserved through yeast ($STN1_{G45}$, $STN1_{D62}$, $STN1_{C70}$, $STN1_{G107}$ and $STN1_{G114}$). Other amino acids were chosen because they were conserved in multicellular eukaryotes ($STN1_{L20}$, $STN1_{I43}$, $STN1_{R50}$ and $STN1_{R120}$) or in plants ($STN1_{L56}$, $STN1_{L92}$, $STN1_{R105}$, $STN1_{T126}$ and $STN1_{E133}$). A few additional residues that showed no conservation were also chosen ($STN1_{T25}$, $STN1_{E26}$, $STN1_{I47}$, $STN1_{T89}$ and $STN1_{I90}$). Glycine residues lack a side chain providing greater conformational flexibility and they can be important for phosphate binding. Cysteine and aspartate are known to be involved in protein binding sites. Leucine and isoleucine can be involved in hydrophobic interactions, and threonine, arginine and glutamic acid can be involved in electrostatic interactions. Generally we opted for alanine mutagenesis, but for a few residues we chose to replace a hydrophobic residue with a charged residue ($STN1_{I43R}$, $STN1_{R120E}$ and $STN1_{R133E}$).

To disrupt TEN1 interactions we designed 17 mutations throughout the 127 amino acid protein. The TEN1 interaction with TER2 may be unique to *A. thaliana* and therefore we have looked for amino acids that could potentially be involved in RNA binding, but are not necessarily conserved. Positively charged amino acids (arginine,

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lysine and histidine) or amino acids that can participate in bases stacking (tryptophan and tyrosine) are commonly involved in interactions with nucleic acids. Clustering of positively charged or ringed amino acids as well as adjacent glycines are predicted to be important for RNA binding (Terribilini et al, 2006). Therefore, we chose amino acids reflecting these criteria for alanine scanning (TEN1$_{K25}$, TEN1$_{R37}$, TEN1$_{R53}$, TEN1$_{K56}$, TEN1$_{R63}$, TEN1$_{R96}$ and TEN1$_{R107/K108}$). Alanine is not generally involved in protein-RNA interactions. To disrupt TEN1 homo-oligomerization we looked to mutate hydrophobic and charged residues that are conserved (TEN1$_{F23}$, TEN1$_{I45}$, TEN1$_{L55}$, TEN1$_{I62}$, TEN1$_{E78}$ and TEN1$_{L90}$) and unconserved (TEN1$_{L22}$, TEN1$_{E42}$ and TEN1$_{E119}$) that span the majority of the protein. The mutant collections will serve as excellent tools for future studies of STN1 and TEN1 in the Shippen lab.

Discussion

For proteins that have multiple binding partners and complex biological functions, disrupting individual protein interactions may help decipher their discrete contributions and provide insight into a complicated pathway. In this study we generated a large collection of STN1 and TEN1 mutants. A major focus was to identify specific residues that disrupt STN1/TEN1 heterodimer formation. We also provide preliminary data for separation of STN1 function. Our ability to define separation of function mutants is surprising as disruption of this interface in fission yeast causes a null phenotype (Sun et al, 2009), suggesting that STN1 and TEN1 function may have evolved to function differently in Arabidopsis. Further investigation into this apparent

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separation of Arabidopsis STN1 function will be required to confirm our results. First, we must confirm that the STN1/TEN1 interaction is truly abolished in vivo. Second, telomere length and chromosome end protection in these complementation lines must be confirmed and additional control mutations tested in vivo. More data to confirm these results and investigate other telomere phenotypes in these mutants are necessary to more fully describe the contribution of the STN1/TEN1 interaction. It will also be interesting to determine if our point mutant proteins, STN1$_{\Delta TEN1}$ and TEN1$_{\Delta STN1}$ are properly localized at the telomere.

Disrupting the STN1 and TEN1 interaction

These data presented here, while preliminary, provide exciting evidence that the functions of chromosome end protection and telomere length maintenance can be separated for STN1. They indicate that the STN1/TEN1 interaction may be important for the prevention of telomere-telomere fusions, but not for telomere length maintenance. It may be that STN1 and TEN1, even when unable to bind each other, are capable of preventing exonucleolytic attack and recombination at the telomere, thereby preventing significant loss of telomere length. However, disruption of the STN1/TEN1 interaction may lead to activation of the DDR and creation of chromosome end–to-end fusions by non-homologous end-joining in G1 (Ferreira et al, 2004; Symington & Gautier, 2011), suggesting that the STN1/TEN1 interaction is required in G1 for telomere protection. These data contrast the findings for the disruption of Stn1/Ten1 in *C. albicans*, which results in a defect in telomere length maintenance similar to the *stn1* and *ten1* null

mutants (Sun et al, 2009). This indicates that STN1 and TEN1 have evolved in Arabidopsis to function differently from yeast, possibly due to interactions with other telomere factors.

Disrupting other STN1 and TEN1 interactions

Defining the residues that promote STN1 and TEN1 interactions in vivo will be an important tool that can provide insight into the specific function of these proteins. In vitro co-IP is a reliable method for assessing protein-protein interactions. In yeast, Stn1 negatively regulates telomerase recruitment to the telomere via interaction with Cdc13 (Grandin et al, 2000; Grandin et al, 1997). Disrupting the STN1/CTC1 interaction will reveal if there is a similar mechanism for telomerase recruitment in Arabidopsis. Disrupting the STN1/CTC1 interaction may also cause loss of TEN1 from the telomere since the CTC1/TEN1 interaction is weak. TEN1 negatively regulates telomerase RAP (Leehy et al, 2013) and TER2 is part of an inhibitory RNP (Cifuentes-Rojas et al, 2012). To determine if TEN1's negative regulation is via its interaction with TER2, the ability to disrupt this interaction will be vital. Finally, members of the Arabidopsis CST complex have evolved a number of interactions with telomere factors that have not been observed in yeast or vertebrates. Determining the importance of these interactions will help to elucidate the mechanisms for chromosome end protection, telomere length maintenance and regulation of telomerase activity for STN1 and TEN1, and in a broader sense how the telomere complex is evolving in plants.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Telomeres are a conserved feature of linear eukaryotic chromosomes, essential for protection and maintenance of genome integrity. Though the telomere sequence and the associated factors differ among plants, vertebrates and yeast, their core functions of protecting the chromosome end from recognition as DNA damage and solving the end replication problem are retained. The gradual telomere loss associated with the inactivation of telomerase is eventually lethal, but the uncapping of the telomere due to mutations in critical telomere associated proteins has immediate and devastating effects on the genome. The focus of this dissertation is on one of the most highly conserved telomere protection complexes, CST (Cdc13(CTC1)/Stn1/Ten1).

The work described in this dissertation provided the first characterization of the CST complex in higher eukaryotes. Indeed, for each of the three CST components, our lab was the first to publish a detailed characterization of the protein in vivo. These studies revealed severe stem-cell related defects caused by profound telomere failure. The CST analysis is a testament to why *A. thaliana* is such a powerful model system for the investigation of telomeres. Since the first publication describing STN1 function in Arabidopsis in 2008 (Song et al, 2008), the study of the CST complex in multicellular eukaryotes has exploded. Within the last year mutations in CTC1 have been shown to cause humans as stem-cell related disease due to telomere defects (Anderson et al, 2012; Polvi et al, 2012; Walne et al, 2012). These findings in humans further validate the

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importance of the CST complex in telomere integrity, and the power of Arabidopsis the model to inform telomere biology for all higher eukaryotes.

The CST complex is conserved and required for telomere integrity

CST was originally thought to be confined to budding yeast, where it serves to protect the telomere from nucleolytic attack, block activation of the DDR and to facilitate telomeric DNA replication by telomerase and polymerase α (Price et al, 2010). However, these functions are associated with shelterin in mammals and fission yeast, negating the obvious need for CST in these organisms (Palm & de Lange, 2008). Discovery of STN1 and TEN1 in *S. pombe* and CST in vertebrates led to the obvious question of functional redundancy between shelterin and the CST heterotrimer complex.

There is still much debate in the field about the role of CST at telomeres. Conflicting reports in mouse and human cells have failed to resolve the role of CST in telomerase regulation and telomere protection (Chen et al, 2012; Gu et al, 2012; Wang et al, 2012). Interestingly, the one function of the CST complex that seems to be highly conserved from yeast to humans is its role in promoting C-strand fill-in by polymerase α/primase after telomerase extension of the G-overhang (Casteel et al, 2009; Huang et al, 2012; Nakaoka et al, 2012; Qi & Zakian, 2000; Wang et al, 2012). Recent data also support the conclusion that human CST promotes replication outside of the telomere by stimulating restart of stalled replication forks (Stewart et al, 2012b). Similarly, Arabidopsis CTC1 interacts with the catalytic subunit of polymerase α, though the function of this interaction has not been investigated (Price et al, 2010).

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Does Arabidopsis CST control C-strand fill-in?

The regulation of C-strand fill-in seems to be a conserved feature of CST (Casteel et al, 2009; Grossi et al, 2004; Nakaoka et al, 2012; Qi & Zakian, 2000; Wang et al, 2012), but in Arabidopsis this function is not clear. In vitro data indicate that CTC1 interacts with pol α, but we found no pol α interaction for STN1 or TEN1 in vitro. To examine the contribution of the complex to C-strand fill-in we could study the effects of concurrent loss of CST members and a mutation in the catalytic subunit of pol α. If CTC1 (or CST) is required for pol α recruitment or activity there should be no change in the telomere length, G-overhang length or genomic instability for these double mutants. Such experiments could help determine whether CST has a conserved role in replication at telomeres across all eukaryotes.

Arabidopsis CST is required for telomere maintenance

Like yeast, Arabidopsis CST binds to ss telomeric DNA and is required for chromosome end protection and telomere length maintenance (Dr. J.R. Lee, unpublished data) (Leehy et al, 2013; Song et al, 2008; Surovtseva et al, 2009). Loss of any of the three members of the complex causes catastrophic loss of telomere length and an increase in the ss overhang, ultimately resulting in telomere recombination and massive genome instability. In contrast, vertebrate CST shows little specificity for ss telomeric DNA and has not been implicated in chromosome end protection (Miyake et al, 2009). Thus, the CST complex appears to be evolving in relation to other telomere-associated factors.

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The data presented in this dissertation indicate that Arabidopsis TEN1 has additional roles outside the context of CST. Unlike STN1 and CTC1 which co-localize with half of the Arabidopsis telomeres, presumably the ones with G-overhangs, TEN1 was only found at a small fraction of telomeres (Leehy et al, 2013). This suggests that TEN1 may have some functions away from the telomere in a capacity yet to be defined. Additionally, *ten1-3* mutants display a significant increase in genomic instability compared to *stn1* and *ctc1* mutants, indicating a more critical role in chromosome end protection for TEN1. Finally, we found that TEN1 is a negative regulator of telomerase repeat addition processivity (RAP), indicating that it may be important for regulating the ability of telomerase to remain associated with the telomere. Notably, knock-down of TEN1 in human cells has been associated with an increase in telomere-related phenotypes (C. Kasbek & C. Price, unpublished data). These data are consistent with conclusions in Arabidopsis, and argue that TEN1 is required for CST mediated telomere integrity, but also makes unique contributions to telomere replication and genomic stability.

Is CST function restricted to the heterotrimer?

Other than the interesting contributions that TEN1 apparently makes to telomerase regulation and genome stability discussed above, mutations in any of the CST components result in same general telomere phenotype, suggesting that loss of one of the components of this heterotrimeric complex is detrimental to function of the whole complex. It is possible that loss of one component causes dissociation of the remaining

proteins from the telomere. Preliminary data from Dr. J.R. Lee in our lab indicate that Arabidopsis CTC1 binds to ss telomeric DNA, and that binding is increased in the presence of STN1 and TEN1. STN1 can also bind DNA independently of CTC1 and TEN1, but this binding is not sequence-specific. We know from studies in *C. albicans* that STN1 is required for TEN1 localization, though STN1 is still able to bind telomeres in the absence of TEN1 (Sun et al, 2009). Additionally, over-expression of STN1 and TEN1 in *S. cerevisiae* can rescue the telomere phenotype of a *cdc13* mutant (Grandin et al, 2001; Petreaca et al, 2006). These data suggest that a Stn1/Ten1 subcomplex is capable of associating with the telomere and protecting it the absence of functional Cdc13, though recruitment of pol α is required for this rescue (Petreaca et al, 2006).

