NOVEL PLAYERS IN TELOMERE MAINTENANCE AND BEYOND

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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May 2017

Major Subject: Biochemistry

ABSTRACT

Telomeres are specialized nucleoprotein caps at the end of linear chromosomes, critical for genome stability. A major function of telomeres is to distinguish chromosome ends from ends of double strand breaks. A second function is to counteract incomplete end-replication via telomerase extension. POT1 (Protection of Telomere 1) is a highly conserved telomere protein known for its essential role in chromosome end-protection and end-replication. *Arabidopsis thaliana* encodes three POT1 paralogs, POT1a, POT1b, and POT1c. AtPOT1a promotes telomerase processivity and therefore is required for telomere length homeostasis. The functions of AtPOT1b and AtPOT1c are less understood.

In this dissertation, I characterized the function of POT1b at telomeres. In contrast to POT1a, I found that POT1b is dispensable for telomere length maintenance and serves as a negative regulator of telomerase. In addition, I tested the hypothesis that TER2/POT1b works in concert with Ku to stabilize the blunt-ended telomeres.

Further characterization of POT1b using biochemical and genetic approaches revealed several unexpected features. First, unlike POT1a, which is primarily localized to the nucleus, POT1b accumulates in the cytoplasm, where its binding partner TER2 also resides. This observation suggests a potential regulatory pathway for TER2 RNP via subcellular trafficking. In addition, I found that early development of POT1b mutants is significantly delayed, indicating that POT1b has a novel role in plant development.

Together, these studies provide insights into the role of AtPOT1b in telomere biology and expand our understanding of POT1 protein function and evolution.

In addition to these studies of POT1 proteins, I examined the role of chromosome remodeler DDM1 (Deficient in DNA Methylation 1) in telomere length maintenance. I showed that plants deficient in DDM1 suffer from abrupt telomere shortening in the sixth generation of the deficiency due to deletional recombination. This telomere rapid deletion (TRD) coincides with increased transposon activation and increased DNA damage sensitivity at the root apical meristem, suggesting that TRD may serve as a mechanism to stimulate programmed cell death, thereby eliminating stem cells with massive DNA damage. These studies open a new avenue for telomere function in promoting genome integrity.

DEDICATION

This dissertation is dedicated to my parents for their unconditional love and support.

ACKNOWLEDGEMENTS

The past six and half years have been a long journey for me. I am extremely grateful for the mentorship given by many intelligent researchers, without whom I could not have enjoyed doing research as much, and for the support and friendship provided by many special people, who are by me side throughout ups and downs.

First, I cannot stress enough how much I appreciate the opportunity to work for my PI, Dr. Dorothy Shippen. Joining the Shippen Lab was the best decision I made in the grad school. I have learnt a lot from her. During these years, I am always fascinated by how passionate Dorothy is about science, how excited she becomes whenever we come up with great ideas, and how she manages to convey complicated scientific concepts in simple but delicate sentences. Dorothy taught me that perseverance, optimism, and focus are merits required for a good researcher, but efficient communication is what it takes to build a great scientist. Dorothy is also more than an academic advisor to me. She is very understanding and caring. Her door is always open and she is always willing to listen to us. When I expressed my willingness to pursue a career in the industry and take business-related classes, Dorothy supported me. She told me, if this is what I want, do it the right way. She encouraged me to get the Business Certificate offered by the Mays Business School. Today, I still remember the thrill I had when I knew I will work in the Shippen Lab for my Ph.D. and my pride of being a Shippen Lab member has never decayed a little till now.

I am also very grateful for the guidance provided by faculty in our department, especially my committees. I know I have not been an outstanding researcher and always need critiques to help me improve. My great committees, Dr. Bryk, Dr. Rye, and Dr. Shan, shared their insights and provided many valuable suggestions with my projects. In addition, Dr. Kapler, Dr. Xiuren Zhang, Dr. He, Dr. Zeng, Dr. Li, and Dr. Kaplan Lab also helped my enormously with my projects by sharing their protocols, equipment and reagents.

Thanks also go to several sharp, patient, and inspiring mentors. Dr. Cheng Lv from Li lab was my very first mentor in our department, who became my good friend after my first rotation with her. Dr. Jung Ro Lee was my mentor in the Shippen Lab during rotation, and we continued to work on the TEN1 project after I joined the lab. We shared an office for three years, and we enjoyed talking about science and beyond with each other. During these years, Jung Ro has been not only a great teacher but also a big brother to me. Another Shippen Lab guru, Dr. Andrew Nelson, from who I inherited the POT1b project, is an unusual scientist in many ways. Andrew is always energetic and positive, and his enthusiasm for science is contagious. Last but not the least, Dr. Chang Shu from the Li lab. He started off mentoring me with experiments. Unexpectedly, the projects evolved into cooking and finally into life decisions. Looking back, I feel blessed working with each mentor and very much cherished these friendship.

I would also like to especially thank other members of the Shippen Lab for their intellectual input during lab meetings and hanging out outside the lab: Katie, Kara, Kyle, Peter, Xintao, Eugene, Vicky, Behailu, Sreya, Borja, and Jerry. Thanks to Dr. Di Liu,

Elena, and Duc for their tremendously help with my projects and for the chances to build my mentoring skill. Special thanks to Callie who has been a great friend of mine all these years. Your perseverance and optimism always impress me. It has been an amazing journey to work with all of you.

I would not been able to enjoy grad school without all my friends. Thank you all for being there for me whenever I needed to talk or do silly things.

Finally, there is no enough words to express how much I am grateful to have endless support from my parents. They suggested me to pursue a Ph.D. and I am glad that I did. There were times that I was in doubt of myself, but they never were. They always have faith in me, and this means the world to me. To my boyfriend, Cong Shao, thanks for putting up with me and stay by my side since high school.

CONTRIBUTORS AND FUNDING SOURCES

This work was supervised by a dissertation committee consisting of Professors Dorothy Shippen, Mary Bryk, and Hays Rye of the Department of Biochemistry and Biophysics and Professor Libo Shan of the Department of Plant Pathology and Microbiology.

The data for Appendix II was in part by JungRo Lee and was published in 2016. All other work for the dissertation was completed independently by the student.

This work was made possible in part by N.I.H. under R01-GM065383 to D. E. S.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Genetic information is stored as nucleic acid sequences, double-stranded DNA (dsDNA) being the most common form. The integrity of genetic material is crucial not only for the survival of the individual, but also its offspring. In a wide range of unicellular organisms, such as yeast and *Escherichia coli,* and (semi-) autonomous cell organelles, such as mitochondria and chloroplasts, dsDNA exists as circular plasmids. However, in most eukaryotes and some prokaryotes, dsDNA is in the form of linear chromosomes. Compared with circular chromosomes, linear chromosomes present several challenges that need to be dealt with to maintain genome integrity.

The ends of linear chromosomes constitute two major dilemmas: the end-protection problem and the end-replication problem. Natural chromosome ends can be mistakenly perceived as double-stranded DNA breaks (DSBs) and thus activate a DNA damage response (DDR) (Lazzerini-Denchi and Sfeir, 2016). This end-protection problem must be solved to prevent its detrimental consequences. Failure to differentiate the chromosome ends from DSBs leads to chromosome end-to-end fusion and compromised genomic stability (McClintock, 1941). Therefore, chromosome termini, also known telomeres, must possess specialized protection mechanisms to dodge the DDR (Arnoult and Karlseder, 2015).

The second challenge at the terminus of linear chromosomes stems from the nature of semiconservative DNA replication by DNA polymerases. In the 1970s, James

Watson, who studied DNA replication in the T7 phage, was the first to hypothesize the end-replication problem (Watson, 1972), in which DNA polymerases are unable to fully synthesize the very end of the lagging strand after removal of the last RNA primer. Based on this theory, gradual loss of chromosome terminal sequences would be expected, leading to shorter chromosomes and hence the loss of essential genetic information. While organisms possessing circular chromosomes do not suffer from these problems, various strategies have evolved to counterbalance the loss of terminal sequences on linear chromosomes (Kobryn and Chaconas, 2001; de Lange, 2004).

Indeed, there is no one simple solution to the end-protection and end-replication problems (Figure 1-1). For example, the genomes of poxvirus, *Borrelia burgdorferi* and *Escherichia coli* phage N15 use a covalently-closed hairpin terminus with or without short palindromic sequences to circumvent the two problems (Cavalier-Smith, 1974; Bateman, 1975; Kobryn and Chaconas, 2001). Unlike the vast majority of eukaryotes, *Drosophila melanogaster* telomeres consist of retrotransposons that are occasionally added to chromosome to compensate for the gradual loss of terminal sequence due to the end-replication problem (Biessmann et al., 1990; Biessmann et al., 1992; Sheen and Levis, 1994). A similar mechanism was observed in *Saccharomyces cerevisiae* mutants lacking the telomerase reverse transcriptase mechanism (see below) for end-replication. Although the majority of these yeast cells die without telomerase, one type of survivor amplifies the Y' repeat elements, to compensate for the loss of terminal sequences (Lundblad and Blackburn, 1993; Yamada et al., 1998).

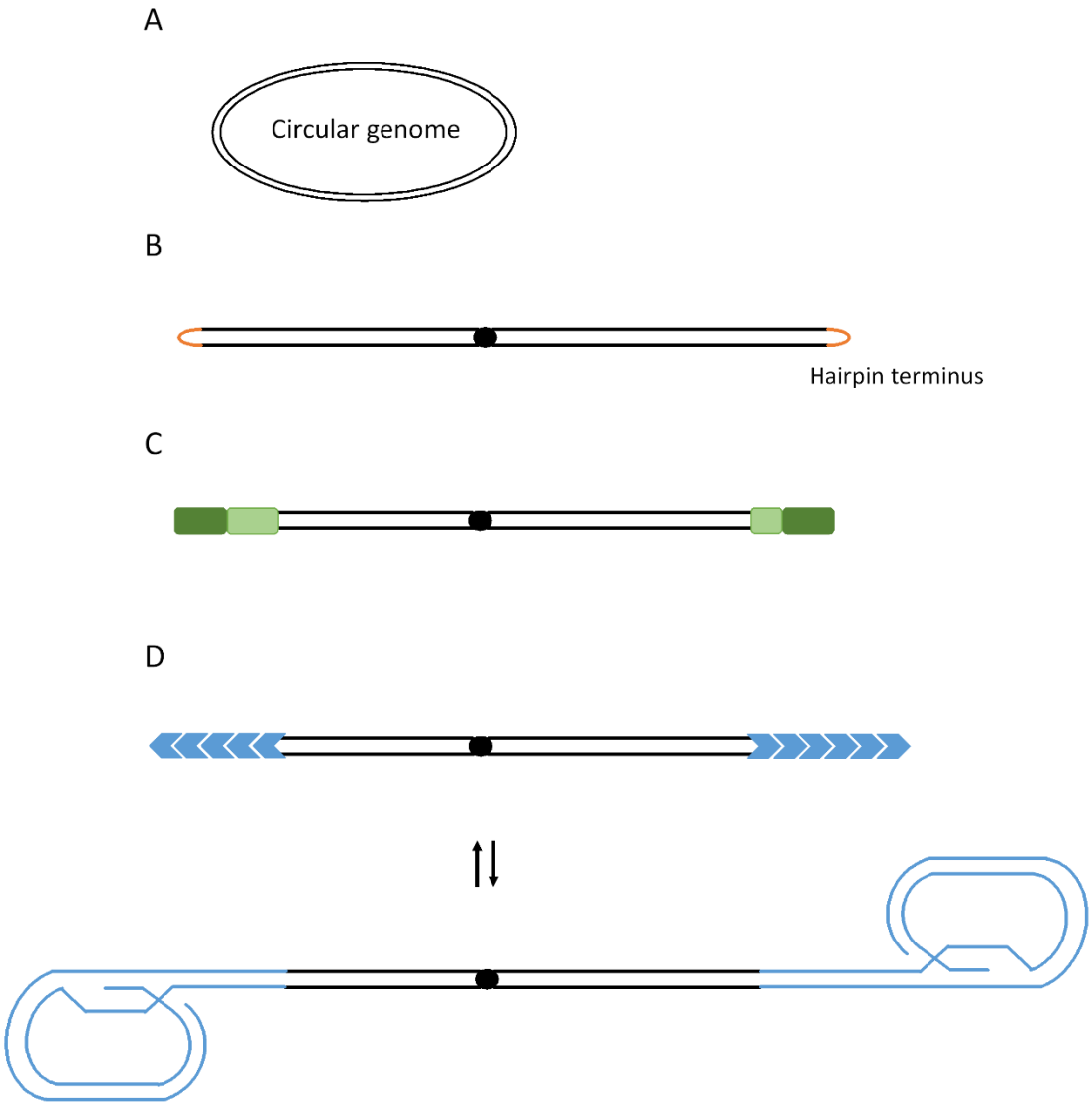


Figure 1-1. Different mechanisms for chromosome end-protection. (A) Circular chromosome used in most bacterium genomes. (B) Covalently-closed hairpin terminus with or without a short palindromic sequences seen in *Borrelia burgdorferi* and *Escherichia coli* phage N15. (C) Retrotransposons at chromosome ends seen in *Drosophila melanogaster*. Green rectangle: retrotransposons. (D) Repeat sequences at telomeres and secondary structures, termed t-loops, are found at the ends of most eukaryotic telomeres. Blue arrows: telomere repeats.

In most eukaryotes, the prevalent form of chromosome end structure is an array of short repeats bound by specialized protein complexes, and maintained by a conserved reverse transcriptase, coined telomerase. In the 1930s, Barbara McClintock, studying chromosomes in maize, and Hermann Müller, studying chromosomes in Drosophila, independently came to the realization that the ends of linear chromosomes possess unique organization that prevents chromosome end-to-end fusions (McClintock, 1938; Meier and Müller, 1938). Since the 1970s, telomeres and telomerase have attracted the attention of generations of scientists, and an explosion of studies have contributed to the understanding of the nature of chromosome ends in eukaryotes.

Telomere structure and features

In the 1970s, Elizabeth Blackburn first defined the terminal sequence of Tetrahymena chromosomes and identified a few hundred base pairs of GC-rich repeats (Blackburn and Gall, 1978) that were later shown to be sufficient to stabilize a linearized plasmid in yeast (Szostak and Blackburn, 1982). Subsequent studies revealed that this GC-rich feature is shared by vertebrate, invertebrate, fungi, ciliate, and plant telomeres. The sequence and number of repeats vary among species (Zakian, 1995). In humans, the telomere repeat is TTAGGG, and the length of the telomere repeat array ranges from 2 to 30 kb (Moyzis et al., 1988; Brown, 1989). Yeasts contain relatively short telomeres, about 300 bp (Runge and Zakian, 1989). The telomere repeat sequence is slightly degenerate $G_{2-3}(TG)_{1-6}$ in *S. cerevisiae* and $TTACAG_{2-3}$ in *Schizosaccharomyces pombe* (Hiraoka et al., 1998). In *Arabidopsis thaliana*, as in almost all plant species, telomeres

are comprised of TTTAGGG repeats, ranging from 2 to 9 kb, depending on the accession (Richards and Ausubel, 1988; Shakirov and Shippen, 2004). *Nicotiana tabacum* has ultra-long telomeres of 40-160 kb with the same repeat sequence as *A. thaliana* (Fajkus et al., 1995).

The telomeric DNA is comprised of ds telomere repeats and a G-rich 3' single-stranded (ss) overhang, known as the G-overhang (Figure 1-2). The generation of the G-overhang is a highly choreographed process with the effort of several nucleases and polymerases (Figure 1-3). When DNA replication is completed, lagging strands are naturally left with short 3' G-overhangs due to the removal of the last RNA primers. The terminus replicated by leading strand synthesis are initially blunt-ended and undergo nucleolytic processing of the C-strand to generate G-overhangs (Makarov et al., 1997; Wu et al., 2010; Wu et al., 2012). Thus, two parameters influence the length of mature G-overhangs without telomerase coming into play: the position of the last RNA primer at the chromosome terminus, and the extent of C-strand processing (Sfeir et al., 2005; Dai et al., 2010). In telomerase-positive cells, telomerase extension of the G-strand is coordinated with fill-in of the C-rich telomeric strand primarily by pol α-primase (Chandra et al., 2001; Fan and Price, 1997; Nakamura et al., 2005; Lue et al., 2014). The length of the G-overhang thus fluctuates during the cell cycle. G-overhang length also varies depending on the organism (Dai et al., 2010). Ciliates and yeast possess short G-overhangs that are 12 to 14 nt (Klobutcher et al., 1981; Jacob et al., 2001; Wellinger et al., 1993; Larrivee et al., 2004), while humans have longer G-overhangs ranging from 35 to 600 nt in length (Makarov et al., 1997; Wright et al., 1997; Stewart et al., 2003). *A.*

thaliana and several other plants are unusual because end of each chromosome has a terminus that contains a 20-30 nt-long G-overhang (Riha et al., 2000), while the other end is blunt-ended (Kazda et al., 2012) (see below). G-overhangs are important regulators of telomere dynamics since they serve as a substrate for telomere-repeat addition (Lingner and Cech, 1996), and they also provides a platform for cell cycle regulated interactions of telomere binding proteins to achieve the complex regulation of telomere metabolism (Wu et al., 2012).

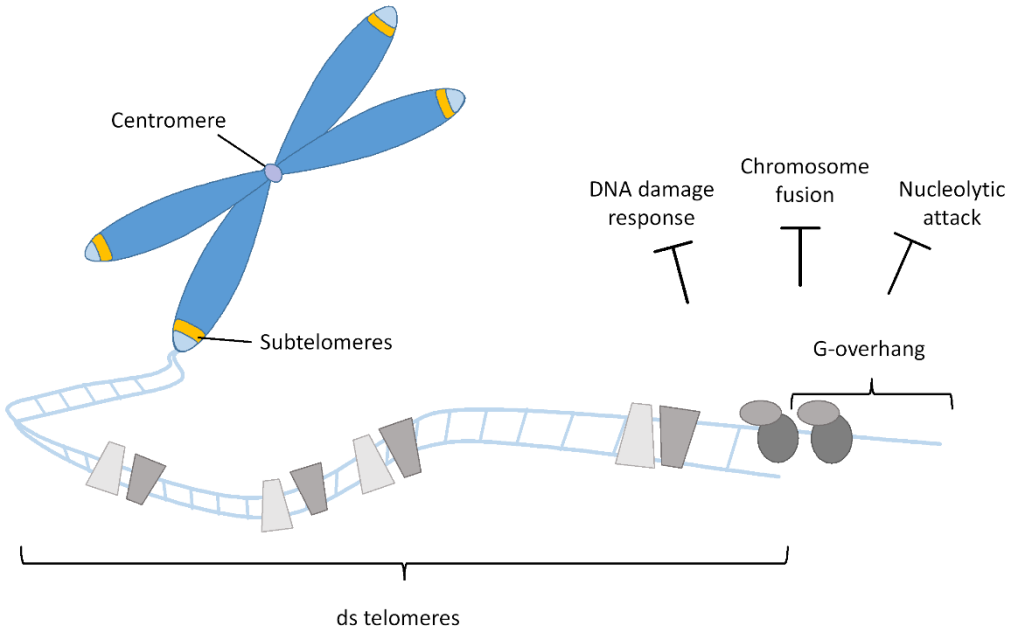


Figure 1-2. Telomere structures. Telomeres consist of tandem arrays of double-stranded short GC-rich repeats that end in a ss 3' overhang (G-overhang). The ds and ss telomeric DNA are associated with specific protein complexes that protect telomeres from DNA damage responses and inappropriate recombination mechanisms, which can lead to telomere shortening or end-to-end chromosome fusion.

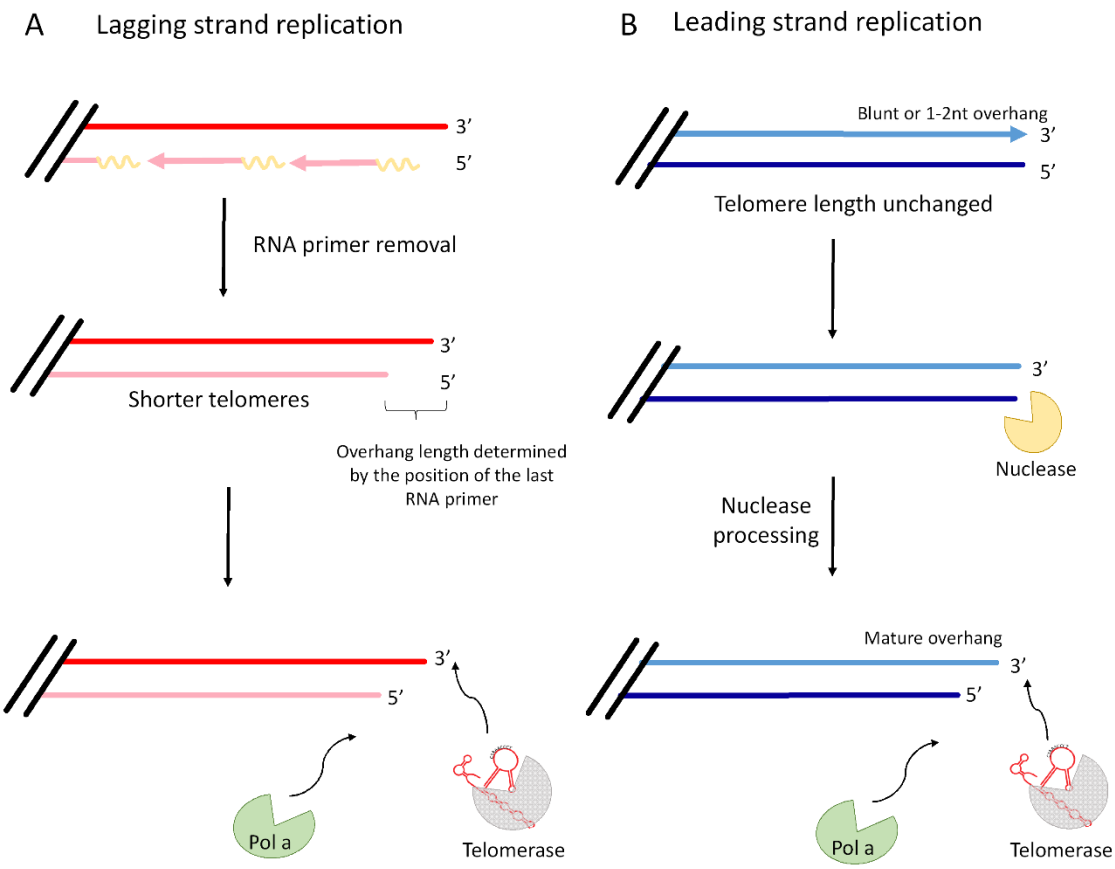


Figure 1-3. The end-replication problem and end processing. (A) During semiconservative DNA replication, the lagging strand uses a short RNA primer (shown in yellow wavy lines) to initiate DNA synthesis. When the DNA synthesis reaches the chromosome terminus, the lagging-strand DNA replication at telomeres (in red) leaves gaps (G-overhang) at the 5' end due to removal of the RNA primers. This gap cannot be filled at the very end, resulting in a shorter telomere. (B) The leading-strand replication at the telomere region (in blue) results in complete replication of the chromosome end. The chromosome end can be further processed by nucleases and form a 3' G-overhang. The G-overhang can be used as a substrate for telomerase extension. After telomere addition, the C-strand will be filled in by Polα-primase.

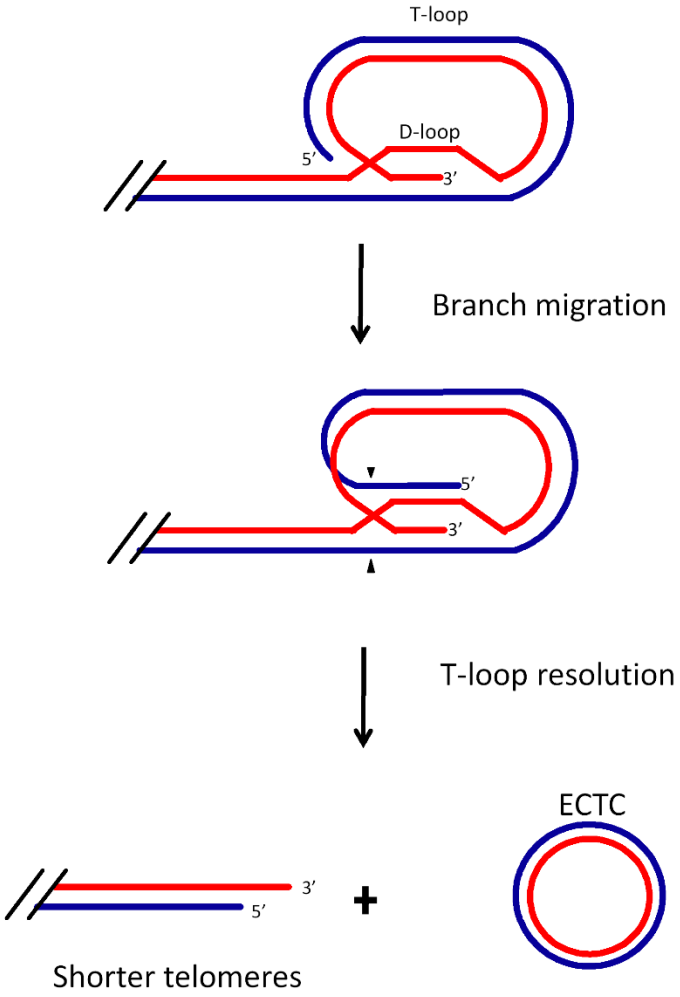


Figure 1-4. T-loop homologous recombination. The 3’ G-overhang folds back and invades into upstream ds telomeric DNA. Branch migration produces an intermediate that resembles a Holliday junction and alters the size of t-loop. Homologous recombination pathways direct the cleavage of t-loop, giving rise to a shortened telomere and an extra chromosomal telomeric circle (ECTC).

G-overhangs also allows for the formation of an alternative conformation of telomeres to promote end-protection (Griffith et al., 1999; Stansel et al., 2001). Due to sequence complementarity, the 3’ G-overhang can be inserted into the duplex region of telomeres to form a higher-order structure, termed the t-loop (Figure 1-4). T-loops were

first observed in isolated mammalian telomeric DNA by electron microscopy. They were lariat-like structures hundreds to thousands of base pairs in size, stabilized by telomere specific proteins (Griffith et al., 1999; Stansel et al., 2001). Later, this telomeric configuration was also observed in garden peas (Cesare et al., 2003), ciliates (Murti and Prescott, 1999), and chickens (Nikitina and Woodcock, 2004) using *in vitro* or *in vivo* methods. A recent study highlighted that functional vertebrate telomeres frequently exhibit the t-loop configuration (10 to 40% of telomeres*) in vivo* (Doksani et al., 2013). The 3' terminus of chromosomes are thought to adopt this native architecture to block access of telomerase (Smogorzewska et al., 2000), and to avoid being perceived by DDR pathways (de Lange, 2009). However, the detailed mechanism of t-loop formation and how the prevalence of t-loops is regulated during the cell cycle remains unknown.

Because of the resemblance between the t-loop and a Holliday junction intermediate, t-loops can be resolved by the homologous recombination (HR) pathway (Wang et al., 2004) (Figure 1-4). In mammals, the players involved in t-loop resolution include XRCC3, the XPF protein, NBS1, and the Werner helicase (Wang et al., 2004; Li et al., 2008). In addition, the ds telomere binding protein TRF2 inhibits t-loop resection (Griffith et al., 1999; Stansel et al., 2001). T-loops deletion can give rise to extrachromosomal telomeric circles (ECTCs), a hallmark for a telomerase-independent recombination-based mechanism for telomere maintenance, called alternative lengthening of telomere (ALT) (Natarajan and McEachern, 2002; Cesare and Griffith, 2004). Resolution of the t-loop can also result in dramatic telomere shortening through a process known as telomere rapid deletion (TRD) (Ancelin et al., 2002; Karlseder et al.,

2002; Wang et al., 2004). TRD has been implicated as a telomere sizing mechanism (see below).

Telomere-associated proteins: protecting chromosome ends and promoting their replication

Besides the sequestration of the 3′ overhang by t-loop formation to provide structural protection, telomere protection is mediated through the binding of multi-subunit protein complexes. This strategy for end-protection is conserved in diverse organisms, however, the composition of the protein caps at telomeres diverges across eukaryotes (Figure 1-5). For example, vertebrate and fission yeast telomeres are capped by shelterin. In contrast, CST (Cdc13/CTC1; STN1; TEN1) protects telomeres in *Arabidopsis* and budding yeast. Both shelterin and CST modulate the architecture of telomeres and block the access of DDR and DNA repair pathways (de Lange 2005; Price et al., 2010; Giraud-Panis et al., 2010). Removal of core capping subunits causes telomere length dysregulation, a powerful DDR, and eventually end-to-end chromosome fusion and genomic instability (de Lange, 2009; Price et al., 2010). How individual components of these complexes contribute to telomere maintenance has been the center of attention in the telomere field and will be discussed below.

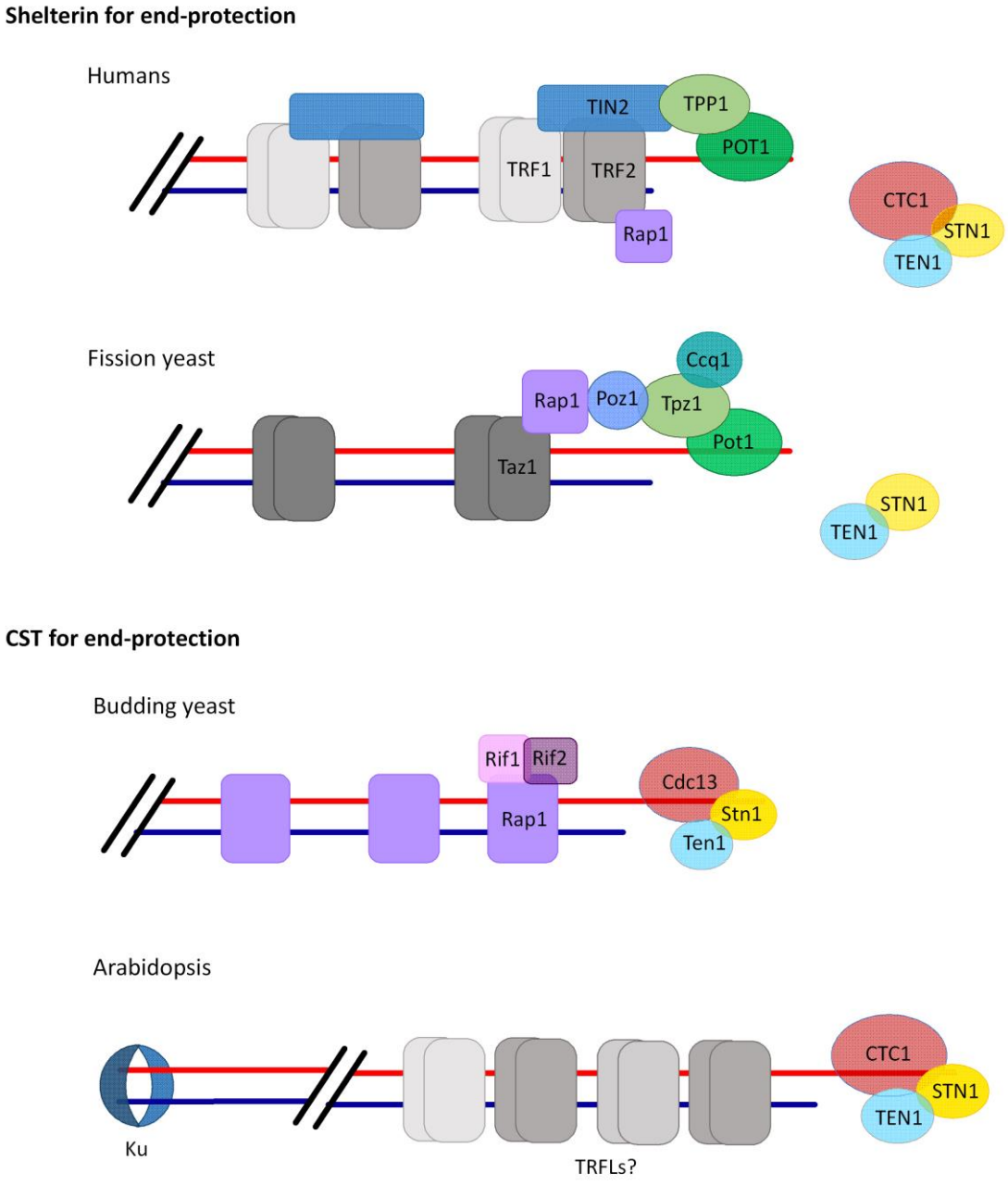


Figure 1-5. Telomere-associated proteins in different species. Diagrams of major telomere-associated complexes, including shelterin in humans and *S. pombe*, and CST in *A. thaliana* and *S. cerevisiae.* CST (subcomplex) also associates with human and *S. pombe* telomeres transiently during the cell cycle. Rap1, Rif1, and Rif2 are responsible for ds telomere protection in *S. cerevisiae.* Ku in *A. thaliana* is responsible for blunt-ended telomere maintenance.

Shelterin—the telomere cap in vertebrates

Mammalian telomeres are associated with shelterin, a six-member protein complex (Palm and de Lange, 2008) (Figure 1-5). Shelterin anchors onto ds telomeric regions through the binding of TRF1 and TRF2 (Telomeric Repeat Factor 1 and 2) (Zhong et al., 1992; Bilaud et al., 1997), and the ss telomeric region through POT1 (Protection of Telomeres 1). TIN2 (TRF1-Interacting Nuclear factor 2) interacts with TRF1 and TRF2 simultaneously and bridges the telomeric duplex binding proteins to the TPP1/POT1 heterodimer at the 3' G-overhang through TPP1 interaction (Kim et al., 1999; Ye et al., 2004a; O'Connor et al., 2006). Finally, RAP1 (Repressor/Activator Protein 1) associates with TRF2. This interaction is critical for non-homologous end joining (NHEJ) inhibition at mammalian telomeres (Li et al., 2000; Sarthy et al., 2009). Individual components of shelterin make unique contributes to telomere maintenance.

TRF1 and TRF2 homodimerize, and both contain a C-terminal Myb DNA binding domain that allows the specific interaction with ds telomeric DNA (Bianchi et al., 1997; Bilaud et al., 1997). TRF1 and TRF2 serve several functions at telomeres. First, TRF1 and TRF2 are essential for telomere length homeostasis. Overexpression of TRF1 or TRF2 in human cells leads to telomere shortening, consistent with a role in negative regulation of telomere length (van Steensel and de Lange, 1997; Smogorzewska et al., 2000). In addition to telomere length regulation, TRF1 and TRF2 contribute to different aspects of telomere architecture. TRF1 can bend telomeric DNA and promote the parallel pairing of telomeric duplex regions *in vitro* (Bianchi et al., 1997; Griffith et al., 1998), while TRF2 promotes formation of the t-loop structure *in vitro* and *in vivo*

(Griffith et al., 1999; Stansel et al., 2001; Doksani et al., 2013). More recent studies highlight a non-redundant role for TRF1 and TRF2 in telomeric chromatin compaction for robust protection (Poulet et al., 2012; Bandaria et al., 2016). Lastly, TRF1 and TRF2 keep DDR and DNA repair pathways suppressed at telomeres. Studies in mouse demonstrated that TRF1-deficient cells suffer from chromosome end-to-end fusions without appreciable telomere shortening (Iwano et al., 2004). Loss of TRF2 activates the ATM (ataxia telangiectasia mutated)-mediated DDR pathway as well as the classical non-homologous end-joining (c-NHEJ) pathway for DNA repair at human and rodent telomeres (Karlseder et al., 1999; Celli and de Lange, 2005; Sfeir and de Lange, 2012), leading to cell cycle arrest and apoptosis or senescence (Karlseder et al., 1999; Takai et al., 2003). TRF2 additionally contributes to end-protection by promoting t-loop formation thereby sequestering 3' G-overhangs (Stansel et al., 2001). Interestingly, TRF1 and TRF2 are found to closely interact with DDR players, potentially for modulation of DDR at telomeres. TRF1 physically associates with Ku70/86, a major player in c-NHEJ (Hsu et al., 2000) and is also a target of ATM kinase (Wu et al., 2007). The function of the ATM and TRF1 interaction is proposed for telomere length control (Wu et al., 2007) and blocking chromosome end-joining (Kishi et al., 2001; Kishi et al., 2002). TRF2 also is found to interact with several components of DNA repair pathways, including Ku70/86 (Ribes-Zamora et al., 2013), ATM, MRE11 complex, and XPF/ERCC1 nuclease (Song et al., 2000; Zhu et al., 2000; Opresko et al., 2002). It is intriguing how shelterin subunits prevent DDR at telomeres and at the meantime interact with major factors in DDR.

The ss telomeric DNA binding protein POT1 was first identified in fission yeast and humans through sequence similarity to the *Oxytricha nova* telomeric binding complex, TEBPα/β (Baumann and Cech, Science 2001). Thereafter, POT1 homologs have been identified in mice, ciliate, plants, and worms. While most organisms encode a single *POT1* gene, *Mus musculus, Caenorhabditis elegans,* Tetrahymena and Arabidopsis endcode two or more *POT1* paralogs (Hockemeyer et al., 2005; Raices et al., 2008; Cheng et al., 2012; Cranert et al., 2014; Beilstein et al., 2015). POT1 proteins contain two conserved N-terminal oligosaccharide/oligonucleotide-binding folds (OB-folds) and a C-terminus with low sequence conservation (Baumann et al., 2002; Lei et al., 2002). The C-terminal domain in human POT1 provides a binding site for the shelterin component TPP1 (Lei et al., 2003). An important function of POT1 is telomere end-protection. POT1 handles the threat from ATR (ataxia telangiectasia and Rad3 related) signaling pathway by competing with replication protein A (RPA), a sensor DNA damage response, for binding of the ss 3' G-overhang (Barrientos et al., 2008; Ray et al., 2014; Hockemeyer et al., 2005; Churikov et al.. 2006; Takai et al., 2011). Knockout of POT1a and POT1b in mouse cells leads to the activation of the ATR pathway, telomere fusions, and senescence (Hockemeyer et al., 2005; Denchi and de Lange, 2007). Consistent with observations in mammals, conditional mutation of *POT1* in fission yeast leads to dramatic telomere erosion accompanied by ATR activation (Pitt and Cooper, 2010). Another conserved role of POT1 is in telomere length control. In human cells, overexpression of a mutant allele of POT1, which has reduced DNA binding, leads to dramatic telomere lengthening (Loayza and de Lange, 2003). *In vitro*

assays indicate human POT1 can negatively regulate telomerase activity (Kelleher et al., 2005). Conversely, overexpression of human POT1 in telomerase positive cells supports a role for POT1 in telomere lengthening (Armbruster et al., 2004; Colgin et al., 2003). These studies indicate that hPOT1 can act as both a positive or negative regulator of telomere length.

One of the most important binding partners of POT1 is TPP1 (Houghtaling et al., 2004; Liu et al., 2004). TPP1 contains an N-terminal OB-fold, a POT1 binding domain and a TIN2-interacting region at its C-terminus, and serves as a bridge connecting the ss telomere binding protein POT1 to telomeric dsDNA through interactions with TIN2 (Liu et al., 2004; Ye et al., 2004b; Wang et al., 2007). TPP1 forms a heterodimer with POT1 (Wang et al., 2007; Xin et al., 2007), resembling the ss telomere binding TEBPα/β heterodimer in the ciliated protozoan *O. nova* (Gray et al., 1991; Horvath et al., 1998; Xin et al., 2007). TPP1/POT1 remodels the telomeric DNA secondary structure by compacting ss telomeric DNA and suppressing guanosine quadruplex formation (Taylor et al., 2011; Zaug et al., 2005; Ray et al., 2014; Hwang et al., 2014). TPP1 association also enhances POT1 binding to telomeric ss DNA (Liu et al., 2004; Wang et al., 2007; Kibe et al., 2010). Finally, TPP1/POT1 heterodimer contributes to telomerase recruitment (Xin et al., 2007; Wang and Lei, 2011) and stimulates telomerase repeat addition processivity (RAP) (see below) (Wang et al., 2007).

Rap1 was initially identified in yeast as an activator or repressor of transcription that binds dsDNA through its myb domain (Kurtz and Shore, 1991; Sussel and Shore, 1991). Later, Rap1 was recognized as a constituent of telomeres. Its presence at

telomeres is conserved in fungi, protozoa, and vertebrates, but interestingly not in higher plants. Mammalian Rap1 does not directly associate with telomeric DNA. It is recruited to telomeres by TRF2 (Li et al., 2000). This association contributes to repression of telomere fusion (Bae and Baumann, 2007; Sarthy et al., 2009). Human Rap1 has been implicated in telomere length regulation with contradicting results using different genetic approaches (O'Connor et al., 2004; Li and de Lange, 2003). However, recent studies agree with previous observations in mouse cells indicating that Rap1 is not essential for telomere length regulation or telomere protection in mammals (Sfeir et al., 2010; Kabir et al., 2014).

TIN2 is a central component of shelterin complex. It was identified as a TRF1-interacting protein (Kim et al., 1999) and later characterized as a bridge between TRF1 and TRF2 (Houghtaling et al., 2004). TIN2 stabilizes the binding of TRF1 and TRF2 at telomeres to prevent DNA damage responses (Kim et al., 2003; Kim et al., 2004; Ye et al., 2004). TIN2 also interacts with TPP1 and therefore connects TPP1/POT1 at the ss telomeric DNA to TRF1 and TRF2/Rap1 at the duplex region (O'Connor et al., 2006). This interaction ensures TPP1/POT1 association with ss telomeric DNA to avoid ATR signaling (Chen et al., 2007; Takai et al., 2011). Depletion of TIN2 in human cells results in reduced TPP1 association with telomeres and compromised telomerase recruitment (Abreu et al., 2010). Consistent with this finding, recent studies indicate that TIN2 is involved in telomere length regulation through telomerase-dependent recruitment (Frank et al., 2015). Thus, TIN2 is a multifaceted protein involved in telomere end-protection and end-replication.

Similar to humans, *S. pombe* uses a shelterin-like complex with seven subunits (Figure 1-5). Taz1 is a homolog of mammalian TRF1/TRF2 that directly binds to ds telomeric DNA (Cooper et al., 1997). The Tpz1 (TPP1 ortholog)-POT1 dimer connects to Taz1 through Rap1 and Poz1 (Kanoh and Ishikawa, 2001). Like human Rap1, SpRap1 mutants lose telomere length control and telomere silencing (Park et al., 2002). Ccq1 interaction with Tpz1 is responsible for telomerase recruitment and end-protection (Harland et al., 2014).

No shelterin-like complex has been identified outside vertebrates and fission yeast, although individual subunits of shelterin are conserved across eukaryotes for telomere end-protection (Figure 1-5 and 1-6). As mentioned previously, *O. nova* encodes orthologs of yeast and vertebrate POT1, TEBPα/β heterodimer for ss telomeric DNA binding (Gray et al., 1991; Fang et al., 1993). Although no TRF1/2 homolog has been identified in budding yeast (Li et al., 2000), Rap1 in budding yeast appears to serve the function of ds telomeric DNA binding to control telomere length and prevent NHEJ at telomeres with the help of Rif1 and Rif2 (Pardo and Marcand, 2005; Miller et al., 2005; Conrad et al., 1990; Longtine et al., 1989; Gilson et al., 1993; Wotton and Shore, 1997; Levy and Blackburn, 2004).

POT1 is a critical and highly conserved component of telomeres (Figure 1-6). The *POT1* gene was duplicated in mice, *T. thermophila*, *C. elegans*, and Arabidopsis leading to more than one *POT1* ortholog (Palm et al., 2009; Jacob et al., 2007; Cranert et al., 2014; Cheng et al., 2012). In mice, POT1a and POT1b have high sequence similarity and play partially redundant roles in telomere maintenance (Hockemeyer et al., 2005;

Palm et al., 2009). Whereas in *T. thermophila*, the two POT1 protein, tPOT1a and tPOT1b, have lower sequence similarity (57% similarity and 44% identity) (Jacob et al., 2007). Similar to mammalian POT1 proteins, tPOT1a regulates telomere length and prevents cell check point activation. However, *tPOT1b* is not involved in telomere maintenance and is not an essential gene. tPOT1b protein has been implicated in chromosome breakage and chromosome rearrangement (Cranert et al., 2014). *C. elegans* encodes four POT1 homologs: MRT-1, POT-1 (CeOB1), POT-2 (CeOB2), and POT-3. Individual POT1 proteins in *C. elegans* contribute to different processes of telomere metabolism, including end processing (Raices et al., 2008), ALT (Cheng et al., 2012), and telomerase activity (Meier et al., 2009).

In plants, a shelterin-like complex has not been identified. However, POT1 homologs have been identified in a wide range of land plants, from green algae to moss to higher plants (Beilstein et al, 2015). In the plant kingdom, the *POT1* gene have undergone gene duplication in some species. For example, three *POT1* genes have been identified in *A. thaliana*: *POT1a*, *POT1b*, and *POT1c* (Rossignol et al. 2007; Shakirov et al., 2005) (Figure 1-6). *A. thaliana* POT1 proteins evolved to be a constituent of telomerase RNPs rather than a telomere binding protein (see below) (Shakirov et al, 2009; Cifuentes-Rojas et al, 2011&2012). TRF-like proteins were found in *A. thaliana* (Karamysheva et al, 2004), however genetic studies revealed that they are essential for telomere maintenance (Fulcher and Riha, 2015) (Figure 1-5).

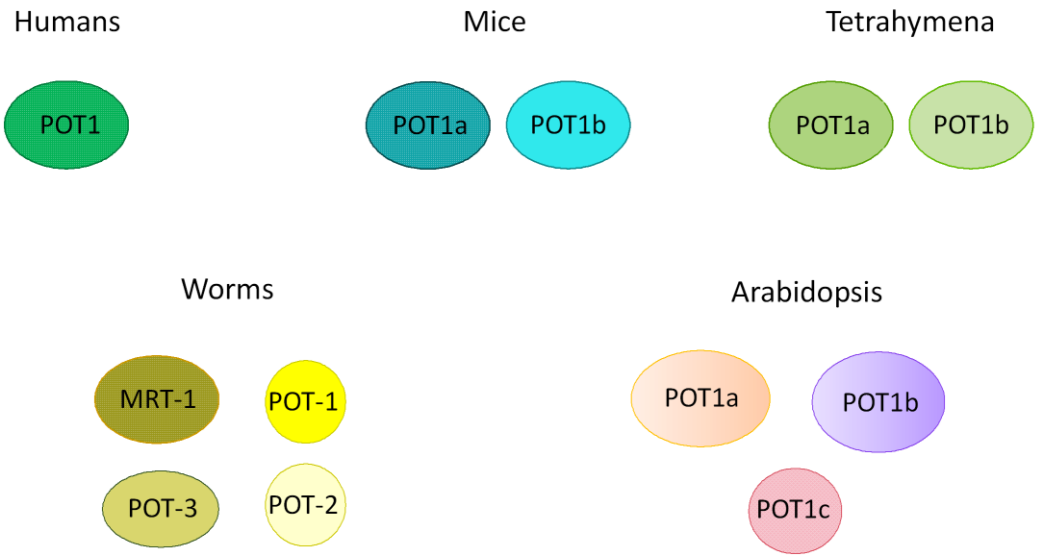


Figure 1-6. POT1 homologs and its duplication in different species. Humans encode one POT1 protein. Mice and Tetrahymena have two *POT1* paralogs. Worms have four POT1-like proteins. POT-1 and POT-2 are single oligosaccharide/oligonucleotide-binding fold (OB-fold) proteins. Arabidopsis encodes three POT1 proteins. POT1c has a single OB-fold.

The CST complex—end protection and telomere replication

The CST (CTC1/ Cdc13; STN1; TEN1) complex provides an alternative solution for telomere end-protection and end-replication regulation among species lacking crucial shelterin components. Initially discovered in the budding yeast *S. cerevisiae*, CST homologs are present in a wide range of organisms, including fungi, mammals, and plants (Giraud-Panis et al, 2010; Price et al, 2010). Components of CST form a trimeric complex and recognize ss telomeric DNA through their OB-folds (Lin and Zakian, 1996; Gao et al, 2007; Miyake et al, 2009). CTC1/Cdc13 is the largest subunit and is predicted to bear more than one OB-fold, while STN1 and TEN1 contain only a single OB-fold.

Sharing structural similarity with heterotrimeric RPA complex, the CST complex is proposed to be a telomere-specific RPA-like complex (Gao et al, 2007; Miyake et al, 2009; Sun et al, 2009). Studies in budding yeast and plants have delineated the function of CST in telomere end-protection: inactivation of individual CST components leads to drastic telomere shortening, extended ss telomeric DNA, extensive recombination, and activation of DDR at telomeres (Gao et al, 2007; Surovtseva et al, 2009; Song et al, 2008; Leehy et al, 2013; Boltz et al, 2012). Disruption of vertebrate CST subunits, however, does not cause immediate telomere erosion, suggesting a minor role in telomere protection (Huang et al, 2012; Stewart et al, 2012b; Gu et al, 2012). Therefore, budding yeast and plants appear to use the CST as their major end-capping complex due to the absence of shelterin-like complex.

Despite these differences in CST function in telomere protection, CST plays a conserved role in telomere replication. CST subunits physically interact with telomerase components and modulate several steps in end-replication (Wu et al, 2012; Chen et al, 2012a; Beilstein et al, 2015). For example, separation-of-function mutations in budding yeast Cdc13 uncovered a role in telomerase recruitment, independent of its end-protection function, via Stn1 interaction (Evans and Lundblad 1999; Nugent et al, 1996; Pennock et al, 2001). Stn1 interferes with telomerase association with Cdc13 by competing with the telomerase accessory factor Est1 for binding sites on Cdc13 (Puglisi et al, 2008; Chandra et al, 2001). In addition, *A. thaliana* TEN1 competes with AtPOT1a for CTC1-STN1 binding, facilitating the switch between telomerase-extendible to non-extendible states (Renfrew et al, 2014). In vertebrates, CST also interacts with TPP1-

POT1 to shut off telomerase (Chen et al, 2012a). CST accumulates at telomeres during late S/G2 phase, where in addition to negatively regulating telomerase, it is proposed to promote G-overhang maturation by C-strand fill-in and resection (Chen et al, 2012a; Wang et al, 2012). Human Ctc1 and Stn1 were originally identified as accessory factors of DNA pol α-primase (Goulian et al, 1990; Casteel et al, 2009). Additional compelling evidence now reveals that interactions between CST components and pol α-primase are conserved for telomere replication, specifically telomere C-strand fill-in (Lue et al, 2014; Derboven et al, 2014). These observations support the conclusion that CST coordinates the action of telomerase and C-strand resection and fill-in machineries to promote telomeric DNA replication.

While the prevailing view has been that CST functions as a stable trimeric complex, recent studies have shed light on the contribution of individual components of CST in telomere biology. For example, the components of the *Candida glabrata* CST complex exhibit a 2:4:2 or 2:6:2 stoichiometry that challenges the conventional trimetric complex model (Lue et al, 2013). In addition, genetic analysis shows that Stn1 or Ten1 can partially complement a *Cdc13* deletion in *S. cerevisiae*, but not *vice versa*. Stn1 and Ten1 apparently have more crucial roles in cell viability than Cdc13 (Holstein et al, 2014). This finding suggests that alternative subcomplexes or assemblies of CST components may have unique functions in telomere biology. *A. thaliana* TEN1, but not STN1 or CTC1, serves as a negative regulator of telomerase activity (Leehy et al, 2013). Moreover, AtTEN1 but not STN1 has protein chaperone activity similar to small heat-shock proteins. This activity is proposed to protect CTC1 against heat induced protein

degradation (Lee et al, 2016). Therefore, it is of interest to investigate the individual contribution of CST components to telomere maintenance and their novel functions outside telomeres. Appendix I presents an interesting observation of *A.thaliana* STN1 and TEN1 localization in chloroplasts in mesophyll protoplasts, suggesting that these proteins may function outside the nucleus.

