Flap Endonuclease 1 Promotes Telomere Replication and Stability by Distinct Mechanisms on the Leading and Lagging Strands

Daniel Cole Teasley
Washington University in St. Louis

Follow this and additional works at: http://openscholarship.wustl.edu/art_sci_etds
Part of the Biology Commons

Recommended Citation

This Dissertation is brought to you for free and open access by the Arts & Sciences at Washington University Open Scholarship. It has been accepted for inclusion in Arts & Sciences Electronic Theses and Dissertations by an authorized administrator of Washington University Open Scholarship. For more information, please contact emily.stenberg@wustl.edu, digital@wumail.wustl.edu.
Flap Endonuclease 1 Promotes Telomere Replication and Stability by Distinct Mechanisms on the Leading and Lagging Strands

by

Daniel C. Teasley

A dissertation presented to the Graduate School of Arts & Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

August 2015
Saint Louis, Missouri
Table of Contents

List of figures........................................................................................................................................... v

Acknowledgments....................................................................................................................................... vii

Abstract..................................................................................................................................................... ix

Chapter 1: Introduction to telomere biology................................................................................................. 1
  Introduction.............................................................................................................................................. 2
  Telomere structure and proteins.................................................................................................................. 3
    Telomere DNA...................................................................................................................................... 3
    Telomere secondary structure................................................................................................................ 5
    Proteins associated with the telomere .................................................................................................... 6
  Telomere replication and length maintenance............................................................................................ 9
    Semi-conservative telomere replication................................................................................................. 10
    Telomerase.......................................................................................................................................... 13
    Recombination at telomeres and alternative lengthening of telomeres .............................................. 15
  Telomere physiology.............................................................................................................................. 18
    Senescence and telomere crisis.............................................................................................................. 18
    Telomeres in cancer cells..................................................................................................................... 19
    Non-cancer telomere-related diseases................................................................................................. 21
  References............................................................................................................................................... 27

Chapter 2: FEN1 ensures telomere stability by ensuring replication fork re-initiation................................. 33
  Introduction.............................................................................................................................................. 34
  Experimental Procedures......................................................................................................................... 37
  Results.................................................................................................................................................... 42
    FEN1 depletion does not impact S phase progression........................................................................ 42
    FEN1 depletion does not impact DNA replication kinetics in vitro.................................................. 43
    FEN1 depletion leads to inefficient replication fork restart............................................................... 44
    FEN1 localizes to the telomere............................................................................................................. 49
    FEN1 depletion leads to the induction of fragile telomeres............................................................... 50
    FEN1 DNA replication fork re-initiation activity is critical to telomere stability.................................. 51
  Discussion............................................................................................................................................... 53
  Acknowledgments.................................................................................................................................. 58
  References............................................................................................................................................... 69

Chapter 3: Flap endonuclease 1 limits telomere fragility on the leading strand.............................................. 80
  Introduction............................................................................................................................................ 81
List of figures

Chapter 1: Introduction and significance

Figure 1.1. A diagrammatic representation of telomere structure and telomere binding proteins 24
Figure 1.2. A depiction of telomere length over the course of cell divisions 26

Chapter 2: FEN1 ensures telomere stability by ensuring replication fork re-initiation

Figure 2.1. FEN1 depletion does not affect S phase progression or in vitro DNA replication 59
Figure 2.2. FEN1 depletion decreases re-initiation of stalled replication forks 61
Figure 2.3. The gap endonuclease activity and C terminus of FEN1 are essential to re-initiate stalled replication forks 63
Figure 2.4. FEN1 mutants localize to the telomere 65
Figure 2.5. FEN1 depletion results in fragile site expression at telomeres 66
Figure 2.6. The gap endonuclease activity of FEN1 is essential for its function at the telomere 67

Chapter 3: Replisome–RNAP collisions on the leading strand induce telomere fragility