To understand the effect of the loss of a single protein on localization of the remaining proteins in Arabidopsis, we can look for telomere localization of the remaining CST components in the *stn1, ctc1* and *ten1-3* mutant backgrounds. Another way this could be examined is by performing telomere chromatin immunoprecipitation (ChIP). We could also examine the formation of sub-complexes in vivo by immunoprecipitation of individual CST components, followed by gel filtration to separate complexes by size. We could then looked for the known interacting proteins pulled down by western blotting or use mass spectrometry to look for other associated proteins. These experiments will demonstrate if the telomere defects in the CST single mutants are caused by loss of telomere binding by the remaining CST components. They will also reveal information about the formation of CST sub-complexes in vivo.

The results presented in Chapter IV demonstrate that the *ten1-3 stn1* double mutant has the same telomere length phenotype as the *ten1-3* and *stn1* single mutants, but the morphological defects for the double mutant are more severe. We know from data presented in Chapter III that TEN1 has a more profound role in maintaining genomic stability and an additional role in regulating telomerase activity. It will be important to examine the effects of the *ten1-3 stn1* double mutant on genomic integrity and telomerase activity. To determine if the double mutant has a synergistic effect on the genomic stability, we can examine rapidly dividing tissue for anaphase bridges. We can then compare the values obtained from the double mutant to the *ten1-3* and *stn1* single mutant siblings. If these genes act synergistically, there should be an increase in anaphase bridges compared to *ten1-3*. In the absence of TEN1, STN1 may cause an increase in telomere recombination, which could be measured by comparing the levels of extra-chromosomal telomere circle (ECTC) formation in the *ten1 stn1* double mutant. Likewise, STN1 may be important for activation of the DNA damage response (DDR) in the absence of TEN1. This possibility can be examined by measuring the level of DDR transcripts in the *ten1-3 stn1* double mutants and comparing them to their single mutant siblings.

Another interesting experiment would be to look at the effect of loss of STN1 and TEN1 on telomerase activity. In budding yeast, Stn1 is a negative regulator of telomerase activity, perhaps in conjunction with Ten1 (Grandin et al, 2001). In the Arabidopsis *ten1-3* mutant, I found a 2.5 fold increase in telomerase activity due to an increase in telomerase RAP. In contrast, there was no significant change in telomerase

activity for the *stn1* mutant. STN1 also interacts with POT1a, a positive regulator of telomerase RAP (X. Song unpublished data) (Surovtseva et al, 2007). Thus, there may be a role for Arabidopsis STN1 in regulation of telomerase activity, which is disguised in the presence of TEN1. We can measure the activity in the *ten1-3 stn1* double mutant by quantitative telomerase repeat amplification protocol (qTRAP) and compare it to single mutant siblings. If the telomerase activity deviates from the *ten1-3* mutant activity it will indicate that STN1 is important for regulation of telomerase activity. A decrease in telomerase activity relative to the *ten1-3* mutant would suggest that STN1 positively regulates telomerase activity possibly by interacting with one of the telomerase RNPs. This model could be further tested using the telomerase processivity assay to test for changes in RAP. These data would help to better understand the additional roles described for TEN1 by understanding how this protein functions in relation to STN1.

What role does the STN1/TEN1 interaction play in telomere maintenance?

In *C. albicans*, abrogating the Stn1/Ten1 interaction causes telomere lengthening, similar to loss of Stn1 or Ten1, suggesting that this interaction is required for telomere length maintenance (Sun et al, 2009). Additionally, Ten1 interaction with Stn1 is required for the recruitment of Ten1 to the telomere. Based on the *S. pombe* Stn1/Ten1 crystal structure (Sun et al, 2009), I mutated residues in Arabidopsis STN1 to disrupt its interaction with TEN1. Preliminary data presented in Chapter V suggests that disrupting the STN1/TEN1 interaction caused a separation of function for telomere length regulation and chromosome end protection. The $STN1_{L147E}$ mutant, which maintained

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some binding to TEN1 in vitro, was able to complement the *stn1* null mutant for both length maintenance and chromosome end protection in vivo. Thus, even partial STN1/TEN1 binding is enough for these functions. Interestingly, all of the STN1$_{\Delta TEN1}$ mutants, we tested that failed to bind TEN1 in vitro, were able to complement the telomere length phenotype of the *stn1* null, but not the chromosome end protection phenotype. This observation suggests that the STN1/TEN1 interaction is required to prevent the activation of the DDR, and disrupting the interaction results in telomere-telomere fusions.

Further studies to confirm that disruption of the STN1/TEN1 interaction causes a separation of function are necessary. Potential caveats that need to be addressed include verifying transgene protein expression in the complemented plants and confirming that the STN1/TEN1 interaction is truly disrupted in vitro. To facilitate these studies, experiments are currently underway using Flag-MYC and Flag-HA tagged STN1 constructs under control of a CaMV 35S promoter to monitor transgenic protein expression.

It will also be interesting to monitor STN1 and TEN1 localization at telomeres in the absence of STN1/TEN1 binding. In fission yeast, Ten1 association with Stn1 is required to recruit Ten1 to the telomere (Sun et al, 2009). Using the tagged proteins fluorescence in situ hybridization (FISH) can be performed to look for telomere localization of STN1 and TEN1. Comparing the co-localization percentage of STN1 and TEN1 in STN1$_{\Delta TEN1}$ to wild type STN1 complementation lines will demonstrate if the STN1/TEN1 interaction is required for telomere localization of these proteins. TEN1

interacts weakly with CTC1, and this interaction may be sufficient for recruiting TEN1 to the telomere. Since telomeres in the STN1$_{\Delta TEN1}$ complementation line do not resemble the *ten1-3* mutant, it is most likely that TEN1 is still associated with the telomere in these plants.

Once the binding and expression are confirmed in vivo, more individual plants for each of the STN1$_{\Delta TEN1}$ mutants need to be tested to determine how the disruption of the protein interaction interface affects telomeres. To more fully characterize the importance of the STN1/TEN1 interaction it will be important to examine the telomere architecture. G-overhang length can be affected by the uncoupling of G and C-strand synthesis and the access of nucleases to the C-strand (Price et al, 2010). To determine if the STN1/TEN1 interaction is important for mediating these functions, one could look for a change in the G-overhang signal in the STN1$_{\Delta TEN1}$ complementation line. The most likely hypothesis for an increase in G-overhang signal is uncoupling of telomerase extension and C-strand fill-in (Chakhparonian & Wellinger, 2003). There may be an increase in G-overhangs for STN1$_{\Delta TEN1}$ mutants, as longer G-overhangs are seen in *stn1* and *ten1-3* mutants (Leehy et al, 2013; Song et al, 2008).

Abnormally long G-overhangs can cause telomere rapid deletion by causing branch migration and the formation of Holliday junctions resulting in ECTC formation (Lustig, 2003). The presence of telomere circles, as by-products of recombination, can be investigated using the t-circle assay (Zellinger et al, 2007). Preliminary data indicate that telomere length is wild type in these STN1$_{\Delta TEN1}$ complementation lines, suggesting that there may be no increase in ECTCs.

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Preliminary data indicates that telomere fuse in the absence of direct

STN1/TEN1 interactions. It would be interesting to clone these products, both to

confirm that they are indeed telomere-telomere fusions and to characterize the state of

these fusions by determining the amount of telomeric and subtelomeric DNA lost prior

to these events occurring. The telomere length data for STN1$_{\Delta TEN1}$ suggest that there may

not be significant loss of telomere length prior to fusion events occurring, but it will

need to be empirically tested. Because PETRA selects allows amplification of only intact

telomeres with a G-overhang, this data does not provide any information on the telomere

length of fused chromosomes. Thus, a subset of telomeres may undergo telomere rapid

deletion, followed by a fusion event. Understanding the architecture of the fusion

junction can provide clues about the events the happen just prior to end-joining. Finally,

the level of anaphase bridges should also be examined in STN1$_{\Delta TEN1}$ complementation

lines, as this analysis will provide a quantitative estimate of genomic instability in the

mutants. Since the *stn1* and *ten1-3* mutants have different levels of genomic instability, it

will be interesting to see if the anaphase bridges more closely reflect those found in *stn1*

or *ten1-3*.

One hypothesis is that telomere fusions and consequent genomic instability arise

due to the inability to prevent the DDR. Lethality in yeast CST mutants is the result of

the activation of the DDR in late S/G2 phase due to increased ss telomeric DNA (Garvik

et al, 1995; Grandin et al, 2001; Grandin et al, 1997). CST is more abundant at the

telomere during late S/G2 phase, indicating that the complex may be most important for

telomere capping during this phase of the cell cycle. Alternatively, this interaction may

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be required for telomere protection in G1, when the machinery for non-homologous end-joining is active (Symington & Gautier, 2011). Data presented in appendix II define a role for CTC1 and STN1 in preventing the activation of the DDR via ATR mediated pathways (Boltz et al, 2012). TEN1 is also important for preventing activation of the DDR (Hashimura & Ueguchi, 2011). To investigate if the STN1/TEN1 interaction is required for the prevention of the DDR, we can measure the mRNA levels of DDR associated transcripts BRCA1 and PARP1, which are upregulated in CST mutants (Boltz et al, 2012; Hashimura & Ueguchi, 2011). If the STN1/TEN1 interaction is required for prevention of the DDR, there will be an increase in these transcripts. We can also examine the effect that disrupting the STN1/TEN1 interaction has on stem cell death, as CST mutants exhibit a stem cell death in roots (Boltz et al, 2012; Hashimura & Ueguchi, 2011).

While investigating the telomere-related defects in the *ten1-3* mutant, we unexpectedly discovered that TEN1 is a negative regulator of telomerase RAP. To understand what role the STN1/TEN1 interaction has on RAP, we can examine the telomerase activity in STN1$_{\Delta TEN1}$ mutants using qTRAP and processivity TRAP. As there is no significant change in telomerase activity in *stn1* mutants, it is unlikely that telomerase activity will be affected by disrupting the binding interface, but this must be tested. If telomerase activity in this mutant is increased, it would will suggest that TEN1's role in the regulation of telomerase activity is mediated by its interaction with STN1, but that loss of the STN1 protein does not confer this same affect due to its own

role in telomerase regulation (see earlier discussion of a potential role for STN1 in modulating telomerase activity).

In addition to mutations in the STN1 interface, parallel experiments should be performed with TEN1 to create TEN1$_{\Delta STN1}$ mutants. In vitro assays to identify such mutants were described in Chapter V and a number of TEN1$_{\Delta STN1}$ constructs are now available to use for in vivo complementation. However, one potential problem with pursuing this goal is the lack of a clean knock-out for TEN1 in *A. thaliana*.

During our initial characterization of TEN1 in Arabidopsis, I analyzed the available T-DNA insertions in TEN1, knocked-down TEN1 using anti-sense constructs and attempted a gene targeting strategy to knock-out TEN1. The T-DNA insertion that was mapped to TEN1 was found to be in the 5' UTR, resulting in only ~50% knock-down of TEN1 mRNA and only a slight perturbation of telomere length. The most penetrant TEN1 anti-sense construct resulted in 80% knock-down of TEN1 mRNA, which caused telomeres to shorten slightly and become more heterogeneous. Unfortunately, the TALEN gene targeting method that we chose was unable to knock-out the TEN1 gene. This method has yet to be proven successful in the creation of knock-outs in Arabidopsis plants. Fortuitously, a point mutation in TEN1 was discovered by the Ueguchi lab in a screen for plants with stem-cell defects (Hashimura & Ueguchi, 2011). I was able to capitalize on this finding to characterize the telomere phenotype of the mutant, *ten1-3*, which is described in Chapter III. Complementation experiments for the TEN1$_{\Delta STN1}$ can be initiated *ten1-3* mutants. These complementation experiments may be more difficult to interpret than the experiments with STN1$_{\Delta TEN1}$, as

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the *ten1-3* mutant does not result in loss of TEN1 protein by western blot. However, TEN1$_{G77E}$ does not interact with STN1 in vitro and is unable to perform the telomere related functions of TEN1. Additionally, the complementation with a wild type TEN1 transgene rescues the defect of the *ten1-3* mutant (Hashimura & Ueguchi, 2011; Leehy et al, 2013). Ideally, TEN1$_{\Delta STN1}$ mutants should be used to complement a complete null for TEN1, and so attempts to identify or create a true *ten1* null mutant should continue.