Ku—a multifaceted complex important for telomere maintenance and end-protection

The Ku70/80 heterodimer (Ku) is best known for its conserved function in classic NHEJ. Ku recognizes a dsDNA terminus with no sequence preference, allowing its association at dysfunctional telomeres. For example, in *A. thaliana* Ku promotes chromosome end joining when telomere protection is compromised (Amiard et al, 2014; Gravel et al, 1998). Paradoxically, Ku also is a versatile constituent of functional telomeres, involved in a wide range of processes to ensure telomere maintenance. In mammals, Ku associates directly with shelterin components to prevent end joining and telomere recombination (Hsu et al, 2000; Wang et al, 2009). *S. cerevisiae* Ku regulates telomere end processing (Vodenicharov et al, 2010; Bonetti et al, 2010a; Bonetti et al, 2010b). In addition, Ku has a conserved influence on telomere length regulation, although the modes of regulation are different among species. In budding yeast, Ku is a positive regulator of telomere length by promoting telomerase recruitment and activity (Boulton and Jackson, 1996; Williams et al, 2014). In contrast, depletion of Ku in *A. thaliana* leads to extensive telomere elongation, implicating a negative role in telomere length maintenance of Ku (Riha et al, 2002). Finally, Ku has been found to interact with

telomerase RNA in mammals, yeast, and plants (see below) (Peterson et al, 2001; Ting et al, 2005&2009; Cifuentes-Rojas et al, 2012). Various functions of Ku at telomeres are still under close scrutiny.

In plants, the functions of Ku have been further extended to protection of blunt-ended telomeres (Kazda et al, 2012). A major focus of this dissertation is analysis of a few players that may assist Ku in the protection of blunt-ended telomeres.

Telomerase, its regulation, and DNTF

The end-replication problem, which is caused by incomplete DNA replication resulting in a loss of terminal sequence (Olovnikov, 1973; Watson, 1972) (Figure 1-3), poses a crucial challenge to telomere length homeostasis and cell viability (Harley et al, 1990; de Lange et al, 1990; Lendvay et al, 1996; Riha et al, 2001; Jaskelioff et al, 2011). Telomere addition by telomerase is the prevalent solution to counterbalance telomere shortening in eukaryotes.

The minimal components of telomerase: TERT and TER

Telomerase activity was first characterized in ciliate *Tetrahymena thermophila* (Greider and Blackburn, 1985), and has continued to be a major focus of investigation. Telomere replication involves a series of steps (Collins, 2011). First, telomerase is recruited to chromosome ends, and recognizes the ss 3' G-overhang as a substrate (Lingner and Cech, 1996). After the RNA template aligns with the G-overhang, telomerase incorporates nucleotides onto the 3' end of DNA by copying the template

sequence with nucleotide addition processivity (Greider and Blackburn, 1989). Once the enzyme reaches the end of the template, it translocates and realigns the newly synthesized 3' end back to the beginning of the template for another elongation-translocation cycle. Multiple rounds of telomere repeat addition are termed "repeat addition processivity" (RAP) (Greider and Blackburn, 1985). Finally, telomerase must disassociate from the telomere for C-strand fill-in by the DNA polymerase α-primase complex (Chakhparonian and Wellinger, 2003).

The minimal makeup of telomerase was narrowed down to a catalytic protein subunit, telomerase reverse transcriptase (TERT), and a template RNA, TER (Lendvay et al, 1996; Lingner et al, 1997; Cong et al, 2002; Feng et al, 1995; Nakamura et al, 1997; Greider and Blackburn, 1989; Singer and Gottschling, 1994). These two components are sufficient to reconstitute telomerase activity *in vitro* (Cohn and Blackburn, 1995; Weinrich et al, 1997; Collins and Gandhi, 1998). TERT and TER have been characterized in a wide-range of organisms (Harrington et al, 1997; Nakamura et al, 1997; Heller et al, 1996; Fitzgerald et al, 1999; Greenberg et al, 1998; Malik et al, 2000). TERT proteins share several conserved domains. TERT has an essential N-terminal domain (TEN) for RNA and DNA binding and a carboxy-terminal extension (CTE) that promotes processivity (Malik et al, 2000; Autexier and Lu, 2006; Collins, 2006). Between these two domains are the RNA binding domain (TRBD) for stable RNP formation and the core reverse transcriptase (RT) domains (Blackburn and Collins, 2011; Lue, 2009). TER molecules exhibit a vast diversity in size and sequence, Nevertheless, TERs from different species share three core structural motifs: a template

region which contains one and a half copies of the telomere repeat sequence, a pseudoknot, and a terminal stem-loop or bulged stem-junction (Blackburn and Collins, 2011). These structural elements make TER more than merely the RNA template; they shape the telomerase active site and provide a scaffold for telomerase accessory proteins to converge at different stages of telomerase action (see below).

Telomerase regulation and cell proliferation

Multicellular organisms have reached a consensus for telomerase regulation during development: higher telomerase activity in actively dividing cells and reduced telomerase activity in somatic cells (Cong et al, 2002). In a single cell or unicellular organisms, telomere synthesis is required for proliferation and is restricted during late S/G2 phase (Greider and Blackburn 1985; Gallardo et al, 2011). This developmental and cell cycle control of telomerase activity mirrors TERT mRNA levels in mice, humans and plants (Greenberg et al, 1998; Meyerson et al, 1997; Takakura et al, 1998; Murofushi et al, 2006; Edqvist et al, 2006). Telomerase activity control is more complicated than merely transcriptional and translational regulation. Posttranscriptional and posttranslational modification of TERT contribute to telomerase regulation (Kilian et al, 1997; Martin-Rivera et al, 1998; Chung et al, 2012). Cellular processes, such telomerase biogenesis, recruitment to telomeres and enzyme activation, also have a profound influence on appropriate spatial and temporal control of telomerase activity (see below). Derepression of telomerase activity in humans and mouse has been associated with cellular immortality and cancer (Shay and Bacchetti, 1997; Kim et al,

1994). Deficiency in telomerase activity, on the contrary, results in limited cell proliferation capacity, aging, and premature cellular senescence due to telomere shortening (Harley et al, 1990, Counter et al, 1992; Hahn et al, 1999; Zhang et al, 1999). Therefore, telomerase regulation is a promising target for cancer therapy and thus a prevalent subject for telomere studies.

Telomerase composition and biogenesis

The interplay of additional proteins associated with the telomerase core components is essential for the complex telomerase regulation *in vivo*. Species-specific telomerase accessory proteins coordinate different layers of regulation for appropriate telomerase activity, including telomerase biosynthesis, trafficking, recruitment, and enzymatic activity. Several well-studied regulation processes will be introduced here.

Telomerase accessory proteins were first characterized by genetic studies of mutants in budding yeast, which showed a progressive decrease in telomere length (Lundblad and Szostak, 1989; Lendvay et al, 1996). Genes responsible for this "ever shorter telomere" (EST) phenotype were identified as constituents of the telomerase holoenzyme (Lundblad and Szostak, 1989; Lendvay et al, 1996). Est2 is the telomerase catalytic subunit in budding yeast. Est1 is an accessory factor that interacts with telomerase RNA, TLC1 (Hughes et al, 2000; Seto et al, 2002). Est1 also interacts directly with Cdc13 at telomeres and is enriched at telomeres during S phase (Qi et al, 2000; Taggart et al, 2002; Wu and Zakian, 2011). Notably, Est1 associates with telomeres prior to S phase where it promotes telomerase recruitment and activates

inactive telomerase (Pennock et al, 2001; Taggart et al, 2002; Chen et al, 2016). Finally, Est3 is enriched at telomeres in late S/G2 phase in an Est1-dependent manner (Tuzon et al, 2011) and is involved in telomerase stimulation through interaction with Est2 (Talley et al, 2011), but the precise role of Est3 is not clear.

Another well-studied telomerase accessory protein is Ku. Ku interaction with TER has been reported in budding yeast, humans, and plants (Peterson et al, 2001; Ting et al, 2005&2009; Cifuentes-Rojas et al, 2012). The nature of this interaction is best characterized in budding yeast. *S. cerevisiae* Ku recognizes a 48 nt stem-loop structure on TLC1 (Peterson et al, 2001). This interaction stabilizes TLC1 (Mozdy et al, 2008; Zappulla et al, 2011) and is required for Est2-telomere association during G1 and early S phase (Stellwagen et al, 2003; Fisher et al, 2004). The Ku-TLC1 interaction also facilitates telomerase activation by facilitating Est1p recruitment and substrate accessibility (Peterson et al, 2001; Williams et al, 2014). Recent biochemical studies provide evidence for mutually exclusive DNA and telomeric RNA binding activity of Ku (Pfingsten et al, 2012), proposing a revised model for the telomerase recruitment function of Ku in which Ku recruits telomerase to the telomere and switches its binding to telomeric DNA instead of TLC1. Finally, Ku is also involved in the nucleo-cytoplasmic trafficking of TLC1 (Williams et al, 2014). Depletion of EST proteins or Ku lead to TLC1 accumulation in the cytoplasm (Gallardo et al, 2008).

Multiple telomerase accessory proteins assist the assembly, trafficking and activation of human telomerase (Nandakumar and Cech, 2013). Mass spectrometry analysis of affinity-purified human telomerase has identified dyskerin, an RNP

maturation factor whose mutation causes the stem cell disorder Dyskeratosis Congenita (DC) (Mitchell et al, 1999; Cohen et al, 2007). A second co-purification identified TCAB1, a protein component of Cajal body (Venteicher et al, 2009). Together, dyskerin and TCAB1 stably associate with core telomerase and contribute to RNP maturation and, in the case of TCAB1, telomerase recruitment to telomeres. Dyskerin directly interacts with TER for RNP maturation (Chen and Greider, 2004; Fu and Collins, 2007). The association between TER and dyskerin is also conserved in plants (Kannan et al, 2008). As in yeast, core components of human telomerase also travel between subcellular compartments in a cell cycle-dependent manner. During most of the cell cycle, human TERT and TER are kept separately in the nucleus, with the majority of TERT in nuclear foci, and TER in the Cajal body (Tomlinson et al, 2006). During S phase, TERT is recruited to nucleoli, and TER-containing Cajal bodies meet TERT at telomeres (Tomlinson et al, 2006; Tomlinson et al, 2008). TER accumulation in the Cajal body is dependent on TCAB1 (Jady et al, 2006). Depletion of TCAB1 reduces TER association with Cajal bodies and also compromises telomerase-telomere association and telomere elongation (Venteicher et al, 2009; Zhong et al, 2011; Stern et al, 2012). TCAB1, but not the Cajal body, is also required for TERT association with telomeres (Stern et al, 2012). Interestingly, mouse TER does not accumulate in Cajal bodies. Instead, it is concentrated in separate nuclear foci (Tomlinson et al, 2010), suggesting that maturation of the mouse telomerase may involve different sets of telomerase accessory proteins.

Once telomerase is brought to the vicinity of telomeres, series of highly regulated molecular processes are initiated, including alternation of the end structure of the

telomere to increase accessibility and activate telomerase. The telomere 3' terminus must be remodeled to release the 3' G-overhang from the t-loop for end-replication (Hockemeyer and Collins, 2015). In mammals, one critical factor for telomerase recruitment and processivity is the shelterin component TPP1. For telomerase recruitment, TPP1 directly interacts with TERT through a highly conserved glutamate and leucine-rich region (the "TEL-patch") (Abreu et al, 2010; Zhong et al, 2012; Nandakumar et al, 2012; Sexton et al, 2012). TPP1 also binds to POT1 to enhance ss telomeric DNA binding (Wang et al, 2007). Further, TPP1/POT1 together expose the 3' G-overhang for optimal telomerase engagement (Zaug et al, 2005; Taylor et al, 2011; Ray et al, 2014; Hwang et al, 2014), acting synergistically to enhance RAP by increasing telomerase translocation efficiency (Wang et al, 2007; Xin et al, 2007; Zaug et al, 2010; Latrick and Cech, 2010). The fission yeast TPP1 ortholog, Tpz1, functions in the same way in telomerase recruitment and activation (Jun et al, 2013). A separation-of-function mutation of Tpz1 continues to allow telomerase recruitment to telomeres through Ccq1, but fails to allow telomerase elongation (Armstrong et al, 2014). These data illustrate the idea that telomerase recruitment does not guarantee repeat addition.

De novo telomere formation at DSBs

Although telomerase has high specificity for telomeric DNA, it can sometimes mistake DSBs for telomeres and add telomere repeats *de novo.* This *de novo* telomere addition (DNTF) requires little or no homology in the substrate to the telomerase RNA template and can generate, through the subsequent association of telomere binding

proteins, a functional telomere serving an alternative way for DSB stabilization (Ribeyre and Shore, 2012). DNTF, also known as chromosome healing, allows resumption of the cell cycle (Michelson et al, 2005). However, DNTF is highly deleterious. Although the break site on the centromere-containing fragment is stabilized by a newly synthesized telomere, the acentric chromosome fragment will be lost during cell division. In humans, terminal chromosome truncation and DNTF are associated with several disorders including Alpha thalassemia, Phelan McDermid syndrome, and mental retardation (Flint et al, 1994; Luciani et al, 2003; Wong et al, 1997). In yeast, chromosome healing often leads to lethality in haploid cells. DNTF that occurs at the cost of losing a chromosome arm is actively suppressed *in vivo* and is quite rare.

Most studies of DNTF have been performed in yeast due to its higher frequency and the workable genetics. Several factors that promote or repress the multi-step DNTF process have been characterized. Telomerase core components, including Est1, Est2, Est3, and TLC1 accumulate at DSBs and are required for DNTF (Bianchi et al, 2004; Negrini et al, 2007; Chung et al, 2010). Although Ku is required for c-NHEJ, it can also promote DNTF by interacting with TLC1 for telomerase recruitment at DSBs (Stellwagen et al, 2003; Bianchi et al, 2004). Accumulation of Cdc13 to DSBs, a process that can be repressed by Mec1-mediated phosphorylation, also promotes telomere addition (Zhang and Durocher, 2010). Meanwhile, the Pif1 5’-3’ helicase inhibits telomere addition at DSBs by destabilizing the telomerase RNA-DNA hybrid (Boule et al, 2005; Zhou et al, 2002). The effective resection of the DSBs by Exo1 and Sgs1 also contributes to the inhibition of DNTF in budding yeast (Zhu et al., 2008; Gravel et al.,

2008). In contrast, molecular events during DNTF in high eukaryotes are less understood. More players need to be identified for a better understanding of the DNTF process. Chapter IV discusses the role of TER2, a telomerase RNA in *A. thaliana,* in inhibition of DNTF.

These studies in yeast together with other works in human cells and plants depict a complex network of biological processes contributing to spatial and temporal regulation of telomerase activity. Breakdown of any process within the network could lead to unwanted telomerase activation and DNTF or telomerase repression that eventually disrupts chromosome stability.

Telomere length homeostasis, TRD, and ALT

Telomere length is maintained at a set range for each species and is under regular surveillance. Telomere length abnormality often hampers cell proliferation: short telomeres trigger DDR, cell cycle checkpoints, and even senescence; aberrant elongation of telomeres also limit cell growth (Lendvay et al, 1996; Riha et al, 2001; McEachern and Blackburn 1995; Fairlie and Harrington, 2015). Telomere length homeostasis is established through the balance between forces: telomerase elongation and erosion (Stewart et al, 2012a). Although telomerase recruitment to telomeres during S phase is ensured by various factors, not all telomeres are extended by telomerase in a single cell cycle (Hug and Lingner, 2006; Teixeira et al, 2004). Shorter telomeres are the preferred substrates for telomerase (Hemann et al, 2001; Shakirov and Shippen, 2004; Teixeira et al, 2004). For example, in yeast only 7% of the telomeres are elongated in a single cell

cycle (Teixeira et al, 2004). These telomeres are extended by about 40 bp, just enough to compensate for incomplete end-replication. In Arabidopsis, analysis of telomere elongation kinetics of individual telomeres showed a fluctuation of telomere length among siblings, indicating an active telomere length surveillance mechanism (Shakirov and Shippen, 2004). Thus, the status of individual telomeres may differ in the same cell, and be accounted for by telomerase preference (Blackburn, 2001). Mounting evidence supports the hypothesis that a dynamic switch exists between the telomerase-extendible and telomerase-nonextendible states during the cell cycle (Teixeira et al, 2004).

The protein counting model provides one mechanistic explanation for the preference of telomerase towards short telomeres (Marcand et al, 1997). In this model, shorter telomeres are associated with fewer telomere duplex associating proteins, such as TRFs in mammals, making them more accessible to telomerase extension. In contrast, longer telomeres with more telomere proteins signal for a telomerase-nonextendible state (Teixeira et al, 2004; Smogorzewska et al, 2000; Marcand et al, 1997). Conformational changes at telomeres, including the assembly and disassembly of t-loops, may contribute to the switch between the two states. Telomerase accessory proteins that promote or block telomerases access to chromosome ends (discussed above) are also likely to contribute to telomere length dynamics.

Oversized telomeres can be trimmed by deletional recombination. The sudden loss of long arrays of telomere repeats, also known as TRD, was originally observed as a sizing mechanism for *S. cerevisiae* telomeres (Lustig, 2003). The t-loop structure, which resembles a Holliday junction intermediate, is resolved giving rise to TRD and an ECTC

byproduct (Wang et al, 2004). ECTCs have been detected in mammals and plants under normal conditions (Wang et al, 2004; Zellinger et al, 2007), and their abundance is elevated in response to telomere dysfunction (Zellinger et al, 2007).

Beside telomere addition by virtue of telomerase activity, telomere length can be maintained by a telomerase independent pathway, termed alternative lengthening of telomere (ALT) (Natarajan and McEachern, 2002; Cesare and Griffith, 2004). Although the ALT mechanism is not preferred in the presence of telomerase, this mode of telomere maintenance is widespread among eukaryotes (Lundblad, 2002; Reddel, 2003). ALT was first found in yeast lacking telomerase (Lundblad and Blackburn, 1993). Although most cells die without telomerase, a small subpopulation of yeast can survive utilizing HR pathways. A similar mechanism has been reported in telomerase-negative human cells (Bryan et al, 1997a &b). While approximately 85% of human cancer cells express telomerase for telomere maintenance, 15% of human cancers lack detectable telomerase and maintain their telomere length through ALT. Thus, ALT appears to be responsible for bypassing the replicative senescence in these settings (Dunham et al, 2000). How ALT is initiated is unknown. Several recombination-based mechanisms pathways are proposed for conducting ALT (Cesare and Reddel, 2010). Sister chromatid exchange (SCE) during DNA replication and HR through strand invasion at the telomeric duplex regions can lead to ALT. Additionally, ECTCs are reported to act as templates for rolling-circle amplification at telomeres (Henson et al, 2002). A recent study revealed several factors that assemble into a break-induced replisome at telomeres to promotes ALT in human cells, independent of ATM and ATR signaling or HR

pathway (Dilley et al., 2016), however, the molecular mechanism of ALT remains enigmatic.

DNA damage at telomeres

Cells face a constant threat from DNA damage elicited by endogenous and environmental factors (Waterworth et al, 2011). Detection and repair of DNA damage is vital for genome stability and hence is executed by multiple overlapping repair pathways. The termini of unprotected telomeres resembles a DNA break and can be perceived by general DDR pathways. The signaling kinases, ATM and ATR, are two central transducers of DNA break surveillance pathways. ATM signaling responds primarily to DSBs, while the ATR pathway respond to RPA bound ssDNA (Shiloh, 2003; Reinhardt and Yaffe, 2009).

Activation of ATM or ATR pathways by spontaneous DNA breaks results in a spectrum of downstream responses, including the recruitment of DNA repair machineries, initiation of cell-cycle checkpoints, and apoptosis or senescence (Maréchal and Zou, 2013). The responses at DSBs are shared by DDR elicited in response to dysfunctional telomeres (Maréchal and Zou, 2013; d'Adda di Fagagna et al, 2003). In mammals, phosphorylation of histone H2AX and local accumulation of foci associated with DDR factors, including 53BP1, Mre11 and NBS1, at DSBs are features of DDR. These foci are also present at telomeres with compromised end-protection, referred to as telomere dysfunction-induced foci (TIFs) (Takai et al, 2003). Additionally, natural DSBs are repaired primarily by the HR and NHEJ pathways. HR, preferred by prokaryotes and

yeast, is based on sequence homology, and is a more faithful repair mechanism. In contrast, NHEJ is more prevalent in higher eukaryotes and is error-prone. Both pathways have been found at dysfunctional telomeres (Arnoult and Karlseder, 2015). HR can lead to telomere sister-chromatid exchange (T-SCE), which could be dangerous due to an unequal exchange of telomere sequences, and t-loop excision leading to TRD (Lazzerini-Denchi and Sfeir, 2016). If recognized as DSBs, telomeres could be fused by NHEJ repair pathways directed by Ku (Riha et al, 2006). Telomere fusion is deleterious; the dicentric chromosomes formed by telomere fusions result in anaphase bridges during mitosis. Subsequently, breakage between the two centromeres causes genome rearrangement, genome instability, and eventually cell death (Lo et al, 2002; Pennaneach and Kolodner, 2009; Pobiega and Marcand, 2010).

How telomere proteins repress DDR

A central question in telomere biology is how telomeres protect themselves from DNA damage surveillance. As previously mentioned, telomere capping proteins and telomere architecture repress ATM- and ATR-mediated DNA damage signaling and multiple DDR pathways. In mammals, TRF2 and POT1 play non-redundant roles in DDR inhibition at telomeres. TRF2 is dedicated to the repression of ATM-mediated DDR. POT1, on the other hand, keeps the ATR pathway under control (Denchi and de Lange, 2007; Sfeir and de Lange, 2012). Besides repressing ATM signaling, TRF2 also facilitates the formation of t-loops to prevent the Ku70/80 heterodimer from synapsing telomere termini. In fission yeast, shelterin components, Taz1 and Rap1, both inhibit

NHEJ (Ferreira and Cooper, 2001; Miller et al, 2005), while budding yeast Rap1 adopts parallel pathways for the inhibition of NHEJ and telomere fusions (Pardo and Marcand, 2005; Marcand et al, 2008). In plants, TERT and the end-protection complex CST is used to suppress DDR. Loss of CTC1 elicits ATR-dependent DDR, manifested by the presence of TIFs, increased telomere fusions, and elevated stem cell death (Amiard et al, 2011; Boltz et al, 2012), whereas *tert* mutants activate ATM signaling (Amiard et al, 2011). Thus, telomeres play a role in tamping down the DDR by capping proteins.

Subcellular localization of telomere proteins

As mentioned previously, telomerase components are not restricted to the nucleus. Spatial control of telomerase by excluding core components from the nucleus has been reported as a mechanism of telomerase regulation. Nuclear exclusion of telomerase components can inhibit appropriate assembly, thereby inhibiting unwanted telomerase activation, or conducting non-telomere functions of the enzyme as discussed below.

Several telomere components have been found to dually localize to the nucleus and the cytosol/organelles (Chiodi and Mondello, 2012; Chen et al, 2012b). The functions of extra-nuclear telomere components are poorly understood, but have been linked to cellular metabolism. For example, human telomerase catalytic subunit TERT contains a nuclear localization signal (NLS), and can be found outside the nucleus (Chung et al, 2012b; Chiodi and Mondello, 2012). The nuclear export of hTERT to the mitochondria occurs in response to oxidative stress (Saretzki, 2009). Mitochondrial

TERT plays a critical role in modulating the level of reactive oxygen species (Ahmed et al, 2008), mitochondrial DNA damage (Santos et al, 2004), cell proliferation (Mukherjee et al, 2011) and apoptosis (Indran et al, 2011). The mammalian shelterin components TIN2 (Chen et al, 2012b), TPP1 (Chen et al, 2007), and POT1 (Chen et al, 2007; Liu et al, 2004) are dually localized in the nucleus and cytosol. Shuttling shelterin components in and out of the nucleus is proposed to promote telomere integrity (Chen et al, 2007). Failure to properly shuttle TPP1 out of the nucleus alters telomere length and induces a DNA damage response at telomeres (Chen et al, 2007). It is unknown how localization contributes to telomere biology. Similarly, the cellular trafficking of telomere proteins remains to be explored.

In Chapter II and Appendix I, data are presented showing that several *A. thaliana* telomere proteins have dual localization.

Epigenetic modification at telomeres

Telomeres are characterized by epigenetic marks in both subtelomeric and telomeric regions (Blasco, 2007; Ottaviani et al, 2008). In mammals, DNA methylation is primarily restricted to cytosines in the CG sequences, which are not present in the telomeric DNA (Goll and Bestor, 2005; Ramsahoye et al, 2000). However, subtelomeric DNA of mammalian chromosomes is heavily methylated (Gonzalo et al, 2006). In addition, post-translational modification of the core histones including H3K9^{me3} and H4K20^{me3} is enriched at both subtelomeric and telomeric regions (Garcia-Cao et al, 2004). These modifications are indications of a heterochromatin state, consistent with

the conclusion that telomeric and subtelomeric regions of mammalian chromosomes have more compact chromatin structure which represses transcription (Blasco, 2007). Despite the lack of DNA methylation in yeast, studies in *S. cerevisiae* are consistent with the findings in mammals, revealing a conserved role of histone modifications in maintaining the heterochromatic state of telomeres and subtelomeres (Thompson et al, 1994; Wyatt et al, 2003; Kimura et al, 2002).

The set of epigenetic marks at *A. thaliana* telomeres are slightly different from these in yeast or mammals. In addition to canonical cytosine methylation of CG sequences, CHG (H=A, T, C), and asymmetrical CHH sequences also can be methylated in plants. The presence of asymmetrical DNA methylation machinery is responsible for DNA methylation at *A. thaliana* telomeres (Cokus et al, 2008), predominantly mediated by the RNA-dependent DNA methylation (RdDM) pathway (Vrbsky et al, 2010). *A. thaliana* telomeres are associated with a combination of heterochromatin marks, including H3K9^{Me2} and H3K27^{Me}, and euchromatin marks, including H3K4^{Me2} and H3K9Ac (Vaquero-Sedas et al, 2011&2012). The mixed epigenetic marks are consistent with the loss of transcriptional inhibition at subtelomeres (Vrbsky et al, 2010), suggesting plant telomeres and subtelomeres are less compact.

A potential role for epigenetic modification in telomeres maintenance has been studied mostly in mammals. DNA methylation and histone methylation are both implicated in telomere maintenance (Blasco, 2007). Mice lacking DNA methyltransferases (DNMTs) have dramatically elongated telomeres and increased telomere recombination, featured by ALT-associated promyelocytic leukemia bodies

(APBs) and elevated telomere sister-chromatid exchanges (Gonzalo et al, 2006). Mouse cells lacking the histone methyltransferases (HMTases) Suv39h1 and Suv39h2 also display abnormally long telomeres and loss of heterochromatin proteins at telomeres (Garcia-Cao et al, 2004). Recent studies in *A. thaliana* also argue that DNA methylation is required for telomere length homeostasis in a telomerase-dependent manner (Vaquero-Sedas and Vega-Palas, 2014; Ogrocka et al, 2014). Further investigation is needed to determine how epigenetic modifications contribute to telomere maintenance, and if the mode of regulation is conserved among species. Chapter V of this dissertation sheds light on this question by discussing the role of a chromosome remodeler DDM1 in *A. thaliana* telomere maintenance.

Arabidopsis as a model eukaryote

Several unique features make *A. thaliana* the reference species and prevalent model organism for all plant scientists. First, *A. thaliana* has a relatively short life span: it takes four weeks from germination to reach the reproductive phase and six to eight weeks to obtain progeny. Secondly, the genome of *A. thaliana* is relatively small (~130Mb), fully sequenced, and well-developed for genetic and transgenic approaches. The Arabidopsis community has invested extensive resources in creating outstanding mutation collections for genetic studies in labs worldwide. Finally, benefiting from 1001 Genomes Project (1001genomes.org) launched in 2008, nearly 1000 naturally inbred lines (accessions) of *A. thaliana* from a wide range of geographic locations have been sequenced (Clark et al, 2007; Genomes Consortium, 2016). This resource provides a rich

collection of genetic variation to elucidate the molecular basis for many traits including adaptation to the environment.

Mutagenesis and CRISPR/Cas9 system in Arabidopsis

Targeting Induced Local Lesions in Genome (TILLING) lines and T-DNA insertion lines have been the two major sources for Arabidopsis mutations. The ethyl methanesulfonate (EMS) -induced TILLING mutation collection has undergone high-throughput genome-wide screening for point mutations. Thousands of TILLING mutations across the genome have been cataloged and are available for analysis.

Another mutation collection is the insertion mutations generated by Agrobacterium-mediated transformation (Krysan et al., 1999). Agrobacterium can inject its transferred DNA (T-DNA) into the host cells, which can integrate into the host genome. Taking advantage of this mechanism, T-DNA lines were created by introducing T-DNAs into Arabidopsis plants and identifying the locus with the T-DNA insertion. These large insertion sequences, depending on the site of insertion, can potentially cause disruption or dysregulation of a gene. Thousands of T-DNA mutations cross the genome are available and have been a prevalent tool for genetic studies in *A. thaliana.*

Other approaches for creating mutations, such as the zinc-finger nuclease (ZFNs) system and transcription activator-like effector nucleases (TALENs) system have had some success in *A. thaliana,* but have obvious drawbacks including limited target choices, high risk of off-targets, and higher cost (Bortesi and Fischer, 2015). However, the CRISPR/Cas system (for ‘clustered regularly interspaced short palindromic repeats’

and CRISPR-associated), is now the prevailing method for introduing mutations into genes of interest and provides an effective and efficient method of genomic editing in plants (Bortesi and Fischer, 2015). CRISPR/Cas was first found as a part of the adaptive immune system in bacteria and archaea. In the CRISPR/Cas system, invading DNA from viruses or plasmids is cut into small fragments, called protospacers. The protospacers can be inserted into CRISPR locus and transcribed into pre-crRNA. The pre-crRNA is further processed into crRNAs, which contain the protospacer sequences. The crRNAs recognize invading DNA or RNA with sequence complementarity and assemble into protein complex with Cas9 to cleave the invading DNA or RNA. This system has been modified for gene editing. In this case, the artificial crRNA can be designed to recognize a natural protospacer sequence in the the gene of interest. The DNA break created by CRISPR/Cas9 is repaired by NHEJ, an imprecise DNA repair pathway, resulting in insertion or deletion of nucleotides causing heritable mutations around the protospacer sequence. This CRISPR/Cas9 technique has been successfully applied in a wide range of organisms, including mammalian cells, zebrafish, and Arabidopsis (Cong et al, 2013; Hwang et al, 2013; Fauser et al, 2014).

Arabidopsis makes a unique contribution to telomere biology

A. thaliana is also an outstanding model for telomere studies. The *A.thaliana* genome, like that of other plant species, is highly plastic (Murat et al., 2012). Recombination is more frequent in plants compared with animals (Gaut et al., 2007). Genome duplication, which is often accompanied by gene shuffling and genome

remodeling, is more prevalent in plants. Transposable elements make up a large portion of plant genomes. Therefore, telomere mutations that are lethal in mammals and yeast can be tolerated in *A. thaliana*, making it possible to study highly conserved telomere components. Additionally, Arabidopsis telomeres are relatively short (2-5 kb in wild-type Col-0 accession) (Shakirov and Shippen, 2004), and 8 out of 10 chromosome arms possess unique subtelomeric sequences, enabling the analysis of individual telomere tracts (Heacock et al, 2004). Similar to that in other multicellular organisms, telomerase activity in *A. thaliana* correlates with cellular proliferation capacity: the highest activity is associated with actively dividing calluses, flowers, early seedlings and cell culture (Fitzgerald et al, 1999). The conservation of telomerase regulation across eukaryotes justified the parallel comparison between telomerase in plants and other multicellular organisms. As expected, homologs for many telomere components in vertebrates and yeast have also been identified in Arabidopsis, providing additional resources for understanding evolutionary perspectives of chromosome end maintenance.

Besides the conserved nature of telomeres, there are several unique features of telomere biology in Arabidopsis. First, telomeres in *A. thaliana* and other flowering plants are asymmetrical (Kazda et al, 2012). About 50% of telomeres are blunt-ended, while the other half have a conventional G-overhang protected by the CST complex (Figure 1-5). The maintenance of the blunt-end telomeres is Ku-dependent (Kazda et al, 2012). In the absence of Ku, the blunt-end telomeres are processed into a G-overhang and protected by the CST complex. The other components of the blunt-end telomere cap are unknown. Ku associates with an alternative telomerase RNA TER2, which could

potentially serve as an RNA scaffold for proteins that protect of blunt-ended telomeres (Cifuentes-Rojas et al, 2012; Xu et al., 2015; Wang and Chang, 2011).

A second potential component of the blunt-end telomere cap is POT1b. *A. thaliana* harbors three POT1 paralogs: *AtPOT1a, AtPOT1b,* and *AtPOT1c* (Rossignol et al, 2007; Shakirov et al, 2005). The duplication of POT1a and POT1b occurred near the origin of the Brassicacease family (~100 mya), whereas *AtPOT1c* represents a partial gene duplication of *AtPOT1a* and emerged very recently (~10 mya) (Beilstein et al, 2015). AtPOT1a and AtPOT1b encode two OB-folds and a C-terminal domain (Surovtseva et al, 2007), while POT1c contains only one OB-fold with over 90% nucleotide similarity to POT1a (A. Nelson and D. Shippen, unpublished data).The *A. thaliana* POT1a and POT1b proteins exhibit relatively low amino acid sequence identity (49%), implicating distinct functions *in vivo* (Shakirov et al, 2005). Plants overexpressing of the second OB-fold and the C-terminal region of POT1a, but not POT1b, experienced telomere length dysregulation. In contrast, overexpression of the first OB-fold of POT1b, but not POT1a, led to telomere deprotection. Genetic complementation assays also support functional divergence between AtPOT1a and AtPOT1b. The *POT1b* gene cannot complement a *POT1a* deficiency (Beilstein et al, 2015). While the function of AtPOT1c remains unclear, notably, both POT1a and POT1b proteins appear to function primarily in telomerase regulation, rather than as telomere capping components (Surovtseva et al, 2007; Cifuentes-Rojas et al, 2012; Renfrew et al, 2014).

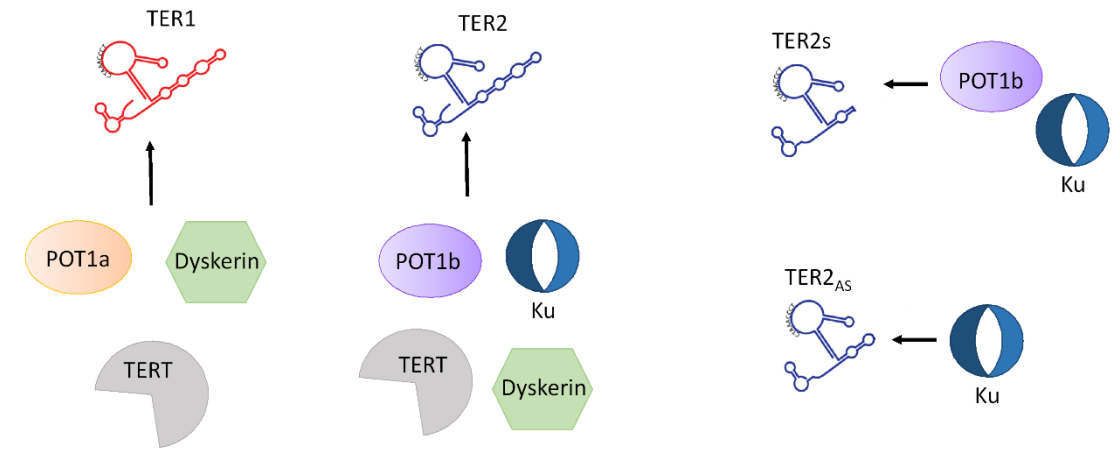


Figure 1-7. TER duplication and alternative RNP assembly in *A. thaliana*. Diagrams of four TER isoforms, TER1, TER2, TER2s, and $TER2_{AS}$. Arrows indicate interactions that have been verified.

AtPOT1a exhibits several functions conserved in vertebrate POT1 and is best studied among the three Arabidopsis POT1 proteins. POT1a specifically recognizes TER1 (Cifuentes-Rojas et al, 2011&2012), the canonical telomerase RNA in Arabidopsis (see below) (Figure 1-7). AtPOT1a accumulates at telomeres during S-phase, but it is not required for telomerase-telomere association (Surovtseva et al, 2007; Renfrew et al, 2014), indicating that, unlike mammalian POT1, AtPOT1a may not be a constitutive component of telomeres and does not play a role in telomerase recruitment (Renfrew et al, 2014). AtPOT1a stimulates telomerase repeat addition processivity and thus is a positive regulator of telomerase activity (Arora et al, 2016). Plants lacking POT1a display progressive loss of telomere tracts, mirroring the phenotype observed in a *tert* mutant (Surovtseva et al, 2007; Riha et al, 2001). AtPOT1a retains the binding

ability to CST complex (Beilstein et al, 2015), an interaction proposed to mediate the switch between the telomerase extendible state and telomerase non-extendible state by competing with TEN1 for STN1 binding (Renfrew et al, 2014). More recently, AtPOT1a was shown to harbor a conserved Phe in the first OB-fold for ss telomeric DNA binding (Arora et al., 2016).

The function of POT1b is less clear. Ectopic overexpression of the POT1b N-terminal OB-fold in wild-type plants led to drastic telomere shortening, chromosome end-to-end fusion and anaphase bridges (Shakirov et al, 2005), implicating a role in end-protection. In addition, AtPOT1b is associated with the alternative telomerase RNA, TER2 (Figure 1-7). The function of POT1b and its binding partners will be discussed in chapters II and III.

Finally, as alluded to above, *A. thaliana* encodes two highly divergent TER genes: TER1 and TER2 (Cifuentes-Rojas et al, 2011). TER1 and TER2 share two regions with high sequence similarity: Conserved Region1 (CR1) containing the template region and Conserved Region 2 (CR2). CR1 and CR2 are adjacent in TER1, but in TER2, CR1 and CR2 are separated by a transposable element (TE) in the majority of Arabidopsis accessions (Xu et al, 2015). TER2 is processed *in vivo* giving rise to two TER2 isoforms: $TER2_{AS}$ and $TER2_{S}$ (A. Suescun and D. Shippen unpublished data; Cifuentes-Rojas et al, 2011) (Figure 1-7). The function of the two shorter TER2 isoforms is currently under investigation.

Under normal growth conditions, TER1 is the most abundant TER isoform and is approximately 10-fold higher than TER2 (Cifuentes-Rojas et al, 2011). However, TERT

has 10-fold higher affinity for TER2 than TER1. TER1 association with AtPOT1a, TERT and the RNA maturation factor dyskerin forms the core of the canonical telomerase to solve the end replication problem (Cifuentes-Rojas et al, 2011). In contrast, TER2 specifically interacts with POT1b, KU, and the RNA maturation factors dyskerin and La protein to form an alternative telomerase RNP (Cifuentes-Rojas et al, 2012; J. Song and D. Shippen, unpublished data). Unlike TER1, TER2 is a highly unstable RNA, however, upon DNA damage, TER2 is stabilized by an unknown pathway, and the spike of TER2 level leads to telomerase inhibition (Xu et al, 2015). The TE in TER2 is essential for this function. The biological significance of the TER2-dependent DNA damage response is still under investigation, but it may provide a route to inhibit DNTF at sites of DSBs as discussed in Chapter IV.

Overview of dissertation

The main focus of this dissertation is to characterize the *A. thaliana POT1b* gene and to dissect its roles in the TER2 RNP. Initial genetic studies of AtPOT1b are presented in Chapter II. Chapter III covers biochemistry and genetic studies of AtPOT1b in context of the TER2 RNP components. A potential biological function of TER2 RNP is presented based on a simple DNTF assay in Chapter IV. Another focus of this dissertation is to investigate how chromatin remodeler DDM1 affects telomere maintenance in *A. thaliana.* Finally, in Chapter V, a novel function of telomeres in promoting genomic stability is presented.

In Chapter II, cytology, biochemistry and genetic approaches are used to study the function of *AtPOT1b*. In marked contrast to AtPOT1a, AtPOT1b is not essential for telomere length maintenance. Instead, it negatively regulates telomerase enzyme activity in flowers. Genetic studies of *pot1b* mutant plants support a role for AtPOT1b in seedling development. Data are presented showing that POT1b localizes to the cytoplasm and interacts with proteins involved in various processes of cell metabolism and response to abiotic stresses. Altogether, these data provide strong evidence for a distinct function of AtPOT1b compared with its paralog AtPOT1a, in plant development and unknown cell processes.

Chapter III discusses the contribution of POT1b, Ku and TER2 to the functions of TER2 RNP: blunt-end telomere capping and telomerase regulation. Data are presented showing that POT1b associates with telomeres and the presence of POT1b may compromise the recruitment of Ku to telomeres. Ku, but not POT1b, can stabilize TER2 RNA in flowers, suggesting distinct contributions of the TER2 RNP subcomplexes. Finally, genetic data are presented indicating that depletion of Ku and TER2 leads to defects in seed formation, consistent with a role for TER2 RNP in early plant development.

Chapter IV elucidates one of the biological functions of TER2 RNP by testing the hypothesis that TER2 inhibits telomerase activity to avoid DNTF at DSBs. DNTF needs to be repressed especially during meiosis, during which DSBs are prevalent, to protect genome integrity. Using an established DNTF assay in tetraploid *Arabidopsis thaliana*, TER2 is shown to repress DNTF.

In Chapter V, data are presented concerning the influence of a nucleosome remodeling protein, DDM1, on telomeres. Unexpectedly, telomeres undergo abrupt shortening in the sixth generation of DDM1 mutants. This precipitous telomere shortening is associated with increased telomere recombination and is also accompanied by increased sensitivity to DSBs and programmed cell death. These data prompt the hypothesis that DDM1 protects against TRD, and in plants lacking DDM1, genomic stability is ensured by eliminating stem cells with extensive DNA damage and dysfunctional telomeres.

In Appendix I, data are presented showing unexpected chloroplast localization of AtSTN1 and AtTEN1. Finally, Appendix II presents published work representing a collaboration with Dr. Jung Ro Lee in the Shippen Lab. This study uncovers an unexpected protein chaperone activity of *A. thaliana* TEN1, supporting a role for TEN1 outside its functions in telomere capping.

This dissertation characterized a distinct POT1 homolog in *A. thaliana* and shed light on novel pathways of telomere capping, telomerase regulation, and plant development. Unanticipated observations in these study also opened the possibility that *A. thaliana* telomere proteins reside in the cytoplasm for cellular processes outside telomere capping. Finally, the importance of telomere regulation in promoting genetic integrity of plant meristems via programmed cell death has been reveal by studies concerning a player in epigenetic modification pathways.

CHAPTER II

ATPOT1B: A MEMBER OF THE POT1 FAMILY WITH NOVEL ROLES IN PLANT DEVELOPMENT AND TELOMERE BIOLOGY

Summary

The ends of a linear chromosome can be mistaken for a DNA double-strand break (DSB) and thus must be protected by conserved nucleoprotein structures called telomeres. The shelterin protein complex plays a key role in telomere protection in mammals. Protection of Telomeres 1 (POT1) is a core component of shelterin that specifically recognizes the single-strand 3' terminus of telomeres to promote telomere length control and to modulate telomere elongation by telomerase. The flowering plant *Arabidopsis thaliana* encodes three POT1 paralogs that evolved to recognize telomerase RNA. While AtPOT1a is well-characterized as a positive regulator of telomerase and is essential for telomere length maintenance, the role of AtPOT1b is unclear. AtPOT1b, distinct from AtPOT1a, assembles into an alternative ribonucleoprotein (RNP) complex that is implicated in negative regulation of telomerase. Here, we isolated several mutant lines of AtPOT1b and used cytological, biochemical and genetic approaches to elucidate the function of AtPOT1b. Knockdown of POT1b caused an increase in telomerase activity in floral tissue, indicating that POT1b negatively regulates telomerase activity. However, telomere length maintenance was unaffected in the mutants indicating a dispensable role in telomere length regulation of POT1b. During seedling development, plants with reduced POT1b had modestly shorter roots than wild-type plants, indicating

that seedling development is delayed. This developmental phenotype is exacerbated in plants that lack telomerase activity. Unexpectedly, we found that a significant fraction of POT1b protein localized in the cytoplasm, and results of a yeast two-hybrid screen showed AtPOT1b interacts with proteins involved in cell metabolism and abiotic stresses. These findings suggest that AtPOT1b may function outside telomere biology. Altogether, these data demonstrate that AtPOT1b distinguishes itself from AtPOT1a in its effect on telomerase activity, its involvement in plant development, and its unexpected cytoplasmic localization.

Introduction

Telomeres are conserved nucleoprotein structures essential for genome stability at the ends of linear chromosomes in eukaryotes. Telomeres serve as physical shields to protect the chromosome ends from being recognized as double-stranded breaks (DSBs) and to prevent incomplete replication of the chromosome terminus (de Lange, 2005). Telomere dysfunction leads to stem cell failure, which profoundly affects growth and development. While mice lacking telomerase, the reverse transcriptase responsible for replication of telomeres, display increased apoptosis, sterility and tumor genesis (Lee et al., 1998; Herrera et al., 1999; Blasco et al., 1997), deletion of telomerase core components in yeast results in the gradual loss of telomere sequences and the onset of growth senescence (McEachern and Blackburn, 1996). Similarly, plants with compromised telomere maintenance suffer a robust DNA damage response (DDR), end-

to-end chromosome fusions, reduced fertility and programmed stem cell death (Riha et al., 2001; Surovtseva et al., 2009; Amiard et al., 2011; Boltz et al., 2012).

Telomeric DNA is comprised of tandem double-stranded (ds) GC-repeats ending in a 3' G-rich overhang (G-overhang). Telomeres are protected by telomere-specific protein complexes, such as the shelterin (vertebrates and fission yeast) and the CST (CTC1/Cdc13; STN1; TEN1) (plants and budding yeast) (de Lange, 2005; Price et al., 2010; Giraud-Panis et al., 2010). Human shelterin has six subunits: TRF1, TRF2, POT1, TIN2, TPP1, and Rap1. Among them, POT1 specifically protects the single-stranded (ss) 3'G-overhang (de Lange, 2005). TPP1 forms a heterodimer with POT1 to recruit telomerase and promote telomerase processivity (Wang et al., 2007; Sexton et al, 2014).

POT1 is the most conserved component of the shelterin complex. First identified in fission yeast, POT1 homologs have been described in ciliates, plants and vertebrates (Baumann and Cech, 2001, Wei and Price, 2004; Raices et al., 2008; Jacob et al., 2007). POT1 proteins typically contain two conserved N-terminal oligosaccharide /oligonucleotide-binding folds (OB-folds) that are essential for binding to ss telomeric DNA and a C-terminal domain for TPP1 binding (Lei et al., 2003). The functions of POT1 have been well-documented in telomere end-protection, telomerase regulation and telomere length control. Mammalian POT1 protects ss telomeric DNA from activating DNA damage signaling and from extensive nucleolytic processing (Hockemeyer et al., 2006; Denchi and de Lange, 2007; Sfeir and de Lange, 2012). POT1 also contributes to telomerase elongation by modulating substrate accessibility (Kelleher et al., 2005; Lei et al., 2005; Ray et al., 2014; Hwang et al., 2014) and by interacting with TPP1 for

telomerase recruitment and activation (Wang et al., 2007; Xin et al., 2007). Finally, POT1 serves as both a positive and a negative regulator of telomere length via telomerase. Overexpression of a mutant allele of human *POT1* that has reduced DNA binding results in profound telomere elongation, implicating POT1 as a negative regulator of telomere length (Loayza and de Lange, 2003). In humans, fusion of POT1 and TERT facilitates telomere elongation (Colgin et al., 2003; Armbruster et al., 2004). Conversely, fission yeast lacking POT1 undergo rapid telomere erosion (Baumann and Cech, 2001).This evidence suggests that POT1 promotes telomere lengthening.

Most organisms harbor only one *POT1* gene, but mice, worms, Tetrahymena and Arabidopsis are exceptions. The two rodent POT1 homologs, POT1a and POT1b, are of high sequence similarity and serve distinct functions in telomere maintenance. MmPOT1a inhibits DNA damage signaling at telomeres, while MmPOT1b regulates the processing of ss telomeric DNA (Hockemeyer et al., 2006). *Caenorhabditis elegans* encodes four POT1-like proteins, which engage in different aspects of telomere metabolism, including telomere length control, telomere end processing and telomere replication (Raices et al., 2008; Cheng et al., 2012). The two Tetrahymena POT1 proteins have more divergent functions. *TtPOT1a* is an essential gene for telomere length regulation. In contrast, TtPOT1b is dispensable for telomere maintenance but uniquely localizes to chromosome break sites participating DNA cleavage during sexual development (Jacob et al., 2007; Cranert et al., 2014).

Within the plant kingdom, two independent gene duplication events occurred in the grasses and in Brassicaceae and gave rise to more than one *POT1* paralog (Shakirov

et al., 2009; Beilstein et al., 2015). The flowering plant *Arabidopsis thaliana* encodes three *POT1* paralogs: *AtPOT1a, AtPOT1b,* and *AtPOT1c* (Rossignol et al. 2007; Shakirov et al., 2005). AtPOT1a and AtPOT1b exhibit relatively low amino acid sequence identity (49%) and were duplicated near the origin of the Brassicacease family (~100 mya) (Shakirov et al., 2005; Beilstein et al., 2015). A third paralog, *AtPOT1c* is unique to *Arabidopsis thaliana*, evolving within the last 10 mya (A. Nelson and D. Shippen, unpublished data). While the function of POT1c remains unclear, both POT1a and POT1b appear to have evolved into telomerase regulatory components, instead of components of the telomere cap (Cifuentes-Rojas et al., 2012; Renfrew et al., 2014).

Genetic and biochemical studies highlight the functional divergence of AtPOT1a and AtPOT1b. First, POT1b cannot complement a *pot1a* null mutation in *A. thaliana* (Shakirov et al., 2005; Beilstein et al., 2015). Second, the POT1a linage, but not POT1b, experienced positive selection, which functions to enhance the POT1a-CTC1 interaction and presumably promote telomere maintenance (Renfrew et al., 2014; Beilstein et al., 2015). Additionally, POT1a, but not POT1b, contains a conserved Phe residue in the first OB-fold that enables it to specifically bind telomeric DNA *in vitro* (Arora et al., 2016). Finally, POT1a and POT1b assemble into two distinct telomerase RNP complexes (Cifuentes-Rojas et al., 2012).

AtPOT1a is the best characterized among the three *A. thaliana* POT1 paralogs. It acts as a positive regulator of telomerase activity and telomere length. Plants lacking POT1a display a progressive loss of telomere sequences due to a dramatic reduction in telomere repeat addition processivity within the telomerase enzyme (Renfrew et al.,

2014). POT1 accumulates at telomeres during the S-phase to promote telomerase activity (Surovtseva et al., 2007). POT1a is also proposed to contribute to the switch between telomerase non-extendible states and telomerase extendible states by competing with TEN1, a negative regulator of telomerase activity (Leehy et al., 2013), for CTC1-STN1 binding (Renfrew et al., 2014). Finally, AtPOT1a binds specifically to the canonical telomerase RNA subunit TER1, which serves as the template for telomere addition (Cifuentes-Rojas et al., 2011& 2012).