Figure 3.1. α-amanitin treatment abrogates expression of mRNAs with short half lives but does not alter steady-state TERRA levels 108
Figure 3.2. FEN1 depletion and transcription inhibition induce replication stress, a DNA damage response, and telomere fragility 110
Figure 3.3. RNA:DNA hybrids are responsible for FEN1 depletion-induced leading strand-specific telomere fragility 112
Figure 3.4. RNA:DNA hybrids are responsible for α-amanitin-induced telomere fragility 114
Figure 3.5. FEN1 flap endonuclease activity is required to limit leading strand-specific telomere fragility 116
Figure 3.6. A model of FEN1’s role following co-directional replisome–RNAP collisions 118

Chapter 4: FEN1 interacts with TRF1

Figure 4.1. FEN1 interacts with TRF1 147
Figure 4.2. FEN1’s interactions with TRF1 and TRF2 are mediated by its C-terminal domain 148
Figure S4.1. Flap endonuclease activity of recombinant FEN1 150
Figure S4.2. Peptide mass fingerprinting of purified TRF1 152
Figure S4.3. Electrophoretic mobility shift assay of purified TRF1 153

Appendix 1: FEN1 loss triggers downregulation of RNase H1
Figure A1.1. RNase H1 protein expression decreases upon FEN1 depletion 186

Appendix 2: FEN1 loss does not alter Pol II occupancy or long RNA:DNA hybrids at the telomere and the actin locus
Figure A2.1 RNA:DNA hybrids decrease at the β-actin 5’-pause site and telomere upon FEN1 depletion 197
Acknowledgments

I must foremost thank my mentor, Sheila Stewart. From my first conversation with her about rotating to long brainstorming sessions over the interpretation of results as a senior student, Sheila has remained a steadfast supporter of my work and development as a scientist. She pushed me to tackle obstacles I did not always want to tackle, while affording me the freedom to see new challenges throughout my time in graduate school, even knowing some of those challenges might be dead ends. Sheila knew when I needed to be pushed, encouraged, or quietly supported, and for that I am appreciative.

I thank my thesis committee members for each bringing something unique to the table; whether at official meetings or in quick emails, they all contributed to the development and refining of my work, each being a great role model. I also thank Jim Skeath, who showed confidence in me as a graduate student, and Melanie Relich, my frequent partner-in-crime at MGG events who made navigating bureaucratic waters an easy task.

My experience in graduate school would have been substantially incomplete without my friends and colleagues in the Stewart Lab. When I rotated in and then joined the lab, Abhishek Saharia provided me with first-rate mentorship; he is still an example for me in my mentorship of students. I also owe gratitude to Mira Pazolli and Julien Duxin, who taught me how to get to the bottom of a scientific problem. Whether discussing a journal article or debating what a result meant, Mira and Julien set an example of how to think critically about my own science. My friend and colleague Elise Alspach was my most reliable and frequent “second opinion” in the lab, and I am thankful for her ear and her thoughts throughout my time here. Hayley Moore, Meghan Ruhland, and Kevin Flanagan added tremendous insight and humor to my days, making the lab a place that was easy to be, even on the longest days. Yujie Fu brought experience and wisdom to bear on my project, always thoughtfully following up on my experiments. Lastly, I thank Bhavna Murali for being a new critic and supporter to challenge long-held assumptions as I neared the end of my graduate work.

I was fortunate during my time in the Stewart lab to mentor multiple scientists. Jane Lock grew as a mentee while I grew as a mentor. Had my first student not been as brilliant and talented as Jane, I might not have sought out new undergraduate students once she graduated. Adrián Rivera was a summer superstar and is already becoming a phenomenal scientist. Shankar Parajuli, who worked with me during his rotation before joining the lab as a graduate student, contributed more substantially to my experiments than anyone else; his skills as a scientist and genuine care for others make the lab a great place to work. My last student, Mai Nguyen, has inspired me with the strongest work ethic I’ve ever seen. Never cutting any corners, Mai has developed into one of the finest scientists I’ve mentored.

I have also been supported by an amazing group of friends who both helped me through challenging moments and reveled with me in more fun times. From my first days in Saint Louis, Brandon and Jessi Kocher have been my closest friends - the kind I could count on anytime for anything. Countless evenings spent with Jamie Kwasnieski,
Marie Strand, and Adam Joyce recharged me after long days. I also want to thank my friends who live afar. Madelynne Manansala has been an unwavering friend who supported me every step of the way over the last years, both professional and personal. Justin Connell, my friend since middle school, and his wife and my friend, Liz Wakefield-Connell, have looked out for my best interests and always been just a message or call away. Christine Liow and Andrew Cunningham have shown me that even if you don’t talk every week, the best friendships are bound by interminable connections. All of these people, as well as many other friends, have made my achievements in graduate school, and life in general, possible.