Are telomeres protected in a double mutant by the remaining CST component?

To understand the entire contribution of CST in Arabidopsis a *stn1 ctc1 ten1-3* triple mutant should be analyzed. While the double mutants (*stn1 ctc1* and *stn1 ten1-3*) are viable and have a similar telomere phenotype, there may be some telomere protection afforded by the remaining CST component. If the remaining complex member makes an independent contribution to telomere protection, this triple mutant would have a more severe phenotype or may even be lethal. These experiments would demonstrate if CTC1, STN1 and TEN1 require heterotrimer formation for their function, or if they can retain some functionality in the absence of other subunits.

The role of the CST complex in telomerase recruitment

The CST complex in yeast is thought to be highly dynamic, with different interacting partners binding at different times in the cell cycle (Price et al, 2010). Telomerase has increased association with the telomere through interactions with CST during late S phase when telomeres are extended (Chandra et al, 2001; Garvik et al,

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1995; Taggart et al, 2002). Cdc13 binds to the G-overhang and recruits telomerase via an interaction with Est1 to extend the chromosome end (Nugent et al, 1996; Qi & Zakian, 2000). This recruitment is blocked by the binding of Stn1 and potentially Ten1 (Chandra et al, 2001; Grandin et al, 2001; Grandin et al, 1997). Phosphorylation of Cdc13 mediates the exchange between Stn1 and Est1 (Gao et al, 2010; Li et al, 2009). In Arabidopsis, POT1a is believed to be a functional homolog of Est1 as it binds to the TER1 subunit in telomerase, is a positive regulator of telomerase activity and interacts with CTC1 (Cifuentes-Rojas et al, 2011; Surovtseva et al, 2009; Surovtseva et al, 2007). Notably, STN1 and POT1a interact in vitro (X. Song, unpublished data), suggesting another possible mechanism for telomerase regulation. To determine the role of these CST interactions in Arabidopsis, we can disruption them and examine how such mutations affect telomere maintenance and telomerase activity. As described in Chapter V, we generated a large collection of point mutations that can be studied to find amino acids that are required for STN1 interaction with CTC1 and POT1a.

To understand what role a STN1/CTC1 sub-complex plays at telomeres in Arabidopsis, we will screen through our STN1 mutant library to identify proteins that disrupt the interaction with CTC1. Unlike budding yeast Stn1 (Petreaca et al, 2006), Arabidopsis STN1 lacks the C-terminal domain that is the binding interface for Cdc13 (Song et al, 2008). For this reason we cannot predict where CTC1 will bind to STN1. However, due to its small size, STN1 is a good target for mutagenesis to map the STN1/CTC1 binding interface.

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After identification of point mutants that disrupt the STN1/CTC1 interaction, genetic complementation in the *stn1* background can be performed. By disrupting the STN1/CTC1 interaction, we can elucidate the function of this interaction in vivo. ChIP could be used with a TERT antibody to determine if there in a change in telomerase recruitment to the telomere when the STN1/CTC1 binding interface is disrupted. It would also be important to determine if there is a separation of function when the STN1/CTC1 binding is disrupted, as our preliminary results suggest for the STN1/TEN1 interaction mutants. Finally, looking at localization of the CST complex members in the absence of STN1/CTC1 binding will determine if this interaction is required to recruit the complex to the telomere, and if not which of the three proteins can still interact with the telomere.

It will be important to confirm the STN1/POT1a interaction in vivo. If this interaction is validated, disrupting the STN1/POT1a interaction will help to elucidate the biological relevance of this interaction. One hypothesis is that POT1a recruits telomerase to the telomere through interactions with STN1 and/or CTC1. By abrogating the STN1/POT1a interaction we can test the STN1/POT1a recruitment hypothesis by correlating decreased binding with changes in telomere length. *pot1a* mutants display gradual telomere shortening that is similar to the telomere shortening phenotype in *tert* mutants (Surovtseva et al, 2007). If loss of STN1/POT1a binding prevents the recruitment of telomerase, then the STN1$_{\Delta POT1a}$ mutant should have a telomere phenotype similar to *pot1a* mutants. If the interaction with STN1 is not required for telomerase recruitment or blocks recruitment, there would not be telomere shortening. A

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more direct method of looking at the recruitment of telomerase to the telomere would be to perform ChIP in the STN1$_{\Delta POT1a}$ background to look for TERT association with the telomere. While there is no change in telomerase activity for *stn1-1*, there is a ten-fold reduction in telomerase activity in the *pot1a* mutant (Song et al, 2008; Surovtseva et al, 2007). Therefore, if the STN1/POT1a binding away from the telomere is inhibitory to telomerase activity, loss of binding in might cause a decrease telomerase activity.

STN1 and TEN1 act in concert with telomerase to maintain telomere length

To determine if telomerase has a role in maintaining telomere length in *stn1* mutants, we examined the effect of concurrent loss of TERT and STN1. Data presented in Chapter IV show that *stn1 tert* double mutants have increased morphological defects and telomere shortening compared to their *stn1* single mutant siblings. These data suggest that STN1 and TERT act in different genetic pathways to maintain telomere length due to the additive effect of losing both. An intriguing alternative hypothesis is that telomerase plays a protective role at telomeres (Khanna et al, 2001; Sharma et al, 2003; Zhu et al, 1999) aside from its catalytic telomere extension role. Loss of two "capping" components in TERT and STN1 could then lead to the additive phenotype we observed.

To determine if telomerase has a protective function in addition to its more carefully characterized catalytic functions, we could complement a *tert* null mutant with a catalytically dead TERT transgene. The transgene would have to be transformed into *stn1 tert* heterozygous plants and then checked for expression of the TERT mutant

protein. Then, the telomere phenotype of a *stn1 tert* double null expressing the catalytically inactive TERT could be compared to the *stn1* null and the *stn1 tert* double mutant for telomere length. If telomere length is the same as the *stn1* mutant it will indicate that the shortening in the *stn1 tert* mutant is likely due to loss of whatever protective role TERT may perform. If telomeres are as short as in the double mutants, it would mean that the catalytic role of telomerase is predominant function of TERT at telomeres. Telomeres with a length that falls between the two extremes would indicate that telomerase is important for both telomere length maintenance and protection in *stn1* mutants.

Due to the increased telomerase activity in *ten1-3* and the novel ability of TEN1 to bind TER2 in vitro, we also wanted to examine telomeres in a *ten1 tert* double mutant. As described in Chapter IV the identification of a double mutant was complicated. The effect of the *ten1 tert* double mutant was even more severe than the *stn1 tert* mutant, with the plants arresting growth at an early stage in seedling development, turning chlorotic and then dying shortly after. No other telomere mutants, or combinations thereof, have been found to cause this morphology, again indicating that TEN1 has additional roles that have yet to be defined. Interestingly, in contrast to *stn1 tert* mutants, these double mutants displayed hallmarks of ALT. Due to the small size of the plants no analysis of G-overhangs could be performed to define the mechanism for ALT. Genetic complementation with a catalytically inactive TERT could also be used in the *ten1-3 tert* background to explore the off telomere more of TEN1, as well as defining a mechanism for telomere elongation in these mutants. If TERT is required for telomere protection in

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a *ten1-3* mutant, it may be possible to recover larger plants that would allow for a more thorough analysis of telomere architecture and genome stability. With larger plants it may also be possible to explore the cell death within the stem cell niche of the root. These data may provide additional evidence to more precisely define the role of TEN1 at the telomere and identify other possible roles away from the telomere.

In *A. thaliana* TEN1 negatively affects RAP (Leehy et al, 2013) and the TER2 RNP negatively regulates TER1 RNP activity (Cifuentes-Rojas et al, 2012). To determine if the TEN1 and TER2 pathways for regulating telomerase activity are related, we looked for direct interaction between TEN1 and TER2. In collaboration with Dr. C. Cifuentes-Rojas, I examined the binding of TEN1 to TER2 in vitro. Preliminary data, in conjunction with data from Dr. J.R. Lee and X. She, indicate that TEN1 preferentially binds to TER2, though we cannot rule out binding with TER1. These data suggest that the two mechanisms for negatively regulating telomerase activity may be related. To test this hypothesis, I have crossed *ten1-3* and *ter2* mutants. If increased RAP is due to loss of TER2 recruitment to the telomeric DNA by TEN1, a *ten1-3 ter2* double mutant would have the same increased telomerase activity and repeat addition processivity as the single mutants.

To identify residues in TEN1 that mediate its RNA binding, filter binding assays should be performed with the point mutant collection described in Chapter V. After assessing the in vitro interactions, TEN1 constructs containing the mutant proteins could be used in genetic complementation assays to determine their TER2 binding and how disrupting this interface will affect telomeres in vivo. These constructs would be

transformed into *ten1-3* heterozygous mutant plants. Pull down of the tagged TEN1 could be done using commercially available antibodies for the tag or our TEN1 antibody, followed by semi-quantitative RT-PCR to assess binding in vivo. Experiments to test the effect on telomerase activity, telomerase RAP, and to assess telomere integrity should be employed to characterize the function of this interaction in vivo.

An alternative model is that TEN1 and the TER2 RNP represent two different pathways for repressing telomerase activity. To investigate specifically the role of TEN1 in modulating telomerase at the telomere, a TEN1-STN1 or TEN1-CTC1 fusion protein could be created and used to complement the *ten1-3 stn1* or *ten1-3 ctc1* double mutant backgrounds and assess the effect on telomerase activity and recruitment by ChIP. This strategy would prevent the dissociation of TEN1 from the telomere. By tethering TEN1, we can examine the effect on telomere length and telomerase activity to determine if TEN1 dissociation is responsible for mediating telomerase access to the telomere. The fusion constructs may have other consequences for the plant, as evidence suggests additional divergent roles for TEN1.

Does TEN1 have additional, non-telomeric roles?

Interestingly, immunofluorescence data suggests that TEN1 may be primarily localized in the chloroplast with a small fraction of the protein in the nucleus to perform telomere-relation functions (Dr. J.R. Lee unpublished data). As previously discussed, *ten1-3 tert* double mutant are chlorotic indicating that TEN1 may be important for chloroplast function. Our data with TEN1 are not the first indication of a telomere-

related protein functioning outside of the nucleus, and vice versa. In Arabidopsis, a splice variant of CP31, a chloroplast RNA editing protein, interacts with telomerase and binds to ss telomeric DNA in vitro and may have a role in telomere protection (Kwon & Chung, 2004; Yoo et al, 2010). Moreover, in human cells, 10-20% of TERT localizes to the mitochondria and has been implicated a variety of protective functions within the mitochondria including oxidative stress protection and blocking apoptotic pathways in response to chemotherapy drugs (Chiodi & Mondello, 2012).

Work done by Dr. J.R. Lee in our lab has shown that TEN1 homo-oligomerizes and that higher molecular weight structures have chaperone activity in vitro. Disrupting TEN1 homo-oligomer formation will allow us to examine the role of this oligomerization in vivo. Therefore, it will be important to determine whether any of the TEN1 mutants in my collection block TEN1 oligomerization. Unfortunately, it would not be possible to test how such a mutant functions in the *ten1-3* mutant as the TEN1$_{G77E}$ protein is more likely to form homo-oligomers in vitro (Leehy et al, 2013). However, the effect on chaperone functions of TEN1 and the effect on binding to other proteins can be tested in vitro for TEN1 proteins that can no longer self-associate.

Does CST act in concert with other telomere capping proteins to promote telomere architecture in Arabidopsis?