Less is known about the function of AtPOT1b. AtPOT1b assembles with TER2, an alternative isoform of TER that represses telomerase activity (Cifuentes-Rojas et al., 2012). Unlike *pot1a* null mutants, plants containing a T-DNA insertion in the second exon of AtPOT1b (*pot1b-1*) display no obvious telomere defects or developmental phenotypes under standard growth conditions (Andrew Nelson's dissertation). However, this *pot1b-1* mutant line is in the Ler-0 accession of *A. thaliana* that lacks full-length TER2. Therefore, it is not feasible to study the function of POT1b in context of its binding partner TER2 using *potb-1* mutants. Unlike POT1a, there is evidence that POT1b may function in telomere protection. Overexpression of a dominant negative allele of *POT1b* containing only the first OB-fold leads to telomere shortening and end-to-end chromosome fusions (Shakirov et al., 2005). Altogether, these data argue that POT1b's function is distinct from POT1a.

Genetic analysis of POT1b has been hindered by the lack of null mutations in the Col-0 accession, where the vast majority of telomere analysis has been conducted and which contains the full complement of TER2 isoforms, TER2, $TER2_S$, and $TER2_{AS}$

(Cifuentes-Rojas et al., 2011; A. Suescun and D. Shippen, unpublished data). Here we used cytological and genetic approaches to investigate *A. thaliana* POT1b with respect to its subcellular localization, its *in vivo* binding partners, and the effect of POT1b mutations on *A. thaliana* growth and development. We report that POT1b accumulates in the cytosol rather than the nucleus, and further that POT1b interacts with cytoplasmic proteins that engage in cell metabolism and plant development. In addition, we describe the generation of new mutations in POT1b in the Col-0 accession of *A. thaliana.* We isolated two POT1b TILLING mutations that cause single amino acid changes in POT1b sequence. One of the TILLING mutations destabilizes POT1b protein and causes increased telomerase activity in floral tissues. A second set of POT1b mutant alleles was created by CRISPR/Cas9 from *Streptococcus pyogenes* that was codon-optimized for *A. thaliana* (Fauser et al., 2014). Analysis of the POT1b knockdown mutants unexpectedly revealed a delay in root growth that is exacerbated by the additional loss of telomerase. Together, these data suggest that AtPOT1b participates in telomerase repression and also functions as a non-canonical POT1 protein with a role in root development.

Materials and methods

Plant materials and growth conditions

The TILLING lines, POT1b$_{P216L}$ (pot2_101A1), POT1b$_{G264E}$ (pot2_176G6), POT1b$_{S273F}$ (pot2_110E1) and POT1b$_{R346W}$ (pot2_101B8) were obtained from the Arabidopsis Biological Resource Center (ABRC). To genotype the POT1b TILLING lines, P216L Fw 5'-ACGTGTTACTCATCTCACTCTG-3' and P216L Rv 5'-

ACTAAAGGCTTCCATCTCTCTGC-3' primers were used for PCR amplification and the products were sequenced to verify the point mutations.

The *pot1b-1* (Ler-0) T-DNA line was isolated from the Cold Spring Harbor Laboratory GeneTrap collection. The primer combination P2GT1F: 5'-AAACCCCAACGATCAGAGAC-3' and P2GT3R: 5'-AGACGAAGAGGTTGTTTCATTGCA-3' was used to genotype the wild type allele. The primer combination P2GT3R and DS3-1: 5'-ACCCGACCGGATCGTATCGGT-3' was used to genotype the mutant allele.

For root length measurements, seeds were plated on 0.5x Murashige and Skoog (MS), 1% Sucrose, 0.75% Agar plates (pH 5.7-5.9) and kept in 4^0C for 2 days for vernalization. The plates were placed vertically to observe root development. Plants were grown at 23^0C in an environmental chamber under long-day conditions (16h light/ 8h dark).

Protoplast preparation and microscopy

Arabidopsis mesophyll protoplasts were isolated as described previously (Sheen, 2001). Protein expression constructs bearing a C-terminal GFP tag were cloned into the pHBT plasmid. After the constructs were subjected to transient transformation into protoplasts, gene expression from the 35S CaMV promoter was allowed for 8 to 10 hours. Protein localization was visualized using the mCherry, DAPI and GFP channels (standard filter set, Nikon) with an inverted Nikon epifluorescence microscope using a 100× objective (Plan Fluo, NA 1.40, oil immersion).

Subcellular protein fractionation and western blot analysis

Subcellular protein fractionation was performed as described previously (Wang et al., 2011). Seedlings were ground to a fine powder in liquid nitrogen. The sample was then homogenized with 2ml/g of lysis buffer (20 mM Tris-HCl, pH 7.5, 20 mM KCl, 2 mM EDTA, 2.5 mM $MgCl_2$, 25% glycerol, 250 mM Sucrose, and 5 mM DTT) and filtered through double-layered Miracloth to obtain total protein. The flow-through was centrifuged at 1500g for 10 min at 4^0C. Then, the supernatant was centrifuged at 10,000g for 10 min at 4^0C. The supernatant was collected as the cytoplasmic fraction. The pellet was washed and resuspended, and then subjected to a sucrose gradient to isolate the nuclear fraction. As quality controls for the fractionation, phosphoenolpyruvate carboxylase (PEPC) antibody was used as a cytoplasmic marker, and histone H3 was used as a nuclear marker. The PEPC antibody and histone antibody were from Abcam (ab34793 and ab1791). POT1a and POT1b peptide antibodies were as previously described (Surovtseva et al., 2007).

For western bloting, total protein was extracted using CelLytic P cell lysis reagent (Sigma-Aldrich).

Yeast two-hybrid screen

AtPOT1b was used as the bait to screen an Arabidopsis seedling cDNA library (a gift from Dr. Libo Shan at Texas A&M University). The yeast strain AH109 was transformed with pBK-PN-POT1b and subsequently with the Arabidopsis seedling cDNA library in a pAD vector by the lithium acetate method (Miller and Stagljar, 2004).

Transformants expressing both the bait and the interacting prey proteins were selected on the amino acid-deficient medium, and the strength of interaction was determined by β-galactosidase activity assay and selection of 3-Amino-1, 2, 4-Triazol (3AT). Positive clones were sequenced and searched in GenBank.

RNA isolation and RT-PCR analysis

Total Arabidopsis RNA was isolated using a Direct-zol RNA MiniPrep kit (Zymo). cDNA was synthesized using qScript cDNA supermix (Quanta Biosciences). Quantitative RT-PCR (qRT-PCR) was carried out as described (Cifentes-Rojas et al., 2012) using SsoAdvance Universal SYBR green master mix (Bio-Rad). RNA from at least three individual plants was used for each genotype and at least two technical replicates were run for each reaction. Expression levels were averaged and normalized to GAPDH. Wild type level was set to one and mutant samples were compared to this value.

Terminal restriction fragment (TRF) analysis

DNA from whole plants was extracted using 2 x CTAB (100 mM Tris-HCl, 1.4 M NaCl, and 20 mM EDTA). TRF analysis was performed using 50 µg DNA digested with *Tru1I*, resolved on a 0.8% agarose gel and hybridized with [^{32}P] 5' end-labeled $(T_3AG_3)_5$ oligonucleotide probe (Fitzgerald et al., 1999).

Telomere repeat amplification protocol (TRAP) and Quantitative –TRAP (Q-TRAP)

Total protein was extracted from 5-day-old seedlings or flowers. TRAP reactions were performed as previously described (Fitzgerald et al., 1996). Quantitative telomere repeat amplification protocol was performed as previously described (Kannan et al., 2008), using a Dynamo HS SYBR Green qPCR kit (Thermo Fisher).

Results

Isolation of POT1b *mutant alleles*

To characterize the function POT1b, we searched for existing *POT1b* mutant alleles in the Col-0 accession. T-DNA insertion collections are the major source of mutants in *A. thaliana* and most commonly used to isolate null mutations. There are three T-DNA insertions at *AT5G06310*: SAIL_38_G01, GK_522D12-020204 and a gene trap line *pot1b-1* (Figure 2-1). The T-DNA insertion in SAIL_38_G01 is in the *Col-3* accession and is located in the 3'UTR of the *POT1b* gene. T-DNA insertion in the 3'UTR has been proven to be less likely to cause null mutation. Therefore, the SAIL_38_G01 is not ideal for the genetic study of POT1b. The GK_522D12-020204 line was annotated to bear a T-DNA insertion in the sixth exon of *POT1b*. Unfortunately, multiple approaches failed to locate the T-DNA insertion in GK_522D12-020204, suggesting the annotation of this mutant line is incorrect. An alternative source of T-DNA lines is found in the Cold Spring Harbor GeneTrap collection (Sundaresan et al., 1995). The *pot1b-1* mutant line in the Ler-0 accession was isolated from the GeneTrap collection (A. Nelson and D. Shippen, unpublished data).

Pot1b-1 contains a T-DNA insertion in the second exon of the *POT1b* gene. The mutation abolishes *POT1b* transcripts and thus is a null mutation. The Ler-0 accession is not ideal for our analysis because it does not encode a full-length TER2, which is the binding partner of POT1b. Instead, the TER2 locus in Ler-0 encodes a truncated TER2 isoform, termed $TER2_{\Delta}$, which lacks the essential intron region for telomerase inhibition (Xu et al., 2015). Since this RNA has not been well-characterized, *pot1b-1* is not an ideal background to study the function of POT1b.

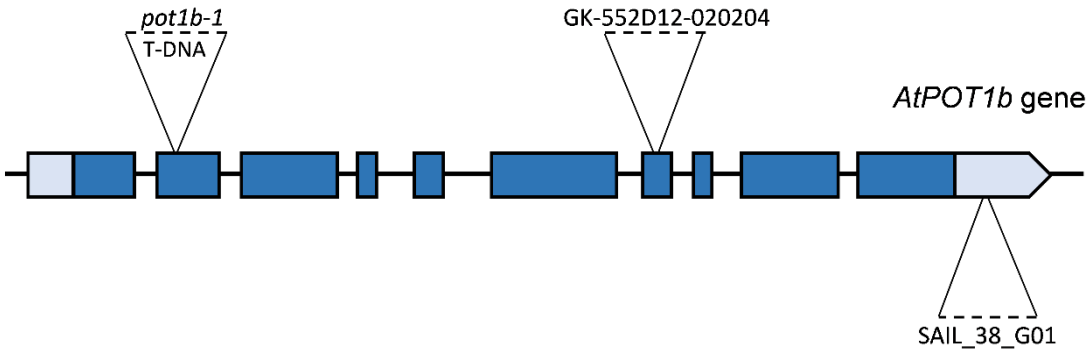


Figure 2-1. Available T-DNA lines in the *POT1b* locus (AT5G06310). Diagram showing the gene structure of AtPOT1b. Exons are displayed as dark blue boxes, introns as lines, and UTRs as light blue boxes. The sites of T-DNA insertion are shown as triangles.

Another resource for Arabidopsis mutations are the point mutation collections known as Targeting Induced Local Lesions in Genome (TILLING) lines. The TILLING line mutations are created by mutagenizing Col-0 seeds with ethylmethanesulfonate (EMS), followed by enzyme and sequencing methods to identify mutations (McCallum et al., 2000; Henikoff et al., 2004). There are 11 TILLING lines that bear a point

mutation in the *POT1b* locus. Among them, four lines contain missense mutations in highly conserved amino acids (Figure 2-2). These mutations are P216L, G264E, S273F, and R346W mutations, all located in the second OB-fold. According to the annotation, POT1b$_{P216L}$ and POT1b$_{S273F}$ lines bear only a single point mutation in the genome, while the POT1b$_{G264E}$ and POT1b$_{R346W}$ contain other mutations at other loci. Therefore, we began our genetic analysis initially with the POT1b$_{P216L}$ and POT1b$_{S273F}$ lines. To verify the genotype of these mutations, PCR reactions were performed to amplify the sequence between exon 5 and exon 9, and the PCR products were sent for sequencing to confirm the point mutation. We were able to identify homozygous mutants of the POT1b$_{P216L}$ and POT1b$_{S273F}$ lines.

Because point mutations may not lead to complete depletion of the protein *in vivo*, we took an additional approach to create a null mutation in *POT1b* using CRISPR/Cas9. The Optimized CRISPR Design tool (crispr.mit.edu) was used to design protospacers that target the *AtPOT1b* coding sequence in the Col-0 accession (Figure 2-3). We chose seven protospacers based on three criteria: targeting the N-terminal of POT1b to avoid partial expression of functional domains, low probability for off-targets hits, and the usage of different PAM sequences to increase the likelihood of success. Individual protospacers were cloned into the CRISPR/Cas expression construct containing codon-optimized *Streptococcus pyogenes* Cas9 (SpCas9). Wild type plants were transformed with Agrobacterium carrying this CRISPR/Cas9 (Fauser et al., 2014).

T1 transformants were identified and the POT1b locus was sequenced.

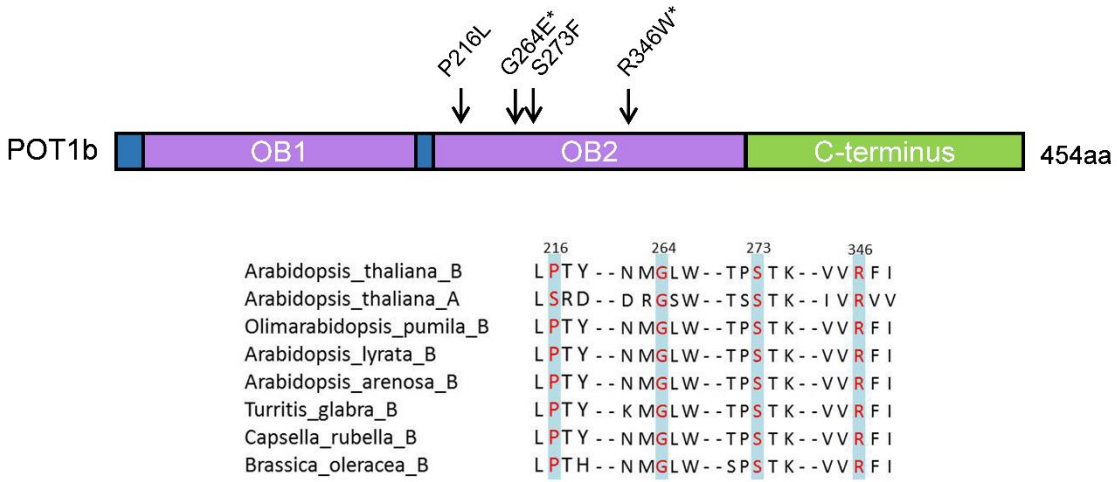
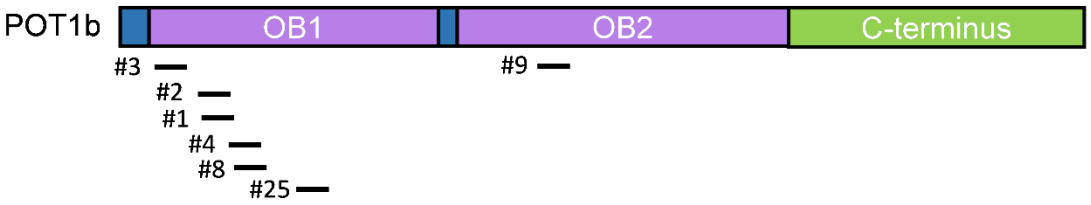


Figure 2-2. Four TILLING lines located in the second OB-fold of POT1b. A diagram of POT1b protein and the location of TILLING line mutations (top). Alignments of POT1b homologs from different species in the Brassicaceae family, along with AtPOT1a from *A. thaliana*. OB: oligosaccharide/oligonucleotide-binding fold. Asterisks indicate additional point mutations are presented in theses mutant backgrounds.

The T1 generations of protospacer 1, 2, 4, and 25 have been selected and genotyped. Only with protospacer 4 did we identify 15 mutant lines among over 160 candidates, suggesting that the efficiency of *Streptococcus pyogenes* Cas9 may differ depending of the protospacer or PAM sequence. Most of these mutant lines have nucleotide addition or deletion of a single nucleotide within the protospacer sequence, which can cause a frameshift mutation. One mutation line contained a deletion of 89 bp in exon 2, which is likely to be a null mutation of *POT1b*. These POT1b CRISPR mutation lines were still in the process of verification and characterization. No telomere related analysis has been done using these mutation lines.

This chapter will describe the analysis of GeneTrap T-DNA line in Ler-0, *pot1b-1*, and the TILLING mutatns in Col-0, POT1b $_{P216L}$ and POT1b $_{S273F}$.



Protospacer	Sequence (5′-3′)	PAM sequence (5′-3′)
1	AGGCCAGATCCCGAATACCA	TGG
2	GATCCATGGTATTCGGGATC	TGG
3	GCTCAACGATGACTCCAATG	AGG
4	GAGCGACGAACTTAACGGGA	AGG
8	GTACGAGCGACGAACTTAAC	GGG
9	ATCTCGGGTTCTACACATAA	AGG
25	TCTTATCGAATCTGAGAGAA	TGG

Figure 2-3. A diagram of POT1b protospacer locations and sequences. OB: oligosaccharide/oligonucleotide-binding fold.

S273F mutation leads to reduced POT1b protein level in vivo

Amino acid substitution caused by a missense mutation can affect protein function in several ways, including decreasing protein stability by disrupting protein structure. During *in vitro* biochemical experiment with POT1b$_{OB1OB2}$ and S273F POT1b $_{OB1OB2}$ variants, we noticed that the S273F POT1b $_{OB1OB2}$ variant did not accumulate to the same concentration as the wild-type protein, suggesting that the mutant protein might be unstable. This observation prompted us to ask if POT1b TILLING mutations affect

POT1b protein level in *A. thaliana.* POT1b protein was assessed by immunoblot analysis using total protein from wild type, POT1b$_{P216L}$, and POT1b$_{S273F}$ flowers (Figure 2-4A). A POT1b monoclonal peptide antibody was used for detecting POT1b (Surovtseva et al., 2007). As a negative control, western blotting was conducted in protein extract from the *pot1b-1* null mutant. Segregating wild type siblings of the POT1b $_{P216L}$ (P216L WT) and POT1b$_{S273F}$ lines (S273F WT-1 and -2) displayed a similar POT1b protein level as Col-0. Notably, POT1b levels were not altered in the POT1b $_{P216L}$ lines. However, two independent POT1b$_{S273F}$ lines (S273F Mut-1 and -2) displayed significantly (~50%) lower POT1b protein levels compared with their wild type siblings. A longer exposure indicated that a residue amount of POT1b protein was present in the two POT1b$_{S273F}$ lines (Figure 2-4A, right), suggesting that the S273F mutation may reduce the stability of POT1b *in vivo*.

To further investigate this possibility, we monitored the level of POT1b mRNA as well as RNA level of POT1a, TER1, and TER2 using qRT-PCR. No significant difference ($P>0.05$) was observed in the steady-state level of POT1b mRNA (Figure 2-4B). In addition, no significant change in POT1a, TER1, and TER2 RNA level was observed in POT1b$_{S273F}$ mutants. TER2, the long-noncoding RNA binding partner of POT1b (Cifuentes-Rojas et al., 2012), was not destabilized by the loss of POT1b. These data support the conclusion that POT1b with S273F mutation is destabilized *in vivo,* and thus POT1b$_{S273F}$ lines is a knockdown line of *POT1b*. Characterization of POT1b function described in this chapter was mainly conducted in POT1b$_{S273F}$ background.

Increased telomerase activity in the flowers of POT1b$_{S273F}$ mutants

Previous studies showed that POT1b specifically associates with TER2 RNP, which has been implicated in telomerase inhibition (Cifuentes-Rojas et al, 2011&2012) To test if POT1b contributes to telomerase regulation, quantitative telomere repeat amplification protocol (Q-TRAP) was adopted to measure the telomerase activity in the $POT1b_{S273F}$ mutants. Compared with wild-type seedlings, $POT1b_{S273F}$ seedling demonstrated similar telomerase activity (Figure 2-5A). The telomerase activity in the $POT1b_{S273F}$ flowers is two-fold higher (P value <0.05, *t* test) than in the wild type, and is increased to the same extent as in *ter2* mutants (Cifuentes-Rojas et al., 2012).

A radioactive TRAP was performed to visualize the telomerase elongation products from $POT1b_{S273F}$ flowers (Figure 2-5B). In TRAP assay, each band represents addition of a seven base TTTAGGG telomeric repeat. High molecular weight products are indicative of longer telomerase product. Compared with the wild type, higher molecular weight products were more abundant in $POT1b_{S273F}$ plants, suggesting that telomerase has increased repeat addition processivity when the level of POT1b is decreased (Figure 2-5B). Therefore, AtPOT1b may function as a negative regulator of telomerase in flowers.

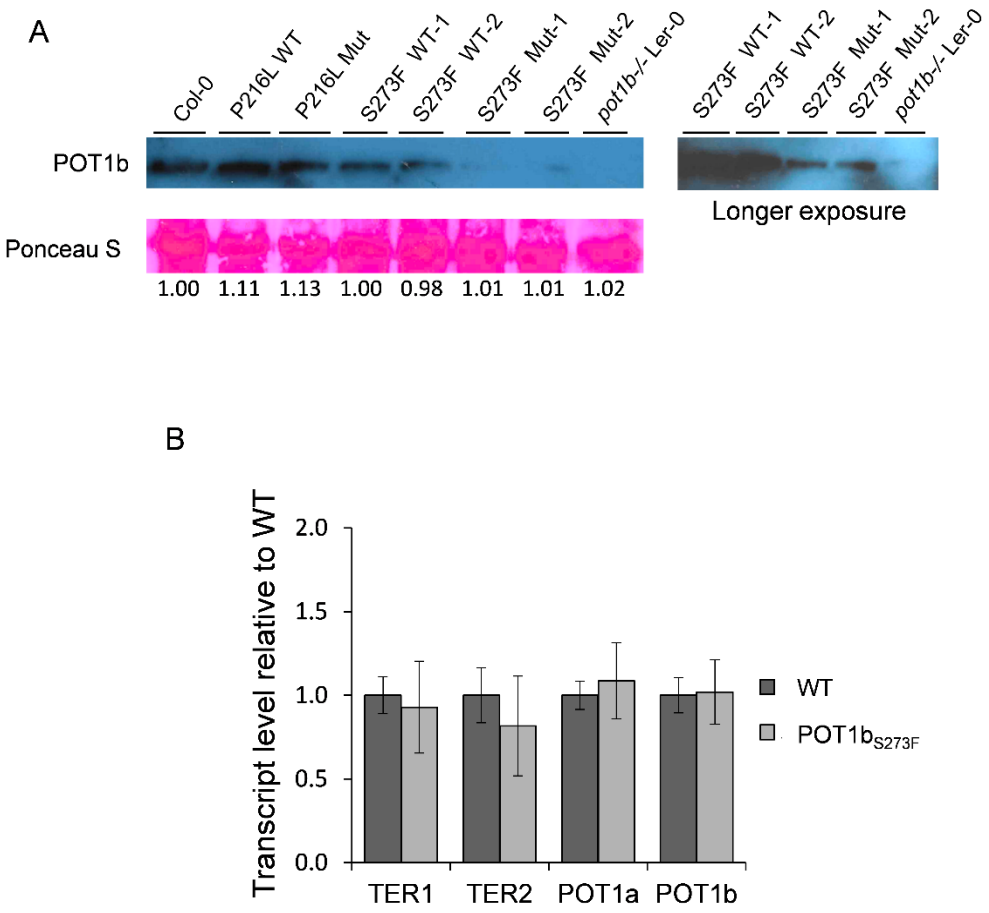


Figure 2-4. POT1b$_{S273F}$ mutation is a knockdown mutant line for POT1b, due to reduced protein stability. (A) Immunoblot results for wild type (WT), POT1b$_{P216L}$, POT1b$_{S273F}$, and *pot1b-1* are shown. Ponceau S stain of rubisco was used for loading control. The blot was probed with a peptide antibody raised against AtPOT1b. Longer exposure for the last four lane was presented on the right. (B) Steady-state transcripts of TER1, TER2, POT1a, and POT1b measured by qRT-PCR. Data were normalized to WT.

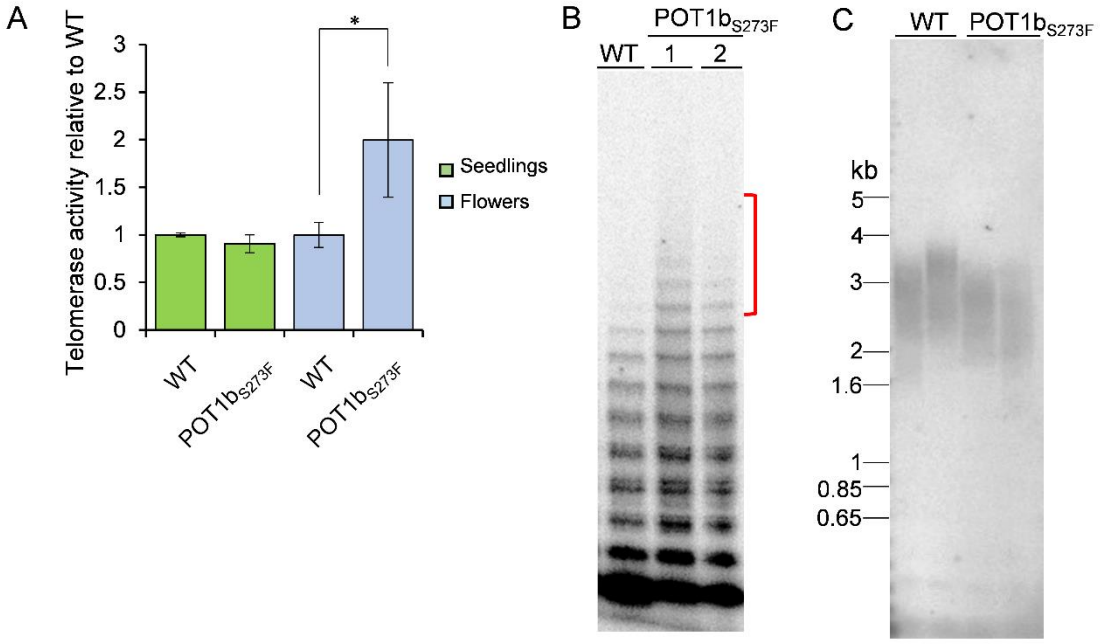


Figure 2-5. POT1b$_{S273F}$ mutation leads to increased telomerase activity, but does not affect telomere length. (A) Telomerase activity in seedlings and flowers measured by Q-TRAP. Data were normalized to wild type (WT) of corresponding tissue type. At least three biological replicates with three technical replicates were used for each data point. Standard deviation between biological replicates was used. (B) TRAP on flower protein extract from WT and POT1b$_{S273F}$. Red bracket indicated the difference in telomerase repeat addition processivity. (C) TRF analysis of POT1b$_{S273F}$. Blot was hybridized with a [32P] 5' end labeled $(T_3AG_3)_5$ oligonucleotide probe. Asterisk denotes P value <0.05 (*t* test).

POT1a is known to stimulate telomerase repeat addition processitivity (Renfrew et al., 2014) and *ter2* mutant is shown to have decreased telomerase activity in flowers (Cifuentes-Rojas et al., 2012). However, the qRT-PCR results shown in Figure 2-4B indicate that the steady-state level of POT1a mRNA, TER1 or TER2 are not altered in POT1b$_{S273F}$ mutant flowers. (Figure 2-4B). These data suggest that telomerase inhibition in POT1b$_{S273F}$ is not associated with dysregulation of telomerase related transcripts,

including TER1, TER2, and POT1a mRNA, and likely reflects a specific decrease in POT1b protein in this background.

No telomere length maintenance defects in POT1b$_{S273F}$ mutants

Since telomerase activity is increased in POT1b$_{S273F}$ mutant plants, we asked if telomere length homeostasis is affected in this line. Terminal Restriction Fragment (TRF) analysis was performed to gauge bulk telomere length of POT1b$_{S273F}$ mutants. The TRF profile of wild type plants spanned from 2 to 5kb as expected (Shakirov and Shippen, 2004), and POT1b$_{S273F}$ showed no difference in telomere length from the wild type (Figure 2-5C). Thus, telomere length maintenance is not affected by the S273F mutation in POT1b.

Delayed early development in POT1b mutants

We extended our characterization of POT1b to its influence on plant development. Fully matured plants (6-weeks old) bearing the POT1b$_{S273F}$ mutation did not display any apparent morphological defects or perturbation in fertility through the first three generations of the mutation (Figure 2-6A). POT1b$_{S273F}$ mutants continue to be wild type-like after successive generations of self-pollination. This observation suggests that the reduced POT1b protein level does not affect plant development, consistent with previous observations in the *pot1b-1* mutant, the adult plants of which were indistinguishable from wild-type plants (Andrew Nelson's dissertation).

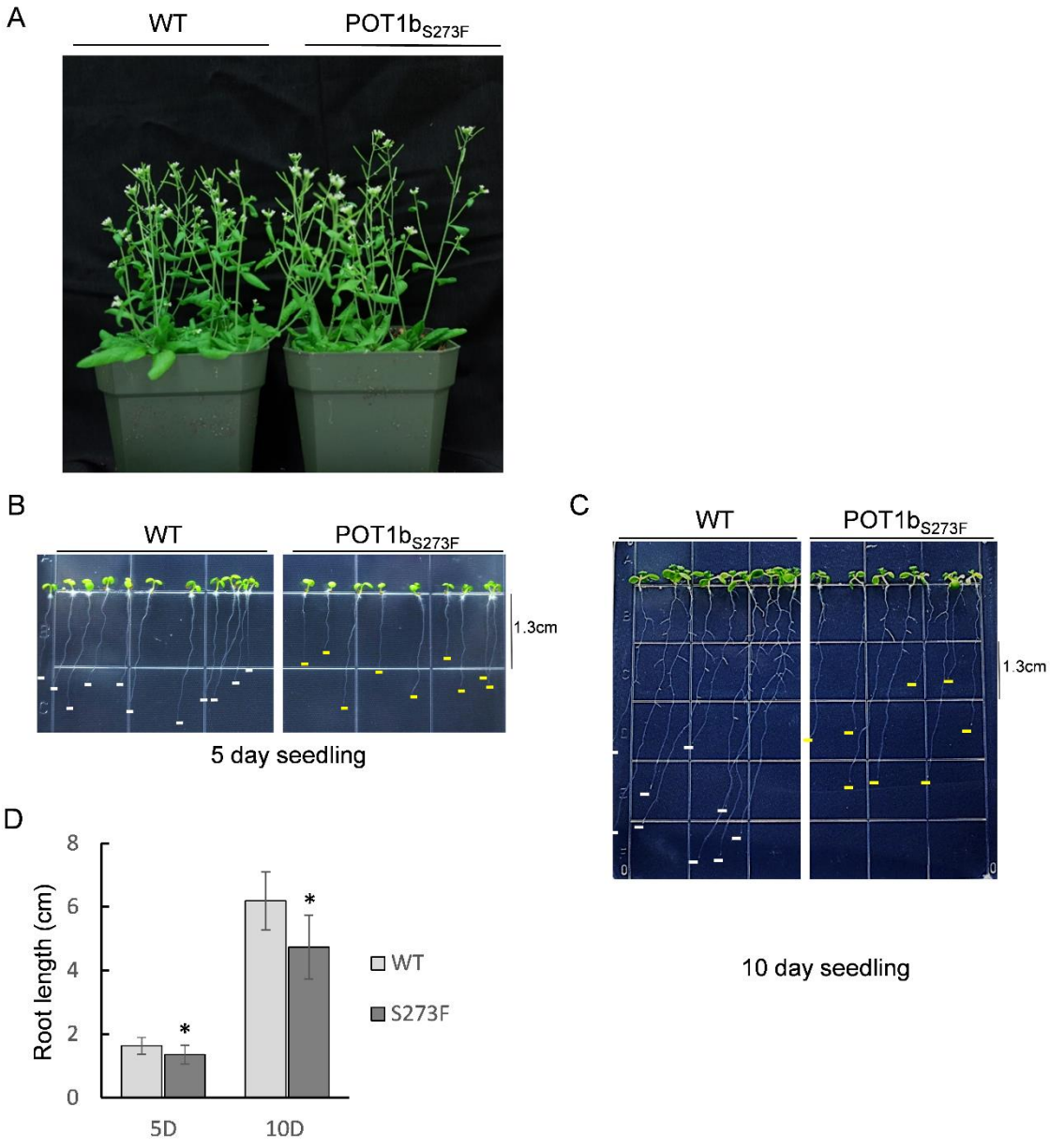


Figure 2-6. POT1b$_{S273F}$ mutant causes a short root phenotype and delayed early development. (A) Six-week-old wild type and POT1b$_{S273F}$ mutant plants grown under the same conditions. Five-day-old (B) and ten-day-old (C) seedlings of wild type and fourth generation of POT1b$_{S273F}$ mutant plants are shown. Yellow and white bars denote the tip of primary roots. (D) Quantification of (B) and (C). Asterisk denotes P value <0.05 (*t* test).

Interestingly, the seedlings of fourth generation POT1b$_{S273F}$ mutants (F4 POT1b$_{S273F}$) were delayed in early development. The root of 5-day-old POT1b$_{S273F}$ seedlings was on average 1.36 (± 0.29) centimeters, while wild-type siblings had longer roots of 1.63 (± 0.26) centimeters on average (Figure 2-6B and D). Similarly, a subset of *pot1b-1* mutant seedlings displayed shorter roots (P value <0.05, *t* test), compared with wild type Ler-0 (Figure 2-7A). In contrast, *ter2* or third generation *pot1a* mutant seedlings did not display any growth delay during early development indicating that the defect is specific to POT1b mutations (Figure 2-7B&C). The short root phenotype of POT1b$_{S273F}$ mutant seedlings became more apparent 10 days after sowing on MS plates (Figure 2-6C). At this stage, the wild type root length is substantially longer than POT1b$_{S273F}$ (P value <0.05, *t* test), with an average of 6.19 ± 0.91 centimeters versus 4.73 ± 1.00 centimeters (Figure 2-6D). These data indicate that POT1b is required for proper seedling development, but not for later development.

The stem cell niche in the root apical meristem (RAM) maintains pluripotency and provides a basis for root growth and development (Miyashima et al., 2013). Programmed cell death (PCD) in RAMs impedes root development and can be detected via cytology approaches (Fulcher and Sablowski, 2009). To test whether PCD played a role in the short root phenotype of POT1b mutants, propidium iodide (PI) staining was used to visualize cell death in RAMs. PI signal accumulation was not observed in RAMs of POT1b$_{S273F}$ or *pot1b-1*, after looking at over 50 roots from 5-day-old POT1b$_{S273F}$ mutant and *pot1b-1* mutant seedlings. Additionally, preliminary data showed that unlike *ter2* mutants (Cifuentes-Rojas et al., 2012), RAMs of POT1b$_{S273F}$ or *pot1b-1* mutants did undergo PCD

4 hours after DNA damage, which was consistent with the observation in the wild type seedlings. Therefore, a reduction in POT1b protein does not lead to cell death in RAMs, and thus may affect early seedling development independent of TER2.

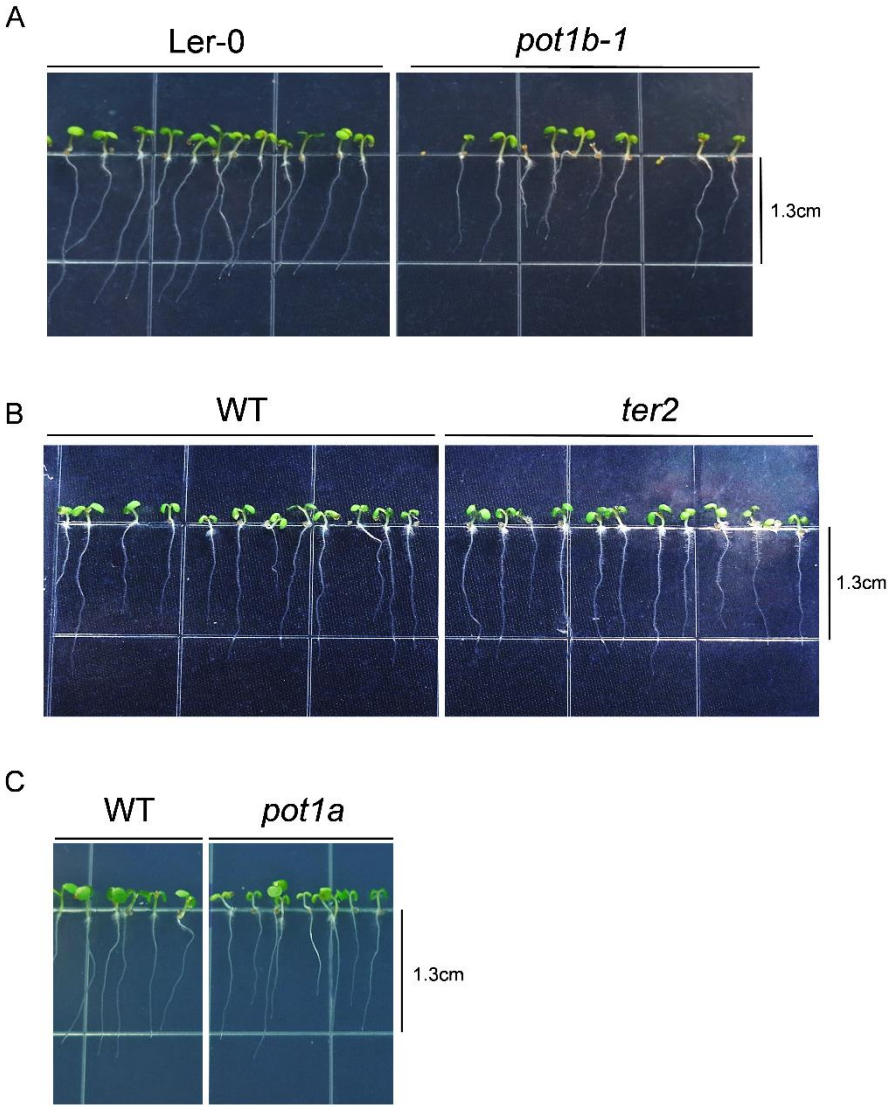


Figure 2-7. Shorter root phenotype specific to POT1b mutations. Five-day-old seedlings of wild type, *pot1b-1* (A), *ter2* (B), and third generation of *pot1a* (C) mutant plants grown under the same conditions. *Pot1b-1* mutants also have shorter roots. No difference in root length was observed in *ter2* and *pot1a* mutants.

The POT1b$_{S273F}$ tert double mutants display a greater delay in early development

To test if POT1b interacts genetically with other factors in telomere maintenance, we crossed the POT1b$_{S273F}$ mutant with *pot1a, tert, ku70* or *ter2* heterozygote mutants. Screening for *pot1a* POT1b$_{S273F}$ and POT1b$_{S273F}$ *ter2* double mutants is still in process, but POT1b$_{S273F}$ *tert* and *ku70* POT1b$_{S273F}$ double mutants were obtained and their characterization is described below.

We monitored the development of second generation POT1b$_{S273F}$ *tert* and *ku70* POT1b$_{S273F}$ double mutants. We have not yet identified a segregating wild type from the POT1b$_{S273F}$ and *tert* cross. Therefore, we compared second generation POT1b$_{S273F}$ *tert* plants with their POT1b$_{S273F}$ heterozygous siblings, *tert* heterozygous siblings, second generation POT1b$_{S273F}$ single mutant siblings and second generation *tert* single mutant siblings (Figure 2-8A). Interestingly, second generation double mutants of POT1b$_{S273F}$ *tert* displayed a more severe delay in seedling development than POT1b$_{S273F}$ single mutants. Four days after germination, the cotyledons of the double mutants were smaller, and the roots were significantly shorter compared with the wild type. Second generation POT1b$_{S273F}$ single mutant and *tert* single mutant seedlings resembled POT1b$_{S273F}$ heterozygous mutant seedlings and *tert* heterozygous mutant seedlings. After eight days, the cotyledons fully opened, and the first pair of rosette leaves of the double mutants emerged, which were significantly delayed compared to the single mutants and heterozygous mutants where the cotyledons opened three to four days after germination (Figure 2-8B). The root length of an 8-day-old POT1b$_{S273F}$ *tert* plant was only slightly longer than the root of a 4-day-old wild type plant. Notably, the double mutants of

POT1b$_{S273F}$ *tert* were ultimately able to develop fully formed vegetative organs and flowers, indicating that the delay was confined to early development. In addition, the delayed seedling development was not observed in the *ku70* POT1b$_{S273F}$ double mutants, indicating that *tert* mutants provide a sensitized background that can aggravate the phenotype of a *POT1b* mutation.

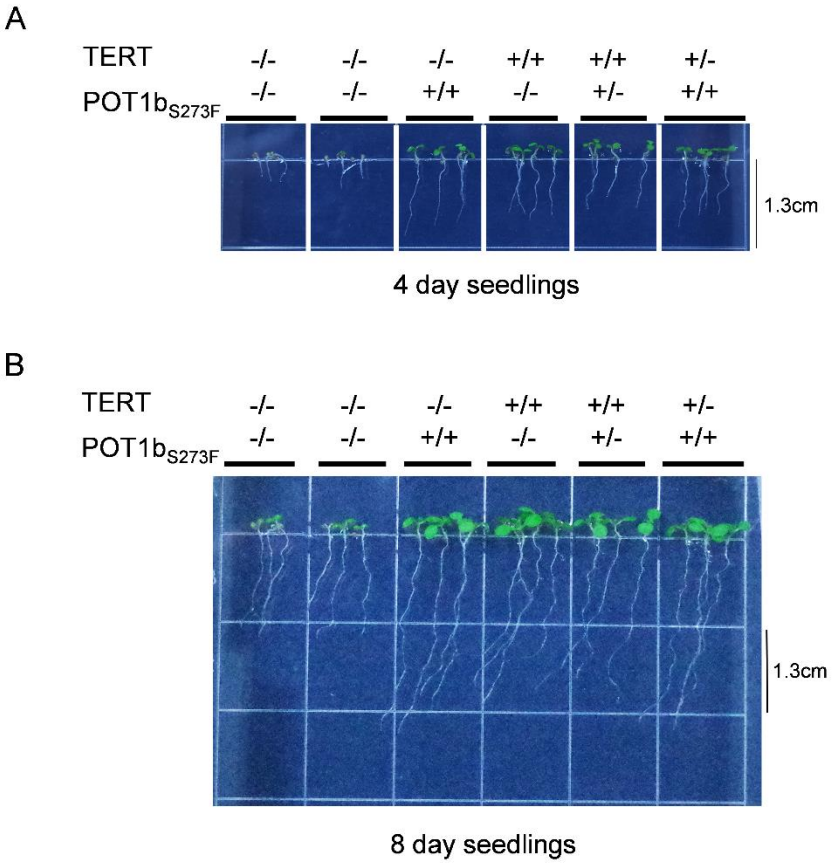


Figure 2-8. The developmental delay is exaggerated in *tert* POT1b$_{S273F}$ double mutants. Four-day-old (A) and eight-day-old (B) seedlings of offspring from a *tert and* POT1b$_{S273F}$ cross with annotated genotypes under the same conditions.

Cytoplasm localization of POT1b: potential novel cellular functions

Preliminary data from the Armstrong Lab (personal communication) demonstrated that overexpressed AtPOT1a and AtPOT1b were found in different nuclear compartments of *Nicotiana benthamiana*, indicating that *A. thaliana* POT1a and POT1b may reside in different cellular compartments. To extend our analysis of AtPOT1b, we investigated the subcellular localization of POT1b initially by using several databases for protein localization prediction. Unexpectedly, analysis of protein sequences using several databases predicted both cytoplasmic and nucleus localization for POT1a and POT1b (Table 2-1). In contrast, Ku70 and TERT, were predicted to be in the nucleus by most databases.

To further evaluate the subcellular localization of POT1a and POT1b, we fused their coding sequences with green fluorescent protein (GFP) under the control of the 35S CaMV promoter. A construct containing only the GFP tag was generated as a negative control. As an additional control, GFP was fused to the nuclear protein Ku70. Arabidopsis mesophyll protoplasts were transiently transfected and GFP fluorescence was visualized 8-10 hours after transformation to mitigate protein over-expression. DAPI staining was used to monitor nuclear localization, while red auto-fluorescence from chlorophyll indicated the localization of chloroplasts. The GFP control was concentrated in the nucleus, with diffuse localization in the cytoplasm, but no appreciable accumulation in chloroplasts (Figure 2-9). As expected, a strong signal of Ku70-GFP was present in the nucleus, with a background signal in chloroplasts. In contrast, POT1b was found in the cytoplasmic area as punctuate fluorescence spots

(Figure 2-9). The POT1b-GFP spots did not overlap with chloroplasts and the size of these spots was much smaller. Unfortunately, multiple attempts to localize POT1a-GFP by this same method were unsuccessful, as we were unable to detect POT1a-GFP expression in protoplasts. Since POT1b is implicated in telomere maintenance and telomerase regulation (Shakirov et al., 2005; Figure 2-5A&B), this finding raises the possibility of non-telomeric functions for AtPOT1b in the cytoplasm.

Considering the caveats of using transient overexpression system in protoplasts, we confirmed cytoplasm localization of endogenous POT1b via biochemical cell fractionation experiment (Figure 2-10). Total protein, cytoplasm, and nuclear fractions from six-day-old seedlings were assayed for POT1b. As a quality control of fractionation, we monitored phosphoenolpyruvate carboxylase (PEPC), a known cytosolic protein, and histone H3, a nuclear protein control. As expected, PEPC was detected at a similar level in the total protein extract and cytoplasmic protein extract, but was not detected in the nuclear fraction. On the other hand, Histone H3 was detected in the total protein extract and the nuclear fraction, but was not present in the cytoplasmic fraction, indicating a successful fractionation. The nuclear fraction contained much less protein overall. Notably, a robust signal of POT1b was detected in the cytoplasmic fraction but not in the nuclear fraction. However, the unequal loading between the total protein and nuclear protein made it hard to determine the fraction of POT1b protein in the nucleus. It is possible that a small fraction of POT1b is present in the nucleus. In contrast, POT1a can be clearly detected in the nuclear fraction, and cytoplasmic POT1a comprises a very small fraction of the total POT1a. These observations are consistent

with the protoplast localization experiments and indicate that a substantial fraction of POT1b is present in the cytosol, while POT1a is primarily localized to the nucleus.

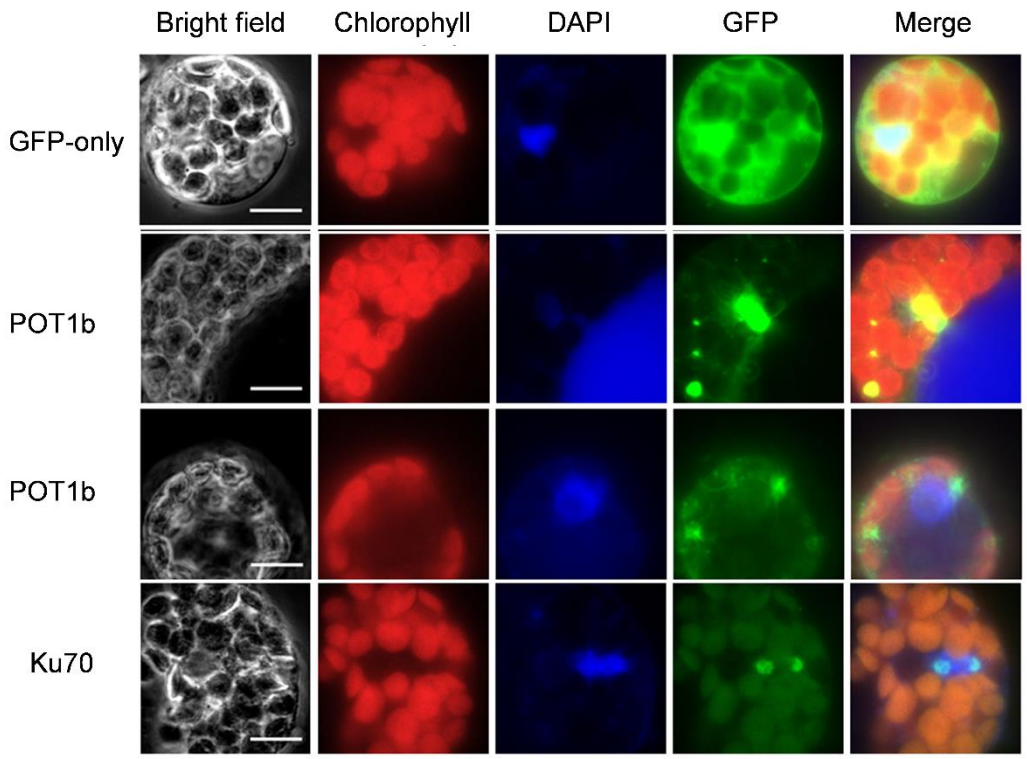


Figure 2-9. AtPOT1b accumulates in the cytoplasm of mesophyll protoplasts. The indicated proteins tagged with GFP were expressed in protoplasts for 8-10 hours. Red autofluorescence of chlorophyII (chloroplast), blue fluorescence from DAPI (nuclear) staining and green GFP fluorescence were monitored separately using fluorescence microscopy. The far right column shows a merge of the three images. Scale Bar = 10 μm.

Name	Accession No.	Localization Predicators			
		Plant-mPLoc	MultiLoc	SUBAcon	BaCelLo
POT1a	AT2G05210	Nucleus	Plasma membrane (0.58), Cytoplasmic (0.15)	Cytosol (0.66)	Nucleus
POT1b	AT5G06310	Nucleus	Plasma membrane (0.75), Cytoplasmic (0.14)	Cytosol (0.879)	Nucleus
Ku70	AT1G16970	Nucleus	cytoplasmic (0.86)	Nucleus (1)	Nucleus
TERT	AT5G16850	Nucleus	Nuclear (0.85)	Nucleus (1)	Plastid

Table 2-1. Compiled localization data for telomere proteins. Listed are the short name of telomere proteins, the *Arabidopsis* gene identifiers (AGIs), the server or database used for subcellular localization prediction and the score for the predication if applicable (Pierleoni et al, 2006; Hooper et al, 2014; Hoglund et al, 2006; Chou et al, 2010).

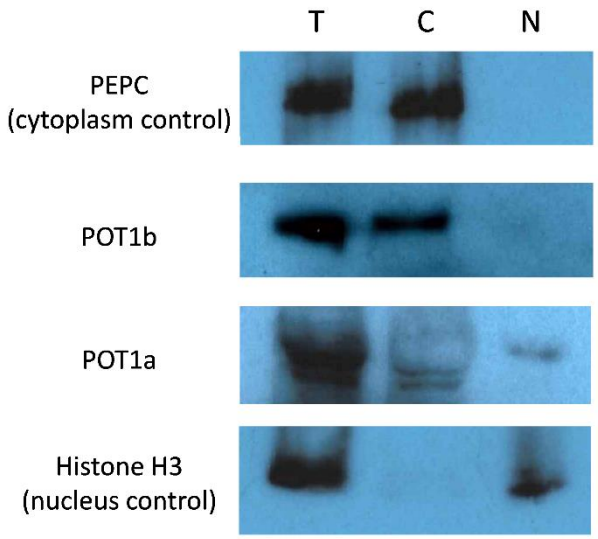


Figure 2-10. Compiled localization data for telomere proteins. Listed are the short name of telomere proteins, the *Arabidopsis* gene identifiers (AGIs), the server or database used for subcellular localization prediction and the score for the predication if applicable (Pierleoni et al, 2006; Hooper et al, 2014; Hoglund et al, 2006; Chou et al, 2010).