This dissertation has covered in detail the chromosome end protection function of the CST complex in *A. thaliana*, but CST is not the only factor implicated in telomere stability. Half of the telomeres in plants are blunt-ended and do not require CST for their

protection, but instead require Ku (Kazda et al, 2012). In addition to CST and Ku, there

is also evidence that POT1c is required for chromosome end protection (Dr. A. Nelson

unpublished data). POT1c is one of three POT1 orthologs in *A. thaliana*. Unlike the

ancestral POT1 in the earliest diverging land plant, *P. patens*, these three *A. thaliana*

POT1 paralogs do not bind to the ss G-overhang, and instead bind to the telomerase

RNAs (Cifuentes-Rojas et al, 2012; Shakirov et al, 2009; Shakirov et al, 2005;

Surovtseva et al, 2007). Like TER2, POT1c is a unique duplication, present in only *A.*

thaliana (Dr. A. Nelson, unpulished data) (Beilstein et al, 2012). Knock-down (KD) of

POT1c by RNAi leads to dramatically shorter, more heterogeneous telomeres, similar to

CST mutants. However, in contrast to CST mutants, POT1c KD causes decreased G-

overhang signals (Dr. A. Nelson, unpublished data). This decrease in signal could

indicate that all the telomeres are blunt-ended, as has previously been reported for half of

the Arabidopsis telomeres, or that a C-overhang is formed, a phenomenon observed in *C.*

elegans (Kazda et al, 2012; Raices et al, 2008). In addition, there are no apparent

telomere fusions or anaphase bridges with POT1c mutants, despite the fact that

telomeres are extremely short (Dr. A. Nelson. unpublished data). These observations

suggest that activation of the DDR is somehow blocked in plants depleted of POT1c,

possibly due to the telomeres still being capped either by the Ku blunt-end capping

complex or binding of CST or another factor to C-overhangs. Preliminary data suggests

a weak interaction between CTC1 and POT1c in vitro, but it will be important to

confirm this interaction in vivo. Moreover, it may be informative to knock-down POT1c

in a CST mutant background and examine the effect on telomeres in vivo. A synergistic

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effect on telomeres would indicate that CST and POT1c make unique contributions to telomere stability and perhaps reveal an ancient connection between CST and the shelterin-like protein POT1c. Such experiments will expand our understanding of telomere capping complexes in *A. thaliana*.

Conclusions

In summary, this dissertation describes the identification and characterization of Arabidopsis CST as a telomere complex important for maintaining telomere repeats, stabilizing telomere architecture and preventing activation of the DDR. This work has helped to expand the field of research on CST from single-cell to multi-cellular eukaryotes, leading to the identification of human disorders related to CST malfunction. TEN1 has been identified as having additional roles in telomerase regulation and promoting genome integrity, in addition to potential non-telomere roles in the chloroplast. A large mutant collection was created that will be an important tool to further define the functions of STN1 and TEN1 interactions both within and outside the CST complex. Finally, these data provide evidence that CST is an ancient telomere complex that is rapidly evolving to promote telomere integrity through new interaction partners.

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APPENDIX I

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 ~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 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 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 G-overhang from harmful activities (de Lange, 2004; Wei and Price, 2003), while double-strand (ds) and single-strand (ss) telomeric DNA binding proteins coat the chromosome terminus to further distinguish it from a double-strand 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 (Gao et al., 2007; Lundblad, 2006). 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 (Guo et al., 2007; Mitton-Fry et al., 2002). 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 (Bianchi et al., 2004; Chandra et al., 2001), while Stn1 is thought to inhibit telomerase action by competing with Est1 for Cdc13 binding (Li et al., 2009; Puglisi et al., 2008). In addition, Cdc13 and Stn1

contribute to coupling of G- and C-strand synthesis through interactions with DNA polymerase α (Grossi et al., 2004; Qi and Zakian, 2000).

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., 2001; Grandin et al., 1997). Telomere protection appears to be facilitated primarily by Stn1 and Ten1, and overexpression of Stn1 and Ten1 can rescue the lethality of Cdc13 depletion (Grandin et al., 2001; Petreaca et al., 2007; Puglisi et al., 2008). Finally, Cdc13 and Stn1 also inhibit telomere recombination (Iyer et al., 2005; Petreaca et al., 2006; Zubko and Lydall, 2006).

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 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 (Hockemeyer et al., 2005; Veldman et al., 2004; Yang et al., 2005; Ye et al., 2004). 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 (Churikov and Price, 2008; Denchi and de Lange, 2007; Guo et al., 2007).

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 (Miyoshi et al., 2008; Xin et al., 2007). 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 CST components 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 from Arabidopsis bind telomeric dsDNA in vitro (Zellinger and Riha, 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. Nelson, Y. Surovtseva and D. Shippen, unpublished data). 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 vivo. Deletion of AtSTN1 results in the rapid onset of growth defects and sterility, coupled with extensive exonucleolytic degradation of chromosome ends, increased telomere recombination, and massive end-to-end chromosome fusion (Song et al., 2008).

Here we report the identification of a new telomere protein, termed CTC1 (Conserved Telomere maintenance Component 1), that physically and genetically interacts with AtSTN1. We show that AtCTC1 localizes to telomeres in vivo and, as for AtSTN1, 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 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 (Casteel et al., 2009; Goulian and Heard, 1990). 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). Cloning, telomere assays and cytology, including FISH, are described in supplemental methods.

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

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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 or CaPO4. The final concentration of

siRNA duplex (see supplemental methods for sequences) was 50 nM (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 of 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 sample. The

normalized values were plotted relative to the mock-transfected control that was set to

100%. All reactions were performed in duplicate.

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 previously uncharacterized 142 kDa protein (Figure A1-1A). RT-PCR demonstrated that CTC1 is widely expressed in both vegetative and reproductive organs (Figure A1-S1A). Further analysis of the CTC1 protein sequence is discussed below.
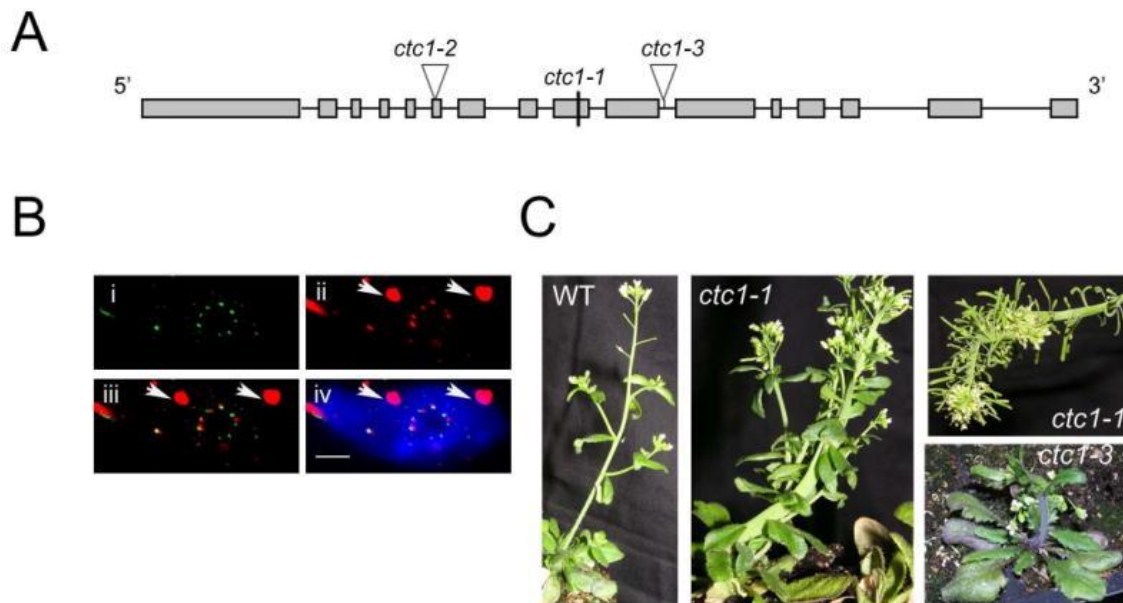
Figure A1-1. Identification of CTC1 in Arabidopsis thaliana.
(A) Top, schematic of the AtCTC1 gene locus. Rectangles represent exons; horizontal black lines are introns. The positions of the point mutation (*ctc1-1*) and T-DNA insertions (*ctc1-2* and *ctc1-3*) are shown. CTC1 associates with telomeres in vivo.

To determine whether CTC1 associates with telomeres in vivo, 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 (Figure A1-1B, Figure A1-S1B 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 (Armstrong et al., 2001) (Song et al., 2008) and, as expected, telomeric FISH signals were positioned in this location. Similarly, CFP-CTC1 was distributed in a punctate pattern surrounding the nucleolus. A merge of these images showed that much of the CFP-CTC1 co-localized with Arabidopsis telomeres (Figure A1-1B and Figure A1-S1B). 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 co-localization with telomeres was retained in non-cycling cells, we examined the apical half of rosette leaves that were at least two 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 (Figure A1-1A). 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 (Figure A1-1A). 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 (Figure A1-S1C).

All three *ctc1* mutants displayed a rapid onset of severe morphological defects in the first generation (Figure A1-1C), 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 (Figure A1-1C). Although most mutants produced an influorescence bolt, this structure was highly variable in size, ranging from very short to wild type (Figure A1-1C, 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, which spanned 2-5 kb in length (Figure A1-2A, lanes 1 to 4), telomeres in homozygous *ctc1-1* mutants were severely deregulated (Figure A1-2A, 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 (Figure A1-S2A).
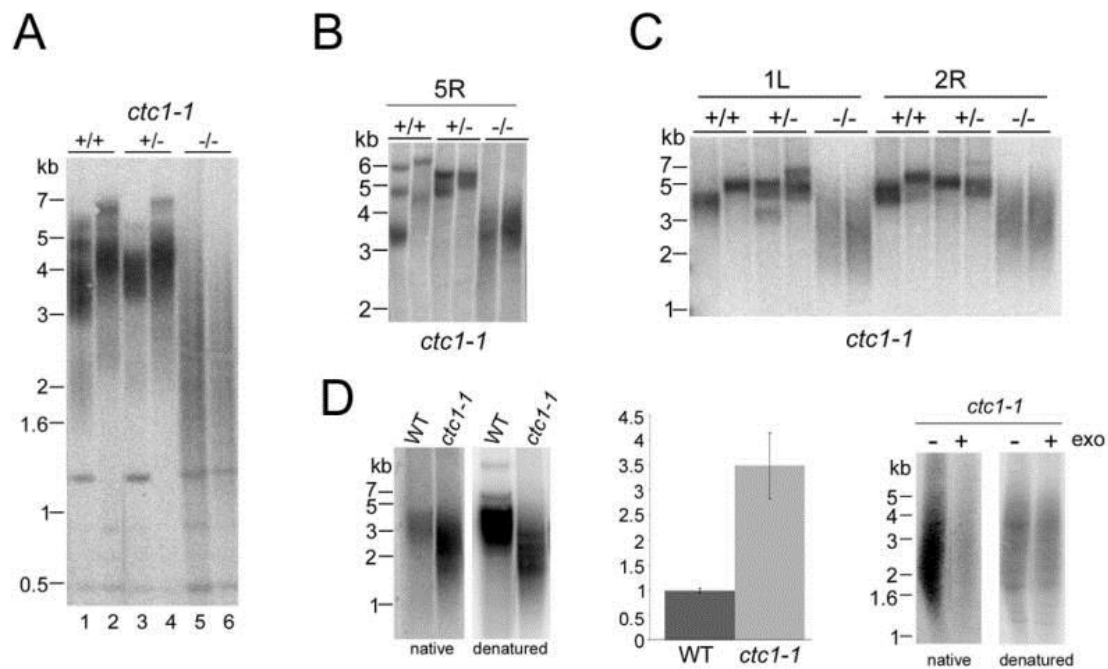
Figure A1-2. Telomere length deregulation and increased G-overhangs in AtCTC1 mutants. (A) TRF analysis of *ctc1-1*. Results are shown for progeny segregated from a parent heterozygous for *ctc1*. (B) Subtelomeric TRF analysis of DNA from *ctc1-1* mutant. DNA blots were hybridized with a probe corresponding 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 (C3TA3)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 8 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 panels A and C, blots were hybridized with a radiolabeled telomeric DNA probe (T3AG3)4. Molecular weight markers are indicated.

We investigated how individual telomeres were affected by CTC1 loss using subtelomeric TRF analysis with probes directed at specific chromosome termini. As expected (Shakirov and Shippen, 2004), sharp bands were produced from wild type telomeres (Figures A1-2B and S2B). In contrast, telomeres in *ctc1* mutants gave rise to a broad heterogeneous hybridization signal spanning 1.5 kb (Figures A1-2B and A1-S2B). Primer extension telomere repeat amplification (PETRA) also generated broad smears in *ctc1* mutants (Figure A1-2C), confirming that the length of individual telomere tracts was grossly deregulated. 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 (Figure A1-S3).

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) (Figure A1-2D). A similar increase in G-overhang signal is observed in Arabidopsis stn1 mutants (Song et al. 2008). Exonuclease treatment reduced the G-overhang signal in *ctc1* mutants by approximately 95%, indicating that the majority of ss telomeric DNA is associated with the chromosome terminus (Figure A1-2D, left panel).