POT1b interacts with cytoplasmic proteins

Finally, to better understand the function POT1b in seedling development, we performed a yeast two-hybrid (Y2H) screen using *A. thaliana* seedling cDNA library to identify POT1b interaction partners. Over 30 positive clones were identified, and 21 different genes were recovered. We increased the stringency for binding conditions by selection on SD plates lacking Threonine, Leucine and Histidine (SD-T-L-H) with 0.5 mM 3-Amino-1,2,4-Triazol (3AT) to assess POT1b-interactioning partners (Figure 2-11). Among the 21 candidates, FBN1b (fibrillin 1b, a lipid binding protein of plastids), CAT3 (catalase 3), and LSU3 (response to low sulfur 3) were initially tested due to their localization and function (Figure 2-11). No self-activation was observed; the strains containing only pBK and pAD did not survive on SD-T-L-H 3AT plates, and neither did the strains containing only pBK-POT1b and pAD. The strains containing pBK-POT1b construct and pAD construct with FBN1b, CAT3 or LSU3 cDNA could survive on SD-T-L-H 3AT plates, suggesting specific binding in the Y2H system. The rest of the candidates need to be further tested using these stringent conditions. Analysis for *in vivo* protein-protein interactions are required for further validation of these POT1b interaction partners.

Among the 21 POT1b interaction candidates, four candidates had more than one hit (see Table 2-2). Notably, eight out of the 21 candidates are predicted or reported to present in the nucleus, while the rest of the candidates are cytoplasmic proteins. These POT1b interaction candidates are predicted to be involved in the stress response and various aspects of cell metabolisms, such as photosynthesis and response to reactive

oxygen species. These observations, together with the cytoplasm localization of POT1b, raise the possibility that POT1b may participate in metabolic processes outside the nucleus.

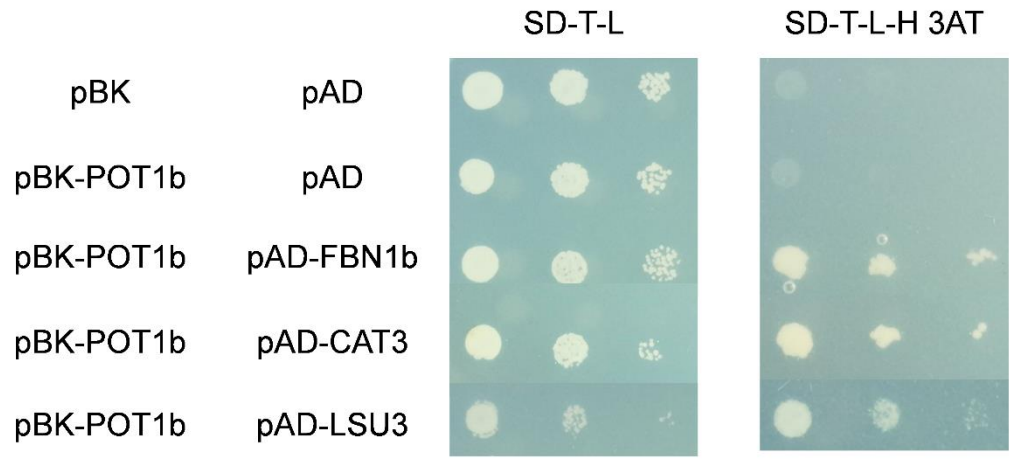


Figure 2-11. Yeast two-hybrid assay to test for POT1b binding candidates. The constructs on the left were transformed into yeast and grew on SD-T-L plates with three different dilutions (10-fold difference between each dilution). The same transformed yeast strains were grew on the SD-T-L-H with 0.5mM 3AT for selection.

Name	Accession #	Hits	Notes
WKN8	AT5G41990	2	Serine/threonine-protein kinase
MYB75	AT1G56650	2	Putative MYB domain containing transcription factor
FAB1C	AT1G71010	2	Phosphatidylinositol-3P 5-kinase
ADF2	AT3G46000	1	Actin depolymerizing factor
PPA 5	AT4G01480	2	Inorganic pyrophosphorytase
RPT2a	AT4G29040	1	26S proteasome subunit
LHCB3	AT5G54270	1	light-harvesting chlorophyll B-binding protein; photosynthesis
PLAC8 family protein	AT1G52200	1	Unknown function
VAB3	AT1G20260	1	V-type proton ATPase subunit B3
LSU1	AT3G49580	1	Response to low sulfur
MS1	AT5G17920	1	5-methyltetrahydropteroyltriglutamate homocysteine methyltransferase
ESM1	AT3G14210	1	Epithiospecifier modifier
SAM1	AT1G02500	1	S-adenosylmethionine synthetase
ADK1	AT5G63400	1	Adenylate kinase; gluconeogenesis
FBN1b	AT4G22240	1	Plastid-lipid associated protein PAP, Structural molecule activity
UXS1	AT3G53520	1	UDP-glucuronic acid decarboxylase
Catalase 3	AT1G20620	1	Catalase activity; breakdown of H2O2 into water and O2
EIN2	AT5G03280	1	Involved in ethylene signal transduction
Catalase 2	AT4G35090	1	Catalase activity
LSU3	AT3G49570	1	Response to low sulfur
Elongation factor 1	AT1G07920	1	GTP binding elongation factor family protein

Table 2-2. List of POT1b interacting candidates in yeast two-hybrid assay.

Discussion

While POT1a in *A. thaliana* is well-characterized as a component of the active telomerase RNP that is essential for telomere length maintenance, knowledge of POT1b

function is very limited. Overexpression experiments of a truncated POT1b implicate POT1b in telomere length regulation and chromosome end protection (Shakirov et al., 2005). However, the merit of this study is undermined by the difficulty of interpreting dominant-negative mutations. In this study, we isolated several new mutant alleles of POT1b and used genetic, biochemistry, and cytology approaches to define the role of POT1b in telomere maintenance and plant development. We show that unlike Pot1a, POT1b is a negative regulator of telomerase activity and is dispensable for telomere length maintenance. Our data further underscored the functional divergence of POT1a and POT1b in *A. thaliana* (Beilstein et al., 2015; Shakirov et al., 2005). Additionally, we report that POT1b is involved in early plant development, potentially working synergistically with TERT. Finally, we show that POT1b, not POT1a, localizes to the cytoplasm and is associated with cytoplasmic proteins of unknown functions.

POT1b negatively regulates telomerase activity

The data presented in this chapter indicate that POT1b does not play an essential role in telomere length control, but it negatively regulates telomerase activity independent of TER2 abundance. Since the duplication of *POT1* occurred ~100 mya (Beilstein et al, 2015) and the duplication of *TER* in *A. thaliana* occurred very recently (Beilstein et al., 2012), it is possible that the role of AtPOT1a and AtPOT1b in telomerase regulation may reflect an ancient function of POT1. This model has been set by the finding in Tetrahymena telomerase holoenzyme (Wan et al., 2015; Jiang et al., 2015). Tetrahymena telomerase holoenzyme contains several OB-fold proteins that can

either positively or negatively regulate telomerase activity. It is possible a similar scenario for telomerase regulation is used by *A. thaliana* in the form of POT1a and POT1b.

To further understand the mechanism and biological significance of POT1b in telomerase inhibition, it is important to test if this regulation is conducted via the TER2 RNP. For example, analysis of *pot1b ter2* double mutant may reveal whether this telomerase inhibition by POT1b is dependent on TER2. Furthermore, since TER2 has been implicated in telomerase inhibition in response to DSBs, monitoring the dynamics of TER2-POT1b interactions *in vivo* with or without DNA damage will provide new insight into the importance of *A. thaliana* POT1b in telomere biology.

AtPOT1b is important in seedling development

We found no morphological differences between adult *pot1b-1* and Ler-0 plants or between adult $POT1b_{S273F}$ and wild-type plants. However, the seedlings of both the *pot1b-1* and F4 $POT1b_{S273F}$ mutants showed reduced root length compared to wild type seedlings under the same conditions. This short root phenotype is an indication of a developmental delay in seedlings, instead of developmental arrest since plants were able to proceed through vegetative growth and reproductive development. Notably, this growth delay phenotype did not occur in *ter2* mutants or *pot1a* mutants, indicating that it reflects a unique contribution of POT1b. Preliminary characterization of the POT1b transcriptional profiles using RT-PCR showed that the POT1b mRNA level peaks in cell culture and root (Shakirov et al., 2005). In addition, RNA-seq analyses of the

transcriptome landscape of *A. thaliana* at different developmental stages revealed that POT1b mRNA is also elevated in the anthers and dry seeds (Klepikova et al., 2015&2016; Yang et al., 2011). POT1a mRNA level is also high in anthers, but is very low in seeds. These expression data are consistent with phenotypic analysis of POT1b mutants and indicate that POT1b is required for plant development, including embryogenesis, seed germination, and seedling development.

It is also possible that the function of POT1b in reproduction and early plant development involves other telomerase components. Notably, a fluorochromatinc reaction of pollen viability in *ter2* mutants demonstrated that TER2 plays a role in male meiocytes (H. Xu and D. Shippen, unpublished data). It is possible that POT1b exerts its functions in plant development in context of TER2 RNP. Genetic and cytology experiments in POT1b mutants will help us to test this hypothesis. In addition, delayed seedling development was also observed in the second generation POT1b$_{S273F}$ *tert* double mutants, but not in *tert* single mutants. *Tert* mutants do not display any morphological defects until the sixth generation, when the telomeres have become critically short and telomere fusion occurs (Riha et al., 2001). Moreover, recent studies from the Shippen lab showed the early onset of this developmental delay in POT1b$_{S273F}$ *tert* double mutants is not caused by accelerated telomere shortening (B. Barbero and D. Shippen, unpublished data). Nevertheless, the early onset of delayed seedling development is dependent on the absence of TERT, since the *ku* POT1b$_{S273F}$ double mutants did not show this phenotype. These observations together open the possibility that POT1b acts coordinately with TERT in plant development, perhaps for zygote formation and seedling development. It

is also possible that the more severe delay of seedling development in the double mutants of POT1b and TERT represents a sensitized background due to the loss of TERT. Understanding the molecular basis of this phenotype and identifying other factors in this pathway are important future goals.

AtPOT1b localizes to the cytoplasm and interacts with cytoplasmic proteins

One of the most unexpected findings from this study is that POT1b accumulates in the cytoplasm. We found that transient expression of POT1b-GFP forms punctuated spots in the cytoplasmic area of the mesophyll protoplasts. A similar localization pattern was reported for proteins in the trans-Golgi network/early endosome vesicles (Gu and Innes, 2011), suggesting that POT1b may localize to vesicles or plastids. Cell fractionation experiments confirmed that a considerable amount of POT1b, but not POT1a, localizes in the cytoplasm.

A yeast two-hybrid screen to identify interaction partners of POT1b and recovered 21 proteins involved in various cell processes. POT1b Y2H binding partners are not limited to nuclear localized proteins; several of the candidates are implicated in cell metabolism that takes place in the cytoplasm, including redox regulation (cytoplasm and mitochondria), photosynthesis (chloroplast), and responses to salt (cytoplasm). If the protein-protein interactions between the Y2H candidates and POT1b can be confirmed, genetic analysis is poised to reveal the function of POT1b in stress responses and cellular processes and thus gradually expand our understanding of POT1b as a unique POT1 homolog in *A. thaliana*.

The duel localization of telomere protein is not unusual. In fact, nuclear export of hPOT1 and mitochondria localization of hTERT have been reported (Chung et al., 2012; Chiodi and Mondello, 2012; Chen et al., 2007; Liu et al., 2004), indicating that telomere proteins may be under spatial control. Spatial control of dual localized proteins is common in plants (Boyle and Brisson, 2001; Krause and Krupinska, 2009). Among the plant proteins known to dually target to the nucleus and to mitochondria or plastids, most are implicated in the regulation of DNA metabolism (Krause and Krupinska, 2009). These data presented in this chapter add POT1b to the collection of dual localized protein in *A. thaliana* and raise interesting questions about the functions of cytoplasmic POT1b.

CHAPTER III

CHARACTERIZATION OF TER2 RNP

Summary

Telomeres are the conserved nucleoprotein structures at the ends of linear chromosomes in eukaryotes. Telomeres protect chromosome ends from DNA damage responses and guarantee the replication of the end sequences. In *Arabidopsis thaliana,* unlike most organisms, telomeres are asymmetrical; half of the chromosomes end in a 3' G-rich overhang protected by the CST (CTC1/Cdc13; STN1; TEN1) complex, while the other half have a blunt end. The DNA repair factor, Ku is required for maintenance of blunt-ended telomeres. Other components of the blunt-end cap remain unknown. A non-canonical telomerase-associated RNA called TER2 associates with Ku and Protection of Telomeres 1b (POT1b) *in vivo* to form an alternative telomerase ribonucleoprotein (RNP) complex, termed TER2 RNP. TER2 has been implicated in telomerase down-regulation in response to double-strand breaks. In this chapter, we explore the possibility that TER2 RNP also functions in blunt-ended telomere capping. We show that similar to *ku* mutants, *ter2* mutants exhibit an elevated G-overhang signal. We also show that Ku and POT1b both associate with telomeres. In addition, POT1b inhibits Ku localization to telomeres, suggesting that POT1b may regulate Ku association with blunt-ended telomeres. We also provide evidence that Ku interacts with TER2, showing that TER2 abundance is decreased in the flower of plants lacking Ku, and thus suggesting that Ku can stabilize TER2 RNA in flowers. Components of TER2 RNP may have a role in

reproductive development. Supporting this conclusion, we reported that simultaneous depletion of Ku and TER2 leads to defects in seed formation. Altogether, these data provide evidence for TER2 RNP as a blunt-ended telomere capping complex, and a factor important for reproductive development.

Introduction

In most eukaryotes, the termini of linear chromosomes are protected by specialized nucleoprotein structures termed telomeres. Telomeric DNA is comprised of tandem arrays of short GC-rich repeats that end in a 3' G-rich single-stranded (ss) extrusion, known as the G-overhang. Telomere specific proteins [e.g. shelterin in mammals and CST (CTC1/Cdc13; STN1; TEN1) in budding yeast and plants] associate with the telomeric DNA and distinguish natural chromosome ends from double-strand breaks (DSBs) (de Lange, 2005; Price et al., 2010). Telomere tracts are maintained by the combined action of the conventional DNA replication machinery and telomerase. After DNA replication, telomerase uses a long noncoding RNA (lncRNA), TER, as a template to extend telomeres. Dysfunction of either telomere proteins or telomerase components perturbs telomere length homeostasis. Critically short telomeres activate a powerful DNA damage response and are prone to end-to-end chromosome fusion, which eventually triggers genome instability. Studies of mice telomeres demonstrate that telomere attrition is associated with limited stem cell proliferation potential (Flores et al., 2005), compromised organ homeostasis (Wong et al., 2003) and premature aging (Chang

et al., 2004), underscoring the critical role of telomere maintenance in promoting genome stability.

In *Arabidopsis thaliana*, the ss region of the telomere is protected by CST (Song et al., 2008; Surovtseva et al., 2009; Leehy et al., 2013). Telomere replication is executed by telomerase using TER1 RNA as template and POT1a as an accessory factor to stimulate repeat addition processivity (Cifuentes-Rojas et al, 2011; Renfrew et al., 2014). Plants lacking individual CST components or telomerase core components exhibit extensive telomere shortening, genome instability, and morphological anomalies (Shakirov et al., 2005; Song et al., 2008; Surovtseva et al., 2009; Leehy et al., 2013; Riha et al., 2001). For example, plants lacking the TEN1 subunit of CST have abnormal phyllotaxy, loss of apical dominance, reduced fertility and increased programmed cell death in root apical meristems (RAMs) (Hashimura and Ueguchi et al., 2011; Leehy et al., 2013). Plants lacking telomerase activity suffer a gradual attrition of telomere sequences, and after six generations, plants start to display growth and developmental abnormalities associated with dysfunctional meristems (reduced fertility, fasciation, and arrest in vegetative growth) (Riha et al., 2001).

A recent study reveals that telomeres in *A. thaliana* and other flowering plants are asymmetrical (Kazda et al, 2012). While one side of chromosome ends in ss telomeric DNA protected by the CST (Song et al., 2008; Surovtseva et al., 2009; Leehy et al., 2013), the other side is blunt-endeded and is protected by Ku, a player in classic non-homologous end joining (c-NHEJ). Ku prevents nucleolytic resection of the blunt-ended telomeres, and deficiency of Ku leads to increased ss telomeric DNA and

increased telomeric circles, consistent with elevated recombination (Riha et al., 2003). Inactivation of both the CST component STN1 and Ku results in more severe defects associated with telomere deprotection, including end-to-end chromosome fusions and profound developmental defects (Kazda et al., 2012).

Little is known about the composition of the blunt-ended telomere cap. One of the candidates for blunt-ended telomere protection is the non-canonical telomerase RNA subunit TER2 (Cifuentes-Rojas et al, 2011). Unlike the canonical TER, TER1, which assembles with POT1a and the RNA maturation factor dyskerin and maintains telomere tracts, TER2 assembles with Ku, POT1b, and dyskerin into an alternative telomerase ribonucleoprotein (RNP) complex (Cifuentes-Rojas et al, 2011&2012). TER2 RNP is a negative regulator of telomerase activity. Plants lacking full-length TER2 display an increase of telomerase activity in flowers, but not in seedlings, suggesting TER2 may modulate telomerase in reproductive organs (Cifuentes-Rojas et al., 2012). TER2 is a highly unstable RNA, but in response to DNA double-strand breaks (DSBs), TER2 stability increases, leading to telomerase inhibition (Cifuentes-Rojas et al., 2012; Xu et al., 2015). How components of TER2 RNP, such as Ku and POT1b contribute to telomerase inhibiton is unclear.

TER2 RNP may also be involved in telomere end-protection, since components of TER2 RNP have been implicated in this process. Analysis of a *ter2 pot1a* double mutant unexpectedly showed that depletion of TER2 accelerated the telomere shortening due to a telomerase deficiency. This excessive loss of telomere tracts cannot be simply explained by the loss of POT1a, but is consistent with a dysfunction in end-protection,

(K. Renfrew and D. Shippen, unpublished data). In addition, overexpression of a dominant negative form of POT1b causes dramatic telomere shortening and end-to-end chromosome fusions, suggesting a role in telomere end-protection of POT1b (Shakirov et al., 2005). Besides blunt-ended telomere protection, *A. thaliana* Ku has been shown to negatively regulate telomere length and to repress telomere recombination (Riha et al., 2003; Zellinger et al., 2007; Kazda et al., 2012). Therefore, three unique components of TER2 RNP, TER2, Ku, and POT1b, are all implicated in various aspects of chromosome end-protection in *A. thaliana*.

In this chapter, the hypothesis that TER2 RNP is a cap for blunt-ended telomeres is investigated. We show a two-fold increase of the G-overhang signal in *ter2* mutants consistent with the results from Ku mutants and supporting the hypothesis that TER2 and Ku function together in blunt-ended telomere protection. We provide evidence showing that POT1b and Ku both localize to telomeres and further that POT1b inhibits Ku from telomere binding. These findings indicate that components of the TER2 RNP are physically associated with telomeres, yet may have distinct functions. Finally, we report that Ku and TER2 act synergistically to promote seed viability. Altogether, these findings support the conclusion that TER2 RNP contributes to telomere stability. They also indicate that individual components of TER2 RNP may have unique contributions and open a possibility of functional subcomplexes of TER2 RNP.

Materials and methods

Plant materials and growth conditions

Plants were grown at 23°C in an environmental chamber under long-day conditions (16h light/8h dark).

T-DNA insertion lines of TER2 (SAIL_556_A04) and Ku70 (SALK_040584) in Col-0 accession were obtained from the Arabidopsis Biological Resource Center (ABRC). Genotyping of *ter2-1* mutant was performed as previously described (Cifuentes-Rojas et al., 2011). Genotyping primers for *ku70* mutants were Ku70LPM1 5'-TTACTTTGTTGTTTCGGGTGC-3' and Ku70RPM2 5'-CTCTTGGCAAGTACACGCTTC-3' to detect for the wild type POT1b allele; Ku70RPM2 and Lba1 5'-TGGTTCACGTAGTGGGCCATCG-3' for mutant alleles (Surovtseva et al., 2007). The *pot1b-1* (Ler-0) T-DNA line was obtained from the Cold Spring Harbor Laboratory GeneTrap collection. The primer combination P2GT1F: 5'-AAACCCCAACGATCAGAGAC-3' and P2GT3R: 5'-AGACGAAGAGGTTGTTTCATTGCA-3' was used to genotype wild type alleles. The primer combination P2GT3R and DS3-1: 5'-ACCCGACCGGATCGTATCGGT-3' was used to genotype the *pot1b-1* mutant allele.

G-overhang Analysis

An in-gel hybridization assay was used to monitor G-overhangs as previously described (Heacock et al., 2007). Six-week-old individual plants were used for genomic DNA extraction. 3' G-overhang signals were normalized using the EtBr signal. The G-

overhang signal obtained from wild type plants was averaged and set as one, and the G-overhang signal from mutant samples were compared to this value.

Chromatin immunoprecipitation (ChIP)

Four to six grams of six day-old seedlings were harvested for each genotype. The ChIP protocol was modified from Saleh et al., 2008. Immunoprecipitation (IP) was performed using a rabbit anti-Ku70 antibody (gift from Dr. Karel Riha at Central European Institute of Technology) or anti-POT1b antibody (Surovtseva et al., 2007) and Protein-A magnetic beads with salmon sperm DNA (Invitrogen). Eluted DNA was subjected to Southern dot blotting on a nylon membrane (GE healthcare). A [^{32}P] 5′ end-labeled $(T_3AG_3)_5$ oligonucleotide probe was used to detect telomeric DNA. The membrane was stripped and hybridized with [^{32}P] 5′ end-labeled rDNA (18S+5S) as a control.

Primer extension telomere repeat amplification (PETRA) and Quantitative telomere repeat amplification protocol (Q-TRAP)

PETRA was performed as described previously (Heacock et al., 2004). 2 µg of genomic DNA was used for each PETRA-T reaction. The PETRA-T reaction was followed by a PETRA-A PCR reaction using a subtelomeric primer to amplify specific chromosome arms. PCR products were subjected to Southern blotting, and telomeric DNA was detected using a [^{32}P] 5’ end labeled $(T_3AG_3)_5$ oligonucleotide probe. For the

Q-TRAP assay, total protein was extracted from flowers. 50 µg of total protein was used for each reaction. Q-TRAP was conducted as described (Kannan et al., 2008).

RNA isolation and RT-PCR analysis

Total RNA from *A. thaliana* floral tissues or 5-day-old seedlings was isolated using a Direct-zol RNA MiniPrep kit (Zymo). cDNA was synthesized with 1µg of total RNA using qScript cDNA supermix (Quanta Biosciences). qRT-PCR was carried out as described (Cifuentes-Rojas et al. 2012) using SsoAdvance Universal SYBR green master mix (Bio-Rad) with GAPDH as a reference gene.

Results

Ter2 mutants have increased G-overhang signals

We hypothesized that TER2 RNP is involved in the maintenance of blunt-end telomeres (Figure 3-1). To test this hypothesis, we examined the architecture of telomeres in plants mutant for TER2. Ideally, a blunt-end assay (Kazda et al, 2012) should be used to directly assess if components of TER2 RNP are involved in blunt-ended telomere capping, but this assay is still under development in the Shippen lab. Therefore, we used an in-gel hybridization assay (G-overhang assay) to measure the amount of ss G-rich telomeric DNA as an alternative method. We first monitored the status of the 3' G-overhang in plants lacking full-length TER2. If TER RNP is required for blunt-ended telomere protection, we expect to observe an increase in G-overhang signal due to 5' to 3' nucleolytic processing of the blunt-ended telomeres as they are

converted into 3' G-overhangs that could be stabilized by the CST complex. The G-overhang signal was elevated in *ku70* mutants by 4-fold, compared to wild type plants (Figure 3-2). The increased G-overhang signal is expected for two reasons. First, *ku* mutants have defects in coordinating telomere C-strand fill-in and telomerase elongation processes (Riha et al., 2003). Second, in *ku* mutants, conversion of blunt-ended telomeres into those with G-overhangs would lead to approximately 2-fold increase in G-overhang signal. Notably, we found a 2-fold increase in the G-overhang signal for *ter2* mutants relative to the wild type level (Figure 3-2). These observations support the hypothesis that TER2 functions in protecting blunt-ended telomeres.

POT1b and Ku are associated with telomeres

If TER2 RNP serves as a blunt-ended capping complex, TER2 RNP components are expected to associate with telomeres. Chromatin immunoprecipitation (ChIP) was used to test if Ku and POT1b are associated with telomeric DNA (Figure 3-3). Ku was also found to associate with telomeres (Figure 3-3A). This association was also abolished in the plants lacking Ku. As a negative control, POT1b-IP was conducted with a POT1b null mutation, *pot1b-1* (Ler-0 accession). We found no telomere association (Figure 3-3B). Strikingly, in wild type seedlings (Ler-0), POT1b was enriched at telomeres. rDNA was used as a control probe for specificity. There was not enrichment of POT1b or Ku at rDNA sequences. In conclusion, the TER2 RNP components, Ku,

and POT1b, are both associated with telomeres in seedlings.

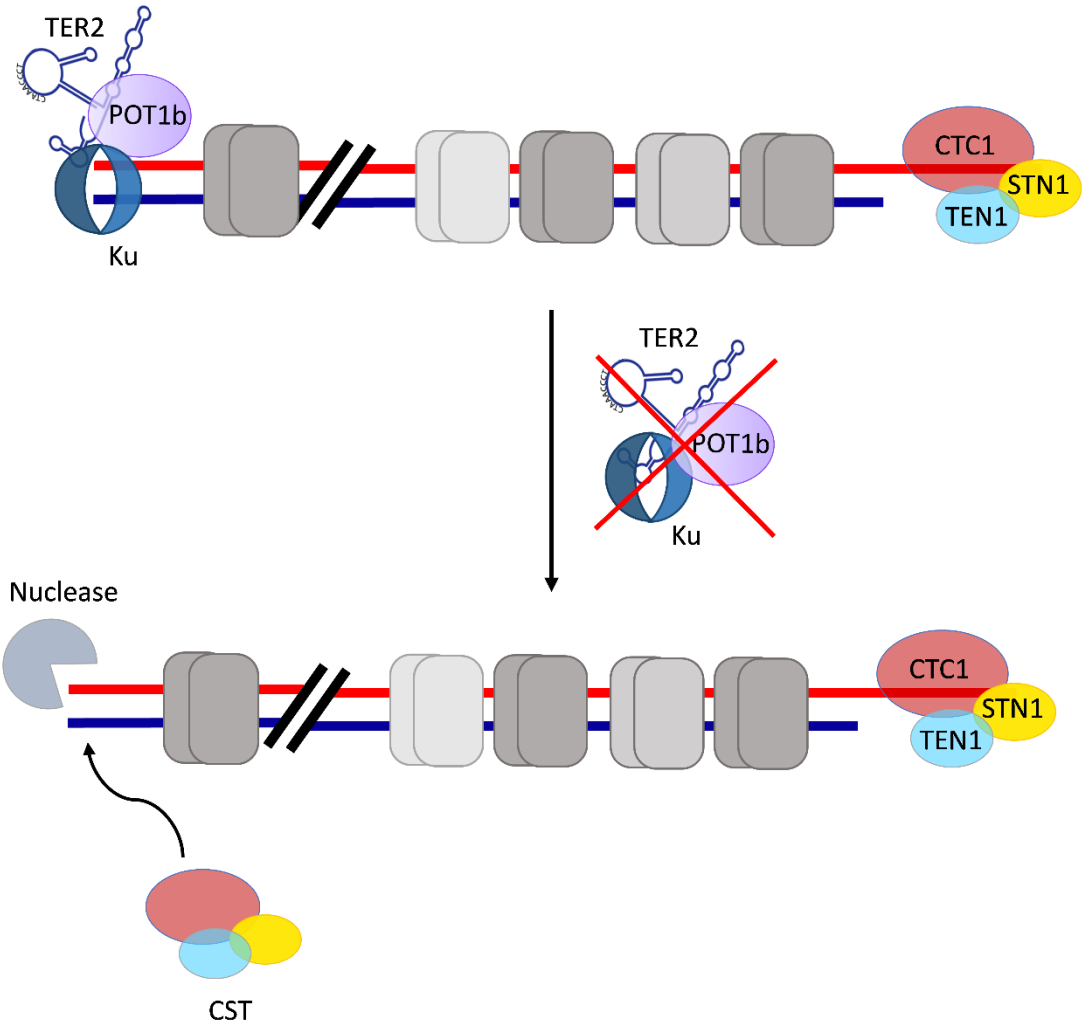


Figure 3-1. Model for telomere capping in *Arabidopsis thaliana.* The blunt-end is protected by Ku and a putative TER2 RNP, while the single-stranded telomere end is protected by the CST. Depletion of the TER2 RNP components leads to nucleolytic processing of the blunt end and conversion to a G-overhang protected by the CST. Gray boxes denote double-stranded telomere binding proteins.

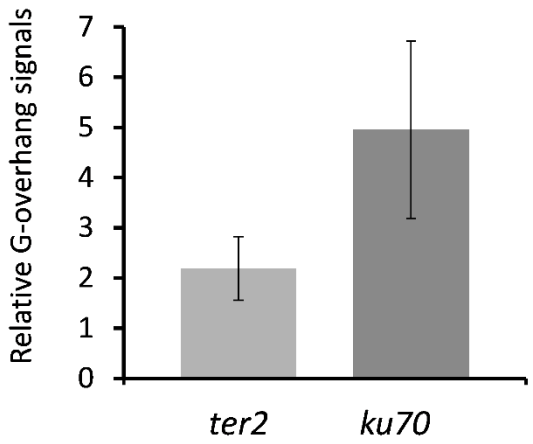
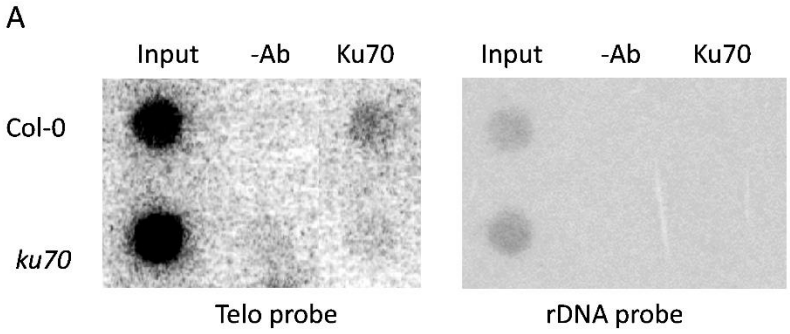
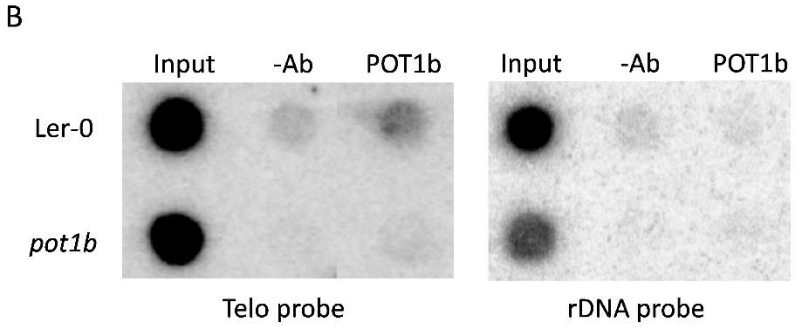
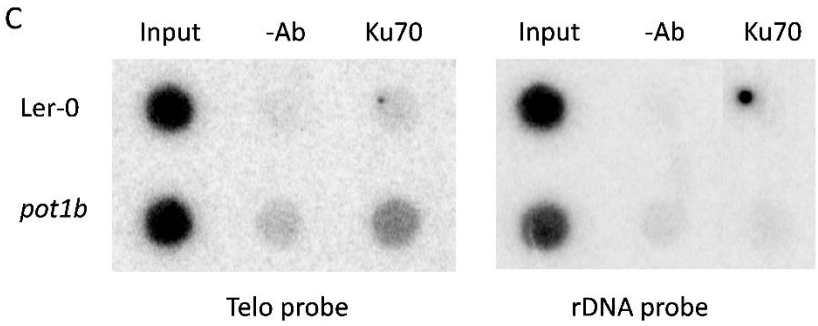


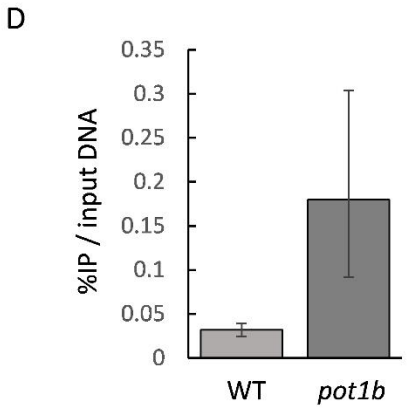
Figure 3-2. Increased G-overhangs in plants lacking TER2 and Ku70. Quantification of G-overhang signals in *ter2-1* and *ku70* mutants relative to the WT (set to 1). The data represents the results from three or more biological replicates.

Figure 3-3. Chromatin immunoprecipitation (ChIP) assay for telomeric DNA association of POT1b and Ku. (A) ChIP was performed on wild type (Ler-0), and *pot1b-1* mutants using an anti-POT1b antibody followed by dot blot analysis with a [^{32}P] 5′ end labeled $(T_3AG_3)_5$ oligonucleotide probe. The membrane was stripped and re-hybridized with a [^{32}P] 5′ end labeled rDNA (18S+5S) oligonucleotide probe. (B) ChIP was performed on wild type (Col-0) and *ku70* using an anti-Ku70 antibody. (C) ChIP was performed on WT (Ler-0), and *pot1b-1* using an anti-Ku70 antibody. (D) Quantification of Ku70 ChIP. IP signal is represented as percent precipitation of input DNA. Error bars represent standard error of the mean from two or three independent biological replicates.


A
Input
-Ab
Ku70
Input
-Ab
Ku70
Col-0
ku70
Telo probe
rDNA probe


B
Input
-Ab
POT1b
Input
-Ab
POT1b
Ler-0
pot1b
Telo probe
rDNA probe


C
Input
-Ab
Ku70
Input
-Ab
Ku70
Ler-0
pot1b
Telo probe
rDNA probe


D
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
%IP / input DNA
WT
pot1b

Finally, we asked how the loss of one TER2 component affects the telomere association of another by monitoring Ku association with telomeres in plants deficient for POT1b (Figure 3-3C). Unexpectedly, we found a 4-fold increase in Ku70 association with telomeric DNA in *pot1b-1* mutants (Figure 3-3D). This observation suggests that POT1b negatively regulates the association of Ku with telomeres, further suggests that subunits of TER2 RNP may compete for telomere binding.

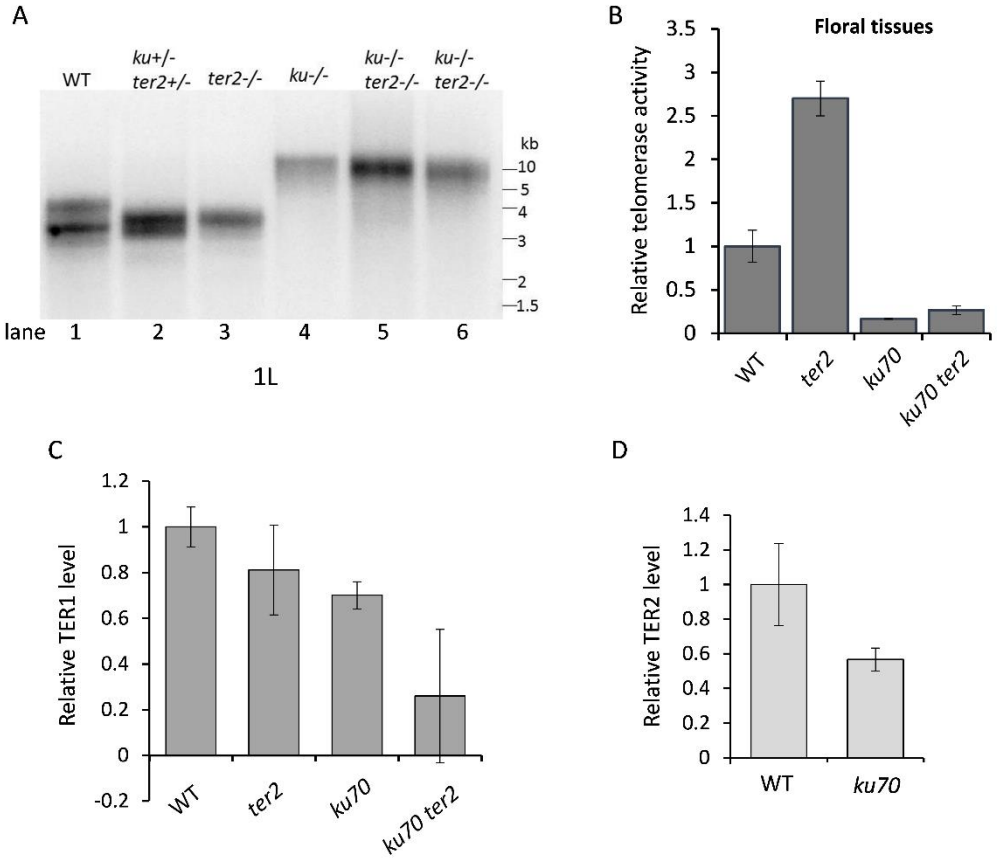


Figure 3-4. Telomere length, telomerase activity and telomeric RNA analysis in the *ku70* x *ter2* cross. (A) Representative data for PETRA. Primer for the left arm of chromosome 1 was used for amplification of telomeric sequences. (B) Quantitative TRAP was used to measure telomerase activity in floral tissues. The average level of wild type telomerase activity was set as 1. Quantitative RT-PCR was used to analyze the steady state level of TER1 (C) and TER2 (D) in floral tissues of different mutant

backgrounds. Averaged wild type RNA level was set as 1. Each data point represents three to five biological replicates, with two technical replicates. The standard deviation between biological replicates is presented by error bars.

Analysis of telomeres and telomerase in ku70 ter2 double mutants

To further investigate the interplay between individual components of TER2 RNP, we used a genetic approach to study double mutants of TER2 RNP components, including POT1b$_{S273F}$ *ter2* double mutants, *ku70* POT1b$_{S273F}$ double mutants, and *ku70 ter2* double mutants. POT1b$_{S273F}$ line is a knockdown mutant with significantly lower (~50%) POT1b protein (see Chapter II). Genetic and biochemical analyses for POT1b$_{S273F}$ *ter2* and *ku70* POT1b$_{S273F}$ double mutants are in progress. Here, we will focus on the analysis of *ku70 ter2-1* double mutants.

Previous studies showed dramatic telomere elongation in *ku* mutants (Riha et al., 2003), but no telomere length perturbation in *ter2-1* mutants (Cifuentes-Rojas et al, 2012). To test if the combined loss of TER2 and Ku affects telomere length homeostasis, we compared the telomere length of *ku70 ter2* double mutants to *ter2* and *ku70* single mutants. Telomere length of individual chromosome arms was measured using the primer extension telomere rapid amplification (PETRA) assay (Figure 3-4A). As expected, telomeres were in the wild type range (2 to 5 kb) in *ter2* mutants (Heacock et al., 2004) (Figure 3-4A, lane 1&3). In contrast, first generation *ku70 ter2* double mutants possess elongated telomeres of up to 10 kb in length, similar to *ku70* single mutants (Figure 3-4A, lane 4-6).

Plants lacking TER2 have been shown to exhibit a two to four fold increase in telomerase activity in floral tissues (Cifuentes-Rojas et al, Gene Dev 2012). If the change in telomerase activity in *ter2* mutants is due to the absence of TER2 RNP, we expect to observe similar pattern of increased telomerase activity in the *ku70 ter2* mutants. However, quantitative telomere repeat amplification protocol (Q-TRAP) revealed that telomerase activity in floral tissues of *ku70* mutants decreased by 5-fold compared to wild type (Figure 3-4B). Telomerase activity was also decreased by 4-fold in the *ku70 ter2* double mutants. Change in telomerase activity was not observed in seedlings of these mutants. Together, these data reveal that the effects of Ku depletion on telomere length and telomerase activity is dominant over the effect of TER2 depletion.

Ku stabilizes TER2 in vivo

One possible explanation for telomerase repression in ku ter2 mutants is a change in telomerase RNA levels. We explored this possibility by monitoring the steady state level of TER1 and TER2 levels in floral tissues using qRT-PCR (Figure 3-4C). TER1 decreased by ~20% in the ter2 mutants and was ~30% lower in ku70 mutants compared to wild type. A more dramatic decrease (approximately five fold) in TER1 was observed in the absence of both Ku and TER2, indicting addictive effects on TER1 stability. We also observed a significant reduce in TER2 level (~ 40%) in the flower of ku70 mutant compared to wild type (Figure 3-4D). Because Ku is known to directly bind TER2 in vivo (Cifuentes-Rojas et al., 2012), these data suggest that Ku may play a role in

stabilizing TER2 in floral tissues.

		Cross A*		Cross B**	
Genotype (F2)	Exp	Obs	Ratio	Obs	Ratio
T-K-	0.562	62	0.689	22	0.733
T-kk	0.188	13	0.144	2	0.067
ttK-	0.188	12	0.133	5	0.167
ttkk	0.062	3	0.033	1	0.033
Sum		90		30	

*Cross A: *ter2/+* ♀ x *ku70/+* ♂
**Cross B: *ku70/+* ♀ x *ter2/+* ♂

Table 3-1. Genotypic ratio of F2 *ku70* x *ter2* cross. Percentage of each genotypes observed and expected in F2 of *ku70* x *ter2* crosses. The parental genotypes of the cross A and cross B is shown below the table. Obs: observed individuals with corresponding genotype. Exp: expected ratio of corresponding genotype. Sum: total number of individual. Ratio: Obs/Sum.

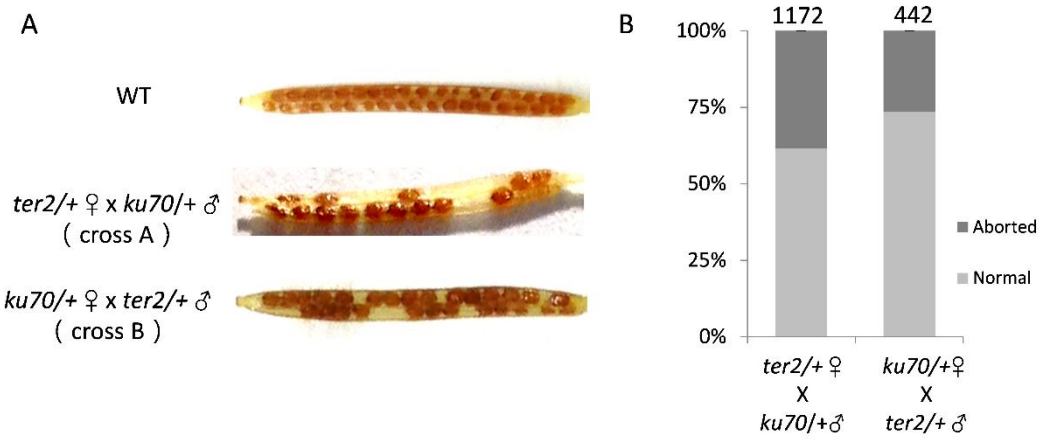


Figure 3-5. Seed abortion in F1 *ku70* x *ter2* cross. (A) Representative figures for siliques from the wild type, F1 generation of cross A, F1 generation of cross B. (B) Quantitation for average percentage of seed abortions in each cross. Numbers above the bar indicate total number of seeds counted.

Meiotic abnormalities in ku70 ter2 double mutants

During isolation of *ku70 ter2* double mutants, we noticed an unexpectedly low recovery rate of double mutant offspring in a *ter2*/+ ♀ x *ku70*/+ ♂ cross (cross A) and its reciprocal cross (*ku70*/+ ♀ x *ter2*/+ ♂, cross B). *Ku70* and *TER2* genes are unlinked in *A. thaliana*: *Ku70* is encoded on chromosome 1 and *TER2* on chromosome 5. A 9:3:3:1 genotypic ratio is expected for the offspring from this dihybrid cross (Table 3-1). However, the frequency of wild type and heterozygous mutants (*ku70*/+ and/or *ter2*/+) combined is higher than expected (68.9% and 73.3% vs 56.2%) in both cross A and cross B. Plants homozygous for either the *ku70 or* the *ter2* mutant alleles were slightly less abundant than expected. Moreover, among the F2 generation segregants of cross A, only three double mutants out of 90 offspring (3.3%) were recovered, only half of the expected frequency (6.2%) (Table 3-1). A similar skewed frequency was observed in the reciprocal cross (cross B) (Table 3-1). Chi-square value for cross A (6.284) and cross B (4.804) suggest that Mendelian inheritance still apply to these two crosses ($P<0.05$). Therefore, these data indicate that *TER2* gene may be haploinsufficient for *ku70* mutants, and *Ku70* gene may also be haploinsufficient for *ter2* mutants.

Adult *ku70 ter2* double mutants, *ku70* mutants, and *ter2* mutants were indistinguishable from wild type plants, suggesting the lower recovery rate of the double mutant did not affect vegetative growth and development. Thus, the defect may be confined to embryo formation or during seed germination. To test if the seed formation is defective, we looked for evidence of seed abortion in the F1 double heterozygous siliques of cross A (53 siliques) and cross B (19 siliques) (Figure 3-5 A&B). The siliques

of double heterozygous cross A yielded 38% (441/1172) aborted seeds on average, while cross B had 27% (114/442) of aborted seeds. These values are significantly higher than wild type where seed abortion is rare (~0%) (House et al., 2010). These observations indicate that Ku and TER2 are involved in plant embryogenesis. Interestingly, the ratio of seed abortion in cross A (38%) and cross B (27%) both exceed the expected ration of double mutants (6.2%), suggesting that *Ku* and / or *TER2* genes are haploinsufficent for embryogenesis.

Discussion

The function of the non-canonical telomerase RNA TER2 in *A. thaliana* is not well understood, although this RNA has been implicated in telomerase regulation and the DNA damage responses (Cifuentes-Rojas et al., 2012; Xu et al., 2015). Previous studies indicate that TER2 is associated with Ku, POT1b, and the RNA maturation factor dyskerin (Cifuentes-Rojas et al., 2012; A. Arora and D. Shippen, unpublished data). A recent finding about Ku's function in blunt-ended telomere maintenance in *A. thaliana* raised the interesting possibility that the TER2 RNP could serve as a capping complex for blunt-ended telomeres. This hypothesis is addressed in this chapter.

Does TER2 RNP cap blunt end telomeres?

In addition to the role of Ku at blunt-ended telomeres, both POT1b and TER2 have been implicated in telomere end-protection. Ectopic overexpression of the first OB-fold of POT1b was associated with telomere erosion and massive telomere fusions

(Shakirov et al, 2005). Genetic studies in *pot1a* mutants showed an exacerbated telomere shortening when TER2 was also absent (K. Renfrew and D. Shippen, unpublished data). This dramatic telomere attrition is not caused by loss of telomerase activity, but is consistent with increased nucleolytic processing, implicating a role of TER2 in the end-protection (Renfrew et al., 2014). If our hypothesis that TER2 RNP is a cap for blunt-ended telomeres is correct, when individual components of TER2 RNP are inactivated, an increase in ss telomeric DNA is anticipated. Second, components of TER2 RNP are expected to associate with telomeric DNA.

To test the first prediction, we monitored the amount of ss telomeric DNA in *ter2* mutants. Our data reveal a 2-fold increase in G-overhang signal in plants lacking full-length TER2, consistent with our hypothesis. To test the second prediction, we used ChIP to assess the association of POT1b with telomeres. As expected, POT1b and Ku associated with telomeric DNA, meeting the primary requirement of a telomere cap. Unexpectedly, we also observe that POT1b inhibits Ku binding to telomeric DNA. The telomere-bound Ku increases by 4-fold in plants deficient of POT1b, suggesting that POT1b controls Ku access to telomeres. It is possible that multiple TER2 subcomplexes are formed *in vivo* and compete for telomere binding during the cell cycle. One possibility is that the Ku-associated subcomplex serves as a blunt-end cap during most of the cell cycle. In this complex, TER2 prevents Ku from sliding off the blunt-end. During the S/G2 phase, POT1b may regulate the disassociation of Ku from the blunt-end, allowing end processing and the access of telomerase.

Ku stabilizes TER2 in floral tissues

To further explore the relationship and functions of individual components of the TER2 RNP, we examined plants deficient in two components of TER2 RNP, Ku70 and TER2. First, we examined the contribution of the two genes on telomere length. Telomere in plants lacking TER2 is maintained in the wild type range (2 to 5 kb) (Cifuentes-Rojas et al., 2012). Plants deficient in Ku70 have long telomeres of over 10 kb in size (Riha et al., 2003). We found that *ku70 ter2* double mutants have elongated telomeres indistinguishable from *ku70* single mutants. Next, we examined the role of the two genes in telomerase activity. Telomerase activity is elevated by ~2.7 fold in the floral tissues from *ter2-1* mutants, as expected (Cifuentes-Rojas et al., 2012). Unexpectedly, both *ku70 ter2* double mutants and *ku70* single mutants demonstrated a dramatic decrease (4-fold and 5-fold) in telomerase activity of floral tissues. These data suggest that the deficiency of Ku overrides the effect on telomeres from TER2 deficiency, which is not surprising since Ku is a multifaceted protein involved in several aspects of telomere metabolism, including telomere end-protection, coordinating end processing, telomere addition at DSBs, and telomere recombination in *A. thaliana* (Riha et al., 2003; Zellinger et al., 2007; Nelson et al., 2011; Kazda et al., 2012).

How are telomeres elongated in *ku70* and *ku70 ter2* where telomerase activity is significantly reduced? Previous studies demonstrated that *ku70* mutants have an increased amount of telomeric circles, a hallmark of deletional recombination at telomeres and alternative telomere lengthening. It is likely that a telomerase-independent

pathway, potentially involving homologous recombination, is responsible for extending telomere sequence (Zellinger et al., 2007; Lustig, 2003).

It is possible that decreased telomerase activity in *ku70* mutants is resulted from a decrease in TER1 or an increase in TER2. QRT-PCR was used to investigate whether the loss Ku70 affects TER1 and TER2 in floral tissues. We did observe a decrease in TER1 level by 30% in the *ku70* single mutants and by 40% in the *ku70 ter2* double mutants. In addition, a decrease in TER2 level was observed in *ku70* mutant flowers, suggesting that Ku could be responsible for TER2 stability in flowers. In addition, previous data from the Shippen lab showed that TER2 peaks in unopened flower buds and gradually decreases as fertilization is completed (H. Xu and D. Shippen, unpublished data). Thus, one possibility is that Ku directly binds to TER2 and is required for its stabilization during meiosis. However, Ku does not associate with TER1 (Cifuentes-Rojas et al., 2011). Recent study showed that TER1 and TER2 form a heterodimer *in vitro,* which is preferred over a homodimer (J. Song and D. Shippen, unpublished data). It is possible that TER2 forms heterodimer with a fraction of TER1 molecules to sequester and stabilize the excess amount of TER1, and that TER1 can be dynamically disassociated from TER2 during the cell cycle for telomerase activation. When Ku is absent, the decrease in TER2 abundance leads to reduced TER1-TER2 heterodimerization. As a result, TER1 level decreases in *ku70, ter2,* and *ku70 ter2* double mutants, and the reduction in TER1 abundance may contribute to the decrease in telomerase activity observed in *ku70* and *ku70 ter2* mutants.