To investigate whether telomeres in *ctc1* mutants are subjected to increased recombination, we used t-circle amplification (TCA) (Zellinger et al., 2007) to look for evidence of extra-chromosomal telomeric circles (ECTC), 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 (Figure A1-3A). 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 (Figure A1-3B). 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.
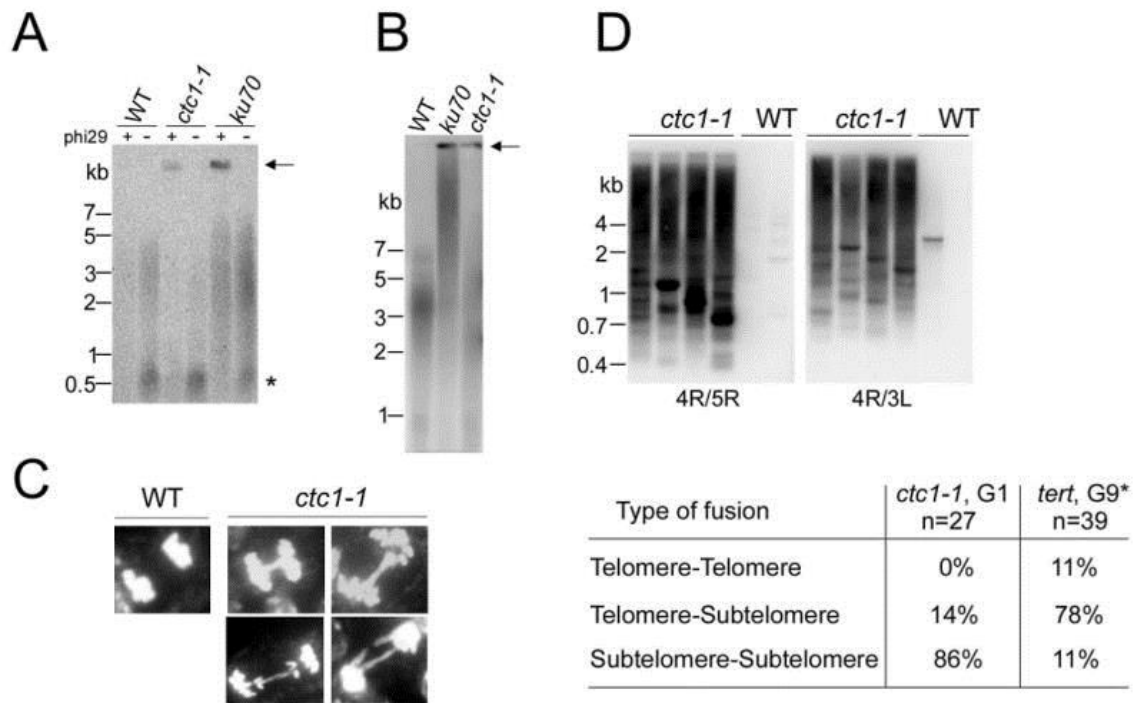
Figure A1-3. *ctc1-1* mutants display elevated telomere recombination and end-to-end fusions. (A) T-circle amplification with *ctc1-1* DNA. Reactions were performed in the presence or absence of phi29 polymerase. ku70 DNA was used as a positive control. (B) Bubble trapping results for *ctc1-1* and ku70 mutants. All blots were hybridized with a radiolabeled telomeric probe. In panels 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.

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 instability in wild type plants, but in all four *ctc1-1* mutants a high fraction of mitotic cells (up to 39%) exhibited anaphase bridges (Figure A1-3C and Table S1). Many anaphases contained multiple bridged chromosomes as well as instances of unequal chromosome segregation (Figure A1-3C). 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. 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.

FISH-labeling to identify chromosome ends present in anaphase bridges from Arabidopsis ctc1-1 mutants.

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 (Figure A1-3D 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 (Figure A1-3D), 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.

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. The *ctc1 stn1* double mutants were viable, and the severity of

morphological defects was similar to the single mutants (Figure A1-S4A). TRF analysis

and PETRA revealed the same heterogeneous, shortened telomere profile in double

mutants as in the single mutants (Figures A1-4A and S4B). Similarly, G-overhang signal

intensity and the level of ECTC were comparable, implying that *ctc1-1 stn1-1* double

mutants did not undergo additional telomeric DNA depletion or increased telomere

recombination (Figures A1-4B and A1-S4C). Finally, the frequency of anaphase bridges

was similar in double mutants and their *ctc1* and *stn1* siblings (Table S2). Altogether

these findings indicate that AtCTC1 and AtSTN1 act in the same pathway for

chromosome end protection.
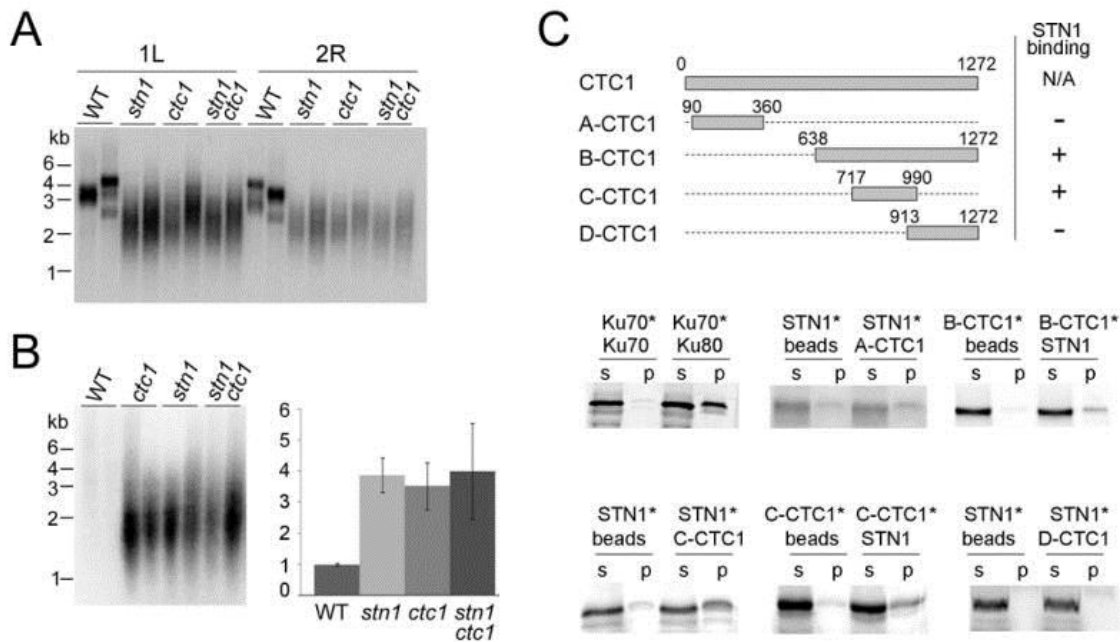
Figure A1-4. AtCTC1 and AtSTN1 function in the same genetic pathway for chromosome end protection and physically interact in vitro.
(A) PETRA analysis of telomere length with DNA from *ctc1-1 stn1-1* double mutants, and their *ctc1-1*, *stn1-1* and wild type siblings. (B) G-overhang analysis using in-gel hybridization. Native gel and quantification results (the average of 6 independent experiments ±SD) are shown. P≤0.005 for all mutant samples compared to wild type, and P≥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, co-immunoprecipitation experiments conducted with recombinant full length AtSTN1 and truncated AtCTC1 fragments A-D. Asterisks indicate 35S-methionine labeled protein; the unlabeled protein was T7-tagged. S, supernatant; P, pellet. Ku70-Ku80 interaction was the positive control.

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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 35S 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 assays (Figure A1-4C). The STN1/C-CTC1 interaction was confirmed in a yeast two hybrid assay (data not shown). These data indicate that AtSTN1 and AtCTC1 directly interact in vitro and hence may also associate with each other in vivo.

Genome instability in human cells depleted of CTC1

TBLASTN and EST database searches revealed CTC1 homologs in a wide range of plant species, and searches using PSI-BLAST and HHpred uncovered putative CTC1 orthologs in many vertebrates (see supplemental material for details). 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 human POT1 (Figures A1-5A and A1-S5).

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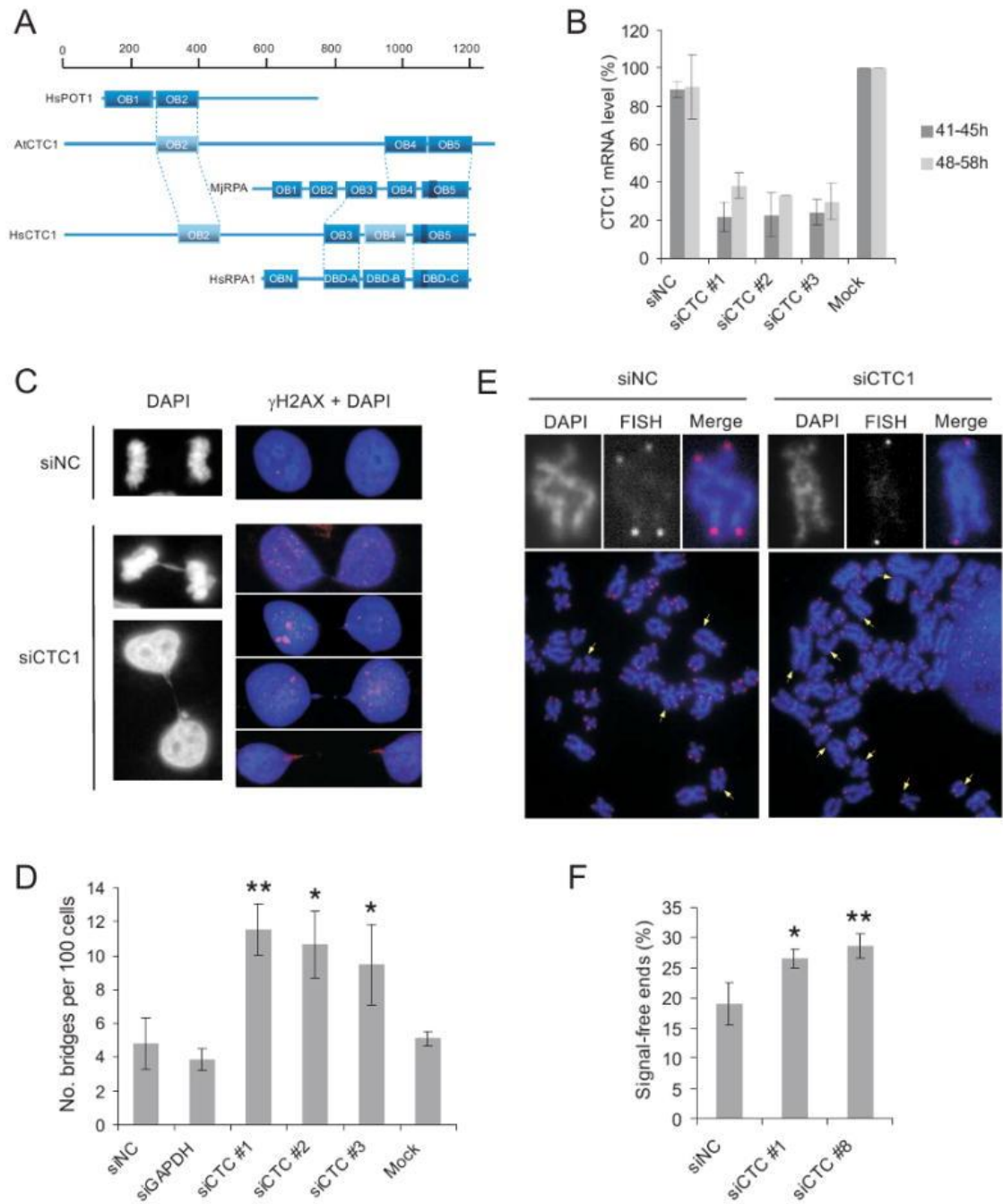**Figure A1-5. Depletion of human CTC1 causes genomic instability and sudden telomere loss.**

(A) Alignment of potential OB-folds in Arabidopsis and human CTC1 with OB-fold domains from POT1 and RPA. Dotted lines connect homologous domains. Dark shading, OB-fold homologies predicted by multiple approaches; light shading, homologies predicted by a single method. Dark rectangle within OB5 of HsCTC1

(Continued) indicates putative Zn finger which is present in human and archaeal RPAs. 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%. NC, non-silencing control; Mock, transfection reagent alone. (C & 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. Mean of three independent experiments ± SEM, stars indicate significance levels (*, p<0.05; **, p<0.01) from one-tailed Student's t-test. (E & 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 non-silencing control or CTC1 siRNA. Stars indicate significance of the increase in signal-free ends; significance levels are depicted as in (D).

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 (Casteel et al., 2009; Martin et al., 2007). 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 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 (Figures A1-5B and A1-6C, data not shown). The effect of CTC1 knockdown was monitored after the cells had recovered from the dual transfection.
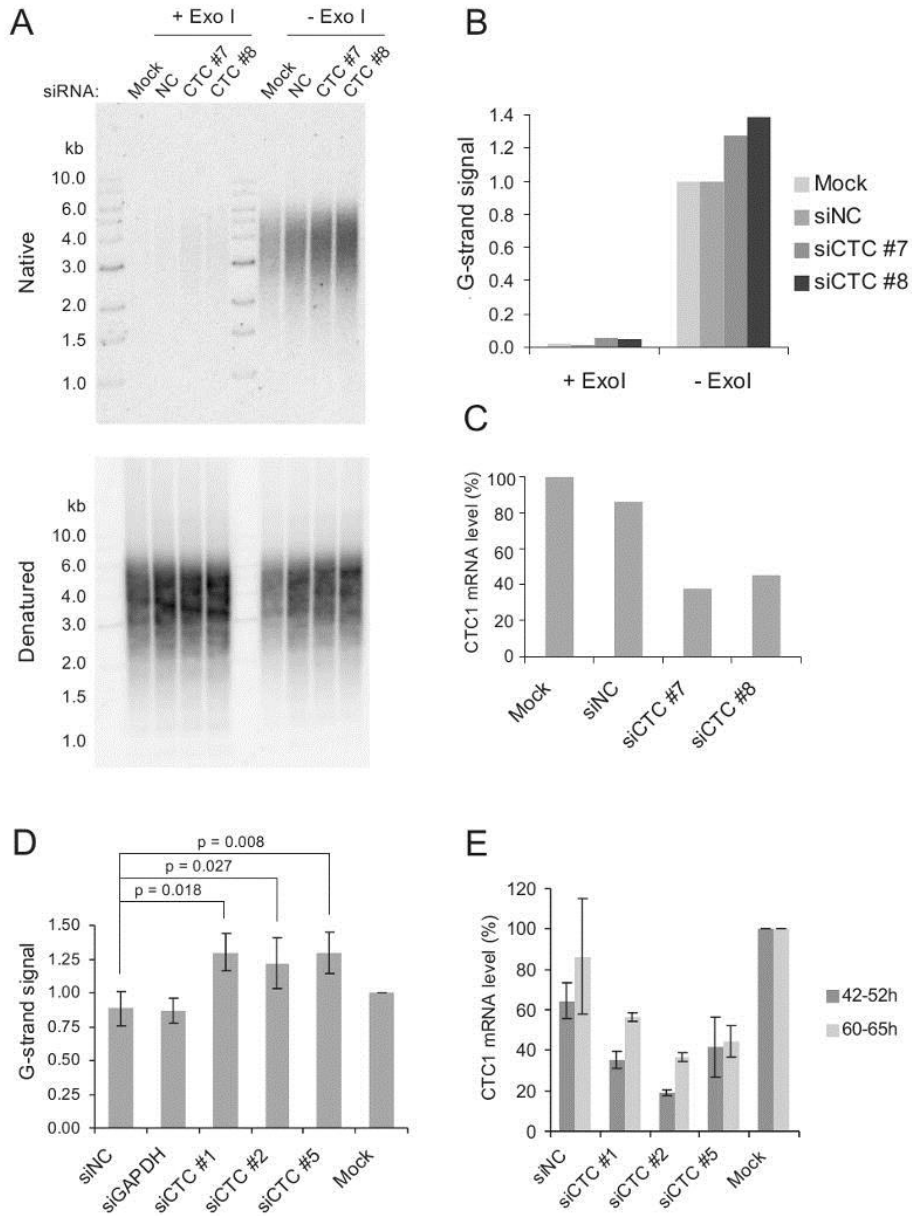
Figure A1-6. Deregulation of the G-strand overhang after CTC1-knockdown in MCF7 cells.
(A) In-gel hybridization of (CCCTAA)4 probe to telomeric restriction fragments under native (upper panel) or denaturing (lower panel) conditions. +Exo I, DNA samples were treated with Exonuclease I prior to restriction 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

(Continued) change in CTC1 mRNA level for experiments shown in (D). See caption to Figure A1-5B for details.FACS analysis of DNA content revealed that CTC1 knockdown affected cell cycle progression in MCF7 cells, with cultures showing an accumulation of cells in G1 and a decrease in the S/G2 fraction (Figure A1-S6A). 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 (Figures A1-5C, A1-5D and A1-S6B). Although the incidence of chromatin bridges was lower in MCF7 cells, there was an increase in the number of cells with micronuclei (Figure S6C). These micronuclei probably reflect anaphase or interphase bridges that were later resolved (Hoffelder et al., 2004). We were unable to determine whether CTC1 knockdown caused 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 (Figure A1-6 and data not shown). In MCF7 cells, the G-strand signal increased by 33 to 41% relative to the non-silencing control siRNA (Figure A1-6). This increase was statistically significant. Treatment with Exonuclease I removed essentially all the G-strand signal from the control DNAs, but a small amount remained in the samples from CTC1 depleted cells (Figure. A1-6A). Thus, removal of CTC1 causes an increase in G-overhang length and may also result in internal regions of ssG-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 (Figure A1-5 E and A1-F). This increase was statistically significant in 4 out of 6 trials, with the greatest frequency of signal free ends correlating with the deepest CTC1 knockdown (Figure A1-S6D). 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 and it is currently unknown whether the full complement of dedicated telomere proteins has been established 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 used a forward genetic approach to identify CTC1, a new telomere protein that is required for genome integrity in higher 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 and (Casteel et al., 2009; Dejardin and Kingston, 2009; Song et al., 2008)). Moreover, we discovered that the mammalian CTC1/STN1 complex corresponds to the recently identified DNA polymerase Alpha Accessory Factor (AAF), previously shown to stimulate Pol α-primase (Casteel et al., 2009). Thus, CTC1 appears to be a specialized replication factor that is required for telomere 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

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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 (Churikov and Price, 2008; Grandin et al., 1997; 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) or 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 functions of their more severely compromised neighbors.

Depletion of the human *CTC1* mRNA revealed a more modest, but significant role for this protein in promoting telomere integrity. 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 to maintain telomere integrity. 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 et al. in

preparation)(Surovtseva et al., 2007). Thus, plant CTC1 may have evolved to function

both in chromosome end protection and telomere replication. In contrast, mammalian

CTC1 may function only in telomere replication.

How CTC1 promotes telomere integrity in higher eukaryotes is unknown, but

important clues come from recent studies of AAF (HsCTC1/STN1) (Casteel et al). 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. Shippen, unpublished data). These findings raise the intriguing

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 higher eukaryotes to possess a Ten1-like protein. Indeed, a putative

TEN1 ortholog has been identified in humans (F. Ishikawa, personal communication)

and Arabidopsis (X. Song, K. Leehy and D. 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 (Grossi et al., 2004; Puglisi et al., 2008; Qi and Zakian, 2000). 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 vivo telomeric function or interactions with telomeric DNA (Casteel et al., 2009; Goulian and Heard, 1990). Thus, this work did not address 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 is consistent with a role in DNA replication or repair. One possibility is that 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 can 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

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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 re-initiate 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 are 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 II

ATR COOPERATES WITH CTC1 AND STN1 TO MAINTAIN TELOMERES AND

GENOME INTEGRITY IN ARABIDOPSIS

Summary

The CTC1/STN1/TEN1 (CST) complex is an essential constituent of plant and vertebrate telomeres. Here we show that CST and ATR (ataxia telangiectasia mutated [ATM] and Rad3-related) 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 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 protect 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 Cdc13/Stn1/Ten1 (CST) 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 replication protein A (RPA) (Gao *et al.*, 2007;Sun *et al.*, 2009). A null mutation in any CST component is lethal, whereas other alleles trigger massive degradation of the telomeric C-strand, causing grossly extended G-overhangs (Nugent *et al.*, 1996; Grandin *et al.*, 1997,2001). Deletion of either the Stn1 or Ten1 orthologue in fission yeast leads to catastrophic loss of telomeric DNA and end-to-end chromosome fusions (Martín *et al.*, 2007).

CST was recently discovered in plants and vertebrates (Song *et al.*, 2008; Miyake *et al.*, 2009; Surovtseva *et al.*, 2009). STN1 and TEN1 are sequence homologues 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, conserved telomere maintenance component 1 (CTC1), is not a sequence homologue of Cdc13, although it shares functional similarities. Like Cdc13, CTC1 physically interacts with STN1, as well as with 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*underlie 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; Price *et al.*, 2010; Giraud-Panis *et al.*, 2010). Recent studies show that *Xenopus* CST is required to prime single-stranded DNA (ssDNA) for replication (Nakaoka *et al.*, 2011). 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 extrachromosomal telomeric

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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 is 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 earlier.

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 kinase ATM (ataxia-telangiectasia mutated) or ATR (ATM and Rad3-related; Sabourin and Zakian, 2008). ATM primarily responds to double-strand breaks, whereas 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), whereas the single-strand–binding proteins mouse Pot1a (Denchi and de Lange, 2007), chicken Pot1 (Churikov *et al.*, 2006), and yeast Cdc13 (Garvik*et al.*, 1995; Ijpma and Greider, 2003; Hirano and Sugimoto, 2007) suppress an ATR-dependent DDR.

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ATR and ATM are also required to maintain normal telomeres. Neither ATM nor ATR has 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.*, 2009, 2011). Similarly, in budding yeast Mec1 (ATR) and Tel1 (ATM) were both 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 although 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, can 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, 2007). Plants doubly deficient in ATM and TERT, the telomerase catalytic subunit, experience an abrupt, early onset of genome instability compared with *tert* single mutants (Vespa *et al.*, 2005). Analysis of individual telomere tracts showed 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 with 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

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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 Murashige and Skoog (MS) medium with 0.7% agar (Caisson Labs, North Logan, UT). Plates were placed in the dark at 4°C for 2–4 d and then moved to long day conditions.
For zeocin treatment, seeds were treated as described. When seedlings were 5–7 d old, they were transferred to liquid MS culture either with or without 20 μM zeocin (Invitrogen, Carlsbad, CA). Seedlings were grown in the dark for 3 d 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, Norcross, GA). To make cDNA, 2 µg of RNA was used with the qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD). cDNA was diluted 1:4 in 10 µg/ml yeast tRNA (Sigma-Aldrich, St. Louis, MO), and 1 µl was used in each quantitative PCR. The SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA) was used following manufacturer's recommendations. Reactions were run on a Bio-Rad CFX96 thermal cycler using 58°C primer annealing and 10-s 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 were 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.* (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 with that of 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′-

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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-d-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 4′,6-diamidino-2-phenylindole (DAPI; Vector
Laboratories, Burlingame, CA). All slides were visualized with a Zeiss (Thornwood,
NY) 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* (Figure A2-
1A) and *atr* (Figure A2-1B) 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
(Figure A2-1C, arrowheads, and Supplemental Figure A2-S1, white arrows), irregularly
spaced siliques (Figure A2-1C, arrows, and Supplemental Figure A2-S1), 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* (Figure A2-1A) or *stn1* mutants (Supplemental Figure A2-S1A).

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 like wild type (Figure A2-1, B and C, and Supplemental Figure A2-S1B). The apparent rescue of morphological defects in *ctc1 atr* and *stn1 atr* mutants is consistent with the conclusion that CST protects against ATR activation.

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 (Figure A2-1, D 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 (Figure A2-1E). We conclude that ATR alters plant growth in response to CST abrogation.

Figure A2-1. Loss of ATR rescues the morphological defects of *ctc1*mutants.
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 that of the *ctc1* single mutant. **(B, 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.