Ku and TER2 play a role in plant development

TER2 is required for telomerase inhibition in flowers, a process that has been proposed to repress telomere addition at DSBs (Xu et al., 2015). In this study, data are presented showing a reduced recovery rate of *ku70 ter2* mutants and increased seed abortion in siliques from heterozygous *ku70 ter2* mutants. Among the F2 generation segregants of *ku70 ter2* crosses, the frequency of observed double mutants was only a half of the expected frequency. The adult double mutant plants are indistinguishable from wild type, implicating a reduced viability of *ku70 ter2* during early development. We further examined seed abortion in siliques from heterozygous *ku70 ter2* mutants and found a significant increase in seed abortion (38% and 27% respectively in two reciprocal crosses). These findings indicate that Ku and TER2 may act synergistically to facilitate embryogenesis. Because telomerase activity is dramatically decreased in *ku70 ter2* double mutants, the defects in seed formation are probably not associated with loss of telomerase repression in the absence of TER2. The precise role of TER2 and Ku70 during Arabidopsis embryogenesis is unclear. Nevertheless, our data support the hypothesis that these two components play a role in meiosis and seed formation.

In conclusion, data presented in this chapter provide evidence supporting a role for TER2 RNP in blunt-ended telomere capping and seed formation in Arabidopsis.

CHAPTER IV

DE NOVO TELOMERE FORMATION IN TER2 MUTANTS

Summary

Telomeres are nucleoprotein structures that differentiate natural chromosome ends from double-strand breaks (DSBs). DSBs can be stabilized by the addition of telomere sequences by telomerase to form *de novo* telomeres. However, *de novo* telomere formation (DNTF) results in chromosome truncations and thus can be highly deleterious. The mechanism of DNTF is poorly understood in higher eukaryotes. Here we used an established DNTF assay in tetraploid *Arabidopsis thaliana* to elucidate the role of TER2 in DNTF. TER2 is a long noncoding RNA shown to down-regulate telomerase activity in response to DSBs. TER2 levels peak in reproductive tissues where DSBs are introduced across the genome, raising the possibility that TER2 plays a role in protecting the genome from DNTF during meiosis. We report that the efficiency of DNTF following integration of telomere repeat containing T-DNA is increased in plants lacking TER2. Due to the small sample size, the difference in DNTF in *ter2* mutants versus wild type plants was not statistically significant. Nevertheless, these data support the hypothesis that TER2-dependent telomerase inhibition in response to DSBs may modulate DNTF during reproduction.

Introduction

The double-strand breaks (DSBs) activate DNA damage responses (DDR) that lead to cell cycle arrest until the damage can be repaired. In contrast, natural ends of linear chromosomes contain telomeres, nucleoprotein structures that form a protective "cap" on terminus to distinguish the ends from DSBs thereby averting actions from DNA damage surveillance machinery (Doksani and de Lange; 2014). In most eukaryotes, telomeres consist of tandem GC-rich DNA repeats that end in a 3' G-rich overhang (G-overhang) and are bound by telomere-specific proteins, including the shelterin complex in vertebrates (de Lange, 2005) and CST complex (CTC1/Cdc13; STN1;TEN1) in budding yeast and Arabidopsis (Price et al, 2010). These complexes facilitate telomere replication and promote end-protection. The loss of a single component of one of these complexes can cause telomere length dysregulation, elicit a powerful DNA damage response, and finally lead to genomic instability (Baumann and Cech, 2001; Gao et al, 2007; Surovtseva et al, 2009; Song et al, 2008; Leehy et al, 2013).

Chromosome termini also face the end replication problem, which leads to loss of sequences at chromosome ends each time the DNA is replicated (Watson, 1972). Telomerase, the telomere-specific reverse transcriptase, provides a solution to the end-replication problem. During the S/G2 phase of the cell cycle (Zhu et al., 1996), telomerase uses the 3' G-overhang on the extreme terminus of the chromosome as a substrate for extension. The reverse transcriptase TERT employs the telomerase RNA subunit as a template for telomere repeat addition, and thus counteract telomere

sequence erosion (Greider and Blackburn 1985; Greider and Blackburn, 1989; Gallardo et al, 2011).

Telomerase activity is fine-tuned through different layers of regulation. In multicellular organisms, telomerase is highest in actively proliferating cells (embryos and stem cells) and is down-regulated in somatic cells (Cong et al, 2002). Developmental control of telomerase largely depends on transcriptional regulation and posttranslational modifications of telomerase core components (Zhu et al., 1996; Xi and Cech, 2014; Yamazaki et al., 2012). However, spatial control of telomerase components also plays a role in regulation of activity and is essential for assembly and recruitment of telomerase holoenzyme to the chromosome ends. In humans, the catalytic subunit of telomerase, TERT, and telomerase RNA, TER, accumulate with additional telomerase accessory proteins, at separate intranuclear sites away from telomeres (Tomlinson et al., 2006; Vogan and Collins, 2015). During S phase the holoenzyme is recruited to telomere ends. Furthermore, substrate accessibility and telomere repeat addition processivity must be achieved for telomerase activation (Wang et al, 2007; Xin et al, 2007; Williams et al, 2014; Chen et al, 2016). Together, these complex mechanisms guarantee appropriate telomerase regulation during the cell cycle and during development for proper telomere maintenance and genome stability. Inadequate telomerase activity results in telomere erosion (Riha et al, 2001; Mochizuki et al, 2004; Lundblad and Szostak, 1989), while constitutive activation of telomerase is a signature of tumorigenesis (Hahn and Meyerson, 2001; Stewart and Weinberg, 2006; Shay and Wright, 2011). Telomerase dysregulation has been associated with several human diseases such as aplastic anemia

and dyskeratosis congenita (Armanios and Blackburn, 2012), and therefore continues to be a prevalent subject for research.

Telomerase has specificity for telomeric DNA sequence, but the enzyme is capable of acting promiscuously to add telomeric repeats to non-telomeric DNA as a way to stabilize broken chromosomes. This process is known as chromosome healing or *de novo* telomere formation (DNTF) and has been observed in many organisms (Pologe and Ravetch, 1988; Wilkie et al., 1990; Lamb et al., 1993; Kramer and Haber, 1993; Flint et al, 1994). DNA double-strand breaks are usually resolved by two major DNA damage repair (DDR) pathways: non-homologous end-joining (NHEJ) and homologous recombination (HR). Because DNTF requires little or no substrate sequence homology to the telomerase RNA template, telomerase-mediated conversion of DSBs to telomeres prevents checkpoint signaling (Flint et al, 1994; Harrington and Greider, 1991; Michelson et al., 2005). Although resolving DSBs allows resumption of cell cycle, DNTF is highly deleterious. The break site on the centromere-containing fragment is stabilized by a newly synthesized telomere, but the acentric chromosome fragment will be lost or recombined during cell division. In humans, terminal chromosome truncation and DNTF are associated with several disorders including Alpha thalassemia, Phelan McDermid syndrome, and mental retardation (Flint et al., 1994; Luciani et al., 2003; Wong et al., 1997). Thus, telomerase action on DSBs needs to be restricted to allow faithful DNA repair.

DNTF is quite rare *in vivo* and is actively suppressed by multiple pathways. Most studies of DNTF have been performed in yeast due to higher frequency of DNTF and

workable genetics. Telomerase core components, including Est1, Est2, Est3, and TLC1 (yeast telomerase RNA) are required for DNTF (Bianchi et al, 2004; Negrini et al, 2007; Chung et al, 2010). In addition, the interaction between Ku and TLC1 is essential for telomerase recruitment to break sites (Stellwagen et al., 2003). Mec1 (ATR in yeast) down-regulates telomerase action at DSBs through phosphorylation of Cdc13, a telomere capping protein in budding yeast, that can accumulate at DSBs and recruit telomerase (Bianchi et al., 2004; Zhang and Durocher, 2010). On the other hand, the Pif1 5'-3' helicase requires phosphorylation by Mec1 to destabilize the telomerase RNA-DNA hybrid and thus dislodge telomerase from a DSB (Schulz and Zakian, 1994; Boule et al, 2005; Makovets and Blackburn, 2009). Effective resection of the DSBs also contributes to the inhibition of DNTF. In budding yeast, Exo1 and Sgs1 nucleases act in two alternative pathways to generate ss DNA at DSBs thereby promoting faithful DNA repair (Zhu et al., 2008; Gravel et al., 2008). In the absence of Exo1 and Sgs1, DNTF is elevated due to increased Cdc13 recruitment (Lydeard et al, 2010; Chung et al, 2010).

The molecular events leading to DNTF in high eukaryotes are less known. Chromosome healing in mammals can be mediated by telomerase-dependent or telomerase-independent pathways (Gao et al, 2008). In response to DNA damage, human telomerase is subjected to phosphorylation to decrease enzyme activity (Kharbanda et al, 2000) and rapid import into the nucleolus (Wong et al, 2002). Thus, telomerase sequestration is another mean to restrain active telomerase from DSBs.

Chromosome healing was first unveiled by Barbara McClintock in her pioneering work on broken chromosomes in maize. In yeast and mammalian cells,

studies of DNTF are based on inducing DSBs adjacent to a telomere "seed" sequence and monitoring the rate of DNTF events (Diede and Gottschling, 1999; Sprung et al., 1999). Studies of plant DNTF have been enabled by introducing a telomere seed sequence via a T-DNA construct with telomere repeat arrays (TRAs) (Yu et al., 2007). Insertion of TRA can be stabilized *in vivo* in two forms. One form is through stable integration of the TRA into the body of the chromosome as a T-DNA. Such integration events cannot be amplified by PETRA reactions due to the absence of a free 3' G-overhang. The second form of integration occurs when the TRA acts as telomere "seed" and blocks full integration of the T-DNA. In this case, one end of the T-DNA is integrated into the chromosome and the other end containing the TRA is bound by telomere proteins and extended by telomerase to make a fully functional telomere *de novo.*

Large deletions caused by DNTF at the telomere seed are often lethal in a diploid plant, but can be bypassed using a tetraploid (4X) plant (Vizir and Mulligna, 1999). Taking advantage of the ability to create tetraploid *A. thaliana,* the Shippen lab developed a method to study DNTF in telomere seeds or TRA introduced into 4X plants to understand the nature of DNTF in plants (Nelson et al., 2011). The DNTF events in 4X Arabidopsis were found throughout the genome, indicating a robust system of DNTF. These studies revealed that both Ku and Lig 4, components of the classical NHEJ (c-NHEJ) pathway, promote DNTF in *A. thaliana*. Unexpectedly, telomerase modestly suppresses DNTF, potentially by competing with proteins for capping the nascent terminus. After integration of seed TRAs, the nascent telomeres function as

native telomeres which are maintained by telomerase and are subjected to the same length regulation (Nelson et al., 2011).

Little is known about how plants control DNTF, but an interesting new mode of regulation for telomerase at DNTF has recently been uncovered in *A. thaliana*. *A. thaliana* encodes two distinct telomerase RNA subunits, TER1 and TER2 (Cifuentes-Rojas et al, 2011). TER1 is a canonical TER required for telomere replication (Cifuentes-Rojas et al, 2011). In contrast, TER2 is a negative regulator of telomerase and assembles into an alternative ribonucleoprotein (RNP) complex with different protein components than TER1 RNP (Cifuentes-Rojas et al, 2012). TER2 is less abundant than TER2, but its levels peak in reproductive tissues (Cifuentes-Rojas et al, 2011; H. Xu and D. Shippen, unpublished data). In *ter2* mutant flowers, telomerase activity is upregulated, indicating that TER2 may negatively regulate telomerase, and further that this function may be important during meiosis. TER2 is a highly unstable RNA, however, in response to DSBs TER2 becomes stabilized and is the most abundant TER isoform (Cifuentes-Rojas et al, 2012; Xu et al, 2015). The increase in TER2 abundance leads to a decrease in telomerase activity (Cifuentes-Rojas et al, 2012). Thus, TER2 has been proposed to cause telomerase inhibition as a mechanism to repress DNTF at DSBs.

Here we tested this hypothesis via the established DNTF assay for *A. thaliana*. We introduced an extremely short TRA of 50 bp into *A. thaliana* plants and monitored DNTF in 4X plants with a mutation in TER2. We report that in *ter2* mutants, the DNTF rate is higher than that in the wild type, supporting a role for TER2 in repressing DNTF *in vivo*.

Materials and methods

Plant materials and growth conditions

The *ter2-1* T-DNA insertion line bearing a T-DNA inserted at the template region has been previously described (Cifuentes-Rojas et al, 2012). Tetraploid wild type Col-0 and *ter2-1* mutants were generated by applying 0.1% colchicine solution to the apical meristem of 7-to 14-day-old seedlings. The ploidy of the plants were confirmed as previously described (Yu et al, 2006).

Plants were grown at 23^{o}C in an environmental chamber under long-day conditions (16h light/8h dark). Transformation by the floral dipping method was performed as previously described (Zhang et al., 2006). Transformants were selected on ½ MS plates with kanamycin (50mg/L).

Plasmid construction

A 50bp TRA was PCR amplified and inserted into the pWY86 construct (termed pWY86-TRA50) as described previously (Nelson et al., 2011). The pWY86-TRA50 was transformed into Stbl2 cells (Invitrogen) to minimize intra-repeat array recombination. The TRA sequence was inserted into the pKGW construct and transferred into *Agrobacterium tumefaciens.*

Primer extension telomere repeat amplification (PETRA)

For each transformant, leaves were used for DNA extraction, and 1 µg genomic DNA was used for PETRA reactions. The PETRA assay was performed as described

previously (Heacock et al., 2004) with a few modifications. In PETRA-A reactions, a PETRA-A primer and a subtelomeric primer were used to amplify endogenous telomeres as a positive control. A PETRA-A primer and a pKGW construct-specific primer were used to amplify *de novo* telomeres. There were three pKGW construct specific primers used in this study. They are pKGW#1 (P1): 5'-ACGTTGCGGTTCTGTCAGTTC-3', pKGW#2 (P2): 5'-GGAATTTATGGAACGTCAGTGGAGC-3', and pKGW#3 (P2): 5'-TCCTGATCGACAAGACCGGCTTC-3'.

Results

To investigate the role of TER2 during *de novo* telomere formation, we utilized the DNTF system that was previously developed for tetraploid (4X) Arabidopsis (Nelson et al, 2011). Briefly, a construct containing a TRA was introduced into 4X Arabidopsis by floral dipping. Telomeres that form after integration of the TRA sequence can be detected via their construct specific sequence (Figure 4-1). The length of initial seed TRA is positively correlated with the frequency of DNTF. DNTF was detected in 54% of plants transformed with ~900 bp TRA, but was detected in 16% of plants transformed with the smallest TRA tested (100 bp). Integration of a longer initial TRA size is associated with higher DNTF efficiency. However, shorter initial TRA may better represent processes during spontaneous DNTF *in vivo* because telomere seed sequences are not essential for DNTF. Therefore, in this study a short TRA of 50 bp (pKGW-

TRA50) was used to closely mimic the *de novo* telomere formation process *in vivo.*

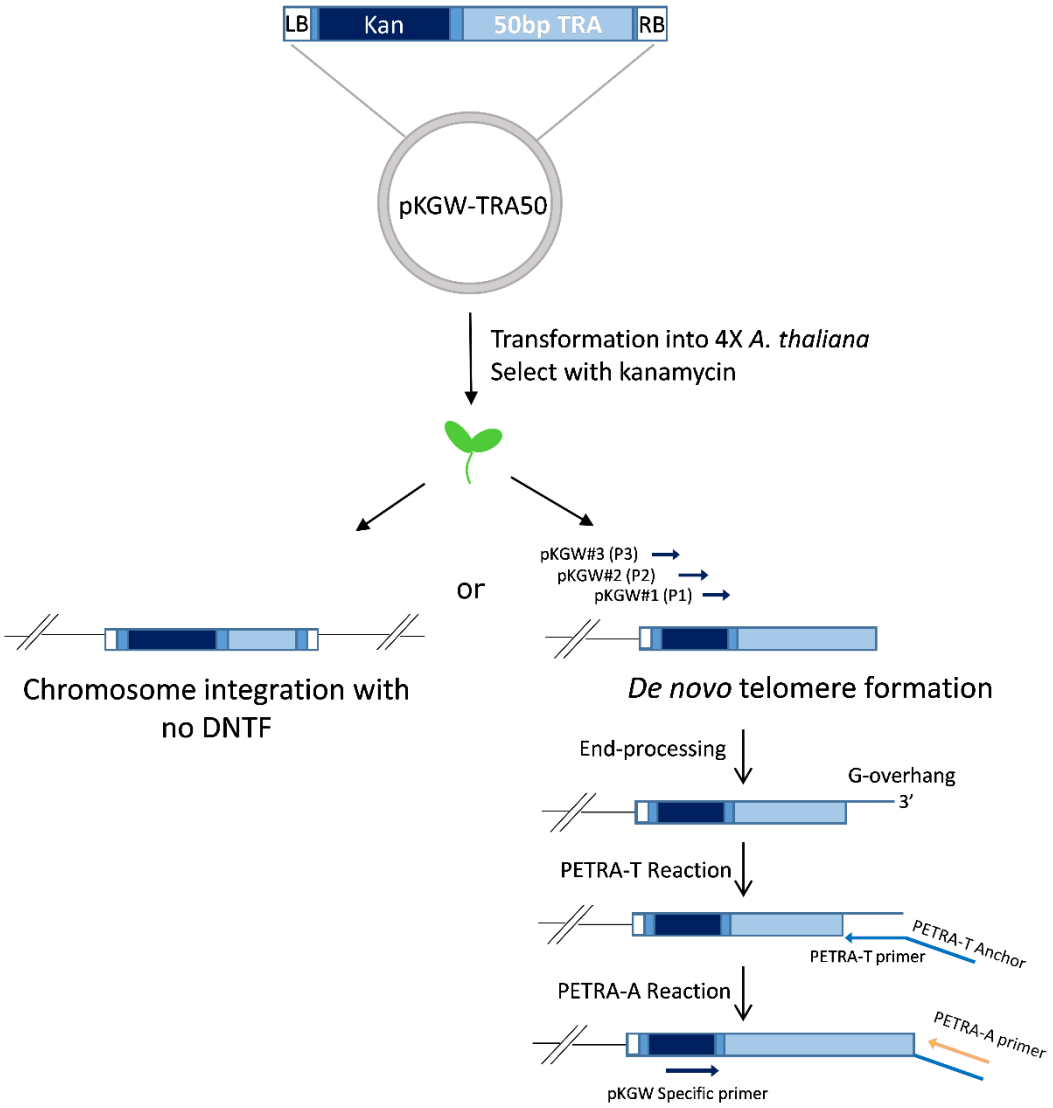
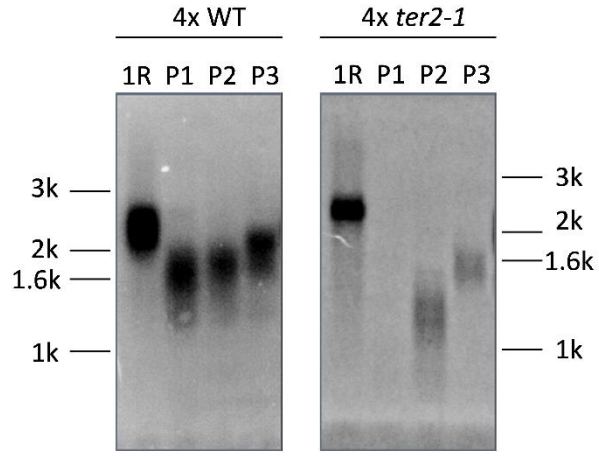


Figure 4-1. Schematic diagram of the DNTF system. A T-DNA construct containing kanamycin selection marker, left border (LB) and right border (RB), and a 50 bp telomere repeat array (TRA) in pKGW is transformed into tetraploid Arabidopsis and selected for transformants. The T-DNA can be fully integrated in the chromosome or be stabilized by *de novo* telomere formation. Transformants are screened for the two outcomes using PETRA assay using pKGW#1 (P1), pKGW#2 (P2), pKGW#3 (P3) and subtelomeric primers to amplify the nascent telomeres with a G-overhang.

A



B

Genotype	TRA length	# of sample	PETRA Positive	DNTF Efficiency	Average (bp)
4X WT	50bp	53	4	7.5%	966±381
4X *ter2-1*	50bp	50	8	17.4%	803±187

Figure 4-2. Detection of DNTF by PETRA. (A) A representative result for PETRA of DNTF in a 4X wild type and a 4X *ter2-1* mutant. Subtelomere primer for the right arm of chromosome 1 (1R) was used as a positive control. The three T-DNA specific primers, P1, P2, and P3 were used to detect DNTF. (B) DNTF efficiency in 4X wild type and 4X *ter2-1* mutants using initial 50 bp TRA. The pKGW-TRA50 was introduced to 4X wild type plants and 4X *ter2* mutants. Transformants were selected on ½ MS plates containing kanamycin for isolation of stable T-DNA insertions. After the kanamycin selection, 53 4X wild type and 50 4X *ter2* transformants were obtained. These transformants were subjected to PETRA analysis to detect DNTF events.

Primer extension telomere repeat amplification (PETRA) reactions were performed four times on each sample using different primers combinations with each transformant (Figure 4-1). In each transformants, a PETRA reaction with a subtelomeric primer was performed as a positive control to amplify the corresponding endogenous

telomere arm. Three primers (P1, P2 and P3), which recognizes the upstream sequences of the seed TRA were used in three PETRA reactions to amplify the nascent telomere (Figure 4-1). Because the P3 primer is further away from the TRA sequence, a larger amplification products from the PETRA reaction are expected. Similar, because the P1 primer targets sequence close to the TRA element, a smaller PETRA products are expected.

The new telomeres can then be amplified by PETRA reactions using a construct specific primer (Figure 4-2A). The percentage of transformants containing a *de novo* telomere over total individuals having an integrated TRA is defined as the DNTF efficiency. Among 53 4X wild type transformants, 4 individuals (7.5%) displayed DNTF (Figure 4-2B). The DNTF frequency in the 4X wild type transformed with 50 bp TRAs was lower than previously reported in the 4X wild type transformed with 100 bp TRAs (7.5% and 16% respectively) (Nelson et al., 2011), as expected for a shorter initial TRA. Among 4X *ter2* transformants, 17.4% transformation events (8 out of 50) led to DNTF. This DNTF frequency is more than 2-fold higher than 4X wild type, suggesting that TER2 is involved in the repression of DNTF. After stabilizing as a telomere, a TRA can also be extended to reach wild type length (Nelson et al., 2011). In both the 4X wild type and the 4X *ter2* transformants, TRAs were extended after DNTF and reached 966 $\pm$ 381 bp and 803 $\pm$ 187 bp on average respectively (Figure 4-2B), indicating that TER2 does not affect the elongation of short nascent telomeres.

To evaluate whether this difference in DNTF efficiency was statistically significant, we performed a Fisher's exact test. The results revealed that the elevated

rate of DNTF for the *ter2* mutants over the wild type was not statistically significant (P value=0.23, Fisher's exact test). We speculate that the lack of statistical support for the hypothesis that TER2 represses DNTF is due to a small sample size (53 for wild type and 50 for *ter2* mutants),

Discussion

Telomerase regulation has long garnered attention for its critical role in promoting genome stability, as well as its potential role in telomerase inhibition based cancer therapy. Mechanism of telomerase regulation are varied and complex. An important aspect of telomerase control is to prevent untimely action of enzyme on DSBs. Although most studies of DNTF have been conducted in yeast, the recent breakthrough of a DNTF assay for *A. thaliana* has enabled analysis of the DNTF mechanism in a multicellular genetically trackable system (Nelson et al., 2011). Two key features made this possible: the use of a TRA as a seed for DNTF allows tracking of nascent telomeres, and the use of tetraploid plants avoids lethality caused by losing chromosome arms during DNTF. The system does have an important caveat that a telomere seed sequence does not truly recapitulate the DNTF *in vivo*, as chromosome breaks may not occur adjacent to a long telomere sequence. Another drawback of this assay is that transformation efficiency in *A. thaliana* is low. Therefore, in order to track the fate of smaller TRAs, a large number of transformants are required due to low frequency of DNTF.

Recent studies in *A. thaliana* demonstrated a pathway of telomerase inhibition by a novel regulatory long non-coding telomerase RNA, TER2 (Cifuentes-Rojas et al., 2012). Several studies suggested that TER2 plays a role in reproductive development. Notably, TER2 levels peak before fertilization (H. Xu and D. Shippen, unpublished data). A fluorochromatic reaction (FCR test) in *ter2-1* mutant pollens demonstrated that TER2 is required for the viability of male meiocytes (H. Xu and D. Shippen, unpublished data). The biological relevance of altered TER2 levels and thus changes in telomerase activity during *A. thaliana* reproduction is not known. DSBs are generated throughout the genome during meiosis. Recombination events are initiated by formation of these DSBs and are essential for genetic diversity. One fascinating hypothesis is that TER2 inhibits telomerase activity to decrease the probability of DNTF at DSBs during meiosis.

In this study, we examined the function of TER2 in DNTF. We found that 4X *ter-2-1* mutants had ~2.3 fold higher DNTF efficiency compared to 4X wild type plants, consistent with the idea that TER2 is a negative regulator of the DNTF process. Two confounding issues exist with this analysis. First, due to the relatively small sample size (53 for wild type and 50 for *ter2*), the ~2.3-fold difference in DNTF efficiency between wild type and *ter2* is not statistically significant (P value = 0.23, Fisher's exact test). Secondly, recent studies from the Shippen labs showed that the *ter2* mutant allele used in this study is not a true knockout mutation of all the TER2 isoforms, but is deficient in the TER2 full-length. Analysis of a true TER2 null mutant may give a different result. Nevertheless, these data suggest that the full-length TER2 represses DNTF at DSBs and

therefore support the hypothesis that TER2-dependent telomerase inhibition may play a role in preventing DNTF and ensuring genomic stability during meiosis.

CHAPTER V

DDM1 PROTECTS AGAINST TELOMERE RAPID DELETION IN ARABIDOPSIS

Summary

Telomeres stabilize linear chromosomes by protecting the ends from eliciting DNA damage responses. Recent studies reveal that epigenetic pathways, including DNA methylation, are crucial for telomere maintenance. Deficient in DNA Methylation1 (DDM1) encodes a nucleosome remodeling protein, essential for maintaining DNA methylation in *Arabidopsis thaliana*. Although *ddm1* mutants can be propagated, in the sixth generation (F6) hypomethylation leads to rampant transposon activation and infertility. Here we examine the role of DDM1 in telomere length homeostasis. We report that bulk telomere length in *ddm1* mutants remains within the wild type range (2 - 5 kb) until F6, when it precipitously drops so that telomeres now span only 2.1 ± 0.3 kb. Plants lacking DDM1 exhibit no dysregulation of the known telomere-associated transcripts, including TERRA. Although the level of telomerase activity becomes more variable in successive generations of *ddm1* mutants, we found no correlation between enzyme activity and telomere length in F6 *ddm1* mutants. Instead, telomere attrition correlates with a significant increase in extrachromosomal telomeric circles and G-overhang signals, arguing that telomeres devoid of DDM shorten due to deletional recombination. Finally, telomere truncation in F6 *ddm1* coincides with the onset of DNA damage hypersensitivity in the root apical meristem. Since DNA damage is known to stimulate homologous recombination, we hypothesize that telomere deletion in F6 *ddm1*

mutants is a byproduct of elevated recombination in response to genotoxic stress. Further, telomere truncation may be beneficial to plants in adverse environmental conditions by accelerating the elimination of stem cells with profound genome instability.

Introduction

Telomeres are nucleoprotein structures at the end of linear chromosomes. The telomeric DNA is comprised of a tandem array of double-stranded (ds) GC-rich repeat sequences that terminate in a 3' G-rich extrusion, known as the G-overhang. Telomeres serve two primary functions: to prevent chromosome ends from being recognized as double-stranded breaks (DSBs), and to allow complete replication of the chromosome terminus by telomerase-mediated synthesis of telomere repeats. These two functions are achieved by telomere bound protein complexes, shelterin in vertebrates and CST (CTC1/STN1/TEN1) in the flowering plant *Arabidopsis thaliana* and budding yeast (de Lange, 2005; Price et al., 2010). Deletion of core subunits of these complexes leads to telomere deprotection, resulting in telomere length dysregulation and activation of a powerful DNA damage response (DDR) that ultimately triggers end-to-end chromosome fusions and genome instability (Sfeir and de Lange, 2012; Miyake et al., 2009; Leehy et al., 2013; Song et al., 2008; Surovtseva et al., 2009). In addition to proteinaceous protection, the 3' G-overhang can invade the duplex region of the telomeres to form a lariat-like structure, termed the telomeric loop (t-loop) (de Lange, 2004). In mammals, shelterin components, such as TRF2, are implicated in the formation and stabilization of

the t-loop (Griffith et al., 1999; Stansel et al., 2001; Doksani et al., 2013). Telomeres adopt this an alternative conformation to dodge DNA damage surveillance to achieve end-protection. The t-loop structure resembles a Holliday junction intermediate and as such can be resolved by homologous recombination machinery (Lustig, 2003). Resolution of the t-loop leads to extrusion of extra-chromosomal telomeric circles (ECTCs) and concomitant truncation of telomere tract in a process, termed telomere rapid deletion (TRD) (Murnane et al., 1994; Li and Lustig, 1996; Bucholc et al., 2001; Wang et al., 2004). Although the precise mechanism for t-loop resolution is unclear, TRD is postulated to be a sizing mechanism that trims long telomeres back into the normal size range (Li and Lustig 1996; Pickett et al., 2009). TRD must be tightly regulated because the loss of extensive telomeric DNA can trigger a wide-range of genome instability, including activation of DDR, telomere fusions, cell-cycle arrest, and apoptosis and senescence (Lustig et al., 2003; Sandell and Zakian, 1993; van Steensel et al., 1998; Longhese, 2008).

Telomere length is influenced by multiple mechanisms and reaches a species-specific length homeostasis. In addition to the disastrous consequences of critically short telomeres, aberrant telomere elongation also impairs cell growth and has recently been implicated in tumorigenesis (McEachern and Blackburn, 1995; Fairlie and Harrington, 2015; Zhang et al., 2015). Telomerase plays an important role in maintaining telomere length homeostasis. Telomerase contains two core components, the catalytic subunit (TERT) and the template RNA (TER) (Lingner et al., 1997; Cong et al., 2002; Feng et al., 1995; Nakamura et al., 1997). Accessory proteins, such as protection of telomeres 1

(POT1) and Ku, play important roles in the appropriate temporal and spatial regulation of telomerase assembly and activity at telomeres (Hockemeyer and Collins, 2015; Nandakumar and Cech, 2013).

A. thaliana has been a useful model for telomere studies due to high structural and functional similarities of telomere binding proteins to their counterparts in animals and its extraordinary tolerance to telomere dysfunction: mutations that cause lethality in mammals are viable in plants (Shakirov et al., 2005; Song et al., 2008; Surovtseva et al., 2009; Leehy et al., 2013). *A. thaliana* telomeres span 2-5 kb in length (for the Col-0 accession). Notably, recent studies uncovered that *A. thaliana* telomeres are unique due to their asymmetry—one end of the chromosome is blunt-ended and the other contains a G-overhang (Kazda et al., 2012). Ku, a central player in the classic non-homologous end joining (c-NHEJ) pathway, is responsible for the maintenance of blunt-end telomeres, as well as repression of aberrant telomere elongation (Riha et al, 2002; Zellinger et al, 2007). Telomeres with a 3' G-overhang are protected by the CST (CTC1; STN1; TEN1) complex (Kazda et al., 2012; Shakirov et al., 2005; Song et al., 2008; Surovtseva et al., 2009; Leehy et al., 2013). Unlike mammals and yeast, *A. thaliana* encodes several telomerase RNA isoforms (Cifuentes-Rojas et al., 2011). The best studied, TER1 and TER2, associate with different telomerase accessory proteins to form distinct ribonucleoprotein (RNP) complexes. TER1 associates with AtPOT1a to form a canonical telomerase RNP responsible for telomere maintenance, while TER2 associates with AtPOT1b and Ku and functions as a negative regulator of telomerase activity (Cifuentes-Rojas et al., 2011 & 2012). Consequently, studies of *A. thaliana* telomeres

have provided interesting insights to conserved and novel mechanisms for telomere maintenance.

Epigenetic modifications are essential for the growth and development of plants. In addition, studies have shown that plant telomeres are associated with epigenetic modifications. Recent studies showed that *A. thaliana* telomeric DNA is methylated by asymmetrical DNA methylation pathways, directed by the RNA-dependent DNA methylation (RdDM) (Cokus et al., 2008; Vrbsky et al., 2010). In addition, subtelomeric regions in *A. thaliana* are associated with both euchromatic features and heterochromatic features (Vaquero-Sedas et al., 2011; Vaquero-Sedas et al., 2012). *A. thaliana* telomeres are associated nucleosomes with a combination of heterochromatin marks, including $H3K9^{Me2}$ and $H3K27^{Me}$, and euchromatin marks, including $H3K4^{Me2}$ and $H3K9^{Ac}$ (Vaquero-Sedas et al., 2012; Vaquero-Sedas et al., 2011). This feature is unexpected since functional telomeres in mammals and yeast are exclusively heterochromatic (Blasco, 2007; Ottaviani et al., 2008). Studies revealed that DNA methylation and histone methylation are independently involved in telomere maintenance in mammals (Blasco, 2007). Mice lacking DNA methyltransferases (DNMTs) have dramatically elongated telomeres and increased telomere recombination, including increased ALT-associated promyelocytic leukemia bodies (APBs) (Gonzalo et al., 2006). Mouse cells lacking histone methyltransferases (HMTases) Suv39h1 and Suv39h2 also display abnormally long telomeres and loss of heterochromatin proteins at telomeres (Garcia-Cao et al., 2004). Recent studies in plants support the conclusion that DNA methylation is required for telomere length homeostasis in a telomerase-dependent manner (Vaquero-

Sedas and Vega-Palas, 2014; Ogrocka et al., 2014). However, the RdDM pathway that directs DNA methylation at telomeres in *A. thaliana* appears to be dispensable for telomere length regulation (Vrbsky et al., 2010). The mechanism by which epigenetic modifications contribute to telomere maintenance remains unclear.

A master regulator of DNA methylation in *A. thaliana* is Deficient in DNA Methylation 1 (DDM1), a conserved SWI2/SNF2 family chromatin remodeler (Brzeski and Jerzmanowski, 2003). In plants, canonical CG sequences, CHG (H=A, T, C) and asymmetrical CHH sequences can be methylated on the cytosine. Methyltransferase 1 (MET1), a homolog of mammalian DNA methyltransferases Dnmt1, is responsible for maintaining CG methylation in plants (Finnegan et al., 1996). Non-CG methylation is mediated by chromomethylases (CMT) and Domains Rearranged Methyltransferase (DRM) proteins (Stroud et al., 2014). *DRM* and *CMT3* genes play a partially redundant role in non-CG DNA methylation: only in *drm1 drm2 cmt3* triple mutants are the CHG and CHH methylation completely abolished, leading to pleiotropic developmental defects (Cao and Jacobsen, 2002a; Cao and Jacobsen, 2002b; Chan et al., 2006). DDM1 does not methylate DNA sequence directly, but is required for heterochromatin formation in *A. thaliana* by promoting DNA methyltransferases' access to heterochromatin (Kakutani et al., 1996, Zemach et al., 2013). Demethylation of up to 70% of the cytosine in genome occurs in *ddm1* mutants (Vongs et al., 1993; Ronemus et al., 1996; Kakutani et al., 1995; Jeddeloh et al., 1999).

A large portion of plant genome is comprised of transposable elements (TEs), and these elements are especially influenced by DNA methylation (Feschotte et al.,

2002). DNA hypomethylation in *ddm1* mutation background leads to increased mobilization of TEs (Singer et al., 2001; Kato et al., 2004; Miura et al., 2001) and strong transcriptional activation of some transposon families (Lippman et al., 2004, Tsukahara et al., 2009). Because TE insertion disrupts genes, influences expression of nearby genes and mediates chromosome rearrangement, derepression of TEs by DNA hypomethylation in plants can have profound effects (Bennetzen, 2000; Vicient, 2010). For example, DNA methylation is required for various steps during plant development, and in its absence morphological anomalies are observed (Finnegan et al., 1996; He et al., 2011). The downstream effects of genome-wide hypomethylation may not be immediately observable. For the first five generations, inbred *ddm1-2* mutant (F1-F5 *ddm1*) plants resemble the wild type *A. thaliana*. However, in the sixth-generation *ddm1* mutants (F6 *ddm1*) plants exhibit marked developmental pleiotropy, including loss of apical dominance, shorter internode lengths, later flowering, increased cauline leaf number, and reduced fertility (Ronemus et al., 1996; Kakutani et al., 1996).

In this study, we investigate the role of DDM1 at *A. thaliana* telomeres. We report that plants lacking DDM1 successfully maintain telomere length in the wild type range for the first five generations, but in F6 *ddm1* telomeres shorten precipitously. The sudden loss of telomeric DNA does not correlate with changes in telomerase activity, but rather coincides with an elevated level of extra-chromosomal telomeric circles, indicative of TRD. *Ddm1* mutants mount a robust DNA damage response and exhibit programmed cell death in root apical meristems. Together, these findings reveal an

unanticipated link between genomic instability caused by DNA hypomethylation, and telomere instability.

Materials and methods

Plant materials

Ddm1-2 seeds were a gift from Dr. Keith Slotkin (Ohio State University). *Cmt3-7 drm1-2 drm2-2* and *drm1-2 drm2-2 met1-3+/-* mutant seeds were a gift from Dr. Xiuren Zhang (Texas A&M University). Plants were grown in soil under long-day conditions (16h light/8 h dark) at 23^0C. For experiments using seedlings, seeds were sterilized using 50% bleach with 0.1% Triton-X 100 and plated on Murashige and Skoog (MS) medium with 0.7% agar (Caisson Labs). Plants were genotyped as previously described (Kankel et al., 2003; Cao and Jacobsen, 2002a; Lindroth et al., 2001).

TRF, TF-PCR and TRAP

DNA from whole plants was extracted using 2 x CTAB (100mM Tris-HCl, 1.4 M NaCl, and 20mM EDTA). TRF analysis was performed using 50 µg DNA digested with *Tru1I*, resolved on an 0.8% agarose gel and hybridized with [^{32}P] 5' end-labeled $(T_3AG_3)_4$ probe (Fitzgerald et al., 1999). Telomere fusion PCR was performed as described (Heacock et al., 2004). The Telo Tool was used for TRF quantification (Gohring et al., 2014). To measure telomerase activity, total protein was extracted from flowers of individual plants (Fitzgerald et al., 1996). Quantitative-TRAP was carried out

as previously described (Kannan et al., 2008), using a Dynamo HS SYBR Green qPCR kit (Thermo Fisher).

G-overhang analysis and telomeric circle amplification (TCA)

An in-gel hybridization assay was used to monitor G-overhangs (Heacock et al., 2007). Single-stranded G-overhang signals were normalized using the EtBr signal. The G-overhang signal obtained from wild type plants was set to one and mutant samples were normalized to this value. TCA was performed as previously described (Zellinger et al., 2007).

RT-PCR and TERRA detection

Total RNA was extracted from plant tissues using a Direct-zol RNA kit (Zymo Research). Reverse transcription was performed with 1µg total RNA with the qScript cDNA SuperMix (Quanta Biosciences). mRNA levels were assessed by quantitative PCR with primers described previously (Leehy et al, 2013; Cifentes-Rojas et al., 2012), using SsoAdvanced Universal Supermix (Bio-Rad). RNA from at least three individual plants was used for each genotype and at least two technical replicates were run for each reaction. Expression levels were averaged and normalized to GAPDH. Wild type level was set to one and mutant samples were compared to this value. TERRA was monitored by northern blot using 10 µg of total RNA. RNA was resolved on a 1% agarose gel, transferred to nylon membrane and hybridized with a ^{32}P 5'end-labeled $(CCCTAAA)_5$ probe.

Propidium iodide staining and cytogenetics

Sterilized seeds were grown in liquid MS culture for 4-5 days. For zeocin treatment, seedlings were transferred to fresh liquid MS culture either with or without 20 µM zeocin (Invitrogen) and treated for 4 h. After zeocin treatment, seedlings were immersed in 10µg/ml propidium iodide solution at room temperature, in the dark for two min, and then rinsed twice with water. Individual roots were separated and transferred to a slide in a drop of water. Images were obtained using a fluorescence microscope with a Zeiss filter set. ImageJ was used to adjust the brightness and contrast of images.

Results

DDM1 is required for telomere length maintenance in A. thaliana

To investigate how epigenetic modification contributes to telomere regulation in *A. thaliana*, we monitored telomere length in *ddm1-2* mutants, which contains a point mutation that causes alternative splicing resulting in a lose-of-function allele (Jeddeloh et al., 1999). Terminal restriction fragment (TRF) analysis was employed to assess bulk telomere length in the second generation (F2) and the terminal generation (F6) of self-pollinated *ddm1* mutants (Ronemus et al., 1996; Kakutani et al., 1996). As expected (Shakirov and Shippen, 2004), wild type (WT) telomere tracts consisted of a heterogeneous profile of products ranging from 2 to 5 kb (Figure 5-1A. Lane 1). Telomeres of F2 *ddm1* mutants also resembled those of wild type (Figure 5-1A. Lane 2-3). In contrast, telomeres were significantly shorter in F6 *ddm1* mutants ranging from

1.5 to 2.5 kb (Figure 5-1A, Lane 4-5).

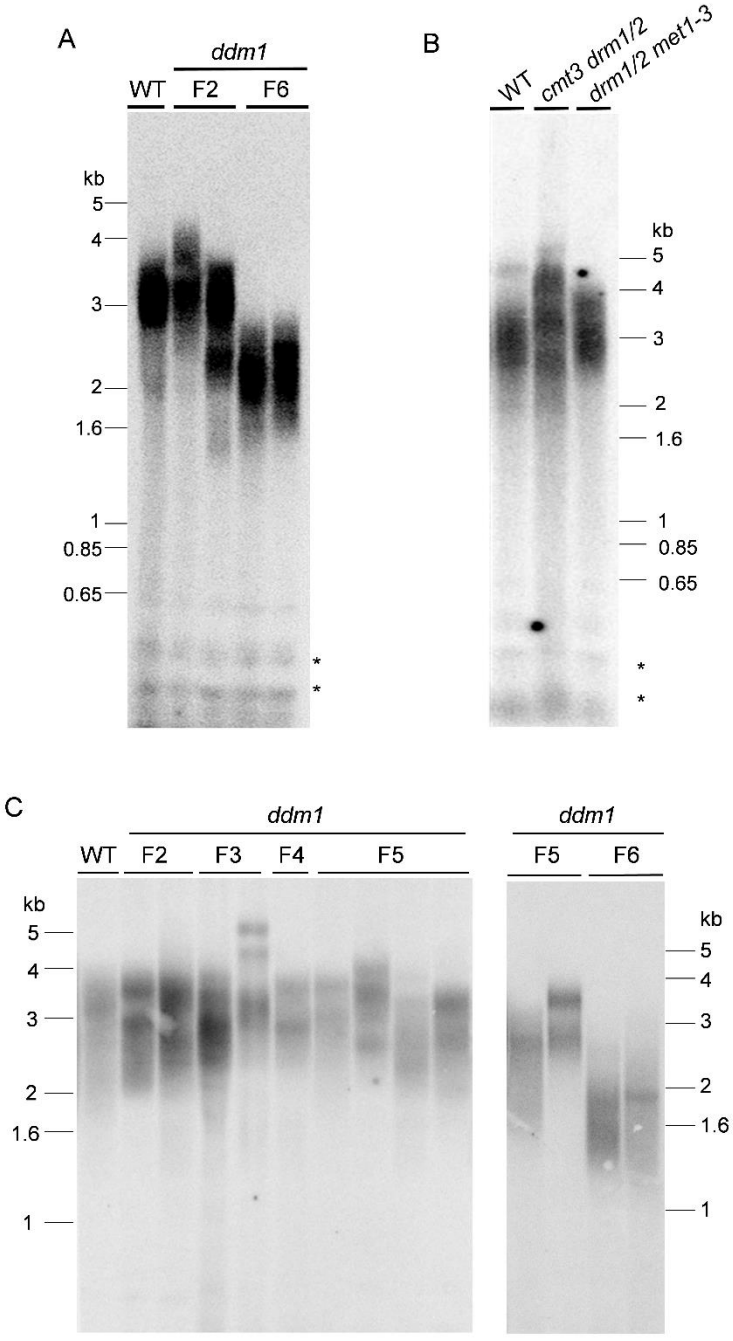
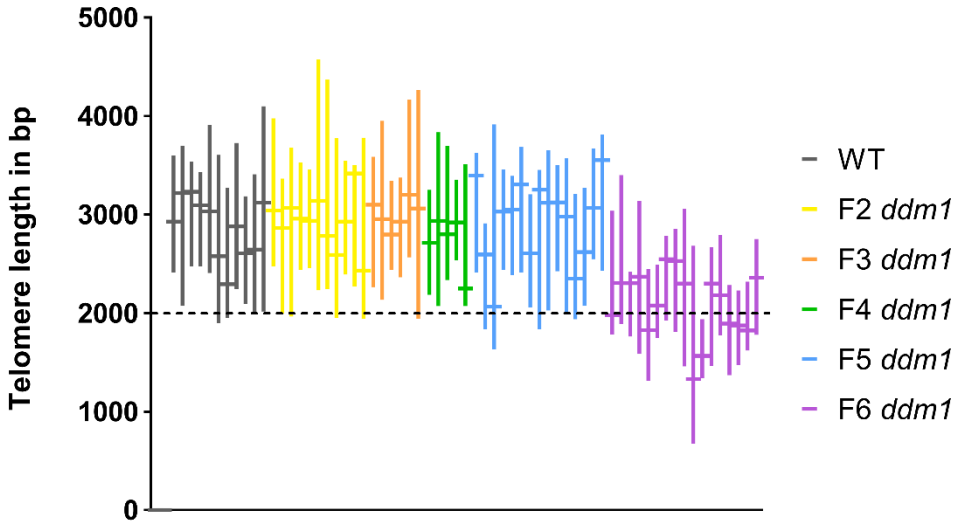


Figure 5-1. Plants lacking DDM1 display precipitous telomere shortening in the sixth generation. (A) Representative data of TRF analysis for bulk telomere length in F2 and F6 *ddm1* mutants. DNA from individual plants were extracted and digested with *Tru1I*. Products were resolved on a 0.8% agarose gel, and Southern blot was performed using telomere G-rich sequence as probe. Asterisks indicate interstitial telomere repeats.

Results from one wild type, two F2 *ddm1*, and two F6 *ddm1* individual are shown. (B) Results of TRF analysis for bulk telomere length in the *cmt3 drm1 drm2* triple mutant and *cmt3 drm1 drm2* triple mutant. (C) TRF analyses of different generations of *ddm1* mutants. Some variation of telomere length is observed among individual plants.

A



B

TRF length	WT	F2 *ddm1*	F3 *ddm1*	F4 *ddm1*	F5 *ddm1*	F6 *ddm1*
Average (bp)	2865	2930	3024	2883	2819	2139
SD (bp)	170	188	179	95	238	335

Figure 5-2. Telomere length quantification of different generation *ddm1* mutants using TeloTool. (A) Graphic representation of the bulk telomere length size range and average length (indicated by a horizontal bar) in *ddm1* mutants. (B) Compiled data for telomere length analysis of each generation. SD: standard deviation.

We examined the kinetics of telomere shortening in the mutants by examining TRF profiles in successive generations of self-pollenated *ddm1* mutants. Telomeres in the F2 - F4 generations were essentially indistinguishable from WT, spanning 2 to 5 kb (Figure 5-1C). In the fifth generation of *ddm1* mutants, the average telomere length (2.8

± 0.2 kb) was similar to wild type (2.9 ± 0.2 kb) (Figure 5-2). However, in F5 *ddm1* the lower boundary of telomere tracts occasionally dipped below the 2 kb wild type range (Figure 5-1C). In F6 *ddm1,* telomere length dropped dramatically (Figure 5-1A and C). Abrupt shortening was associated with the vast majority of individuals tested (Figure 5-2, upper panel). The average length of telomere tracts in F6 *ddm1* mutants was restricted to 2.1 ± 0.3 kb (Figure 5-2), more than two S.D. below the mean size of WT or *ddm1* mutants in prior generations. On average, telomeres shortened by more than 600 bp from F5 to F6. Loss of telomeric DNA was especially remarkable for long telomeres, which were depleted by up to 2 kb in a single generation.

To investigate whether telomere shortening is associated with loss of asymmetrical DNA methylation at telomeres, we examined telomere length in *cmt3 drm1 drm2* triple mutants, in which non-CG methylation is significantly reduced genome-wide, including at telomeres (Cao and Jacobsen, 2002a; Chan et al., 2006; Cokus et al., 2008). The *drm1 drm2 met1* triple mutant, which losses CG methylation and exhibits compromised non-CG methylation was also tested (Zhang and Jacobsen, 2006; Cokus et al., 2008). As expected, morphological abnormality were evident in the second generation of these mutants, including severely compromised reproductive ability, developmental retardation, reduced plant size, and curled leaves. These plants are almost sterile, therefore we only analyzed the second generation of the mutants. However, no telomere shortening was detected (Figure 5-1B), indicating that telomere attrition is not a general response to genome wide perturbation in DNA methylation.