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 (Figure A2-2A, lanes 1, 4, and 6), whereas G1 *ctc1* telomeres were shorter and more heterogeneous (Figure A2-2A, lane 7). The absence of ATM did not affect telomere length in G1 *ctc1* mutants (Figure A2-2A, lanes 8 and 9). In both G1 *ctc1* and G1 *ctc1 atm* mutants, telomeres ranged from 1 to 5 kb, with a peak signal at 2 kb. In contrast, telomeres were consistently shorter in G1 *ctc1 atr* mutants than in G1*ctc1* (Figure A2-2A, lanes 2, 3, and 7), with some signals trailing below 1 kb (peak, 1.5 kb). Similar findings were obtained with G1 *stn1 atm* (Figure A2-2A, lanes 13–16) and G1 *stn1 atr* mutants (Figure A2-2A, lanes 19, 20, 23, and 24).

Primer extension telomere repeat amplification (PETRA) was used to precisely measure telomere length on individual chromosome arms. In this assay, wild-type telomeres range from 2 to 5 kb and typically appear as one to three bands, depending on the chromosome arm (Figure A2-2B; Heacock *et al.*, 2004). As with bulk telomere analysis, PETRA showed that the telomere profiles of *atr* (Figure A2-2B) and *atm* (Supplemental Figure A2-S2, A and B) were similar to that of wild type, whereas telomeres from G1 *ctc1* and G1 *stn1* migrated as a broad smear ranging from 1.5 to 4 kb

(Figure A2-2B). PETRA confirmed that telomere tracts were similar in G1 *ctc1* and G1 *ctc1 atm* mutants (Supplemental Figure A2-S2A). In contrast, telomeres in G1 *ctc1 atr* mutants were shorter by an average of 300 base pairs compared with G1 *ctc1*mutants (Figure A2-2, B and C). The same result was obtained for *stn1* mutants in both *atm*-deficient (Supplemental Figure A2-S2B) and *atr*-deficient (Figure A2-2B) 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 signals, threefold to six-fold greater than wild type (Supplemental Figure A2-S3). G-Overhang status was wild type in *atr* mutants. Furthermore, the loss of ATR did not exacerbate the G-overhang phenotype in *ctc1* mutants (Supplemental Figure A2-S3). 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.
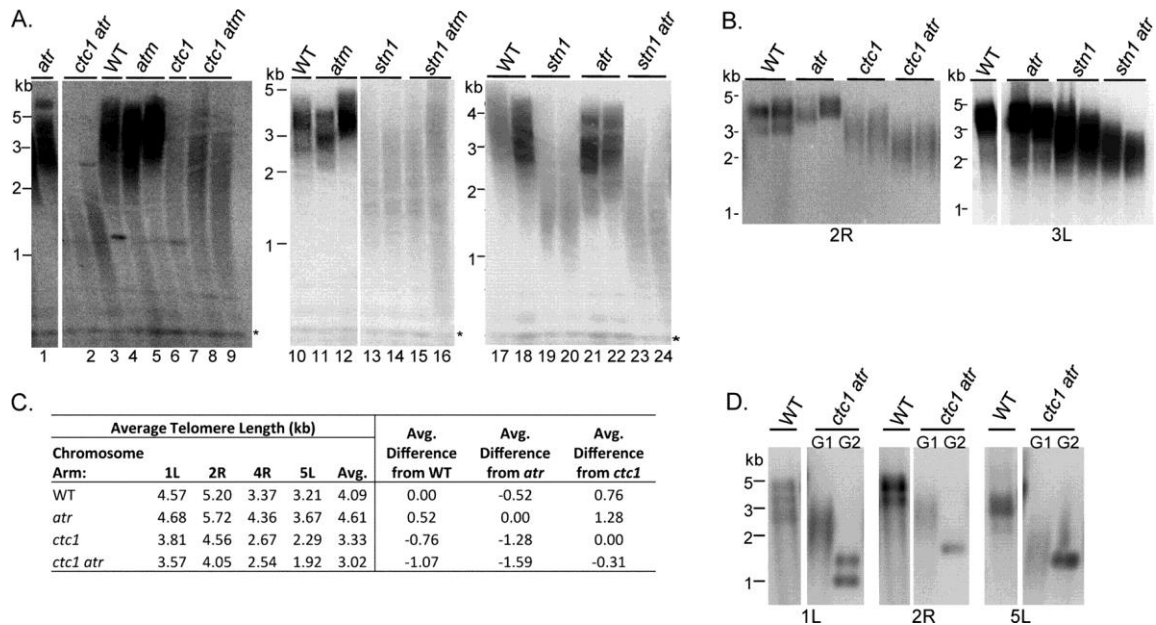
Figure A2-2. ATR, but not ATM, contributes to telomere length maintenance in *ctc1* and *stn1* mutants.

(**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 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.

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Because G2 *ctc1 atr* mutants have much more severe morphological defects than G1 *ctc1 atr* (Figure A2-1, D 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 1 kb) than their G1 parents (Figure A2-2D). This attrition is more than three times greater than the telomere shortening in G1 *ctc1 atr* mutants versus their *ctc1* siblings (300 base pairs; Figure A2-2, A–C), and more than two times higher than G2 *stn1* mutants versus their G1 parent (~400 base pairs; unpublished data). In conjunction with telomere shortening, the profile of telomere fragments switched from heterogeneous, smeary bands in the G1 *ctc1 atr* parents to very homogeneous, sharp bands in the G2 *ctc1 atr* offspring (Figure A2-2D). PETRA assays conducted with five generations of *atr* mutants revealed no change in telomere length (Supplemental Figure A2-S2C), 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 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 the 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 (Figure A2-3 and data not shown). 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 (Figure A2-3). This decrease persisted in subsequent plant generations, with G4 *atr* mutants also exhibiting dramatically reduced enzyme activity. A similar decrease in TRAP activity was not observed in *atm* deficient plants (Figure A2-3). 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 (Figure A2-3). Of note, 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 A2-3). 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 (Figure A2-3). This

observation suggests that the repression of telomerase activity in G2 *atr* mutants may reflect the activation of a DDR triggered by replicative stress. Taken together, 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 nonhomologous end-joining (NHEJ) pathway, which is activated by ATM and indirectly by ATR (Denchi and de Lange, 2007; Deng *et al.*, 2009). Therefore we used telomere fusion PCR (TF-PCR) to ask whether the accelerated telomere shortening in plants lacking CST and ATR correlates with an increased incidence of telomere fusions. TF-PCR uses 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 or *atr* (Supplemental Figure A2-S4, B and D) or *atm* (Supplemental Figure A2-S4, A and C) mutants. In contrast, massive chromosome end-joining events, represented by abundant heterogeneous smears, were associated with the loss of CTC1 (Supplemental Figure vS4, A and B) or STN1 (Supplemental Figure A2-S4, C and D). When either ATR (Supplemental Figure A2-S4, B and D) or ATM (Supplemental Figure A2-S4, A and C) was absent in *ctc1* or *stn1* mutants, TF-PCR products were still detected.
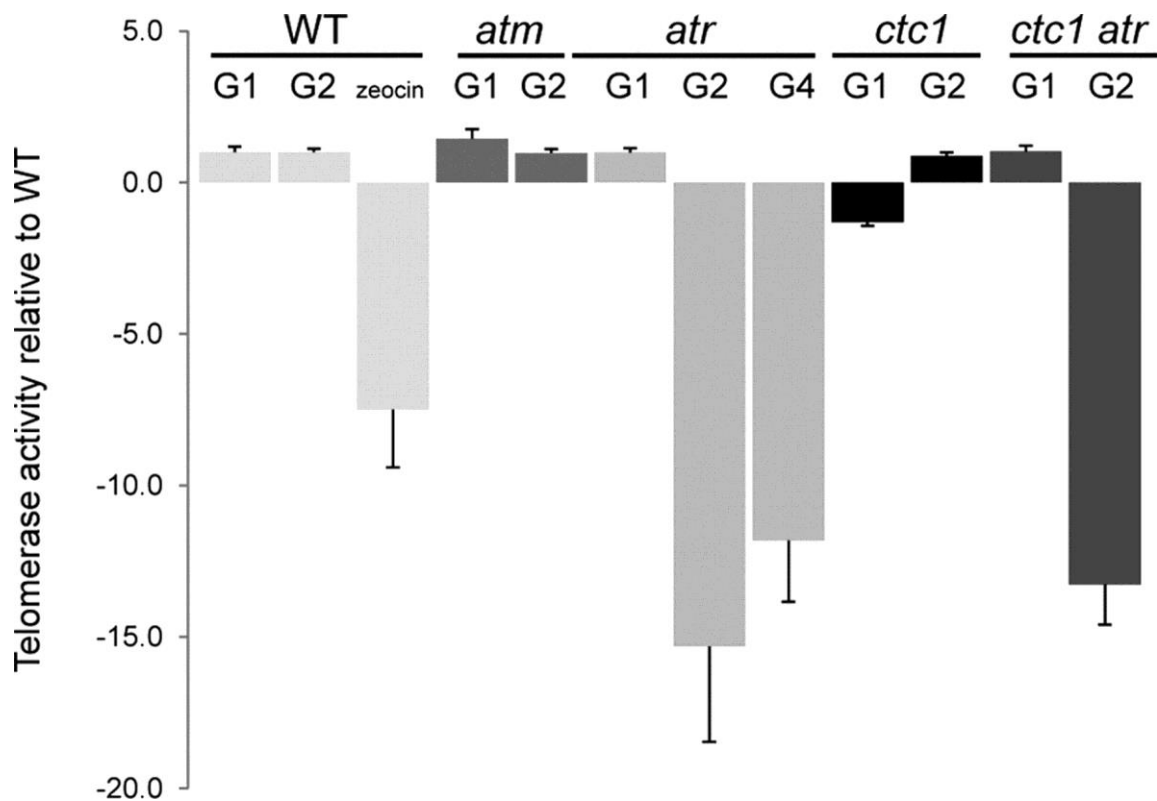
Figure A2-3: ATR stimulates telomerase activity.
Quantitative TRAP results for first-generation (G1), second-generation (G2), and fourth-generation (G4) mutants of different genotypes are shown. Q-TRAP was also performed on wild-type seedlings treated with 20 μM zeocin for 3 d. All samples were from 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 SD. n = 2 for all genotypes except G1 WT, n = 5; zeocin-treated WT, n = 6; G1 *ctc*1, n = 4; G2 *atr*, n = 3; and G4 *atr*, n = 4.

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 (Figure A2-4A). 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 with few or none in wild type and *atr* and *atm* mutants (Figure A2-4B). 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* and *ctc1* (Figure A2-4B). Remarkably, 70% of anaphases in the triple G1 *stn1 atr atm* mutants contained bridged chromosomes (Figure A2-4B). 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.

A.
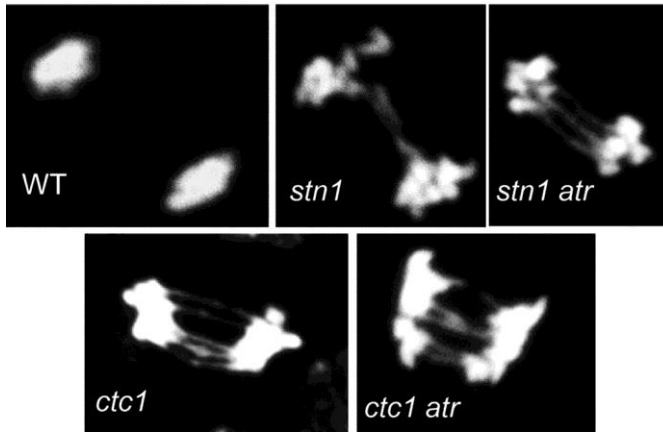
B.

Genotype	Anaphases		
	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 A2-4: 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.