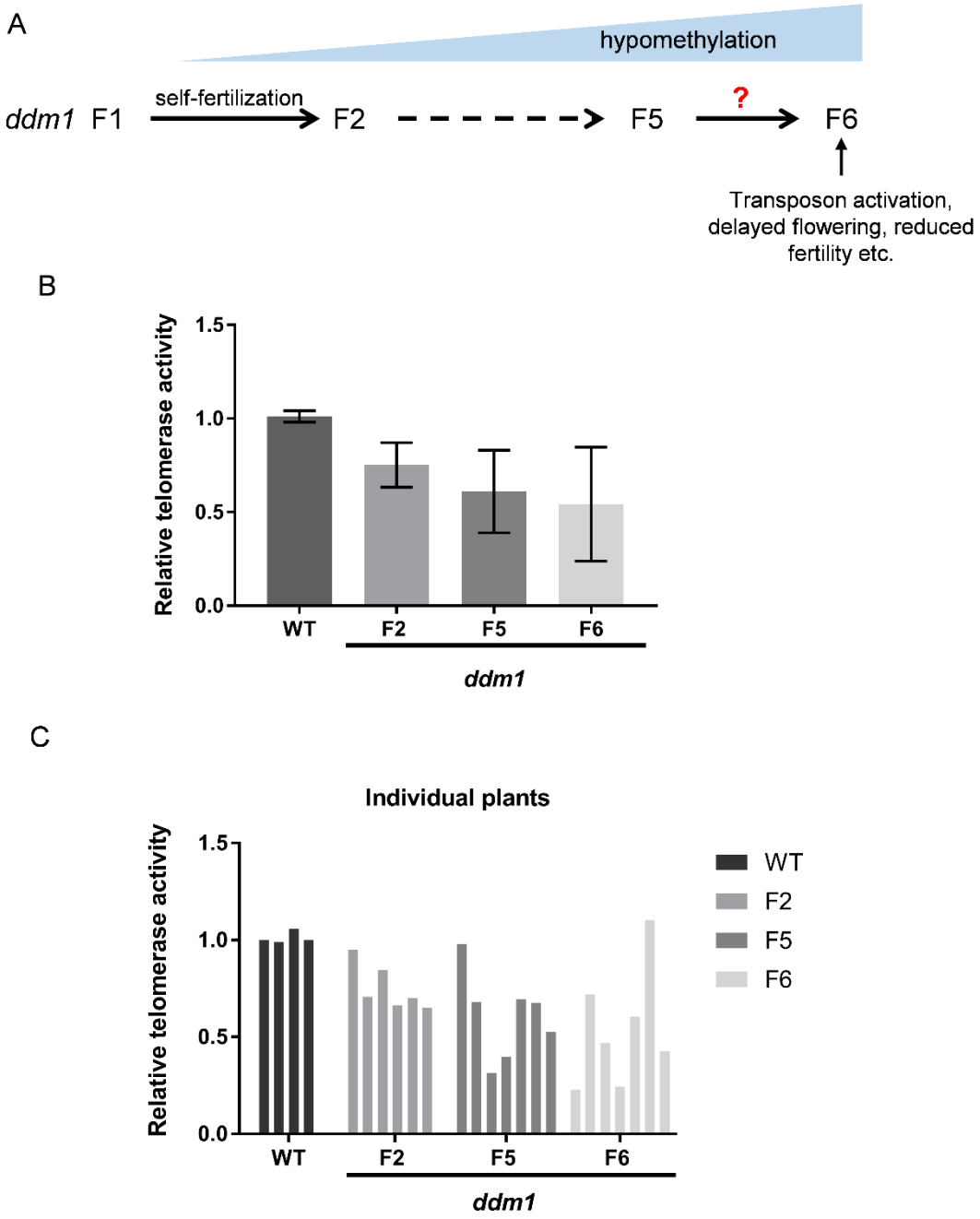


Figure 5-3. Telomerase activity in *ddm1* mutants. (A) Diagram of increased hypomethylation in self-pollinated *ddm1* mutants and the onset of severe morphological phenotypes in sixth generation mutants. (B) Quantitative TRAP results showing relative telomerase activity from flowers of *ddm1* mutants. Averaged wild type level of telomerase activity was set as 1. Each data point represents three to five biological

replicates (individual plants of the same genotype and generation), with two technical replicates. The standard deviated between biological replicates is represented by error bars. (C) Relative telomerase activity in each individual wild type and *ddm1* mutant plants.

Telomere shortening in ddm1 mutants does not correlate with the absence of telomerase activity or changes in telomere-related transcripts

In plants lacking TERT, the catalytic subunit of telomerase, the first appearance of morphological abnormalities occurs in the fifth or sixth generation (Riha et al., 2001), when telomeres shorten below the critical length threshold of 1kb (Heacock et al., 2004). Later generation *tert* mutants display asymmetric leaf growth, decreased germination efficiency, and vegetative growth arrest, phenotypes attributed to stem cell deficiency from dysfunctional telomeres (Riha et al., 2001). Because the pleiotropic developmental defects of F6 *ddm1* mutants mimic late generation *tert* mutants (Figure 5-3A), we asked whether telomerase deficiency is associated with telomere shortening in F6 *ddm1* mutants. Quantitative telomeric repeat amplification protocol (qTRAP) was used to monitor telomerase activity in flowers. In F2 *ddm1*, telomerase activity was decreased by approximately 25% relative to WT (Figure 5-3B). Telomerase activity declined further in F5 and F6 mutants, with plants exhibiting only 61% and 54% of the wild type activity level, respectively. Variability in the amount of telomerase activity in individual F5 and F6 plants increased relative to earlier generation mutants or wild type plants (Figure 5-3C). Notably, however, we found no correlation between the level of telomerase activity in F6 plants and telomere length in individual plants.

We next asked if the abundance of telomere-related transcripts was altered in

ddm1 mutants using quantitative RT-PCR (qRT-PCR). We measured steady-state transcript levels of CST components, CTC1 (Surovtseva et al., 2009), STN1 (Song et al., 2008) and TEN1 (Leehy et al., 2013) as well as Ku70 (Riha et al., 2002; Riha et al., 2003), POT1a, a telomerase processivity factor (Surovtseva et al 2007; Renfrew et al., 2014), the canonical telomerase RNA subunit TER1 (Cifuentes-Rojas et al., 2011) and the telomerase regulatory lncRNA TER2 (Cifuentes-Rojas et al., 2012). We found no significant differences (P >0.05) in the level of the transcripts in F2, F5 or F6 *ddm1* mutants (Table 5-1). Microarray data using inflorescence from wild type and F6 *ddm1* mutants is consistent with our observation by qRT-PCR (K. Slotkin, personal communication).

Name	Relative expression level					
	F2 *ddm1*		F5 *ddm1*		F6 *ddm1*	
	Average	SD	Average	SD	Average	SD
CTC1	1.69	±1.02	1.87	±0.15	0.89	±0.15
STN1	1.92	±0.50	1.66	±0.09	1.38	±0.34
TEN1	1.03	±0.57	1.12	±0.51	1.03	±0.32
RAD51	1.72	±1.03	2.16	±1.15	1.54	±0.72
POT1a	1.84	±0.07	2.14	±0.03	1.63	±0.27
TER1	1.94	±0.77	1.89	±0.86	1.11	±0.20
TER2	2.32	±1.55	1.85	±0.42	0.94	±0.36
Ku70	1.53	±0.45	1.57	±0.45	1.33	±0.24

Table 5-1. Steady state transcript levels of telomere and telomerase-related genes in *ddm1* mutants. Averaged wild type levels of mRNA are set as 1, and all transcript levels in *ddm1* mutants were converted to these values. Data presented are mean ± standard deviation (n >=3). No significant difference (P>0.05, student t-test) was observed between wild-type samples and individual generation of *ddm1* mutants among all transcript tested.

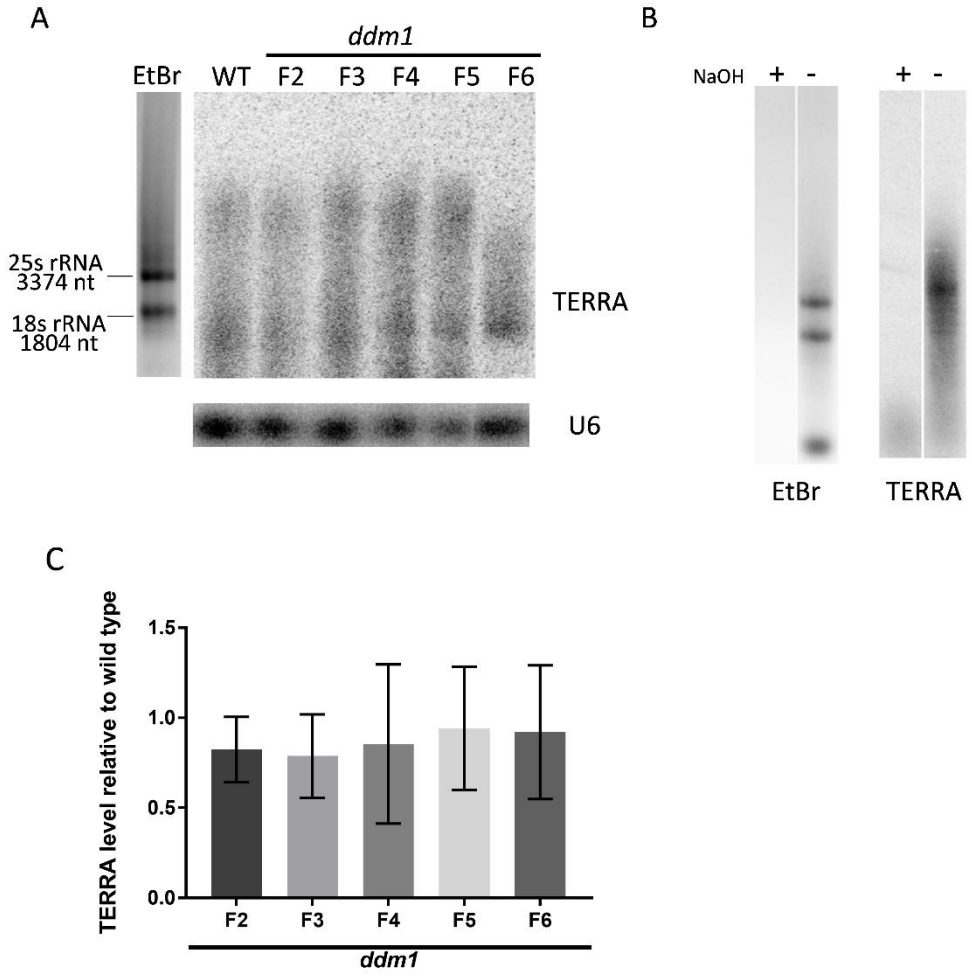


Figure 5-4. The level of telomere transcript TERRA is not changed significantly in *ddm1* mutants. (A) Northern blot for TERRA detection was carried out using total RNA from two-week-old seedlings. EtBr gel image indicates the molecular weight of 25s and 18s rRNA of the corresponding gel. U6 transcript serves as loading control. (B) NaOH treatment for the total RNA indicates no DNA contamination. (C) Quantification of TERRA signal of the F2 F5 and F6 *ddm1* mutants. Signals are normalized to wild type and three biological replicates are used. Error bars represent the standard deviation.

Subtelomeric DNA is methylated in *A. thaliana* (Vrbsky et al., 2010), and a combination of euchromatic and heterochromatic histone modification has been identified (Vaquero-Sedas et al., 2011&2012). Genome-wide DNA hypomethylation in *ddm1* mutants may affect epigenetic modifications at subtelomeric regions and

consequentially influence the production of TERRA (Arnoult et al., 2012). TERRA is a population of long non-coding RNAs transcribed from subtelomeres and telomeres that is implicated in telomere length regulation (Pfeiffer and Lingner, 2012; Arora et al., 2014). Northern blotting was used to determine if TERRA is altered in *ddm1* mutants. TERRA was detected as a heterogeneous smear sensitive to NaOH treatment (Figure 5-4A and B) (Vrbsky et al, 2010). The size distribution of TERRA transcripts was similar in the wild type and in *ddm1* mutants except in F6 *ddm1*, where higher molecular weight transcripts significantly were reduced. This change in the TERRA profile correlates with the shorter telomere repeat arrays in F6 *ddm1* mutants. The overall TERRA hybridization signal was similar between *ddm1* mutants and the wild type (Figure 5-4C). We conclude that telomere shortening in F6 *ddm1* mutants is not associated with a substantial change in telomere-related RNA transcripts.

DDM1 is required for repression of telomere recombination

Telomere repeat arrays are heterogeneous in wild type *A. thaliana* (Fitzgerald et al., 1996; Riha et al., 2001). However, the telomere profile of *ddm1* mutants occasionally displayed sharp TRF bands, representing discrete population of telomeres, which are not typically observed in wild type samples (Figure 5-1C). The unusual telomere profile of *ddm1* mutants has also been observed in *stn1* and *ten1* mutants (Figure 5-1C) (Song et al., 2008; Leehy et al., 2013). Such bands are associated with telomere rearrangements and specifically telomere recombination. To explore this possibility further, we first looked for evidence of end-to-end chromosome fusions in *ddm1* mutants using telomere

fusion PCR (TF-PCR) (Figure 5-5). In this assay, subtelomere-specific primers are used to amplify covalently linked telomere fusions, and the PCR products are detected on a Southern blot using a telomere probe (Heacock et al., 2004). As expected, a robust TF-PCR signal was detected with DNA from the positive control derived from a *stn1* mutant (Song et al. 2008), while no fusion products were obtained with wild type (Figure 5-5). Similarly, we failed to detect TF-PCR products in *ddm1* mutants using several subtelomere primer combinations (Figure 5-5). To confirm these results, cytology of mitotic chromosomes spreads were used to gauge the occurrence of anaphase bridges in *ddm1* mutants. The dicentric chromosomes formed from telomere fusions do not segregate properly during mitosis, can be observed during anaphase as chromatin bridges. Analysis of over 50 anaphases in F5 and F6 *ddm1* mutants revealed no bridged chromosomes. The absence of telomere fusion in *ddm1* mutants was not unanticipated since even in F6, the average length of telomeres in *ddm1* mutants does not fall below the critical end-protection threshold of 1kb (Heacock et al., 2004).

Aside from telomere fusion, dysfunctional telomeres can also trigger TRD. A hallmark of TRD is the formation of extra-chromosomal telomeric circles (ECTCs), which are extruded during t-loop resolution (Wang et al., 2004; Li et al., 2008). ECTCs can be detected by a Southern blot based t-circle amplification (TCA) assay. High molecular weight ssDNA produced by rolling circle amplification of ECTCs can be detected on alkaline agarose gel electrophoresis using a telomeric probe (Zellinger et al. 2007). As a positive control, we assayed T-circle formation in *stn1* heterozygous and homozygous mutants. T-circle formation was elevated by approximately two-fold in

stn1-/- mutants compared to stn1+/- and wild type plants (Figure 5-6A) (Song et al., 2008). F5 and F6 *ddm1* plants also exhibited increased ECTC production, but this was not observed in F2 mutants.

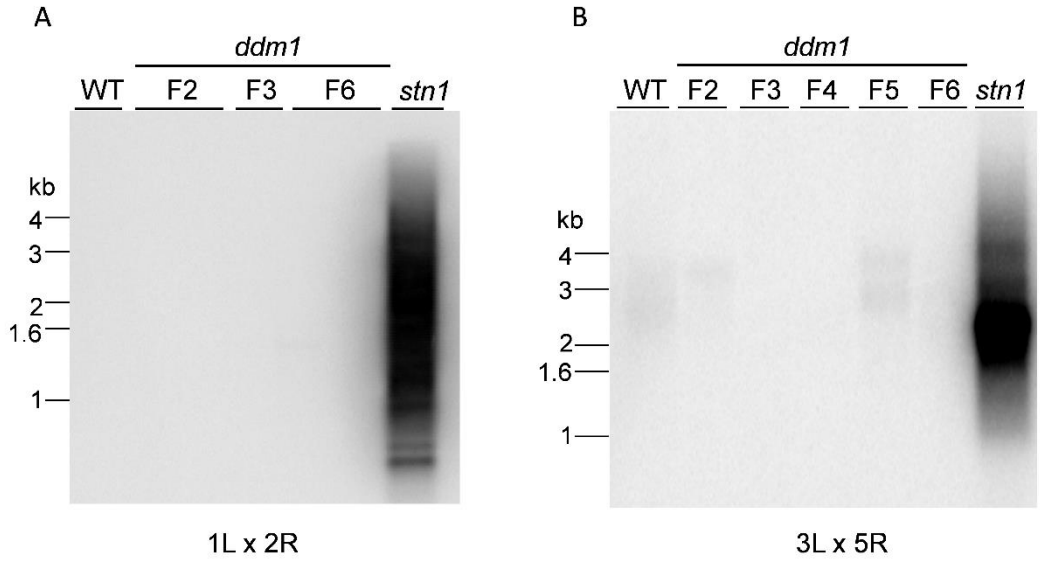


Figure 5-5. No chromosome end-to-end fusion in *ddm1* mutants. Representative data for telomere fusion PCR with *ddm1* mutants using subtelomeric primer 1L and 2R (A) and 3L and 5R (B). *Stn1* mutant serves as a positive control. PCR products were resolved in a 0.8% Agarose gel and were probed with a [^{32}P] 5' end labeled $(T_3AG_3)_4$ oligonucleotide probe. Representative results of at least three independent experiments are shown.

Elevated telomere recombination is typically coincident with increased single-stranded DNA at the chromosome terminus. Therefore, we monitored the status of the G-overhang in *ddm1* mutants using the in-gel hybridization method. Compared with WT, *ddm1* mutants displayed an increased G-overhang signal. The G-overhang signal was not significantly increased in F2 (1.4 ± 0.6), however it was substantially higher in F5 (3.4 ± 1.7) and more so in F6 (4.4 ± 1.5) (Figure 5-6B; $P<0.05$). Taken together, the elevated levels of ECTC and G-overhangs are consistent with increased TRD in F6

plants lacking DDM1.

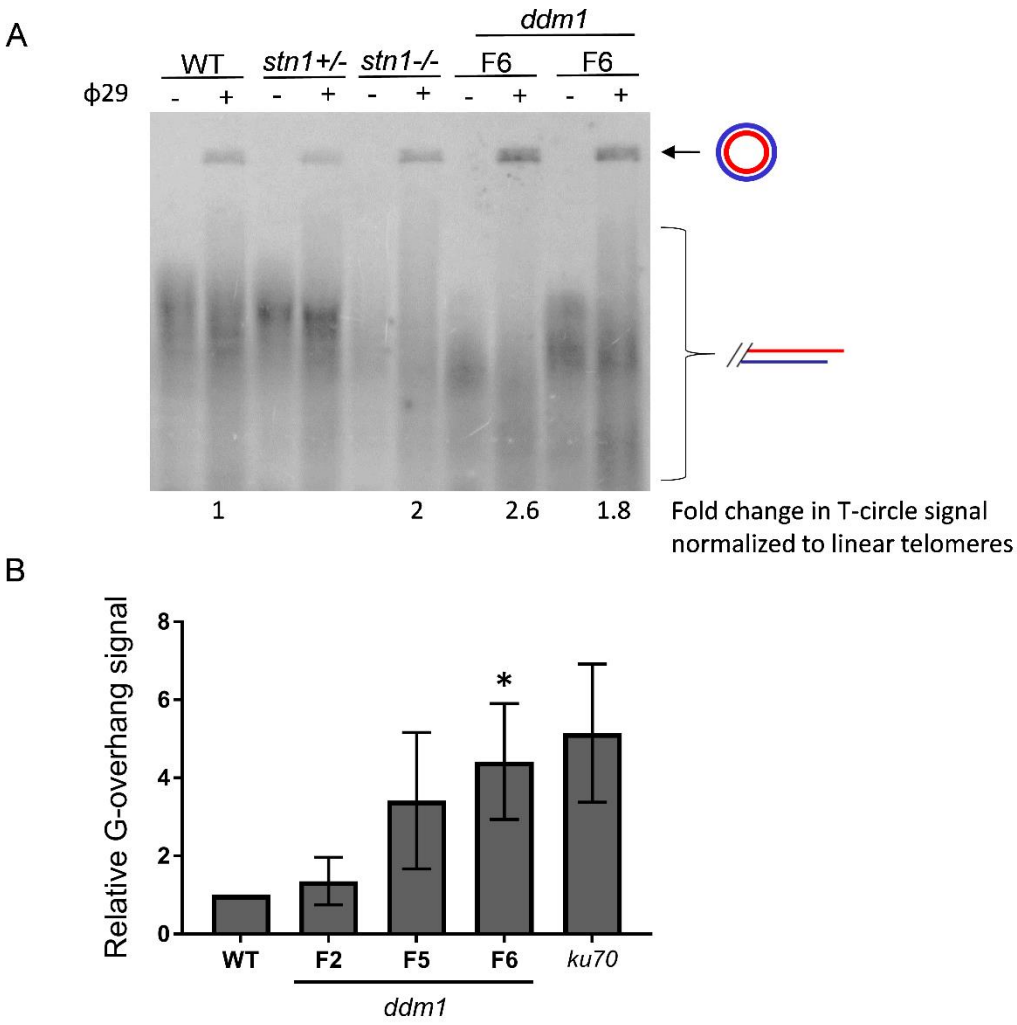
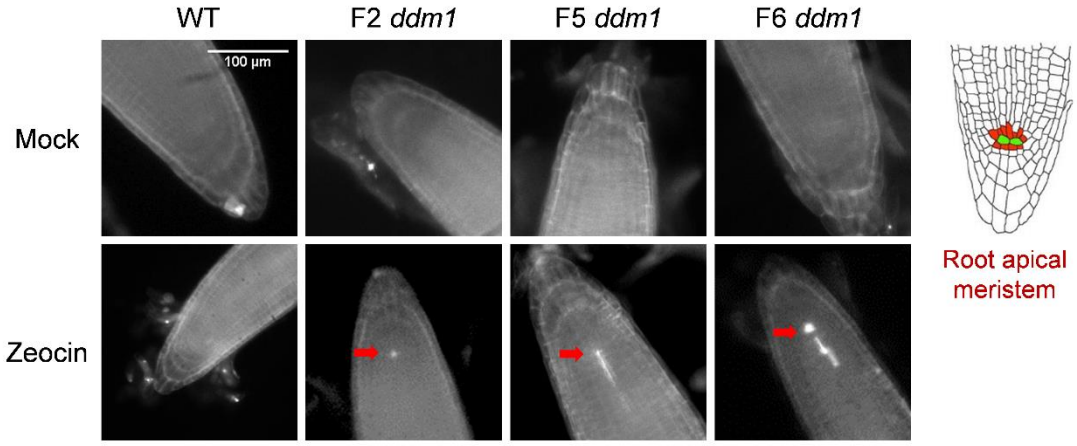


Figure 5-6. TCA analysis and in-gel hybridization analysis for G-overhangs in different generation of *ddm1* mutants. (A) TCA was carried out with wild type, *stn1* heterozygous (+/-) and homozygous (-/-) mutant and F6 *ddm1* mutant DNA in the presence (+) or absence (-) of phi 29 polymerase to amplify ECTCs. Circular and linear telomere repeats are indicated. The ECTCs signal is normalized to WT and is indicated under the corresponding lane. (B) Quantification of the G-overhang signal for G-overhang. DNA isolated from WT, *ddm1* mutants and *ku70* mutant. *Ku70* mutant serves as a control for increased G-overhang signal. Data are represented as mean ± SD. Asterisk showed a statistically significant difference (p-value <0.05, student's *t*-test) between wild type and F6 *ddm1*.

A



B

Genotype	Mock			Zeocin		
	Total	PI-positive	Ratio	Total	PI-positive	Ratio
WT	47	2	4.26	82	3	3.66
F2 ddm1	37	0	0.00	92	8	8.70
F5 ddm1	51	1	1.96	78	7	8.97
F6 ddm1	46	2	4.35	84	19	22.62

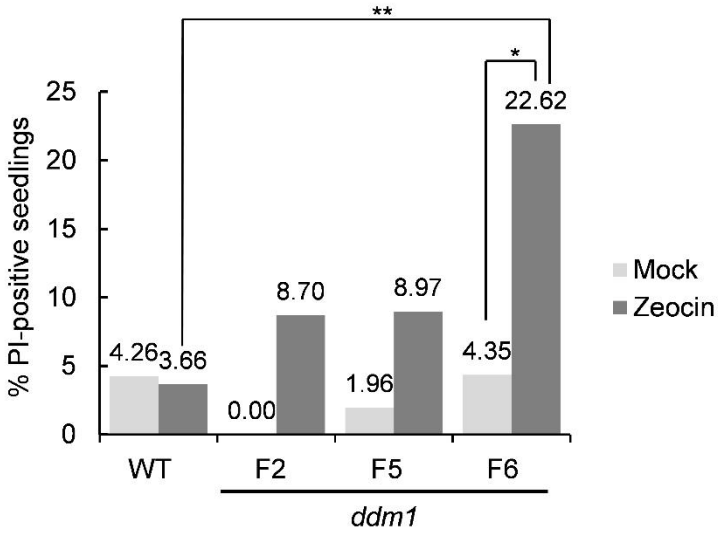


Figure 5-7. The F6 *ddm1* mutant plants are hypersensitive to DNA damage. (A) Representative images of root tips of 5-day-old wild type and *ddm1* mutant seedlings stained with propidium iodide (PI) for programmed cell death in the absence (a, b, c and d) or in the presence (e,f,g and h) of Zeocin. Scale bar is indicated in (a). Red indicates the root apical meristem cells and green indicates the quiescent center. (B)

Quantification of percentage PI-positive RAM in WT and *ddm1* mutants. Graphic demonstration (lower panel) of percentage PI-positive RAM is shown in the lower panel. Asterisk denotes p-value <0.05 (Fisher exact test). Double asterisks denote a p-value < 0.005 (Fisher exact test).

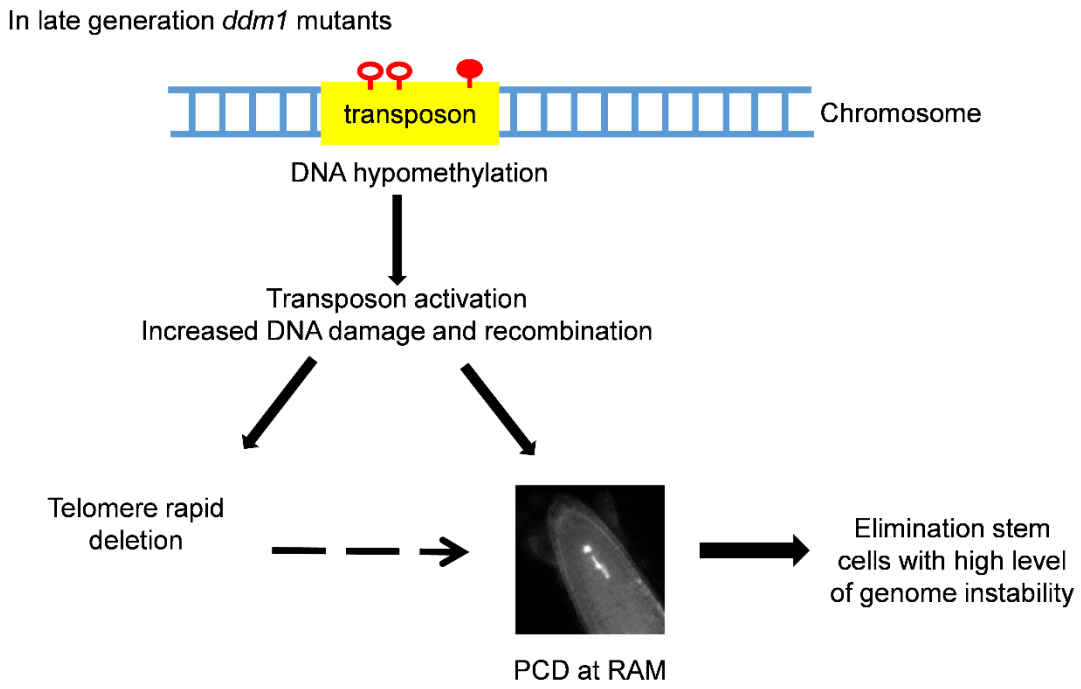


Figure 5-8. Model demonstrating the interplay between genomic recombination and telomere maintenance and stem cell PCD. Genome-wide DNA hypomethylation in late generation *ddm1* mutants causes elevated transposon activity and recombination. TRD is used as a "cleansing" mechanism in response to genome collapse by accelerating PCD in damaged stem cell niches. Closed and open lollipops, respectively, indicate demethylated cytosine and methylated cytosine.

Root apical meristems in the F6 ddm1 mutant are more sensitive to DSBs.

Cells within the stem cell niche are hypersensitive to DNA damage (Fulcher and Sablowski, 2009). DDM1, DNA methyltransferases, and RdDM factors are all upregulated in shoot apical meristems to reinforce TE silencing and stable epigenetic inheritance (Baubec T et al., 2014). Plants deficient in DDM1 exhibit a higher basal

level of DSBs and accumulate more DNA damage under genotoxic stress than wild type (Yao et al, 2012; Questa et al., 2013). Hence, it is possible that the abrupt telomere shortening we observe in F6 *ddm1* mutants is part of a global response to genome-wide chromosome instability.

We investigated the role of DDM1 in promoting stem cell viability by monitoring the frequency of programmed cell death (PCD) in the root apical meristem (RAM) of 5-day-old *ddm1* mutant seedlings using propidium iodide (PI) staining (Fulcher and Sablowski, 2009; Figure 5-7A). In the absence of genotoxic stress, 2/47 (4.26%) wild type seedlings displayed positive PI staining. Similarly, few if any of the RAM from F2 (0/37, 0%), F5 (1/51, 1.96%) and F6 (2/46, 4.35%) *ddm1* mutants were PI-positive (Figure 5-7B), indicating that the intrinsic genome instability associated with the loss of DDM1 does not trigger PCD in the RAM.

To further dissect the DNA damage response in plants lacking DDM1, we treated seedlings with zeocin, a radiomimetic drug that induces DSBs (Fulcher and Sablowski, 2009). We reasoned that exogenous DNA damage may exacerbate the basal level of genome instability of *ddm1* mutants when TEs are activated. WT individuals showed no substantial difference in PI staining in the absence (2 PI-positive roots/47 total roots, 4.26%) or in the presence of 20 μM zeocin (3/82, 3.66%) presumably due to the active DNA damage repair pathways and low dose of the drug (Fulcher and Sablowski, 2009). However, the percentage PI-positive seedlings increased modestly in F2 (8/92, 8.70%) and F5 (7/78, 8.97%) *ddm1* mutants, consistent with a hypersensitive DNA damage response. Strikingly a large fraction of the F6 *ddm1* seedlings contained PI-positive

RAMs (19/84, 22.6%; P<0.05, Fisher exact test) following zeocin treatment (Figure 5-7B), suggesting stem cells in F6 *ddm1* mutants are hypersensitive to DNA damage. We conclude that F6 *ddm1* root apical meristems have a considerably lower tolerance to genotoxic stress than in the earlier generations and WT. These findings are consistent with genome wide chromosome instability in F6 *ddm1* mutants, and imply a mechanistic link between this elevated response and abrupt telomere shortening.

Discussion

Although aberrant telomere elongation and recombination are associated with mammalian cells bearing mutations in DNA methyltransferase and histone H3K9 methyltransferases (Garcia-Cao et al., 2004, Gonzalo et al., 2006), there is scant evidence directly linking epigenetic modifications to changes in telomere maintenance and end protection. In this study we investigated how the loss of a master regulator for heterochromatin formation, DDM1, impacts telomere structure and maintenance in Arabidopsis.

Abrupt telomere shortening in the sixth generation ddm1

We showed that telomeres are stable during the first five generations of DDM1 mutants, but they abruptly and dramatically shorten in the sixth generation. Telomere shortening in F6 *ddm1* corresponds to a length of 800 bp on average relative to WT. The average telomere length in F6 *ddm1* falls in the minimum (~2 kb) of the wild type size range (Shakirov and Shippen, 2004), but the shortest telomeres do not reach the critical 1

kb size threshold that triggers telomere fusion (Heacock et al., 2007). As expected, no end-to-end chromosome fusions were observed in the F6 *ddm1* mutants. Thus, the phenotype of F6 *ddm1* telomeres is distinct from the uncapping phenotype of the CST mutants (Shakirov et al., 2005; Song et al., 2008; Surovtseva et al., 2009; Leehy et al., 2013), indicating that no massive nucleolytic attack accrued in the F6 *ddm1*.

Previous studies which compared telomere length and telomere DNA methylation in different generations of *met1*+/- and *ddm1-8* mutant plants (bearing a T-DNA insertion at the C-terminus of DDM1), concluded that telomere shortening in DNA hypomethylaiton mutants were associated with the loss of DNA methylation at telomeres (Ogrocka et al., 2013).These authors observed that a subset of either *met1* heterozygote mutants or *ddm1-8* mutants had telomere length below 2 kb, as early as the second and third generation (F2 and F3). In F4, however, telomere shortening of *ddm1-8* was not as distinct. The discrepancy in that finding and ours may be due to the difference between the two mutant alleles (*ddm1-8* vs *ddm1-2*). Ogrocka et al. correlated a reduced DNA methylation at telomere sequences in these mutants with telomere shortening. However, a recent study failed to detect substantial DNA methylation at *A. thaliana* telomeres (Vega-Vaquero et al., 2016).

Our results are also inconsistent with a direct role for DNA methylation in telomere length control. The function of DDM1, a SWI2/SNF2 family chromatin remodeler, is to promote genome-wide DNA methylation (Vongs et al., 1993; Jeddeloh et al., 1999). Profound loss of DNA methylation at TEs and other repetitive elements occurs in the first generation *ddm1-2* mutants. DDM1 contributes to all three classes of

DNA methylation by providing the accessibility for DNA methyltransferases to H1-containing heterochromatin for TE repression (Zemach et al., 2013). The loss of DNA methylation directs redistribution of H3mK9 and H3mK4 at heterochromatin regions, and therefore results in a loss of the heterochromatin state and strong transcriptional activation of TEs (Lippman et al., 2004). One possibility is that TE repression by DNA methylation is required for telomere maintenance.

CG-methylation is almost entirely abolished in the *ddm1* and *met1* mutants (Cokus et al., 2008). DRM1, DRM2, and CMT3 are together responsible for non-CG methylation (Cao et al., 2003). The *cmt3 drm1 drm2 met1* quadruple mutant is embryonic lethal (Chan et al., 2006).

Therefore, we examined the role of DNA hypomethylation on telomere length by assessing *drm1 drm2 met1* triple mutant in which CG-methylation is absent and non-CG methylation is compromised and *drm1 drm2 cmt3* triple mutants, in which methylation at CHH and CNG sequences are deficient, including telomere repeats (Cao et al., 2003). Our results showed that telomere length in both mutants was similar to WT. These findings correlated with our data for the first five generations of *ddm1* mutants, in which the methylation of CG, CNG and CHH sequences are all significantly compromised (Zemach et al., 2013). Thus, we conclude that genome-wide DNA hypomethylation is not sufficient to cause telomere length dysregulation.

Altogether, our findings indicate that neither genome-wide nor telomere DNA hypomethylation directly lead to telomere length dysregulation.

Deletional recombination: a possible mechanism for telomere truncation in late generation DDM1 mutants

We considered several explanations for abrupt telomere shortening in F6 *ddm1* mutants. Loss of telomerase is one. We found that telomerase activity decreased steadily in *ddm1* mutants and by F6, was 54% of the WT activity level with higher individual variability. Although, haploinsufficiency of TERT, the telomerase catalytic subunit, leads to telomere attrition in mammals (Hauguel and Bunz, 2003; Zhang et al., 2003; Armanios et al., 2005; Liu et al., 2000; Du et al., 2007), this is not the true for *A. thaliana* (Fitzgerald et al., 1999). Telomeres of heterozygous *tert* mutants span 2 to 4 kb as WT. Complete loss of TERT leads to a loss of telomere sequence of 500 bp per generation and a distinct TRF profile with several sharp bands. Aside from the steady decrease in telomerase activity, telomere truncation observed in *ddm1* mutants is distinct from the telomere phenotype caused by inactivation of telomerase, indicating that reduced telomerase activity in the late generation *ddm1* mutants does not account for the abrupt telomere shortening.

DDM1 plays a minor role in gene methylation (Lippman et al., 2004), but its profound impact on chromatin may influence gene expression. The steady-state transcript levels of telomerase components (including TER1, TER2 and POT1a) and TERRA do not correlate with the precipitous loss of telomeres in the F6 *ddm1* mutant. We also did not observe a significant difference among different generations of the *ddm1* mutants in the steady-state transcript levels of known telomere capping proteins. We cannot rule out the possibility that a change in modifications of telomere capping protein

and telomerase components at the protein level or other unknown mode affects telomerase association with or activity on telomeres in F6 *ddm1* mutants.

The precipitous nature of the telomeric DNA loss is consistent with genome-wide TRD in the F6 *ddm1* plants (Vespa et al., 2007; Watson et al., 2005). Yeast uses TRD in order to trim oversized telomeres down to wild-type length (Li and Lustig, 1996). Similar phenomena were observed in human and yeast, with an increase in ECTCs generated by t-loop resection (Wang et al., 2004; Iyer et al., 2005; Lustig, 2003). We hypothesize that the abrupt telomere shortening observed in F6 *ddm1* is via TRD and tested this hypothesis by monitoring the ECTCs in *ddm1* mutants. Only the late generations (F5 and F6) of the *ddm1* mutant exhibited an increased G-overhang signal and extra-chromosomal telomere circles, but earlier generations of *ddm1* mutants did not. Thus, changes terminal DNA architecture and the production of ECTC indicate that telomeres are significantly modified in F6 *ddm1* mutant, consistent with TRD.

Increased genome instability in F6 ddm1

Although early generation *ddm1* mutants are viable and fertile (Kakutani et al., 1996), in F6 *ddm1-2* mutants display gross morphological and developmental abnormalities, including decreased plant status, twisted leaves, loss of apical dominance, and significantly reduces in fertility (Ronemus et al., 1996; Kakutani et al., 1996). These phenotypes are attributed to rampant activation of TEs (Richards, 1997). Stem cell death is likely to underlay the partial or complete sterility, loss of apical dominance, and perhaps other defects in F6 *ddm1* mutants. Strikingly, the onset of these abnormalities

coincides with abrupt telomere shortening, and thus a unifying hypothesis to account for stem cell dysfunction and telomere truncation is an accumulation of massive genome instability in F6 *ddm1*.

Stem cell niches are crucial for maintaining genome integrity. For example, RAMs are hypersensitive to DNA damage, and thus undergo PCD after DNA damage to avoid the risk of accumulating mutations (Fulcher and Sablowski, 2009). We monitored the frequency of PCD in RAMs of *ddm1* mutants as a readout for genome stability. Under normal conditions, there was no significant difference ($P>0.05$) in the frequency of PCD in RAMs between WT and *ddm1* mutants, even in F6. However, when DSBs were induced by zeocin, RAMs of F6 *ddm1* mutants were much more susceptible to DNA damage and underwent PCD at a significantly higher level than WT (22.62% and 3.66% respectively, $P<0.05$). This observation implies that the genome of F6 *ddm1* mutants are intrinsically unstable, and that additional DNA damage triggers PCD in RAMs.

A working model

These studies reveal a remarkable interaction between global genome instability, telomere truncation, and stem cell failure. The onset of stem cell defects due to increased TE activation, precipitous telomere shortening and elevated telomere recombination, and increased sensitivity to DNA damage of RAMs all coincide in F6 *ddm1* mutants. Loss of DNA hypomethylation and the TE activation it induces pose a serious threat to genome stability in self-pollinated *ddm1* mutants; one consequence is to elevate homologous

recombination across the genome (Bennetzen, 2000; Vicient, 2010) (Figure 5-8). While a high rate of HR can benefit plants by enhancing their capacity to adapt to adverse conditions (Molinier et al., 2006; Boyko et al., 2010; Kovalchuk et al., 2003), elevated HR may also stimulate TRD. Previous studies in *ten1* and *ctc1* mutant seedling demonstrated the onset of TRD after heat shock (Lee et al., 2016), implicating TRD in the stress response. A shortening in response to environmental stresses may undermine telomere homeostasis; precipitous decrease in telomere length jeopardizes telomere stability and as a consequence leads to loss of genome integrity (Lendvay et al., 1996; Hemann et al., 2001). Plants with critically short telomeres suffer a robust DDR, end-to-end chromosome fusions, and programmed stem cell death (Riha et al., 2001; Amiard et al., 2011; Boltz et al., 2012). We hypothesize that TRD is used as a "cleansing" mechanism in response to genome collapse by accelerating PCD in damaged stem cell niches. This seemingly deleterious outcome of telomere truncation may be beneficial overall for the integrity of stem cell pool by culling inviable stem cells.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Eukaryotic linear chromosomes use a conserved telomere structure for solving the end-replication problem and the end-protection problem. In most species, telomere replication is dependent on the telomerase reverse transcriptase. Although the replication machinery is conserved, distinct accessory proteins and mechanisms have evolved for accurate temporal and spatial control of telomerase action. Proper telomerase control guarantees telomerase recruitment and activation at shorter telomeres. On the other hand, telomerase activity must be down-regulated in other circumstances to avoid chromosome healing at double-strand breaks (DSBs) or to prevent cells from bypassing replicative senescence. Despite decades of research, understanding of the complex machinery of telomerase regulation is only the tip of the iceberg.

In addition to telomere maintenance by telomerase, telomeric DNA is protected by telomere capping proteins and by the formation of secondary structure from the surveillance of DDR and nucleolytic attack. The end-capping mechanisms for chromosomes that bear 3’ G-overhangs have been characterized in detail; the key players have been identified, and their major contributions to telomere end-protection are illustrated for many species. However, relatively little is known about the mechanism of chromosome end-protection in plants where half of the telomeres consist of blunt ends.

Although telomeres are made of tandem GC-rich repeats, associated with specialized proteins, they have a lot in common with the rest of the chromosomes. For example, telomeric DNA in higher eukaryotes is associated with nucleosomes and different histone variants (Makarov et al., 1993). Epigenetic marks found at telomeres contribute to telomere maintenance (Blasco, 2007). Unprotected telomeres resembles DSBs and attract players in DNA damage responses and DNA repair pathways. These observations raise a series of questions starting with: are telomere binding proteins specialized for telomere functions only? Do telomere components function outside telomeres for other cellular processes? Can telomeres respond to genome instability?

In this dissertation, the characterization of Arabidopsis POT1b revealed several unexpected features of this telomere-associated protein and shed light on some important unanswered questions. Additionally, the study of a chromosome remodeler in *A. thaliana*, DDM1, presented a preliminary model for the response of telomeres to genome instability.

POT1b has multiple roles in telomere biology

In most species, POT1 has been characterized as a conserved telomere component that binds to the telomere 3' G-overhang and coordinates telomere end-protection and end-replication (Lei et al., 2003; Baumann and Price, 2010; Colgin et al., 2003; Kelleher et al., 2005). Duplication of the *POT1* gene in many species gave rise to multiple *POT1* orthologs that serve divergent functions at telomeres and even outside telomeres. *Arabidopsis thaliana* harbors three POT1 paralogs: AtPOT1a, AtPOT1b, and

AtPOT1c. AtPOT1a retains several canonical functions of POT1 protein, such as promoting telomerase activity and interaction with the CST (Renfrew et al., 2014; Surovtoseva et al., 2007; Beilstein et al., 2015). Complementation analysis and biochemistry analyses have shown that AtPOT1b is divergent from AtPOT1a, however, the function of AtPOT1b remains largely unknown (Beilstein et al., 2015; Arora et al., 2016; Shakirov et al., 2005). Is AtPOT1b like mice POT1b which contributes to telomere maintenance, or like Tetrahymena POT2 which functions outside telomeres? In this study, evidence were presented for functions of AtPOT1b both at telomeres and outside telomeres.

Does POT1b play a role in telomere end-protection and telomere length regulation?

POT1 proteins have been implicated in various processes that revolve around telomere end-protection and end-replication. It is possible that AtPOT1b plays a role in telomere biology. Previous studies of *pot1b-1* mutations (in a Ler-0 background) and studies of $POT1b_{S273F}$ mutations in Chapter II showed that POT1 is not essential for telomere length maintenance. However, previous genetic studies suggest a role of POT1b in end-protection. Ectopic overexpression of the first OB-fold of POT1b causes drastic telomere shortening and end-to-end chromosome fusion, probably due to nucleolytic attack (Shakirov et al., 2005). It is possible that the overexpression of a dominant-negative POT1b allele competes with endogenous POT1b for its binding partners that are involved in telomere end-protection. Similar conflicting phenotypes have also been reported for TER2 mutants. Plants lacking full-length TER2 do not

display any telomere length defects. However, an additional loss of TER2 in plants deficient of POT1a causes exacerbated telomere shortening (K. Renfrew and D. Shippen, unpublished data). The accelerated telomere shortening due to inactivation of TER2 does not correlate with a change in telomerase activity (Renfrew et al., 2014), but is probably caused by nucleolytic attack, implicating a role in telomere end-protection of TER2. Since TER2 is a binding partner of POT1b, one interpretation for these observations is that the TER2-POT1b may play a redundant role in telomere end-protection. Mechanistic explanations are still missing for this conundrum, and the function of AtPOT1b in end-protection remains a puzzle.

Recent paradigm-shifting studies of Ku in *A. thaliana* may provide an explanation for the role of TER2 and POT1b in telomere biology (Kazda et al., 2012). Kazda et al. demonstrated that half of telomeres in plants are blunt-ended, and that Ku, another component of TER2 RNP (Cifuentes-Rojas et al., 2012), is an essential component for blunt-ended or short (1 to 3 nt) G-overhang-containing telomeres in *A. thaliana.* Blunt-end telomeres may represent the products of lagging strand replication, after which Ku immediately associates with the chromosome end to prevent further nucleolytic processing. When Ku is absent, protection of blunt-ended telomeres is compromised. Exposed blunt-ended telomeres become accessible to nucleases for terminal resection, which convert the blunt-end into a G-overhang. The new G-overhang is expected to be recognized by the CST complexes, solving the end-protection crisis caused by depletion of Ku.

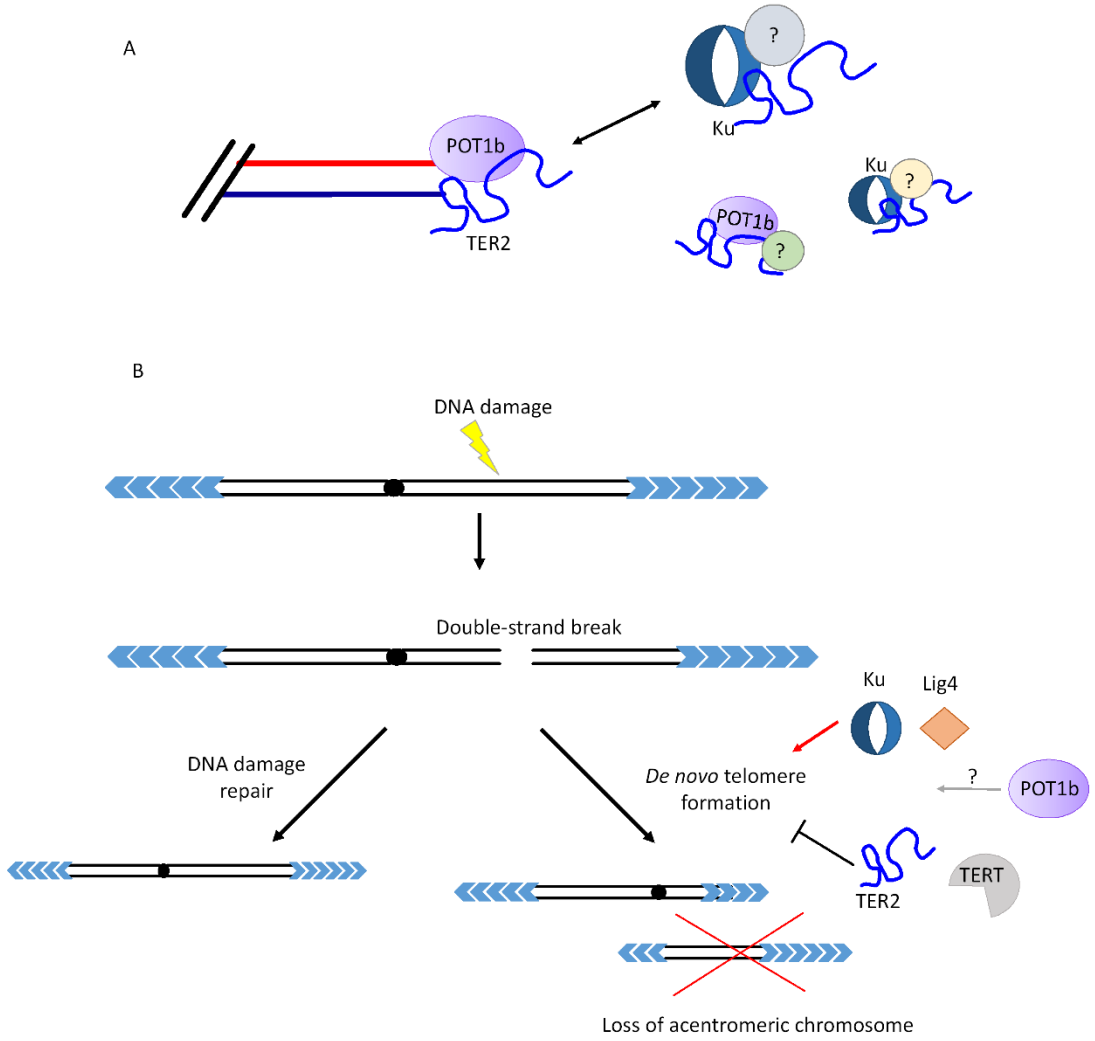


Figure 6-1. Model for TER2 RNP functions. (A) Components of TER2 RNP are associated with telomere ends, potentially protecting blunt-end telomeres. POT1b inhibits KU association with telomeres. Different TER2 subcomplexes may coexist for distinct functions. Spheres denote unknown proteins. (B) Schematic of *de no* telomere formation (DNTF) in *A. thaliana.* DSBs can either be repaired by DNA damage repair pathways, or at lower frequency, be stabilized by the formation of nascent telomeres. Ku and Ligase IV promotes DNTF, while TER2 and TERT inhibits DNTF. The function of POT1b in DNTF is unknown.

Are there other components involved in the capping mechanism for blunt-ended telomeres? TER2 and POT1b are the two top candidates for the blunt-end capping

complex, due to their physical association with Ku (Cifuentes-Rojas et al., 2012). In budding yeast, Ku interacts with the telomerase RNA, TCL1, through a conserved hairpin structure (Peterson et al., 2001; Stellwagen et al., 2003). This Ku-TCL1 interaction contributes to telomerase recruitment by Ku unloading telomerase RNP to the telomere terminus and subsequently switching to telomere DNA binding (Pfingsten et al., 2012). The Ku-TER interaction is also present in *A. thaliana*, and interestingly this interaction occurs with TER2, not TER1 (Cifuentes-Rojas et al., 2011). Additional studies suggest that POT1b also specifically associates with TER2 *in vivo* (Cifuentes-Rojas et al., 2012). Thus, TER2 may act as a scaffold to bring POT1b and Ku to the blunt-ended telomeres (Figure 6-1A).

To investigate this hypothesis, several experiments must be undertaken. First, the blunt-end assay and the G-overhang assay can be used to determine if TER2 and POT1b are essential for blunt-ended telomere maintenance (Kazda et al., 2012). Preliminary data in Chapter III demonstrated that plants lacking full-length TER2 have increased G-overhang levels, indicating an increase in terminal DNA resection and consistent with observations in the *ku* mutants that blunt ends are converted to G-overhangs (Kazda et al., 2012). Second, the association of TER2 or POT1b with telomeres needs to be verified by fluorescence *in situ* hybridization (FISH) or chromatin immunoprecipitation (ChIP). ChIP analysis presented in Chapter III showed that POT1b and Ku are both associated with telomeres. FISH experiments for TER2 localization at telomeres should help determine whether TER2 is associated with the telomeres. A remaining question is then which components are required for the blunt end cap. This can be tested using a

genetic approach to monitor the telomere structure in the absence of individual component of the TER2 RNP. Preliminary data in Chapter III unexpectedly revealed that in the absence of POT1b, Ku associates with more telomeres, implicating a role of POT1b in controlling Ku access at telomeres (Figure 6-1A). Based on this observation, it is possible that Ku and POT1b independently associate with TER2 to form distinct TER2 subcomplexes that dynamically interact with telomeres for different functions during the cell cycle. For example, it is possible that POT1b turns away Ku molecules that are not TER2 bound or help disassociate TER2-Ku from the blunt-end during the S/G2 phase for end processing and telomerase action. Preliminary data from the Shippen lab suggests that POT1b and Ku interact weakly in a co-immunoprecipitation (CoIP) assay using *in vitro* expressed proteins in the rabbit reticulocyte lysate (RRL). A separation-of-function mutation of POT1b lacking a Ku binding site will provide more insights into the nature of the Ku-POT1b interaction.