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 the DDR (*RAD51,BREAST CANCER SUSCEPTIBILITY 1* [*BRCA1*]) and (*poly [ADP-ribose] polymerase 1* [*PARP1*]; Doucet-Chabeaud *et al.*, 2001; Lafarge and Montané, 2003;Yoshiyama *et al.*, 2009). Quantitative real-time (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 with wild type (3.7- and 1.9-fold, respectively; Figure A2-5). In addition, *RAD51* expression was 1.5 times higher in *ctc1* mutants (Figure A2-5), but the difference was not statistically significant. These results suggest that the CST complex protects against a DDR.
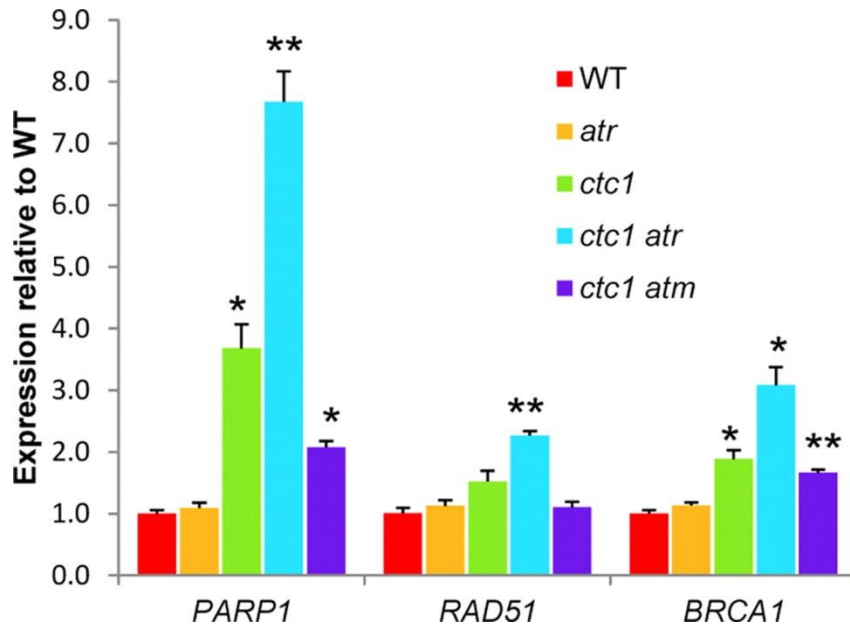
Figure A2-5: Loss of CTC1 activates a transcriptional response, which is alleviated by ATR.
Quantitative RT-PCR results are shown for the DDR transcripts *PARP1, BRCA1*, and *RAD51* in floral organs. Expression levels are relative to wild type, and data for first-generation (G1) mutants are shown. For each genotype, n = 3, except for *ctc1 atm*, n = 2. *p < 0.05 relative to wild type; **p < 0.005 relative to wild type (Student's t test). Error bars represent SEM.

We next asked whether ATM or ATR is 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

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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 at*r mutants relative to wild type, *atr*, or *ctc1* (Figure A2-5). 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 (RAMs) of seedlings, using propidium iodide (PI) staining (Figure A2-6A). 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 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 (Figure A2-6A, ii). Similarly, G2 *atr* seedlings showed no PI staining (Figure A2-6A, iii). In contrast, strong PI staining was observed in G2 *ctc1* RAM (Figure A2-6A, iv) or G2*stn1* RAM (Figure A2-6A, v), consistent with activation of a robust DDR. We next asked whether ATR is responsible for cell death in CST mutants (Figure A2-6A, vi). Strikingly, the number of

PI positive cells in G2 *ctc1 atr* dropped to an average 1.75 cells/root, compared with 5.75 and 4.35 cells/root for *stn1* and *ctc1*, respectively (Figure A2-6A, vi, and B). 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 (Figure A2-6A, vii and 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. Furthermore, we speculate that the decrease in PCD in *ctc1 atr* mutants leads to an accumulation of cells exhibiting DDR and increased number of end-to-end chromosome fusions.
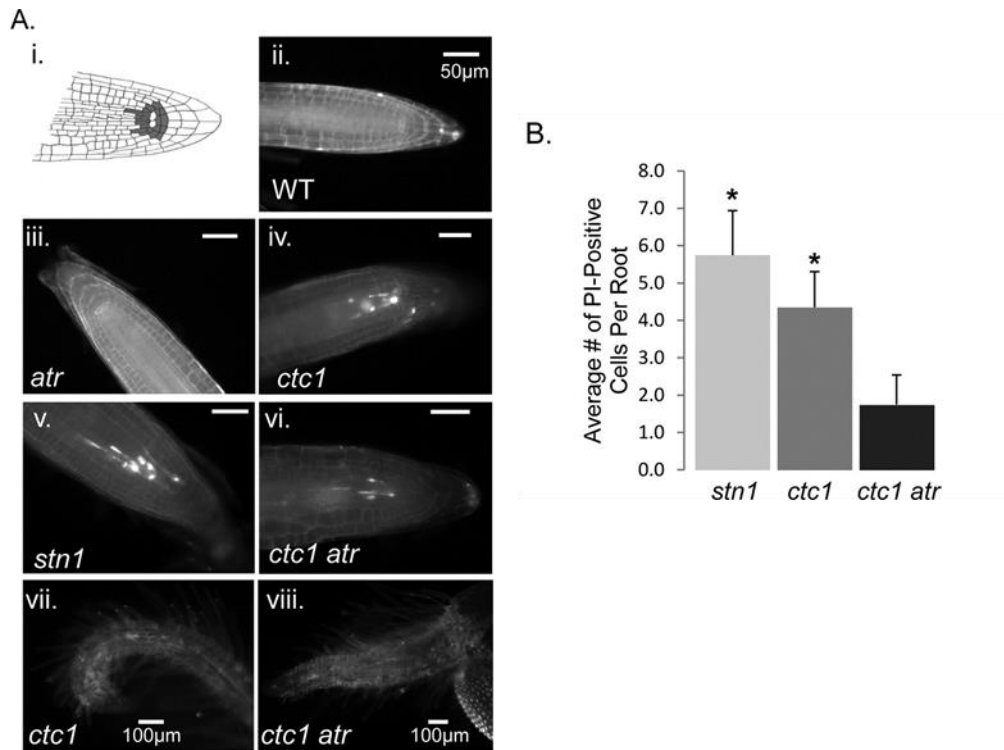
Figure A2-6: ATR activates programmed cell death of the root apical meristem (RAM) of ctc1 mutants.
(A) Representative images of G2 seedling root tips stained with propidium iodide (PI).
(i) Diagram of a root tip. Stem cells and adjacent daughter cells are shaded gray. White cells in the RAM center are quiescent center cells. WT (ii) and *atr* (iii) roots are PI negative, but the RAMs of ctc1 (iv) and *stn1* (v) mutants have numerous PI-positive (dead) cells. (vi) Fewer PI-positive cells are present in *ctc1 atr* mutants. (vii and viii) 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). *p < 0.05 (Student's t test). Error bars represent SEM.

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 with *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 (Figure A2-7B).
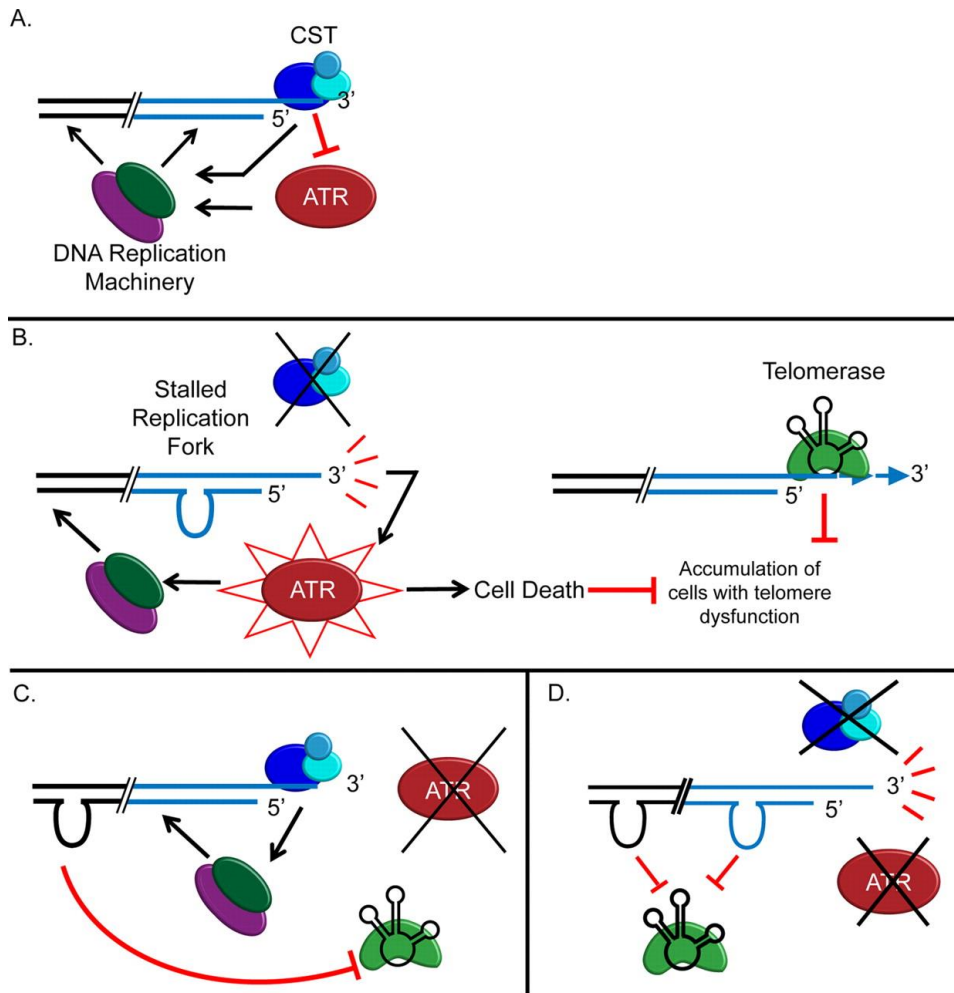
Figure A2-7: Model depicting CST and ATR cooperation in maintaining telomeric DNA and genome integrity in Arabidopsis.
(A) In wild-type plants, CST interacts with the 3′ overhang to protect the chromosome terminus from telomere shortening, end-to-end chromosome fusions (Song et al., 2008; Surovtseva et al., 2009), and activation of ATR-dependent DDR (this study). ATR facilitates replication fork progression. Similarly, CST is believed to stimulate replication fork restart within the telomeric duplex via interaction with DNA polymerase alpha (Price et al., 2010; Nakaoka et al., 2011). 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 the text for details.

While this article was under review, Amiard *et al.* (2011) published a study that verifies and complements our findings concerning the role of CST in suppressing an ATR-mediated DDR. 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. They 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 later discussion). Of note, telomere fusions accumulate even in the absence of both ATM and ATR when CST is compromised (Amiard *et al.*, 2011; this study). A third PIKK family member in vertebrates—DNA-dependent protein kinase catalytic subunit (DNA-PKcs)—functions in NHEJ (Lieber *et al.*, 2003) and could potentially serve as backup mechanism to trigger telomere fusion. Plants lack an obvious DNA-PKcs orthologue, and thus the ATR/ATM–independent response elicited by telomere dysfunction is unknown. Further complicating matters, uncapped telomeres engage both canonical and no-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

uses PARP1 and DNA ligase III, is activated if the canonical DNA-PKcs/Ku pathway is

nonfunctional (Audebert *et al.*, 2004; Wang *et al.*, 2006). It is unknown whether 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 (Figure A2-5).

Cooperation of CST and ATR in telomere maintenance

Figure A2-7 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 provide 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; Figure A2-7A). Although our findings do not

specifically address whether CST directly contacts the G-overhang, they are consistent

with this conclusion and with the present 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. Multigenerational 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 ~300 base pairs shorter in *ctc1 atr* mutants than when ATR is intact. However, in the next generation, telomere shortening is much more aggressive, and up to 1 kb more telomeric DNA is lost. We hypothesize that this biphasic response reflects two distinct contributions of ATR in promoting telomere maintenance (Figure A2-7, B and C).

Emerging data indicate that ATR and CST cooperate to facilitate DNA replication through the telomeric duplex (Price *et al.*, 2010; Stewart and 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). Of note, 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-α (Casteel *et al.*, 2009; Price *et al.*, 2010). Consistent with this model, *Xenopus* CST is required for priming replication of ssDNA (Nakaoka *et al.*, 2011). Taken together, these findings indicate CST and ATR cooperate in relieving replicative stress within the telomere duplex (Figure A2-7, B and C). When both CST and ATR are compromised, replication fork stalling is increased (Figure A2-7D), 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. Although the extent to which ATR and CST modulate replication of the telomeric duplex in plants is unknown, preliminary data suggest that the

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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 (Figure A2-7C). 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

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dysfunction does not inhibit telomerase. Sustained repeat incorporation onto compromised chromosome ends would be advantageous if it delays the onset of complete telomere dysfunction. Of note, *ctc1 tert* telomeres shorten more rapidly than in either single-mutant background (Boltz and 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). Similarly, 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.