In addition to protection of the blunt end telomeres, genetic studies indicate that the TER2 RNP may also contribute to telomere maintenance and regulation of the DNA damage signal at telomeres. Preliminary data from a study of *pot1a ter2* double mutants indicated that TER2 may promote telomere recombination. Chromosome end-to-end fusion due to critically short telomeres is suppressed in the third and fourth generations of *pot1a ter2* double mutants compared with the *pot1a* single mutants (S. Bose and D. Shippen, unpublished data). It is possible that POT1b in the TER2 RNP is somehow involved in the signaling to the DNA damage response (DDR) when telomeres are dysfunctional. This hypothesis can be tested by comparing the frequency of telomere

recombination in plants lacking POT1b protein along with CST components, the absence of which leads to massive chromosome end-to-end fusions (Song et al., 2008; Surovtseva et al., 2009; Leehy et al., 2013). Continuing studies in the Shippen Lab will test this hypothesis.

Together, these studies will increase our understanding of the unusual telomere architecture and machinery for telomere maintenance in *A. thaliana*.

Does POT1b play a role in telomerase regulation?

While AtPOT1a assembles with TER1 into a canonical telomerase RNP for telomere replication, AtPOT1b associates with an alternative TER2 RNP, which is a negative regulator of telomerase activity (Cifuentes-Rojas et al., 2011&2012). The TER2 RNP is implicated in telomerase inhibition upon DNA damage (Cifuentes-Rojas et al., 2012). Previous studies revealed that *A. thaliana* down-regulates telomerase activity after DSBs are induced. This regulation is dependent on the rapid stabilization of TER2 (Xu et al., 2015). This finding provides a fascinating new mechanism for telomerase repression at sites of DSBs.

Does POT1b influence telomerase regulation in context of TER2 RNP? Data presented in Chapter II demonstrated that POT1b negatively regulates telomerase activity in flowers, but not in seedlings. This pattern of confined telomerase regulation recapitulates what was observed in the *ter2* mutants (Cifuentes-Rojas et al., 2012), supporting the hypothesis that components of the TER2 RNP are involved in the developmental regulation of telomerase. Notably, TER2 abundance does not change in

flowers of POT1b knockdown mutants, indicating that POT1b does not influence telomerase by modulating TER2 abundance. It is even possible that POT1b regulates telomerase independent of TER2. To test if POT1b and TER2 are in the same generic pathway for developmental control of telomerase, I propose to analyze telomerase activity in plants lacking both POT1b and TER2. *In vitro* telomerase activity assays conducted with a wild type extract supplemented by recombinant POT1b or TER2 can be used to further test this hypothesis. If POT1b is involved in telomerase inhibition in context of TER2 RNP, POT1b may also respond to DSBs. This hypothesis can be tested monitoring telomerase activity after DNA damage in plants lacking POT1b.

The biological significance of TER2-mediated telomerase regulation during development is still under investigation. TER2 peaks in reproductive tissues, especially in unfertilized flowers (H. Xu and D. Shippen, unpublished data), while POT1b mRNA accumulates in dry seeds and anthers (Klepikova et al., 2015&2016; Yang et al., 2011). A central feature of meiosis is programmed DSBs for meiotic recombination. Similarly during germination, desiccated seeds face enormous stresses, including reactive oxygen species, high temperature, and increased humidity, accompanied with high level of DNA damage (Waterworth et al., 2016). Thus, one appealing hypothesis is that TER2 RNP is upregulated in reproductive tissues and proliferating tissues to inhibit *de novo* telomere formation (DNTF). Multiple regulatory pathways have evolved to limit DNTF at the sites of DSBs to promote faithful DNA repairs. The TER2 RNP in *A. thaliana* may serve as one of the mechanisms to down-regulate telomerase activity in confined developmental stages for DNTF inhibition (Figure 6-1B).

In Chapter IV, a potential role for TER2 in controling DNTF is analyzed using an established DNTF assay in tetraploid *A. thaliana* plants. By introducing a "seed" telomere sequence, DNTF was monitored in *ter2* mutants. The preliminary data suggest an increase in DNTF efficiency, supporting a role of TER2 in DNTF inhibition. The sample number was small, so additional analysis of more DNTF events is needed to make rigorous conclusions. In addition, similar analyses need to be done in tetraploid *pot1b* mutants to address whether POT1b contributes to DNTF inhibition. Since Ku promotes DNTF in plants (Nelson et al., 2011), it will be intriguing to elucidate if and how a putative TER2-POT1b RNP inhibits DNTF in *A. thaliana* (Figure 6-1B).

What is the molecular mechanism of TER2/POT1b-mediated telomerase down-regulation? The fact that TERT has higher affinity for TER2 over TER1 provides one possible mechanism of telomerase inhibition by TER2 (Cifuentes-Rojas et al., 2011), in which upon DNA damage, an increasing amount of TER2 molecules compete with TER1-bound telomerase and thus reduce the number of active telomerase complexes. Recent studies in the Shippen lab revealed that TER1 and TER2 form a heterodimer *in vitro* and further that heterodimerization is preferred over homodimerization (J. Song and D. Shippen, unpublished data). This observation provides another mechanism for rapid telomerase inhibition upon DNA damage. When DSBs accumulates *in vivo*, TER2 level increases allowing TER1-TER2 dimer to form, which blocks the template or active site of the TER1 RNP. The latter model is favorable considering the very rapid inhibition of telomerase after the onset of DSBs (Cifuentes-Rojas et al., 2012). In addition, different processing intermediates of TER2 have been identified to associate with

POT1b or Ku (A. Suescun and D. Shippen, unpublished data). Thus, it is possible that distinct subcomplex assemblies of TER2 and its processed isoforms serve different functions in telomerase regulation.

Finally, how does POT1b contribute to TER2-dependent telomerase suppression? What factors are involved in this process? Genetic analyses can provide insight in this regard. In addition, B. Barbero in the Shippen lab is undertaking mass spectrometry of affinity-purified POT1b to identify novel binding partners that may provide clues about POT1b functions and interactions in telomerase regulation.

A possible role for POT1b in plant early development

Data in Chapter II showed that after four rounds of self-pollination, seedlings carrying a point mutation that causes a reduced POT1b protein level ($POT1b_{S273F}$) displayed shorter root length than the wild type seedlings. One possible explanation for this phenomena is that POT1b is required for early development of plants. The fertility and morphology of the adult plants did not appear to be affected, therefore this phenotype reflects developmental delay, not arrest. The short root phenotype was not observed in *ter2, tert* or *pot1a* single mutant seedlings, indicating a unique contribution by POT1b. Preliminary data from Chapter II and recent observation by B. Barbero showed that this delayed early development worsens in $POT1b_{S273F}$ mutants that also lack TERT. Importantly, the developmental phenotype does not correlate with a change in telomere length, suggesting the conclusion that POT1b plays a novel role in early plant development.

Seedling development of *A. thaliana* spans approximately 2 weeks and can be divided into two major of stages defined by the changes in morphology: seed germination and leaf development (Boyes et al., 2001). Seed germination sequentially consists of seed imbibition, radicle emergence, and hypocotyl and cotyledon emergence, and is completed within 5.5 days after sowing on plates. Since the growth delay associated with POT1b mutation is evident even before the 5^{th} day, seed germination may be the development stage that requires POT1b. During desiccation, metabolic programs and gene expression are specifically regulated for desiccation tolerance, dormancy competence, and last but most important, successful germination of dry seeds (Angelovici et al., 2010). A recent finding unveiled a striking transcriptional DSB damage response during germination, indicative of massive genotoxic stress (Waterworth et al., 2015&2016; El-Maarouf-Bouteau et al., 2011). Activation of ATR and ATM signaling is proposed to promote faithful transmission of genetic information through the control of germination potential (Waterworth et al., 2016). One interesting possibility is that POT1b is also under the transcription control and contributes to DNA damage response or telomerase inhibition during seed germination. Indeed, RNA sequencing data from several groups indicates that POT1b mRNA is enriched in dry seeds, supporting this hypothesis (Klepikova et al., 2015& 2016). Preliminary data from the Shippen lab showing that POT1b can bind ATR *in vitro* provide further support for a role of POT1b in DDR during early development (Y. Surovtseva and D. Shippen, unpublished data).

Several experiments must be undertaken to explore the role of POT1b in seed germination. Since the POT1b mutants are viable and fertile, could the delayed early development mean confined mitotic arrest in apical meristems due to DNA damage? Detailed analysis of root anatomy will provide information about the mitotic activity of the meristems and the stem cell niches. Additionally, immunostaining in roots using cell-cycle markers could be informative for meristem activity. In parallel, the transcript level and protein modification of players in cell-cycle regulation can be analyzed by RT-PCR and immunoblotting, respectively, to demonstrate if the cell cycle is arrested during seed germination due to the deficiency of POT1b protein. To test whether POT1b affects DNA damage signaling during seed germination, the activation of ATM and ATR can be examined by monitoring their down-stream signaling molecules, such as PARPs, BRCA1, and Rad51. The level of γ-H2AX should also be tested. Finally, delayed seeding development was only observed after the fourth generation of POT1b mutation, strongly suggesting the involvement of epigenetic regulation. Epigenetic modification, including DNA methylation levels, can be analyzed for known genes that control germination and root development. To test this hypothesis, *pot1b* mutant will be crossed with Col-0 as either paternal parent or maternal parent, and the offspring of this backcross will be analyzed.

Unexpected subcellular localization for telomere proteins

In Chapter II and Appendix I, microscopy data showed that three telomere proteins in *A. thaliana*, POT1b, STN1, and TEN1, are dually localized: in the nucleus

and in the cytoplasm. Cytoplasm localization of telomere proteins is not unprecedented. For example, human shelterin components, TIN2, TPP1 and POT1, accumulate in the cytoplasm (Chen et al., 2007; Chen et al., 2012b; Liu et al., 2004). Nuclear export of TPP1 controls the amount of TPP1 and its binding partner POT1 in the nucleus for proper telomere length control and for tamping down the DNA damage response at telomeres (Chen et al., 2007). Notably, the telomerase catalytic subunit TERT in humans has also been found in mitochondria (Saretzki, 2009). Mitochondrial TERT plays a critical role in modulating the level of reactive oxygen species (Ahmed et al., 2008), mitochondrial DNA damage (Santos et al., 2004), cell proliferation (Mukherjee et al., 2011), and apoptosis (Indran et al., 2011; Santos et al., 2006).

Spatial control of dually localized proteins is common in plants (Boyle and Brisson, 2001; Krause and Krupinska, 2009). In response to developmental or environmental cues, these proteins can transiently shuttle back to the nucleus to activate biotic and abiotic signaling pathways. Among the plant proteins known to dually target to the nucleus and to mitochondria or plastids, most are implicated in the regulation of DNA metabolism (Krause and Krupinska, 2009). Thus, it is of interest to further investigate how the dual localization of POT1b, STN1, and TEN1 in the cytoplasm contributes to functions outside telomere maintenance.

POT1b resides outside the nucleus

Chapter II presented microscopy and immunoblot analysis indicating that unlike POT1a, which resides in the nucleus for telomere maintenance, POT1b is primary

localized to the cytoplasm. Again, this data supports the conclusion that AtPOT1b is functionally distinct from AtPOT1a, and further that it may have functions outside the nucleus. What is the function of cytoplasmic POT1b? To address this question, a yeast two hybrid (Y2H) screen was performed to identify POT1b binding partners. Y2H candidates for POT1b interaction are listed in Chapter II and are implicated in metabolic processes in different cellular compartments, including photosynthesis (chloroplast), stress responses (cytoplasm and mitochondria), and transcriptional regulation (nucleus). Notably, POT1b-GFP fusion protein formed punctate spots in the cytoplasm of the mesophyll protoplasts. This pattern of localization is consistent with secretory vesicles or plastids, which are involved in photosynthesis or storage of metabolic products. Further experiments to look for co-localization of POT1b and the Golgi apparatus will determine if POT1b is involved in the secretory pathway. We have conducted initial verification of the interaction between POT1b and several of its Y2H binding partners, but further verification for the candidates, including *in vivo* CoIP, needs to be done to confirm these interactions.

It is possible that there are two distinctive populations of POT1b: a cytoplasmic POT1b population and a nuclear fraction for telomere interaction. Preliminary data from the Shippen lab showed that POT1b binding partner, TER2, can be detected more in the cytoplasm, although TER2 also appears in the nucleus (A. Suescun and D. Shippen, unpublished data). Interestingly, one of the TER2 isoforms, $TER2_{AS}$, is not enriched in the cytoplasm. This pattern of different localization of TER2 and $TER2_{AS}$ raises a possibility that a portion of TER2 is excluded from the nucleus to form a TER2-POT1b

subcomplex. If this is true, nuclear export of TER2-POT1b RNP may provide a novel mechanism to regulate telomerase and protect telomeres. For example, it is possible that excessive amount of TER2-POT1b RNP may retain in the cytoplasm to be recruited to the nucleus in response to environmental or genotoxic stress. In contrast, Ku, a major binding partner of TER2, is exclusively localized to the nucleus, raising the possibility that POT1b is excluded from the nucleus to ensure TER2-Ku association at the blunt-ended telomeres. Or is it possible that TER2 is sequestered in the cytoplasm by POT1b to avoid TER1-TER2 dimerization and thus allow the action of telomerase in normal S phase? To approach these questions, *in vivo* pull-down of cytoplasmic POT1b can be undertaken to determine whether TER2 and POT1b form a complex in this compartment. Cytology experiments for POT1b localization during developmental stages or under various stresses will help to reveal the function of POT1b.

STN1 and TEN1 accumulate in chloroplasts

Another unanticipated observation presented in the Appendix I is that TEN1 and STN1 are primarily localized in *A. thaliana* chloroplasts. Although we observed no obvious defects in the leaves of *ten1* and *stn1* mutants (Leehy et al., 2013; Song et al., 2008), a detailed analysis of chloroplast anatomy and physiology will be needed to determine whether TEN1 and STN1 function in this compartment. The function of AtTEN1 has just been expanded to include a protein chaperone role, which responds to heat stress (Lee et al., 2016). Numerous molecular chaperones are implicated in chloroplast protein import (Flores-Perez and Jarvis, 2013; Hendrick and Hartl, 1993), so

it is conceivable that AtTEN1 chaperone activity is deployed to stabilize a protein target in chloroplasts.

Since both AtTEN1 protein and mRNA are rapidly responsive to temperature, and perhaps other environmental stimuli, it is possible that the complex regulation of TEN1 and its chaperone function define a novel regulatory pathway linking environmental stress and cellular metabolism to genome stability (Lee et al., 2016). An alternative explanation for the chloroplast localization of TEN1 and STN1 is that sequestration in this organelle provides a mechanism to regulate telomere structure or metabolism in the nucleus.

A possible role of telomeres in programmed cell death to promote genome integrity

In plants, root and shoot meristems are especially sensitive to DNA damage and thus require specific maintenance to avoid the risk of accumulating mutations. Plants evolved programmed cell death (PCD), mediated by ATM and ATR pathways, as a stringent mechanism for genome integrity of the stem cells (Fulcher and Sablowski, 2009). In Chapter VI, a model is proposed in which abrupt telomere shortening contributes to PCD in root apical meristems (RAMs). The progressive DNA hypomethylation of *ddm1* mutants culminates in the sixth generation with rampant transposon activation and morphological defects, including sterility and reduced apical dominance. Additionally, telomeres undergo abrupt shortening, potentially mediated by deletional recombination that triggers DNA damage sensitivity, and increased

programmed cell death in the stem cell niches. Recent findings in plants lacking TEN1 illustrated that telomeres respond to heat stress and undergo telomere rapid deletion (TRD) (Lee et al., 2016). Together with the observations in Chapter VI, I propose that telomeres are part of an environmental sensor that perceives genotoxic stress, extreme temperatures, and likely other assaults and responses by increased deletional recombination resulting in telomere shortening. The abrupt telomere shortening can lead to uncapped telomeres, while trigger PCD thereby eliminating stem cells with genomic instability. In this context, TRD can promote genome integrity by propagation of undamaged plant stem cells.

Conclusions

In summary, this dissertation has provided new insight for the consequences of gene duplication of a critical and highly conserved telomere protein, POT1. The data highlighted the remarkable functional divergence of the two *A. thaliana* POT1 paralogs. Data are presented showing that POT1a and POT1b localize to different subcellular compartments, play different roles in telomere maintenance, and contribute to different specialized cellular processes. As AtPOT1a harbors the highly conserved functions of POT1, such as telomere length control, stimulating telomerase, and association with CST, it is an essential gene for telomere maintenance. In contrast, AtPOT1b is not essential for telomere length regulation, but data from this dissertation reveal a role for POT1b in negatively regulating telomerase in flowers and possibly in blunt-ended telomere capping. Data are also presented indicating novel roles for POT1b beyond

telomeres in early plant development and unknown functions in the cytoplasm. Thus, AtPOT1b appears to be a non-conventional POT1 protein, with interesting and novel functions that remain to be fully elucidated.

Another interesting model discussed in this dissertation is that TRD can be used as a "cleansing" mechanism for eliminating damaged stem cells. Data presented in this dissertation demonstrated that the genome of plants with dramatically reduced DNA methylation and transposon activation are intrinsically unstable. Telomere deletional recombination has been demonstrated in these plants. Telomere truncation could stimulate programmed cell death in the damaged meristems and further guarantees the faithful transmission of genetic information by eliminating inviable stem cells. Thus, telomeres may be capable to respond to genome recombination and serve a role in promoting stem cell integrality.

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APPENDIX I

ATSTN1 AND ATTEN1 LOCALIZE TO THE CHLOROPLAST

Rationale

The prevailing view has been that CST functions as a trimeric complex, but mounting evidence indicates that sub-complexes of CST and individual subunits dynamically interact with each other and in some cases, exchange for telomerase subunits and the conventional DNA replication machinery to promote telomere stability (Chen et al., 2012a; Grossi et al., 2004; Qi and Zakian, 2000). For example, yeast Stn1 inhibits telomerase binding to Cdc13 (Chandra et al., 2001), arguing that CST components must be modified in some fashion for replication, perhaps via phosphorylation of Cdc13 (Li et al., 2009) and Stn1 (Liu et al., 2014). In some genetic backgrounds, STN1 and TEN1 stabilize chromosome ends in a Cdc13-independent manner (Holstein et al., 2014; Petreaca et al., 2006). Moreover, relative to human cells deficient in STN1 or CTC1, cells lacking TEN1 exhibit more severe growth defects, a higher frequency of chromosomes lacking telomeric DNA and more anaphase bridges (Kasbek et al., 2013).

Data from *Arabidopsis* also support the conclusion that TEN1 makes unique contributions outside the context of the CST heterotrimer. Like *stn1* and *ctc1* mutants (Song et al., 2008; Surovtseva et al., 2009), *ten1-3* mutants display dramatic telomere shortening, extended G-overhangs and end-to-end chromosome fusions (Leehy et al., 2013). Telomere failure culminates in defective stem cell proliferation and sterility (Hashimura and Ueguchi, 2011; Leehy et al., 2013). However, *ten1-3* mutants suffer even

more genome instability than *stn1* or *ctc1* mutants (Leehy et al., 2013; Song et al., 2008; Surovtseva et al., 2009). In addition, TEN1 associates with a significantly smaller fraction of *Arabidopsis* telomeres than CTC1 (Leehy et al., 2013; Surovtseva et al., 2009), suggesting that TEN1 only transiently engages the chromosome terminus. Unlike STN1 and CTC1, which physically associate with enzymatically active telomerase, TEN1 negatively regulates telomerase repeat addition processivity (Leehy et al., 2013). TEN1 competes with POT1a, a positive regulator of telomerase processivity, for interaction with STN1: binding of TEN1 and POT1a by STN1 is mutually exclusive (Renfrew et al., 2014). Consequently, STN1 is proposed to dynamically exchange TEN1 for POT1a when telomerase extends telomeres (Renfrew et al., 2014; Surovtseva et al., 2007).

Here we report unanticipated chloroplast localization of AtSTN1 and AtTEN1 revealing their remarkably dynamic nature. We show that although TEN1 and its binding partner STN1 play a critical role in modulating telomere maintenance and stability, these proteins primarily localize to chloroplasts. These findings suggest that AtSTN1 and AtTEN1 play a novel role in the cytoplasm.

Materials and methods

Protoplast preparation and microscopy

Arabidopsis mesophyll protoplasts were isolated as described previously (Sheen, 2001). Protein expression constructs bearing a C-terminal GFP tag were cloned into the pHBT plasmid. After transient transformation into protoplasts, gene expression from the 35S CaMV promoter was allowed for 8 to 10 hours. Protein localization was visualized

using the mCherry, DAPI and GFP channels (standard filter set, Nikon) with an inverted Nikon epifluorescence microscope using a 100× objective (Plan Fluo, NA 1.40, oil immersion).

Results

Immunolocalization experiments with purified *A. thaliana* nuclei indicate that all three *A. thaliana* CST components co-localize with telomeres (Leehy et al., 2013; Song et al., 2008; Surovtseva et al., 2009). Unexpectedly, however, analysis of protein sequences using several databases predicted chloroplast or cytoplasmic localization for AtSTN1 and AtTEN1 (Table I-1). In contrast, these same programs had CTC1 in the nucleus. To evaluate the sub-cellular localization of AtTEN1 and AtSTN1, we fused their coding sequences with green fluorescent protein (GFP) under the control of the 35S CaMV promoter. A construct containing only the GFP tag was generated as a negative control. As an additional control, GFP was fused to the nuclear protein Ku70, a key component of the non-homologous end joining DNA repair pathway and regulator of telomere length in *A. thaliana* (Riha et al., 2006). Arabidopsis mesophyll protoplasts were transiently transfected and GFP fluorescence was visualized 8-10 hours after transformation to mitigate protein over-expression. DAPI staining was used to monitor nuclear localization, while red auto-fluorescence from chlorophyll indicated the localization of chloroplasts. The GFP control was concentrated in the nucleus, with diffuse localization in the cytoplasm, but no appreciable accumulation in chloroplasts (Figure I-1). As expected, a strong signal of Ku70-GFP was present in the nucleus, with a background signal in

chloroplasts. Remarkably, both STN1-GFP and TEN1-GFP showed the opposite localization profile. These proteins were predominantly found in chloroplasts, and displayed only weak nuclear staining (Figure I-1). Since the critical roles of STN1 and TEN1 in telomere metabolism are executed in the nucleus, these findings raise the possibility of non-canonical functions for AtSTN1 and AtTEN1 in chloroplasts.

Name	**Accession No.**	**Localization Predicators**		
		Plant-mPLoc	**MultiLoc (score)**	**SUBAcon (score)**
CTC1	AT4G09680	Nucleus	Plasma membrane (0.53), nuclear (0.17)	Nucleus (0.95)
STN1	AT1G07130	Chloroplast	Peroxisomal (0.92)	Nucleus (1.00)
TEN1	AT1G56260	Chloroplast	Cytoplasmic (0.93)	Cytosol (0.99)
Ku70	AT1G16970	Nucleus	Cytoplasmic (0.86)	Nucleus (1.00)
TERT	AT5G16850	Nucleus	Nuclear (0.85)	Nucleus (1.00)
POT1a	AT2G05210	Nucleus	Plasma membrane (0.58), cytoplasmic (0.15)	Cytosol (0.66)

Table I-1. Localization prediction for Arabidopsis telomere proteins. Data obtained from Chou and Shen, 2010; Hoglund et al., 2006; Hooper et al., 2014.

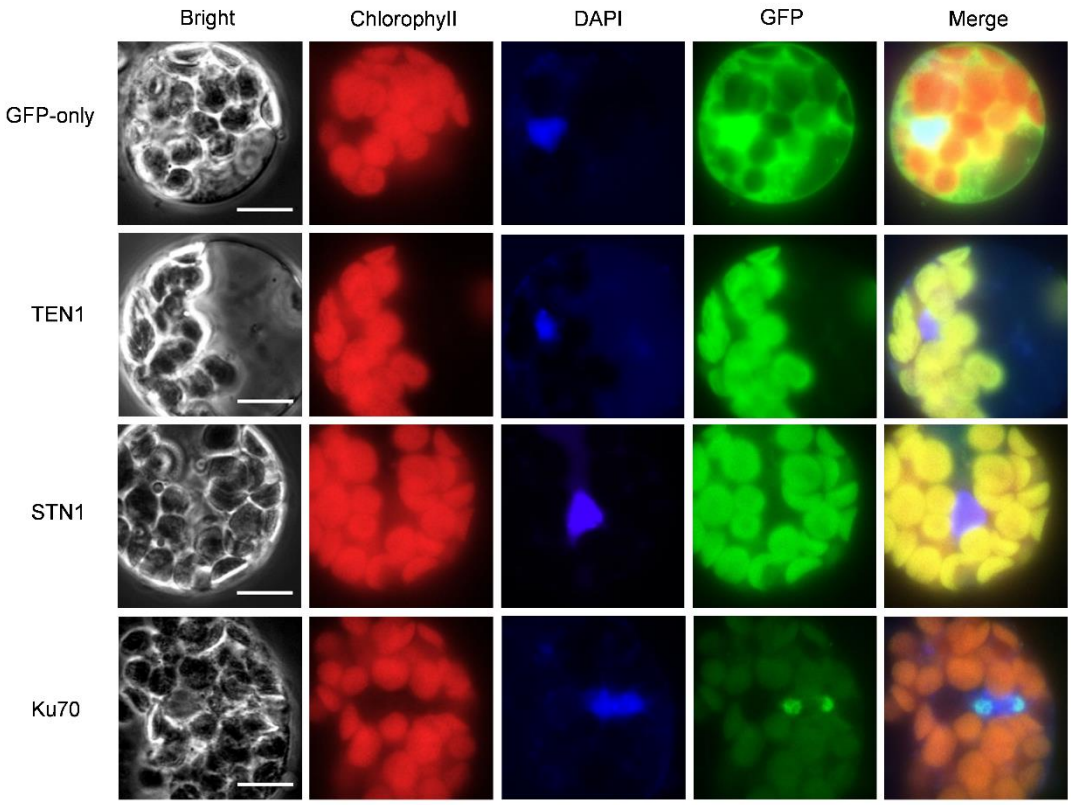

Figure I-1. AtTEN1 and AtSTN1 localize to chloroplasts in mesophyll protoplasts. The indicated proteins tagged with GFP were expressed in protoplasts for 8-10 hours. Red autofluorescence of chlorophyII (chloroplast), blue fluorescence from DAPI (nuclear) staining and green GFP fluorescence were monitored separately using fluorescence microscopy. The far right column shows a merge of the three images. Scale Bar = 10 μm.

APPENDIX II

DYNAMIC INTERACTIONS OF ARABIDOPSIS TEN1: STABILIZING TELOMERES IN RESPONSE TO HEAT STRESS

Summary

Telomeres are the essential nucleoprotein structures that provide a physical cap for the ends of linear chromosomes. The highly conserved CST (CTC1/STN1/TEN1) protein complex facilitates telomeric DNA replication and promotes telomere stability. Here we report three unexpected properties of Arabidopsis thaliana TEN1 that indicate it possesses functions distinct from other previously characterized telomere proteins. First, we show that telomeres in ten1 mutants are highly sensitive to thermal stress. Heat shock causes abrupt and dramatic loss of telomeric DNA in ten1 plants, likely via deletional recombination. Second, we show that AtTEN1 has the properties of a heat-shock induced molecular chaperone. At elevated temperature, AtTEN1 rapidly assembles into high molecular weight homo-oligomeric complexes that efficiently suppress heat-induced aggregation of model protein substrates in vitro. Finally, we report that AtTEN1 specifically protects CTC1 from heat-induced aggregation in vitro, and from heat-induced protein degradation and loss of telomere association in vivo. Collectively, these observations define Arabidopsis TEN1 as a highly dynamic protein that works in concert with CTC1 to preserve telomere integrity in response to environmental stress.

Introduction

Telomeres are among the most highly dynamic structures in the genome. They form a platform for terminus binding proteins (termed shelterin in vertebrates) (de Lange, 2005) that sequester chromosome ends, protecting them from eliciting a DNA damage response. Telomere proteins also present the chromosome terminus as a substrate for replicative enzymes. Telomeric DNA consists of tandem arrays of G-rich repeats that terminate in a single-stranded 3′ overhang (G-overhang). During much of the cell cycle the G-overhang is proposed to be concealed in a t-loop, wherein the single-strand terminus invades the telomeric duplex to form a Holliday junction-like structure (Griffith et al., 1999) inaccessible to telomerase (Smogorzewska et al., 2000). Failure to stabilize t-loops leads to the abrupt loss of telomeric DNA via recombinational deletion in a process termed telomere rapid deletion (TRD) (Lustig, 2003; Wang et al., 2004). TRD must be contained to avert catastrophic telomere shortening and replicative senescence. Telomere length homeostasis is achieved by a highly orchestrated, but poorly understood, series of conformational changes that sequentially convert the G-overhang into telomerase-extendable and non-extendable states (Blackburn, 2001; Teixeira et al., 2004). This binary switch is controlled by long-range protein interactions (Loayza and de Lange, 2003; Marcand et al., 1997), dynamic shifts among core components of telomere complexes (Jun et al., 2013) and post-translational modification (Garg et al., 2014; Liu et al., 2014; Miyagawa et al., 2014; Zhang et al., 2013).

One telomere protein complex under intensive scrutiny is CST (Cdc13/CTC1, Stn1, and Ten1). CST bears structural similarity to replication protein A (RPA) (Gao et

al., 2007; Sun et al., 2009) and associates with the G-overhang via an oligosaccharide-oligonucleotide binding fold (OB-fold) within the Cdc13/CTC1 subunit (Mitton-Fry et al., 2004). Stn1 and Ten1 each harbor single OB-fold domains and form *a stable heterodimer* (Petreaca et al., 2006). Mutation of budding yeast CST components causes degradation of the telomeric C-strand and hence increased length of the G-strand (Garvik et al., 1995; Grandin et al., 2001; Grandin et al., 1997), phenotypes attributed to defects in telomeric DNA replication as well as chromosome end protection (Nugent et al., 1996; Xu et al., 2009). Cdc13 coordinates telomeric DNA replication by first facilitating G-strand synthesis through interactions with telomerase, and then C-strand synthesis via association with DNA polymerase α/primase (Qi and Zakian, 2000). Unlike yeast CST, which stably and sequentially engages telomerase and DNA pol-α, vertebrate CST only transiently associates with telomeres, where it represses telomerase repeat addition processivity and stimulates Pol-α to facilitate the switch from G-strand to C-strand synthesis (Chen et al., 2012a; Lue et al., 2014). Vertebrate CST is also implicated in restoring replication fork progression following replication stress (Kasbek et al., 2013; Wang et al., 2012).

The prevailing view has been that CST functions as a trimeric complex, but mounting evidence indicates that sub-complexes of CST and individual subunits dynamically interact with each other and in some cases, exchange for telomerase subunits and the conventional DNA replication machinery to promote telomere stability (Chen et al., 2012a; Grossi et al., 2004; Qi and Zakian, 2000). For example, yeast Stn1 inhibits telomerase binding to Cdc13 (Chandra et al., 2001), arguing that CST

components must be modified in some fashion for replication, perhaps via phosphorylation of Cdc13 (Li et al., 2009) and Stn1 (Liu et al., 2014). In some genetic backgrounds, STN1 and TEN1 stabilize chromosome ends in a Cdc13-independent manner (Holstein et al., 2014; Petreaca et al., 2006). Moreover, relative to human cells deficient in STN1 or CTC1, cells lacking TEN1 exhibit more severe growth defects, a higher frequency of chromosomes lacking telomeric DNA and more anaphase bridges (Kasbek et al., 2013).

Data from Arabidopsis also support the conclusion that TEN1 makes unique contributions outside the context of the CST heterotrimer. Like *stn1* and *ctc1* mutants (Song et al., 2008; Surovtseva et al., 2009), *ten1-3* mutants display dramatic telomere shortening, extended G-overhangs and end-to-end chromosome fusions (Leehy et al., 2013). Telomere failure culminates in defective stem cell proliferation and sterility (Hashimura and Ueguchi, 2011; Leehy et al., 2013). However, *ten1-3* mutants suffer even more genome instability than *stn1* or *ctc1* mutants (Leehy et al., 2013; Song et al., 2008; Surovtseva et al., 2009). In addition, TEN1 associates with a significantly smaller fraction of Arabidopsis telomeres than CTC1 (Leehy et al., 2013; Surovtseva et al., 2009), suggesting that TEN1 only transiently engages the chromosome terminus. Unlike STN1 and CTC1, which physically associate with enzymatically active telomerase in *A. thaliana*, TEN1 negatively regulates telomerase repeat addition processivity (Leehy et al., 2013) and is not associated with active telomerase (Renfrew et al. 2014). In addition, AtTEN1 competes with POT1a, a positive regulator of telomerase processivity, for interaction with STN1; binding of TEN1 and POT1a by STN1 is mutually exclusive

(Renfrew et al., 2014). Consequently, STN1 is proposed to dynamically exchange TEN1 for POT1a when telomerase extends *A. thaliana* telomeres (Renfrew et al., 2014; Surovtseva et al., 2007).

Here we report several unanticipated properties of AtTEN1 that reveal its remarkably dynamic nature. We demonstrate that TEN1, but not STN1, protects Arabidopsis telomeres from thermal stress-induced rapid telomere shortening *in vivo*. We provide evidence that AtTEN1 responds to heat stress by assembling into high molecular weight complexes with protein chaperone activity and finally we show that TEN1 has the capacity to protect CTC1 from heat-induced degradation *in vivo*. These findings provide new insight into AtTEN1 function and interactions, and suggest that this protein plays a novel role in the plant response to the environment.

Material and methods

Plant materials, growth conditions, and treatments

The *ctc1-3*, *stn1* and *ten1-3* mutants were described previously (Leehy et al., 2013; Song et al., 2008; Surovtseva et al., 2009). *Arabidopsis thaliana* were grown at 23°C under long day conditions (16 hours light; 8 hours dark) on either 0.5x MS plates or on Sunshine soil mix.

Plasmid construction and yeast two-hybrid analysis

Arabidopsis TEN1 was PCR-amplified from an Arabidopsis cDNA library using *AtTEN1* F primer containing a *BamH*I site and the initiation codon and with AtTEN1 R

primer containing both an *Xho*I site and the stop codon, respectively. N- or C-terminal truncated *AtCTC1* constructs comprising amino acid residues 1 to 384, and 385 to 1272 were amplified from pET28a-AtCTC1 as a template. PCR products were subcloned into the pGEM-Teasy vector (Promega). Inserts were digested with *BamH*I and *Xho*I, and ligated into the corresponding sites of pET-28a vector (Novagen) for expression in *E. coli*. All of the constructs were verified by sequencing.

For yeast two-hybrid analysis, pBD-GAL4-AtTEN1, pAD-GAL4-AtSTN1 and pAD-GAL4-AtCTC1 were generated and yeast two-hybrid assays were performed using SD/-Leu/-Trp/-His/-Ade selection medium with 10 mM 3-aminotriazole (3-AT). After transformation, positive clones were subjected to the o-nitrophenyl--D galactoside (ONPG) assay for β-galactosidase activity in yeast to check binding strength as described (Clontech, Palo Alto, CA).

Purification of recombinant TEN1 and CTC1

E. coli BL21(DE3) pLysS transformed with pET-28a encoding wild type or mutant AtTEN1 and AtCTC1 proteins were cultured at 30°C in LB medium supplemented with kanamycin (50 μg/ml) until the OD of the culture at 600 nm reached 0.3. After additional incubation at 4°C for 30 min, 0.4 mM of isopropyl-β-D-thiogalacto-pyranoside (IPTG) was added to the culture and incubated for 14 h at 16°C. Cells were harvested by centrifugation and stored at -70°C until use. Frozen cells were suspended in phosphate-buffered saline (PBS) buffer, and disrupted by sonication. Soluble crude extract was loaded into a Ni-NTA column. Histidine tagged protein was affinity-purified

by Ni-NTA agarose. Purified AtTEN1 was dialyzed and used for biochemical analysis, and for preparation of polyclonal antibody.

Protein identification

Recombinant AtTEN1 protein was digested with trypsin, and subjected to MS analysis using MALDI-TOF-MS. All MALDI-TOF-MS spectra were searched against the NCBI protein database using the MASCOT search program (http://www.matrixscience.com).

SEC, co-IP, and yeast two-hybrid assays

SEC on FPLC (Amersham Pharmacia, ÄKTA) was performed with a Superdex 200 HR 10/30 column equilibrated at a flow rate of 0.5 ml/min at 25°C with 50 mM HEPES, (pH 8.0) buffer containing 100 mM of NaCl. Protein peaks (A_{280}) were isolated and concentrated using a Centricon YM-10 (MILLIPORE, USA). Protein interactions of AtTEN1, AtSTN1, and AtCTC1 were tested using a co-IP assay (Leehy et al., 2013) and two-hybrid analysis (Lee et al., 2006).

Chaperone assays and bis-ANS fluorescence measurements

Chaperone activity was measured using MDH and CS substrates as described (Jang et al., 2004; Lee et al., 2009). Porcine heart mitochondrial MDH, CS, dithiothreitol (DTT), H_2O_2 were purchased from Sigma. Turbidity due to substrate aggregation was monitored in a DU800 spectrophotometer (Beckman, CA) equipped with a thermostatic

cell holder. F-I and F-II fractions of TEN1 taken in 50 mM HEPES buffer were incubated with 10 μM of bis-ANS. Hydrophobic domain exposure of TEN1 was examined by measuring the binding of bis-ANS to each fraction with a FM25 spectrofluorometer (Kontron, Germany) as described (Jang et al., 2004). Bis-ANS was from Molecular Probes.

Protein stability assays

AtCTC1 N-term, AtCTC1 C-term and MDH were incubated at RT and 45°C for 30 min with or without recombinant AtTEN1 or AtSTN1. Heat-treated samples were centrifuged, and stable and unstable fractions were displayed on an SDS-PAGE gel. To monitor protein stability *in vivo,* four week-old seedlings from WT, *stn1*, *ten1-3* and *ten1-3* complementation lines (Hashimura and Ueguchi, 2011) were incubated at RT or at 42°C for 30 min. Total protein was extracted and equal amounts (45 μg) were resolved by SDS-PAGE followed by immunoblotting with AtCTC1-antibody. The oligomeric status of TEN1 in WT Arabidopsis was assessed at RT and upon heat shock by immunoblotting of total protein with an AtTEN1-antibody (Leehy et al., 2013).

Cryo-EM and image processing

Following SEC, F-II fractions of AtTEN1 were frozen in vitreous ice on a Quantfoil R2/1 holey carbon grid with a FEI Vitrobot. Fifty cryo-EM images were acquired at an effective magnification of 81081X using a FEI TECNAI F20 cryo-electron microscope operated at 200kV. A total of 300 particles was selected and

particles were averaged into 16 reference-free class-averages using EMAN2 (Tang et al., 2007). After careful screening, four classes of different particle sizes were compiled and shown in the figure.

Telomere analysis

DNA was isolated using 2x CTAB as described previously (Leehy et al., 2013). The heat shock seedling sample was collected after heat treatment at 42°C for 1 hr. To determine the length of specific telomere tracts, PETRA (Heacock et al. 2004) was performed with 2 μg of DNA. Quantitative telomere repeat amplification protocol (qTRAP) was performed as previously described (Leehy et al., 2013). For telomere ChIP, 5 g of 2 week-old plant tissues were harvested after heat treatment and ChIP was performed as described (Saleh et al., 2008). Filter-binding assays were performed using a [^{32}P] 5′ end labeled $(T_3AG_3)_5$ oligonucleotide probe. A [^{32}P] 5′ end labeled rDNA (18S+5S) was used as a control probe.

Results

AtTEN1 and AtCTC1 protect against heat-induced telomere truncation

To extend our analysis of *A. thaliana* CST components, we employed the AtGenExpress Visualization Tool to examine RNA expression profiles under various abiotic stimuli (Table II-1). The data indicated that heat shock, cold shock and oxidative stress trigger a prompt increase in *AtTEN1* transcripts. Notably, this response is specific for *TEN1* as *CTC1*, *STN1*, and other telomere-related transcripts are largely unaffected.

Among the abiotic stressors tested, heat shock had the greatest impact on *TEN1* expression. *AtTEN1* mRNA increased 2.3 fold after 3 hours at 38°C, then returned to the basal level when plants were transferred to 25°C. By comparison, mRNAs in the Hsp70 family are elevated by 2- to 13-fold under the same conditions. This observation prompted us to investigate whether TEN1 might play a role in the plant response to thermal stress. Specifically, we asked if heat shock induced a change in telomere structure or integrity in *ten1-3* plants. *ten1-3* is not a null allele, but this mutation destabilizes TEN1 protein *in vivo* and abolishes STN1 binding *in vitro* (Leehy et al., 2013). Two week-old wild-type and *ten1-3* seedlings were placed at 42°C for one hour, and then returned to 23°C to recover. Plant samples were pooled to obtain sufficient material for analysis, and telomere length was assessed by primer extension telomere repeat amplification (PETRA). This PCR-based method assesses telomere length on individual chromosome arms (Heacock et al. 2004). Unlike the telomeres of wild-type seedlings, which range from 2-5 kb, *ten1-3* telomeres are more heterogeneous and on average 1-2 kb shorter (Leehy et al., 2013) (Figure II-1A). Immediately following heat shock, telomere length was unchanged in *ten1-3* mutants, but during the 18-hour recovery period, telomere tracts became more homogenous and shortened by an additional 1.5-2 kb, with most telomeres accumulating at the bottom end of the size range (Figure II-1A). This result was not unique to a single telomere tract (Figure II-1D and II-2A). Furthermore, heat-induced telomere shortening was detected in plants deficient in CTC1, but not STN1 (Figure II-1B, II-1C, Figure II-2B and II-2C). We

conclude that TEN1 and CTC1, but not STN1, are needed to stabilize *A. thaliana* telomeres in response to heat shock.

Name	Accession No.	Relative expression level after treatment									
		38°C				4°C			Oxidative		
		0.25h	3h	3h + 1h recovery	3h + 3h recovery	0.5h	3h	12h	0.5h	3h	12h
TEN1	AT1G56260	1.306	2.276	1.506	1.188	1.544	1.303	1.030	1.434	1.462	1.060
STN1	AT1G07130	0.710	0.596	0.853	0.953	0.577	1.075	0.652	0.768	0.908	0.959
CTC1	AT4G09680	1.068	1.312	1.387	0.934	0.984	0.966	1.288	0.976	0.995	0.981
TERT	AT5G16850	0.991	1.131	0.946	0.992	1.003	0.962	1.135	0.871	0.968	0.890
Ku70	AT1G16970	0.645	0.415	1.319	0.835	0.655	0.564	0.541	0.581	0.540	0.573
Ku80	AT1G48050	1.099	0.399	1.133	0.903	0.961	0.793	0.665	0.976	1.007	0.925
POT1a	AT2G05210	1.687	1.658	1.357	1.337	1.465	1.156	0.859	1.184	1.499	1.424

Table II-1. Transcriptome data for A. thaliana telomere-related transcripts in response to abiotic stressors. Data as reported by Killian et al. 2007 were obtained from the AtGenExpress Visualization Tool using the Affymetrix ATH1 microarray. The untreated basal levels of the mRNAs are set to 1.

A time course experiment showed that heat-induced telomere shortening occurred very rapidly in *ten1-3* mutants. One hour after heat shock, a broader size distribution of telomeres was observed (Figure II-D and Figure II-2D). After three hours, only a faint signal could be detected in the range of untreated telomeres; the majority of telomeres were shorter than the shortest telomeres in untreated *ten1-3*. By 18 hours, telomere appeared to be stabilized at the shorter length set point (Figure II-1D).

We performed quantitative telomere repeat amplification protocol (Q-TRAP) to measure telomerase activity following heat shock. Previously we showed that telomerase activity is elevated in *ten1-3* flowers, reflecting an increase in repeat addition processivity (Leehy *et al*, 2013). In contrast, telomerase activity was not substantially different in *ten1-3* seedlings compared to wild type (Figure II-1E), suggesting that TEN1-mediated control of telomerase is developmentally regulated. Heat stress reduced

telomerase activity slightly in both wild type and *ten1-3* mutants. Enzyme activity levels rebounded 18 hours post-treatment in wild type, increasing 3-fold relative to untreated samples (Figure II-2E). This rebound effect was not observed *ten1-3* seedlings, and telomerase levels declined further to 4.5-fold the level of untreated *ten1-3* mutants. Why *ten1-3* mutants fail to exhibit this rebound effect for telomerase is unknown. Nevertheless, the data indicate that the abrupt heat-induced telomere shortening in *ten1-3* seedlings is not due to abrogation of telomerase activity.

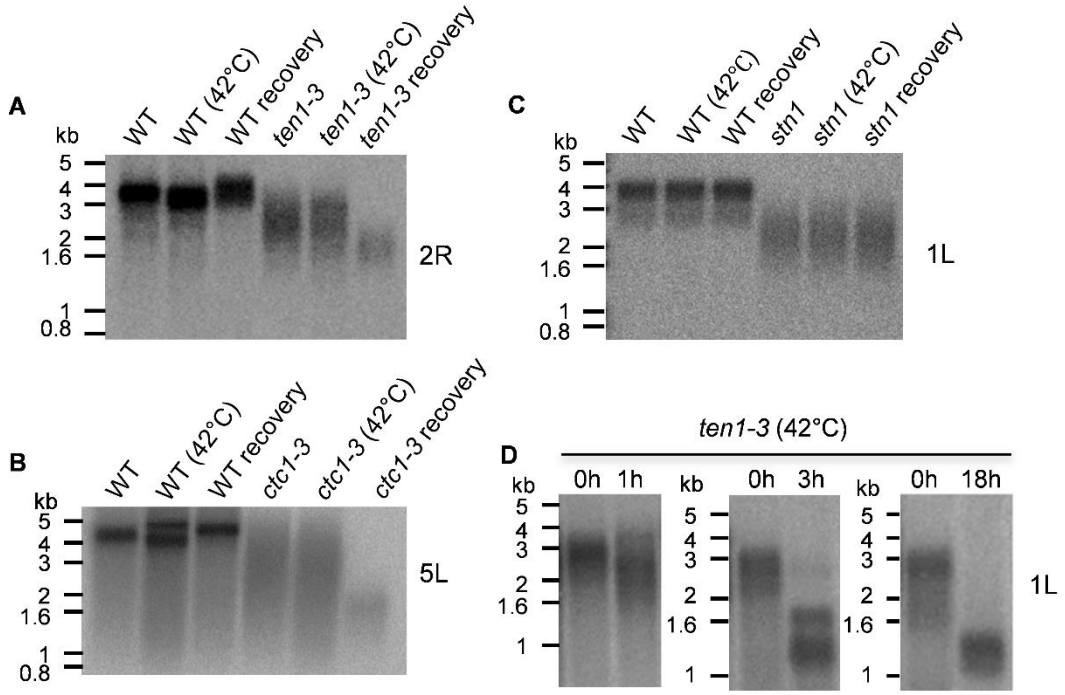

Figure II-1. Plants lacking AtTEN1 or AtCTC1 undergo rapid telomere shortening upon heat shock. (A-D) PETRA was used to measure telomere length of specific chromosome arms following heat shock. Wild type (WT) and mutant plants were subjected to 42°C for 1 hour and then returned to 23°C to recover. PETRA results for *ten1-3* (A and D), *ctc1-3* (B) and *stn1-1* mutants (C) are shown. The telomere monitored, right arm of chromosome 2 (2R), left arm of chromosome 5 (5L) or left arm of chromosome 1 (1L) are indicated. Blots were hybridized with labeled $(TTTAGGG)_5$.

(D) PETRA time course analysis of *ten1-3* telomere length upon heat shock. Representative results of at least three independent experiments are shown.

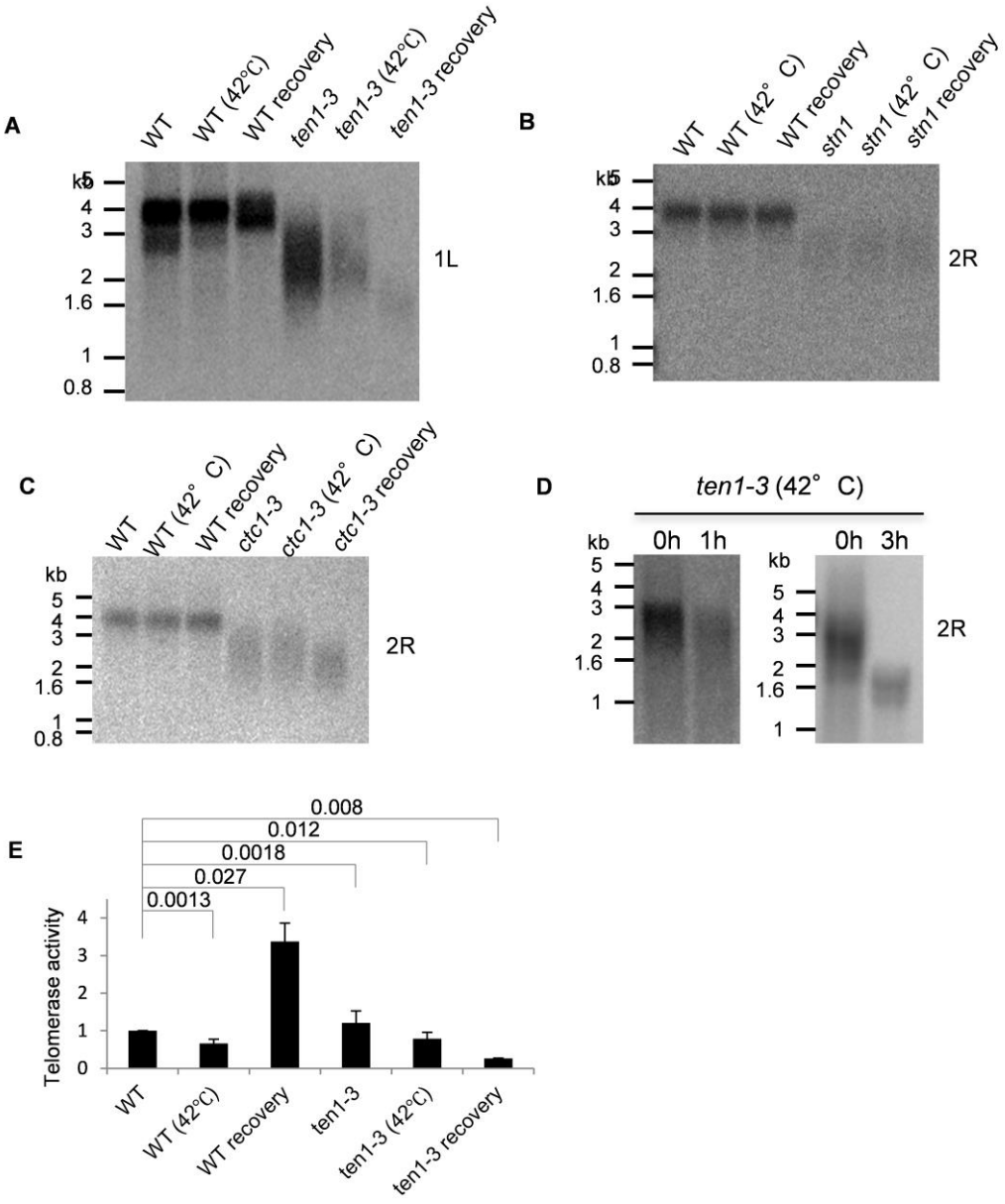

Figure II-2. Heat-induced telomere shortening in *ten1-3* and *ctc1-3* mutants.
(A-D) PETRA was used to evaluate telomere length following heat shock. 1L and 2R represent individual subtelomeric primers. Blots were hybridized ^{32}P labeled $(TTTAGGG)_5$. (D) PETRA of a time course experiment where DNA was isolated 1 hr or 3 h after heat shock. (E) Telomerase activity in two week-old seedlings measured by Q-

TRAP. Data were normalized to WT; each data point represents three biological replicates. *p* values are indicated.

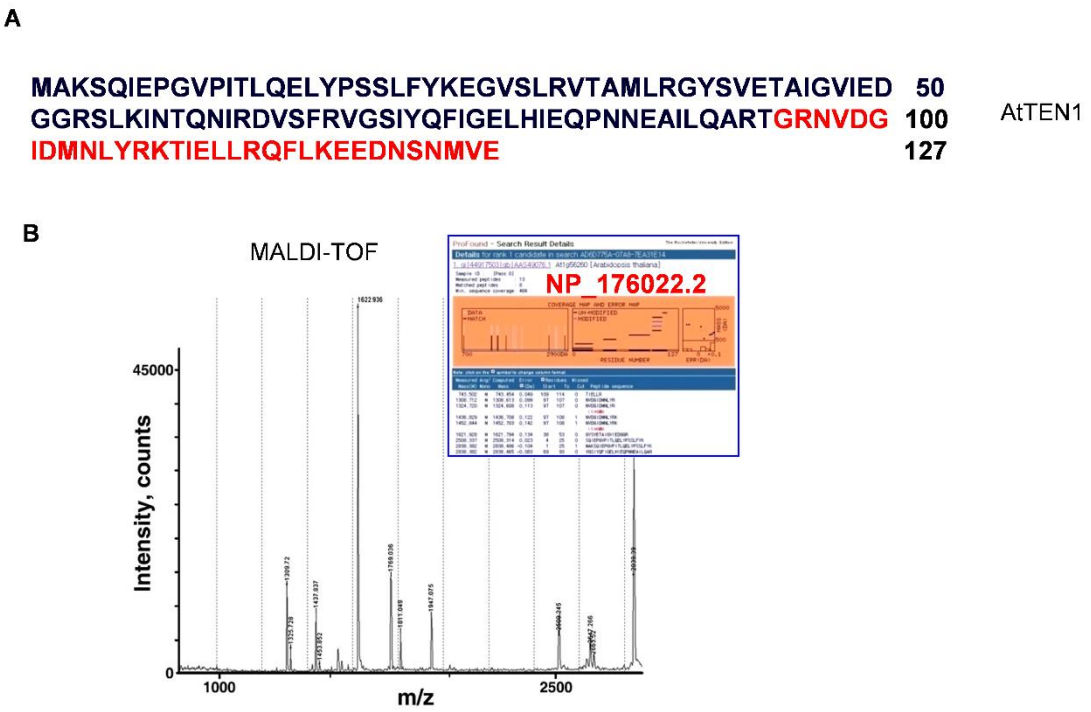

Figure II-3. Characterization of AtTEN1 protein. (A) FoldIndex prediction for AtTEN1. Red indicates a predicted disordered segment in the C-terminus. (B) Mass spectrometry results for purified AtTEN1. Purified protein was subjected to sMALDI-TOF analysis, which identified the protein as AtTEN1.

AtTEN1 exhibits chaperone activity on model protein substrates

PSIPRED and FoldIndex indicated that AtTEN1 forms a single Oligosaccharide Binding-fold with a disordered C-terminus (Figure II-3A) (Leehy et al., 2013; McGuffin et al., 2000; Prilusky et al., 2005). Notably, STN1 and TEN1 orthologs from yeast and vertebrates are not predicted to contain a similar unstructured domain. Since disordered and homo-oligomeric structures as well as thermo-sensitive phenotypes are characteristic

of protein chaperones (Jang et al., 2004; Tompa and Csermely, 2004), we asked if AtTEN1 could act as a molecular chaperone. Recombinant AtTEN1 protein was expressed in *E. coli* and purified to homogeneity as determined by mass spectrometry (Figure II-4A and Figure II-3B). Analysis by SDS-PAGE revealed a single band of 16 kDa, the expected molecular weight of a monomer (Figure II-4A). In contrast, native-PAGE and size exclusion chromatography (SEC) revealed a discrete high molecular weight (HMW) complex of ~160 kDa (Figure II-4B and II-4C). Immunoblotting with an AtTEN1 antibody confirmed that this species was indeed TEN1 (see Figure II-5C).

To assess chaperone activity, we asked whether AtTEN1 could function as a "holdase" chaperone by protecting the model protein substrates malate dehydrogenase (MDH) and citrate synthase (CS) from heat-induced aggregation at 43°C as measured by light scattering (Jang et al., 2004). Incubation of the substrates with increasing amounts of chaperone protein prevents thermal aggregation, which is measured by monitoring turbidity. In the absence of ATP, TEN1 efficiently suppressed thermal aggregation of MDH and CS at a 1:1 molar ratio of substrate to TEN1 (Figure II-4D and II-6). In contrast, denatured TEN1 was unable to stabilize the model substrates (Figure II-4D). More importantly, STN1 did not prevent protein aggregation even in a five-fold molar excess over substrate (Figure II-4D and II-6), indicating that the thermal stability afforded by TEN1 is a specific property of this protein. To verify the chaperone activity of AtTEN1, we compared the activities of AtTEN1 with the well-known chaperone proteins, AtTDX and cPrxI. AtTDX is a plant-specific thioredoxin (Trx)-like redox protein that functions as both a disulfide reductase and a chaperone (Lee et al., 2009).

cPrxI is from yeast and is categorized as a cytosolic 2-Cysteine peroxiredoxin. cPrxl serves as a highly efficient molecular chaperone and a peroxidase; the dual functions are mediated by structural changes in response to different redox states (Jang et al., 2004). We found that AtTEN1 chaperone activity was 1.5 fold higher than the AtTDX (Lee et al., 2009) and similar to the cPrxI (Jang et al., 2004). Thus, we conclude that AtTEN1 can function as a bona-fide chaperone to stabilize denatured model protein substrates.

Heat shock promotes AtTEN1 assembly into higher order spherical structures with increasing chaperone activity

A well-conserved feature of molecular chaperones is their tendency to reversibly assemble into higher order oligomers (Haley et al., 1998; Hendrick and Hartl, 1993). Therefore, we asked if heat-dependent structural changes were associated with AtTEN1. Native-PAGE revealed a marked structural alteration in TEN1 30 minutes after heat shock at 43°C (Figure II-5A). Consistent with native PAGE analysis, AtTEN1 exists predominantly as a HMW complex of ~160 kDa when subjected to SEC (Figure II-4C and II-5B). Following heat shock an extra peak appeared in the void fraction (F-I) (Figure II-5B). Both F-I and F-II fractions were analyzed again by immunoblotting with AtTEN1 antibody after native-PAGE. Unlike the TEN1 complex in F-II, the new ultra-HMW fraction (F-I) did not enter a 10% native gel (Fig. 3C, top). On SDS-PAGE both the HMW and ultra-HMW fractions resolved into a single band with a molecular weight of 16 kDa, corresponding to monomeric TEN1 (Figure II-5C, bottom).

The fluorescence intensity of 1,1′-bi(4-anilino) naphthalene-5,5′-disulfonic acid (bis-ANS) binding to AtTEN1 was greater for the F-I fraction than the F-II fraction (Figure II-5D), indicating that more hydrophobic patches on TEN1 are exposed by heat treatment in ultra-HMW complexes. Increased surface hydrophobicity correlates with increasing chaperone activities of proteins (Jang et al., 2004; Lee et al., 2009). As predicted, chaperone assays based on bis-ANS fluorescence, revealed that the F-I fraction exhibited 3.5-fold higher activity than the F-II fraction (Figure II-5E).

Cryo-electron microscopy was used to investigate the architecture of HMW AtTEN1 (Figure II-5F). The protein concentration of F-I was insufficient for analysis and so F-II was examined. Analysis of 300 particles revealed four distinct size classes ranging from 9 to 13 nm in diameter (Figure II-5F). The 2D architecture of the TEN1 particles is remarkably similar to the small heat shock-related αβ-crystallin chaperones from vertebrates (Braun et al., 2011), which like AtTEN1 assemble into a heterogeneous array of globular structures in response thermal stress. Taken together, these data indicate that the chaperone activity of AtTEN1 is associated with its ability to assume discrete higher order oligomeric structures.

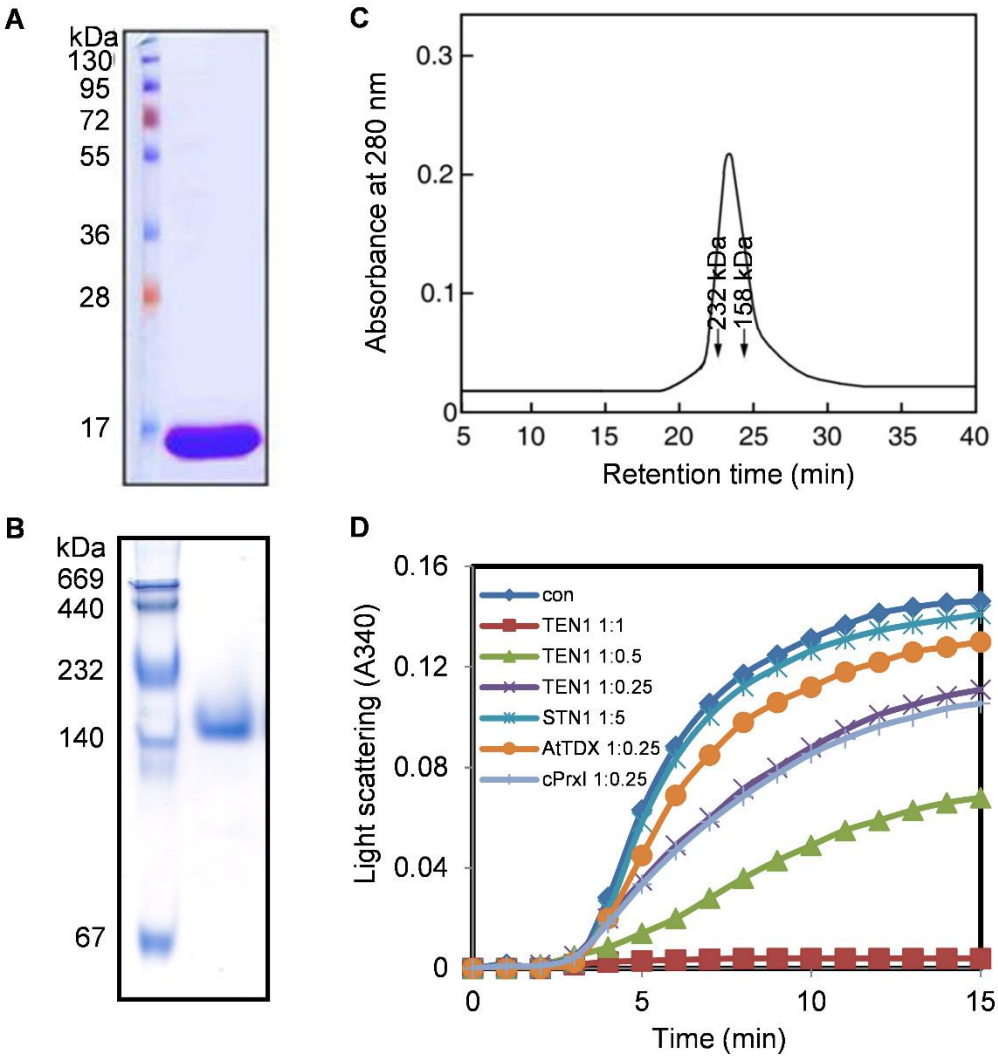

Figure II-4. High molecular weight AtTEN1 complexes and chaperone activity on a model protein substrate. (A) *E. coli* expressed AtTEN1 was resolved by 12% SDS-PAGE and stained by Coomassie Blue. (B) TEN1 HMW complexes were visualized by 10% native-PAGE. Molecular weight makers in kDa are shown. (C) Analysis of AtTEN1 by size exclusion chromatography (SEC). SEC was performed by FPLC using a Superdex 200 HR 10/30 column as described in the Methods. Catalase (232 kDa) and aldolase (158 kDa) markers are indicated. (D) Chaperone activity was measured by using 1.5 µM malate dehydrogenase (MDH) as a substrate. Thermal-aggregation of the substrate was examined in the presence of the proteins indicated. Reactions with AtTEN1 were conducted at molar ratios of substrate to AtTEN1 at 1:0.25, 1:0.5 and 1:1 at 43°C. Also shown are data for *A. thaliana* thioredoxin-like chaperone (AtTDX), yeast peroxiredoxin (cPrxl) and a negative control reaction with only MDH substrate (con).

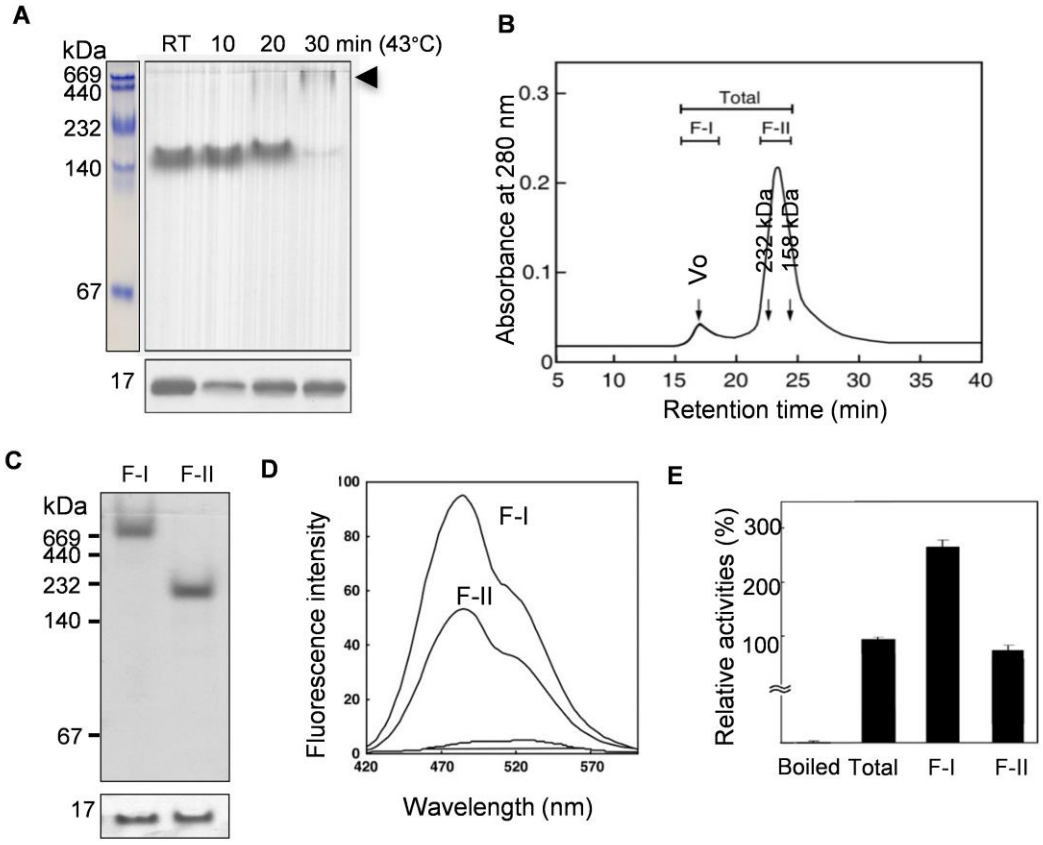
A
kDa
RT 10 20 30 min (43°C)
669
440
232
140
67
17
B
Absorbance at 280 nm
Total
F-I
F-II
Vo
232 kDa
158 kDa
Retention time (min)
C
F-I F-II
kDa
669
440
232
140
67
17
D
Fluorescence intensity
F-I
F-II
Wavelength (nm)
E
Relative activities (%)
Boiled Total F-I F-II
F
TEN1
αB-crystallin
smaller
(9nm)
larger
(13nm)

Figure II-5. AtTEN1 assumes higher order oligomeric structures. The oligomeric status of AtTEN1 was analyzed by chromatographic, electrophoretic and cryo-EM techniques. (A) AtTEN1 was subjected to heat treatment at 43°C for the times indicated followed by western blotting with AtTEN1 antibody after separating the protein by 10% native (top) or SDS-PAGE (bottom). (B) Size exclusion chromatography (SEC) was performed on heat-treated protein for 20 min as in panel A. Molecular weight standards are indicated. Purified proteins were divided and collected into two fractions (F-I and F-II). (C) The F-I and F-II fractions were concentrated and subjected to western blotting with AtTEN1 antibody after resolution by 10% native or SDS-PAGE. (D) F-I and F-II fractions were concentrated, and changes in TEN1 hydrophobicity were measured using a bis-ANS probe, which binds to hydrophobic clusters of aminoacyl residues. (E) Relative activity of chaperone function was analyzed based on bis-ANS fluorescence. The activities of SEC fractions were compared to total protein, whose activity was set to 100%. Denatured (boiled) AtTEN1 protein was included as a negative control. Data represent means of at least three independent experiments. (F) 2D class-averages of cryo-EM data for TEN1 F-II complexes are indicated with a representative of each class. The fractions of molecules in each class are 26%, 27%, 23% and 24%, from top to bottom. The size of each class-average is 21nm x 21nm. The 2D class average of the 13nm-diameter TEN1 oligomer shows features similar to the αB-crystallin image. A 2D projection of the αB-crystallin generated from its density map obtained from the EM databank (Accession ID: EMD 1776) is shown in the right column. The projection image of αB-crystallin is 25nm x 25nm.

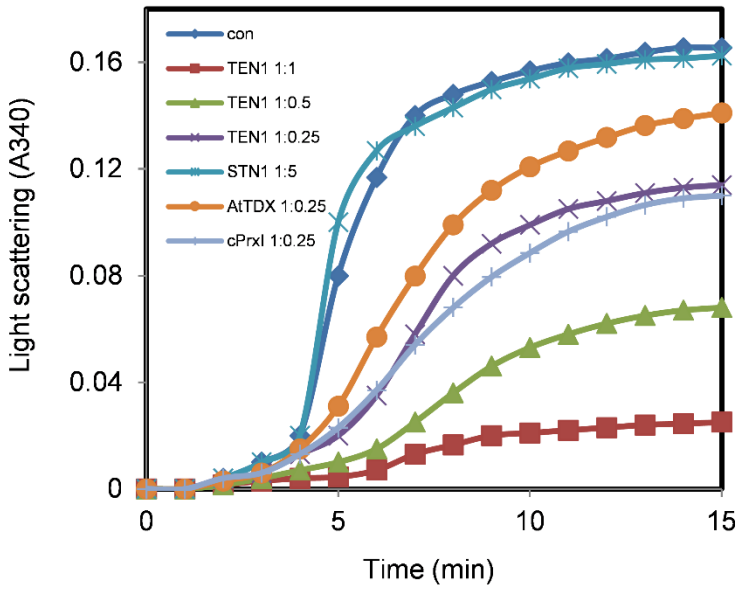

Figure II-6. Chaperone activity of *A. thaliana* TEN1 protein. Chaperone activity was measured by using 1.5 µM citrate synthase (CS) as a substrate. Thermal-aggregation of the substrate was examined in the presence or absence of AtTEN1 at molar ratios of CS to TEN1 of 1:0.25, 1:0.5 and 1:1 at 43°C. Also shown are data for *A. thaliana* thioredoxin-like chaperone (AtTDX), yeast peroxiredoxin (cPRxl) and a negative control reaction with only CS substrate (con).

Arabidopsis TEN1 protects CTC1 from thermal-induced aggregation in vitro and protein degradation in vivo

Since AtTEN1 has protein chaperone activity, we asked if it could stabilize CTC1. We previously reported robust interactions between *A. thaliana* TEN1 and STN1 (Leehy et al., 2013) and STN1 with CTC1 (Surovtseva et al., 2009). A weak interaction between AtTEN1 and AtCTC1 was observed by *in vitro* co-immunoprecipitation (co-IP)

(Figure II-7A) and yeast two-hybrid analysis (Figure II-7B), consistent with previous studies showing weak interaction between yeast Ten1 and Cdc13 (Grandin et al., 2001).

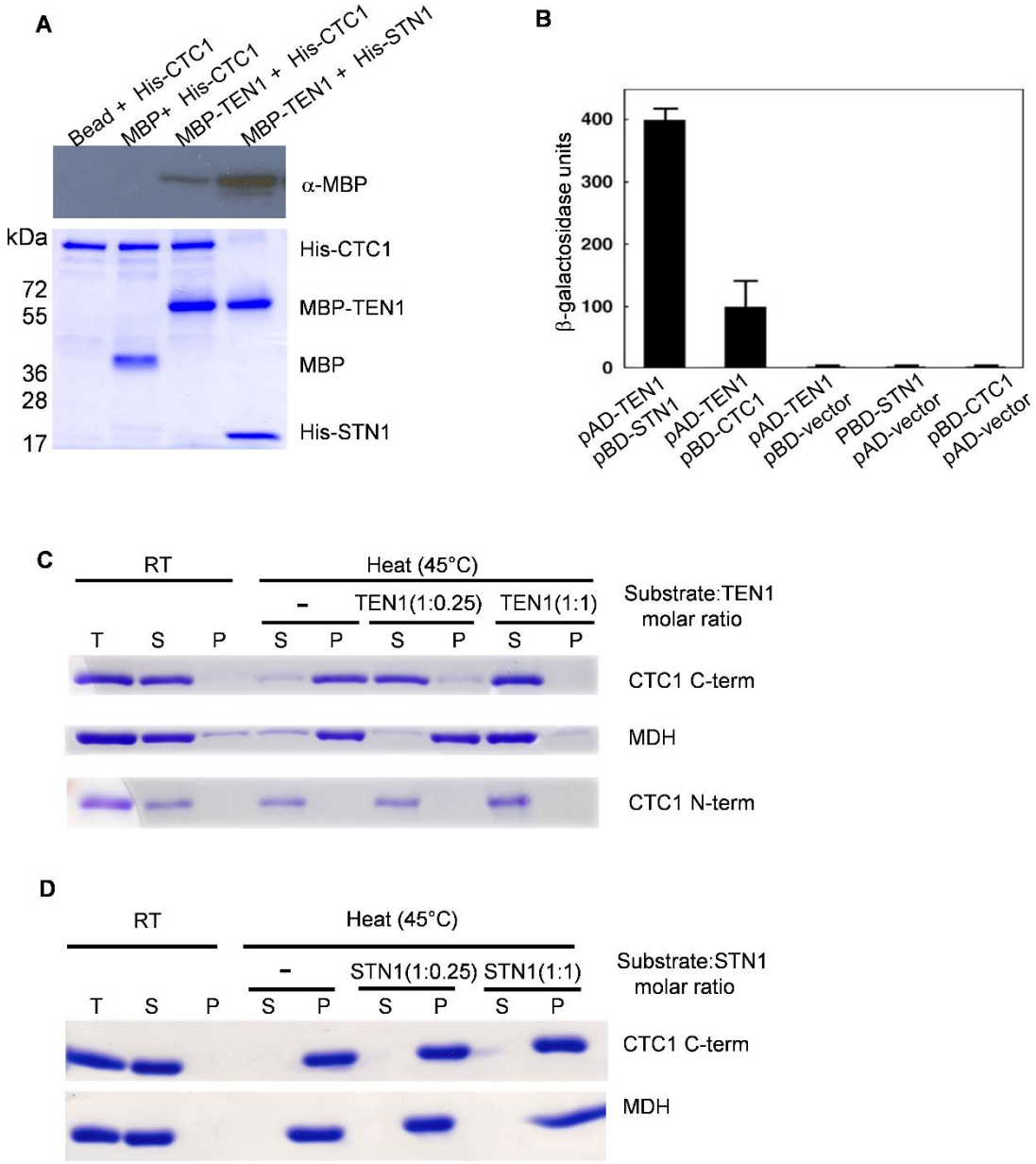

Figure II-7. AtTEN1 protects CTC1 from thermal-induced aggregation *in vitro*. (A) Co-IP western blot data with recombinant MBP-fusion CST proteins and His-tagged CST proteins are shown. Immunoprecipitation of MBP-fusion TEN1 using His tagged

CTC1 and STN1 recombinant proteins by anti-His antibody conjugated agarose beads. Co-IP protein input controls with same amounts included (lower panel). (B) Results of yeast two-hybrid assays for CST interactions. Numbers indicate arbitrary units to show relative activities of protein-protein interactions using ONPG activity. pAD and pBD denote an activation domain or a binding domain containing vector. TEN1, STN1 and CTC1 with empty vectors are included as controls for self-activation. Values represent means of at least three independent experiments. (C, D) *In vitro* protection assay. (C) AtTEN1 protects AtCTC1 from heat-induced aggregation. CTC1 N-term (1-384 a.a.), CTC1 C-term (385-1272 a.a.) and MDH were incubated at RT and 45°C for 30 min with or without AtTEN1. (D) AtSTN1 does not protect CTC1 from heat-induced aggregation. CTC1 C-term (385-1272 a.a.) and malate dehydrogenase (MDH) were incubated at RT and 45°C for 30 min with or without STN1. For panel C and D, equal amounts of total protein (T) were centrifuged and divided into soluble- (S; stable) or pellet- (P; unstable) fractions. Proteins were resolved by SDS-PAGE followed by Coomassie staining.

We were unable to express full-length AtCTC1 in *E. coli.* Therefore, we generated two constructs that covered the amino (CTC1 N-term (1-384 aa)) and carboxy (CTC1 C-term (385-1272 aa)) regions of the protein. MDH and the two CTC1 constructs were expressed in *E. coli* and subjected to heat denaturation with or without AtTEN1. MDH and CTC1 were soluble at room temperature (RT), but following heat shock most of the MDH and CTC1 C-term became insoluble (Figure II-7C and D). In contrast, CTC1 N-term was heat stable (Figure II-7C). Addition of AtTEN1 at a molar ratio of 1:1 relative to substrate protected both MDH and CTC1 C-term from aggregation. When the ratio of substrate to AtTEN1 was reduced to 1:0.25, CTC1 C-term was solubilized, but not MDH, arguing that AtCTC1 is preferentially protected by AtTEN1 (Figure II-7 C). To test if heat stabilization of CTC1 simply reflected an interaction with a binding partner, we asked if AtSTN1 could stabilize the CTC1 C-term since the binding site for AtSTN1 lies within this region (Surovtseva et al., 2009). In marked contrast to AtTEN1, AtSTN1 failed to prevent CTC1 C-term aggregation even at a 1:1 molar ratio of AtSTN1 to CTC1 C-term (Figure II-7D). This result is consistent with results from our chaperone

activity assays for AtTEN1 and AtSTN1 on model protein substrates. Taken together, these findings provide additional support for a thermal protection function for AtTEN1, and not AtSTN1. The results also argue that AtCTC1 is a specific *in vitro* substrate for AtTEN1 activity.

We next asked if AtTEN1 stabilizes CTC1 *in vivo.* Immunoblotting with an AtCTC1 antibody detected two very diffuse bands in wild type plants (Figure II-8A and II-9B). The upper bands ranged in size from ~95 to 150 kDa, encompassing full-length AtCTC1 (~142 kDa). Two diffuse lower molecular weight bands of 55 kDa and 75 kDa were also visible, likely representing proteolytic AtCTC1 breakdown products (Figure II-8A). A similar profile was observed in *stn1-1* and *ten1-3* mutants, although the 72 kDa product was absent. As expected, CTC1 was not detected in *ctc1-3* mutants (Surovtseva et al., 2009) (Figure II-8A), verifying the specificity of the AtCTC1 antibody.

In wild-type plants, thermal stress decreased the relative abundance of AtCTC1 breakdown products and increased the fraction of full-length CTC1 (Figure II-8A). The same result was observed in *stn1* mutants, demonstrating that AtSTN1 is not required for AtCTC1 stability *in vivo*. In marked contrast, no full-length AtCTC1 was detected in *ten1-3* mutants upon heat shock, only a series of bands ranging from ~100 kDa to 55 kDa. Conversely, in a *ten1-3* genetic complementation line where ectopic expression of AtTEN1 protein is ~2 fold higher than AtTEN1 in wild-type plants (Leehy et al., 2013), full-length CTC1 was stabilized and the 55 kDa breakdown product decreased. These

observations indicate that CTC1 is protected from thermal-induced degradation by TEN1 *in vivo*.

We next assessed the oligomeric status of endogenous AtTEN1 after thermal stress. In the absence of heat shock, AtTEN1 migrated as a discrete band between 232 and 140 kDa on native PAGE (Figure II-8B). Because human and yeast CST complexes exhibit an apparent molecular mass of ≥500 kDa in SEC (Lue et al., 2013; Miyake et al., 2009), we suspect this AtTEN1-containing complex does not represent a trimeric Arabidopsis CST. Strikingly, under the same heat shock conditions that destabilized AtCTC1, AtTEN1 formed extremely diffuse ultra-HMW complexes ranging from 160 to at least 669 kDa (Figure II-8B). 18 hours after heat-treated seedlings were restored to 23°C, the ultra HMW complexes diminished significantly, and by 36 hours, AtTEN1 returned to its pre-heat shock size (Figure II-8B). SDS-PAGE indicated that the steady state level of AtTEN1 was essentially unchanged throughout the time course (Figure II-8B, bottom). Whether the lower MW AtTEN1 complexes that accumulated during the recovery period represent disassembled ultra HMW complexes or new AtTEN1 synthesis is unknown.

In conjunction with formation of ultra HMW AtTEN1 complexes, heat shock disrupted AtTEN1 binding to AtSTN1 *in vitro* (Figure II-8C) and *in vivo* as shown by a heat denaturation experiment performed with tobacco leaves transiently expressing AtTEN1 and AtSTN1 (Figure II-8D). These results indicate that thermal stress triggers a major conformational shift in AtTEN1, which is coincident with decreased binding to AtSTN1, increased chaperone activity, and AtCTC1 stabilization.

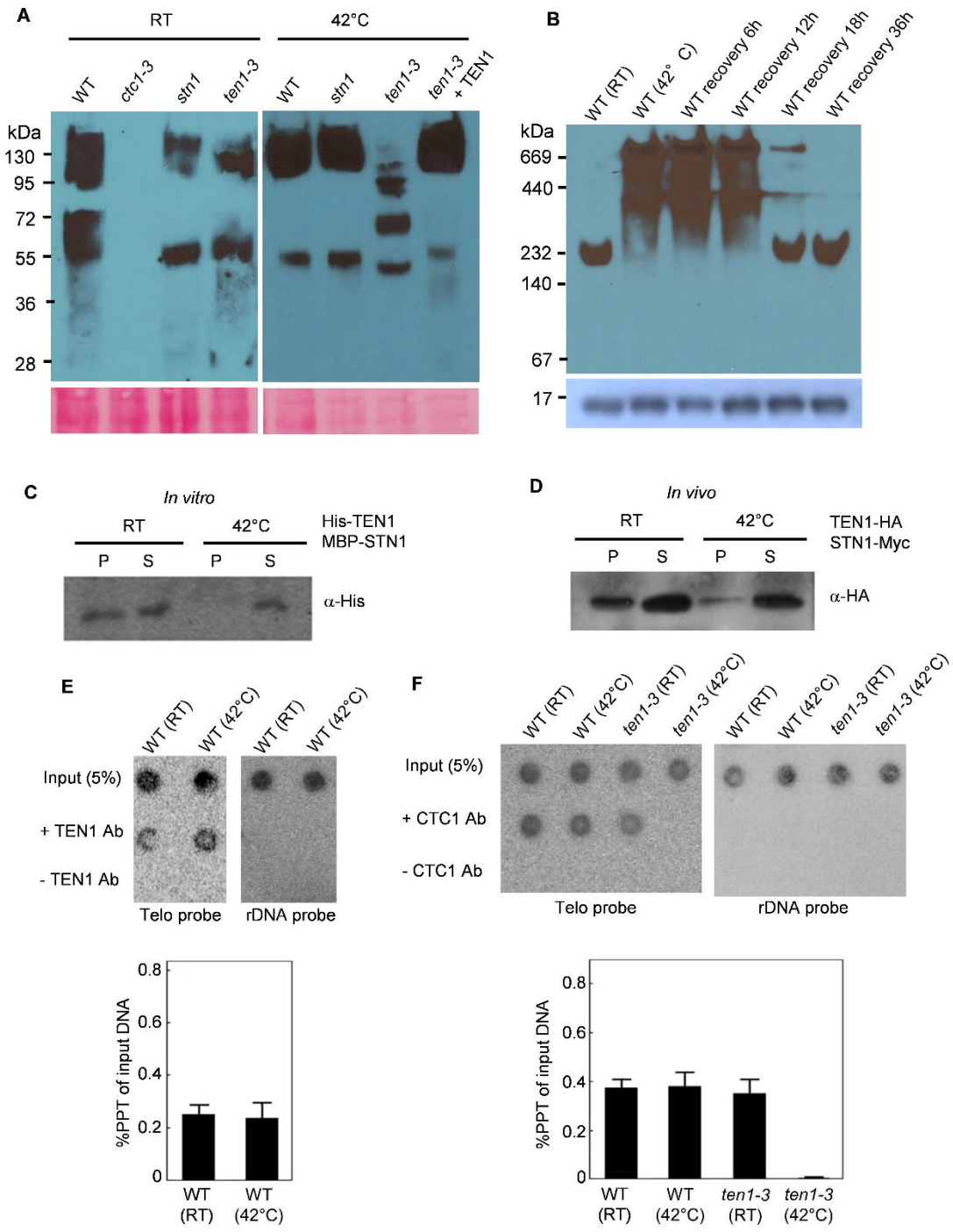

Figure II-8. AtTEN1 promotes CTC1 stability and telomere association following heat shock. (A) Heat stress destabilizes AtCTC1 *in vivo*. WT, *stn1*, *ten1-3* and an AtTEN1 complementation line of *ten1-3* mutant plants were incubated at RT or at 42°C

for 30 min. Equal amounts of total protein were resolved by SDS-PAGE followed by western blotting with AtCTC1 antibody. An image of the *Ponceau S* stained membrane is shown in the bottom panel. (B) AtTEN1 assembles into higher molecular weight oligomers in response to heat shock. Western blot analysis of AtTEN1 was performed on total protein isolated from plants kept at RT or subjected to heat shock at 42°C for 30 min. Extracted protein was resolved by 10% native-PAGE. (C-D) Temperature-dependent interaction of AtTEN1 with STN1 *in vitro*. (C) Results of co-IP assays performed with *E. coli* expressed His-TEN1 and MBP-STN1 in the presence or absence of heat treatment. IP was performed with anti-MBP antibody followed by western blotting with anti-His antibody. S, supernatant; P, pellet. (D) AtTEN1-HA and AtSTN1-Myc were transiently expressed in tobacco leaves and TEN1 was pulled–down by with anti-Myc. The western blot was probed with anti-HA antibody. (E-F) Telomeric DNA association of AtCTC1 and AtTEN1. ChIP was performed on WT and *ten1-3* mutants treated or untreated with heat shock using anti-TEN1 antibody (E) and anti-CTC1 antibody (F) followed by dot blot analysis with a [^{32}P] 5′ end labeled $(T_3AG_3)_5$ oligonucleotide probe. A [^{32}P] 5′ end labeled rDNA (18S+5S) was used as a control probe. Quantification of TEN1 and CTC1 telomere ChIP data (lower panel). IP signal is represented as percent precipitation of input DNA. Error bars represent standard error of the mean from three independent biological replicates.

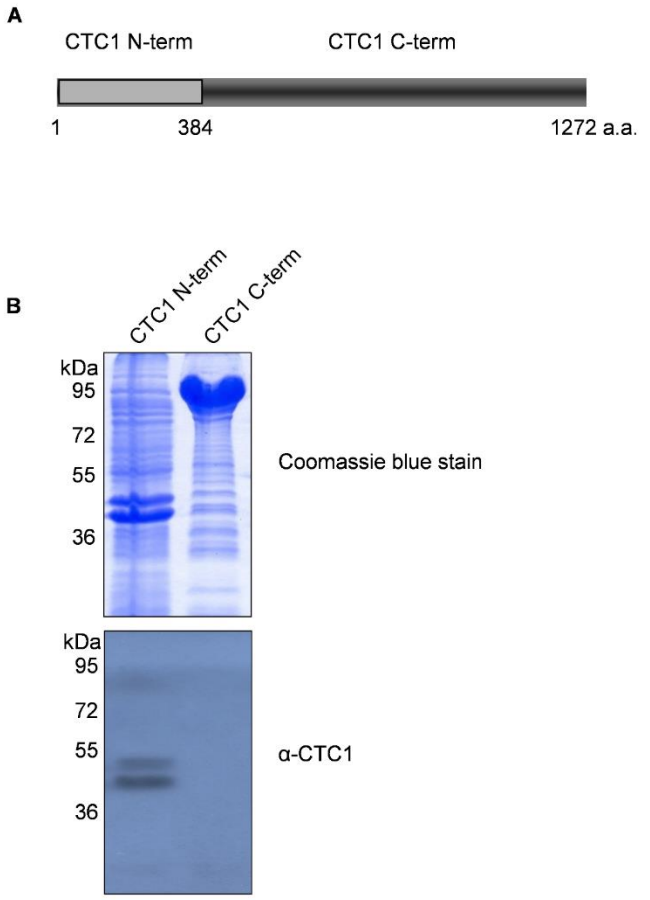

Figure II-9. Specificity of the CTC1 polyclonal antibody. (A) Diagram of CTC1 N-term and C-term expression constructs. (B) Western blot results for CTC1 N-term and C-term recombinant proteins induced by IPTG in *E. coli* are shown. A Coomassie blue stained loading control is included. Molecular size markers in kDa are on the left. The blot was probed with a polyclonal antibody raised against Arabidopsis CTC1 N-term.

Arabidopsis TEN1 stabilizes the association of CTC1 with telomeres following heat shock

Since heat shock causes AtTEN1 to assemble into ultra HMW oligomers, we asked if telomere-bound AtTEN1 increases under these conditions. Chromatin

Immunoprecipitation (ChIP) revealed no significant difference in the level of telomere-associated AtTEN1 before or after heat shock (Figure II-8E), suggesting that HMW TEN1 complexes do not accumulate at telomeres. Finally, we asked if the abrupt telomere shortening associated with heat stress in *ten1-3* mutants reflects the loss of CTC1 from telomeres. There was no difference in the telomere association of CTC1 in wild type in the presence or absence of heat shock (Figure II-8F). In addition, the level of telomere-bound CTC1 in *ten1-3* mutants was similar to wild type at room temperature, indicating that TEN1 is not required for CTC1 localization at chromosome ends. In contrast, CTC1 binding to telomeric DNA was completely abolished when *ten1-3* plants were subjected to heat shock (Figure II-8F). We conclude that AtTEN1 stabilizes telomere-bound AtCTC1 under heat stress to promote telomere integrity.

Discussion

The telomere is a remarkably dynamic region of the genome that fluctuates in each cell cycle from a sequestered, fully protected state to an open conformation accessible to the replication machinery. The molecular basis for these conformational switches is largely unknown. In this study we provide evidence that a core constituent of the CST complex is itself a highly dynamic protein with molecular chaperone activity.

Chaperone activity of AtTEN1

Our results show that AtTEN1 has the biochemical, biophysical, and structural properties of a small heat shock-like protein chaperone. AtTEN1 prevents thermal

aggregation of model protein substrates *in vitro* with activity that is robust and comparable to other anti-aggregation chaperones. As is typical for small heat shock chaperones (Haslbeck et al., 2005; Hendrick and Hartl, 1993; Sun and MacRae, 2005), thermal stress triggers an increase in AtTEN1 hydrophobicity and elevates its chaperone activity. In addition, the heat-induced biochemical changes within AtTEN1 are accompanied by a large conformational shift, resulting in AtTEN1 dissociation from AtSTN1 and assembly into ultra-HMW homo-oligomeric spheres. In contrast to the archetypal small heat shock proteins that form homogeneous globular structures consisting of either 12 or 24 subunits (Haslbeck et al., 2005), our cryoEM data indicate that HMW TEN1 oligomers more closely resemble αβ-crystallins of the vertebrate eye lens (Braun et al., 2011). In response to heat shock, αβ-crystallins assemble into a heterogeneous population of spherical frameworks ranging in size from 6- or 12-mers to 24- or 48-mers (Braun et al., 2011; Peschek et al., 2013). Both conventional sHsps and αβ-crystallin harbor a large internal cavity (Haslbeck et al., 2005; Peschek et al., 2009; Raman and Rao, 1997), which also may be present in AtTEN1. It is possible that the larger AtTEN1 particles (13nm) provide an interior scaffold to stabilize substrate proteins, while smaller AtTEN1 complexes represent assembly intermediates. 3D reconstruction analysis will be necessary to test this hypothesis.

Although canonical sHsps bind a wide range of target proteins, αβ-crystallins preferentially stabilize certain classes of substrates such as aquaporin 0 (AQP0) (Swamy-Mruthinti et al., 2013). AtTEN1 may also have preferential targets *in vivo*, one being AtCTC1. We found that AtTEN1 not only protects AtCTC1 from thermal-induced

aggregation *in vitro* and protein degradation *in vivo*, but also is required to stabilize telomere-bound AtCTC1 in response to heat stress. In contrast, AtSTN1 fails to exhibit chaperone activity on model substrates, and cannot protect AtCTC1 from thermal aggregation, indicating that AtTEN1-mediated protection of AtCTC1 is specific. Importantly, because AtSTN1 binds AtCTC1 with higher affinity than AtTEN1 (Chen et al., 2013; Miyake et al., 2009), the thermal stabilization of AtCTC1 by AtTEN1 is consistent with a catalytic chaperone activity rather than stabilization as a stoichiometric member of the CST complex.

A chaperone-related function for AtTEN1 is attractive given the central role of CST in coordinating the exchange of macromolecular DNA replication complexes on chromosome ends during S phase. While chaperones have previously been implicated in the assembly and disassembly of large telomere-related complexes (DeZwaan et al., 2009; Forsythe et al., 2001; Grandin and Charbonneau, 2001), the specificity of these interactions, and their precise role in stimulating telomere maintenance is unclear. Our data define AtTEN1 as a multi-functional protein that forms a stable binary complex with AtSTN1, but in the presence of heat shock can dissociate from AtSTN1 and assemble into HMW homo-oligomers with chaperone activity. We note that the intrinsically disordered domain within AtTEN1 is predicted to encompass the STN1 binding interface, based on the yeast and human TEN1-STN1 crystal structures (Bryan et al., 2013; Sun et al., 2009). This observation can explain our failure to obtain AtTEN1 mutants that separate STN1 binding from homo-oligomerization: if the two outcomes are

mutually exclusive, AtSTN1 binding could negatively regulate the chaperone activity of AtTEN1.

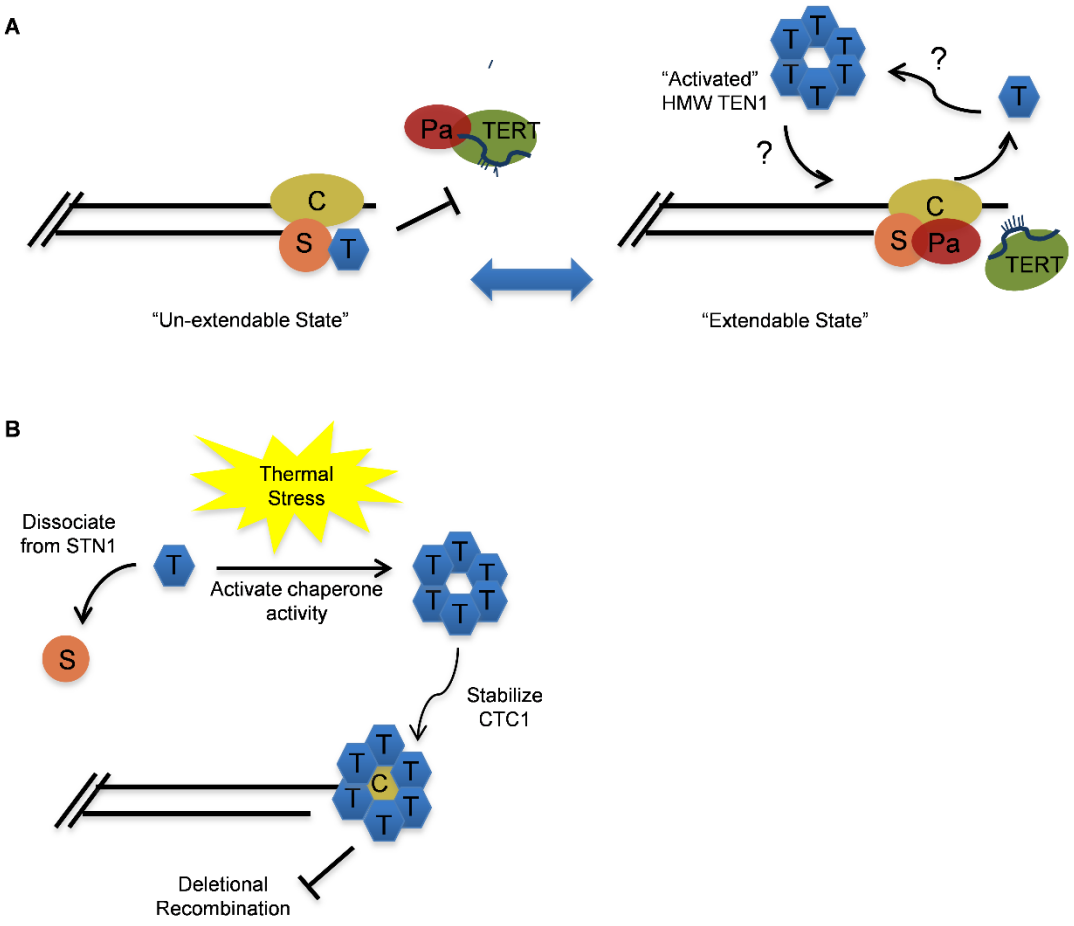

Figure II-10. A speculative model for dynamic interactions of AtTEN1. (A) AtTEN1 may play a role in regulating the switch of telomeres from the telomerase un-extendable to the extendable state. The CST complex is proposed to form a protective cap for *A. thaliana* telomeres. Unlike STN1 and CTC1, TEN1 is not associated with enzymatically active telomerase. Thus, the exchange of TEN1 for POT1a may help to convert telomere ends into a telomerase extendable state. Once dislodged from STN1, TEN1 is free to form "activated" higher order structures (illustrated for simplicity as a hexamer) with chaperone activity. Association of high molecular weight (HMW) TEN1 with CTC1 could facilitate conformational changes in CTC1 that promote the access of telomerase or DNA Pol-a to the chromosome end. (B) In response to thermal stress, TEN1 is dislodged from STN1 and assembles into "activated" HMW complexes that stabilize CTC1 and protect telomeres from deletional recombination (TRD). Abbreviations: C, CTC1; S, STN1; T, TEN1; Pa, POT1a.

A role for AtTEN1 in controlling telomere dynamics

Figure 6 presents a speculative model for how dynamic interactions of AtTEN1 affect telomere maintenance and stability. We previously showed that AtTEN1 is a negative regulator of telomerase (Leehy et al., 2013), and further that binding of TEN1 and the telomerase processivity factor POT1a with STN1 is mutually exclusive (Renfrew et al., 2014). Thus, exchange of AtTEN1 for POT1a could help to convert telomeres to a telomerase-accessible conformation (Figure II-10A). After STN1 disengages, AtTEN1 is free to assemble into higher order complexes with chaperone activity. "Activated" AtTEN1 has the potential to stabilize different conformations of CTC1 to coordinate several protein exchanges. For example, once the telomeric G-strand is extended by telomerase, CTC1/STN1 must swap telomerase for DNA Pol-αto enable synthesis of the C-rich telomeric strand (Huang et al., 2012; Qi and Zakian, 2000). In addition, when telomere replication is complete, Pol-α must be dislodged to generate a fully protected, inaccessible chromosome terminus. We suspect that direct interactions of AtTEN1 with CTC1 are transient. We detected only weak binding between these proteins *in vitro*, and AtTEN1 occupancy at telomeres is significantly lower than CTC1 (Leehy et al., 2013; Surovtseva et al., 2009). Even after heat shock when TEN1 forms ultra-HMW oligomers, there is no substantial increase in telomere-bound TEN1. CST remains the best candidate for a chromosome capping complex in *A. thaliana*, since functional shelterin orthologs have yet to be defined (Fulcher and Riha, 2015; Nelson and Shippen, 2012). Consequently, reestablishing the STN1-TEN1 interaction may be an integral part

of terminating the telomere replication cycle and establishing a fully protected, telomerase inaccessible state (Figure II-10A).

TEN1 as a guardian for telomere integrity

In addition to a potential role in modulating telomere replication, our data indicate that TEN1 protects *A. thaliana* telomeres from thermal stress by stabilizing CTC1 (Figure II-10B). In the absence of TEN1, telomeres in *A. thaliana* seedlings shorten abruptly and dramatically following heat shock. We considered the possibility that heat-induced telomere shortening was caused by depletion of telomerase activity. Although we observe a slight decrease in telomerase activity upon heat stress, *tert* mutants completely devoid of telomerase activity lose telomeric DNA at a rate of 1 kb across an entire plant generation (Riha et al. 2001), less DNA than is lost in the 18-hour window following heat shock. An alternative explanation for abrupt telomere shortening is that heat stress induces replication fork stalling in the telomeric duplex region, which can trigger double-strand DNA breaks and loss of telomeric DNA (Baird, 2008). However, plants at this developmental stage undergo cell division on average only once every 18 hours (Beemster and Baskin, 1998), and thus replication fork stalling is unlikely to account for the precipitous loss of telomeric DNA. The most likely explanation for heat-induced telomere shortening is DNA processing by a non-replicative recombination mechanism such as TRD.

How could thermal stress lead to TRD? Plant genomes are exquisitely responsive to environmental assault and a variety of abiotic stressors including radiation (Lebel et

al., 1993), heavy metals (Rahavi et al., 2011), and elevated temperature (Boyko et al., 2010) as well as biotic stresses in the form of pathogen attack (Kovalchuk et al., 2003) increase the frequency of homologous recombination (HR). Elevated HR can persist for multiple plant generations even after the stress is eliminated (Molinier et al., 2006). Whereas a high rate of HR increases the ability to adapt to adverse conditions, it may also stimulate TRD. Thus, a telomere-associated chaperone such as AtTEN1 would be a useful weapon for plants to avert TRD in a hostile environment. Studies in human cells support the conclusion that telomere protein complexes evolved multiple interconnected strategies to stabilize and actively restore the integrity of chromosome ends in response to environmental assault. Human telomeres, for example, are less susceptible to UV induced photo-adducts than bulk chromosomal DNA (Parikh et al., 2015). Further, the shelterin components POT1, TRF1, and TRF2 physically interact with and stimulate factors necessary for repair of oxidative and UV damage (Miller et al., 2012; Parikh et al., 2015). In Arabidopsis, both AtTEN1 protein and mRNA are rapidly responsive to temperature, and perhaps other environmental stimuli. We hypothesize that the complex regulation of AtTEN1 and its chaperone function define a regulatory pathway linking telomere protein to environmental stress and genome stability in plants.