I want to thank my family as well, for being endlessly supportive during my long journey. My parents watched me move 800 miles away to chase a desire to gain and create knowledge, never flinching despite knowing I might not make it home for every holiday. They still support me every day, always hoping and wishing for my success. My brother and sister each give me strength and show me that family is a constant and a necessity. I am also grateful for my new family in Saint Louis; my parents-in-law Anne and Bill and new brothers- and sisters-in law welcomed me into their family. They make Saint Louis feel like home.

Lastly, I thank my wife and best friend, Carolyn. My persistence and effort in finishing graduate school is exceeded only by the persistence and effort I spent convincing her to date me – thankfully, it took considerably less time than a Ph.D. From those first dates together, through our engagement, and into our marriage, Carolyn has driven me to do better every day. She has shown patience with long, irregular hours and intellectual exhaustion, never failing to believe in my abilities and what lies ahead. Without the strength that she has given me to look beyond the rocky parts of the road to see the bigger picture, my work would not have been possible. Her love and support make me excited for the future we will share together.

I am grateful to the National Institutes of Health Ruth L. Kirschstein NRSA Training Grant in Cellular, Biochemical, and Molecular Sciences (T32 GM007067) for funding portions of my graduate work.
ABSTRACT OF THE DISSERTATION

Flap Endonuclease 1 Promotes Telomere Replication and Stability by Distinct Mechanisms on the Leading and Lagging Strands

by

Daniel C. Teasley

Doctor of Philosophy in Biology and Biomedical Sciences Molecular Genetics and Genomics

Washington University in St. Louis, 2015

Professor Sheila A. Stewart, Chairperson

High fidelity DNA replication is essential for genomic stability and cell survival; this fact is underscored by the redundancy present in DNA replication and repair pathways. The complexity of these pathways is most evident at challenging DNA templates, such as those with repetitive sequence and transcribed loci. Among these challenging templates are telomeres, which are terminal, highly repetitive sequences that maintain genomic stability by preventing aberrant end-to-end chromosome fusions. In the absence of accurate, complete telomere replication, genomic instability results, ultimately leading to cell death or transformation. Here, we describe two unique roles in telomere stability for the DNA replication and repair protein flap endonuclease 1 (FEN1). First, we find that FEN1 maintains telomere stability by facilitating replication fork reinitiation on the lagging strand- replicated telomere. In the absence of FEN1, sister telomere loss (STL) occurs at lagging strand-replicated telomeres. Genetic knockdown-rescue experiments demonstrated that FEN1’s nuclease activity, interactions with DNA repair proteins via its C-terminus, and gap endonuclease activity are essential for preventing STL. Similarly,
an analysis of FEN1’s ability to reinitiate stalled replication forks revealed that it is dependent on the same activities as its ability to prevent STL, suggesting that FEN1’s role in reinitiating stalled replication forks is responsible for its ability to suppress STL on the lagging strand. Second, we show that FEN1 maintains telomere stability by limiting telomere fragility on the leading strand-replicated telomere. Strikingly, this activity is biochemically and genetically distinct from FEN’s role in preventing lagging strand-specific STL; FEN1’s ability to suppress telomere fragility depends only on its flap endonuclease activity, while its C-terminal interactions and gap endonuclease activity are dispensable. We show that FEN depletion-induced telomere fragility is increased by RNA polymerase II inhibition and rescued by ectopic ribonuclease H1 expression, suggesting that FEN1 limits leading strand-specific telomere fragility by processing RNA:DNA hybrid/flap structures that arise following co-directional replisome–RNAP collisions at the telomere. Notably, this is the first known role for FEN1 in leading strand DNA replication, and the first molecular mechanism for telomere fragility at the leading strand. Lastly, we demonstrate that while FEN1 interacts directly with the shelterin protein TRF1, which is required to prevent telomere fragility, this interaction does not contribute to FEN1’s ability to suppress telomere fragility. Together, these data indicate that FEN1 has two functionally separate roles in maintaining telomere replication and stability: preventing STL on the lagging strand by facilitating replication fork reinitiation, and suppressing telomere fragility on the leading strand by processing intermediates that result from replisome–RNAP collisions.
Chapter 1:

Introduction to telomere biology

Daniel C. Teasley and Sheila A. Stewart

This chapter is in press in The Encyclopedia of Cell Biology, 2016. © Elsevier
Introduction

All eukaryotic genomes are organized into linear chromosomes. As a result, each chromosome possesses termini that must be protected from two distinct problems not present in organisms with circular genomes. The first of these is the end replication problem, a consequence of the mechanism of lagging strand DNA synthesis. During lagging strand synthesis, when the last Okazaki fragment on the lagging strand template is processed to remove the ribonucleotide primer, an irreparable 5' gap remains. This gap on the lagging strand, as well as nucleolytic processing of the chromosome ends, results in a progressive shortening of the linear chromosome with each cell division (Zakian, 2012). Telomeres are the means by which cells avoid the loss of genetic information that would occur as a result of the end replication problem—instead of a progressive loss of protein-coding sequences, a progressive loss of non-coding telomere sequence occurs instead. In serving as a stopgap to the end replication problem, telomeres also act as a molecular clock that regulates cellular lifespan. In the absence of elongation mechanisms, telomeres eventually erode to lengths that are no longer sufficient to protect against loss of genetic information. Once telomeres become critically short, they trigger cellular senescence to prevent continued division that might result in a loss of genetic information and genome instability (Frias et al., 2012). The second potential problem resulting from linear chromosome ends arises from the fact that all cells possess exquisitely sensitive mechanisms to recognize free DNA ends as DNA damage. By coordinating the activities of DNA repair proteins, telomeres protect natural chromosome ends from DNA repair events that would result in chromosome fusions (de Lange, 2004). Since inappropriate chromosome fusions lead to genome instability, telomere integrity is essential for cell proliferation (Frias et al., 2012).
The molecular events that telomeres are associated with have consequences reaching far beyond the proliferation of a given cell. Telomere dysfunction has been linked to a number of diseases including dyskeratosis congenita, aplastic anemia, and emphysema (Armanios and Blackburn, 2012). Despite the wide range of organismal effects that can occur as a result of telomere dysfunction, perhaps no disease is more strongly linked to telomere biology than cancer. Telomere attrition is classically considered to be a tumor suppressive mechanism due to the fact that shortened telomeres cause checkpoint activation and cellular senescence (Xu et al., 2013). Because senescence is a potent obstacle to transformation, cells that become neoplastic must stabilize their telomeres. Along similar lines, telomere shortening or loss has the potential to cause substantial genome instability resulting in apoptosis or mitotic catastrophe (Xu et al., 2013). Despite these strong tumor suppressive effects, telomere dysfunction can also act as a tumor promoting mechanism. Since genome instability can lead to oncogenic translocations, gene amplification, and loss of heterozygosity of tumor suppressor genes, telomere dysfunction can also enhance the transformation process (Xu et al., 2013). These opposing roles – preventing and promoting cancer – underscore the complexity of the molecular activities that maintain telomeres, and the activities that take place in response to telomere shortening.

**Telomere structure and proteins**

**Telomere DNA**

The telomere sequence in eukaryotic organisms is canonically composed of short, G-rich repeats. Indeed, all identified vertebrate telomeres are composed of the sequence 5′-(TTAGGG)ₙ-3′ oriented toward the chromosome terminus (McEachern et al., 2000).
Similar highly repetitive, G-rich sequences exist in other organisms; for instance, *Tetrahymena thermophila* telomeres consist of 5'-(TTGGGG)n-3' repeats (Fulcher et al., 2014). Telomeres are composed largely of double stranded DNA (dsDNA), with a relatively short 3' single strand DNA (ssDNA) overhang of the G-rich strand (Sfeir, 2012). The length of the overhang can vary – in humans it is typically between 30 and 500 nucleotides – but its presence is essential, as evidenced by the fact that it is actively produced by resection following DNA replication (Novo and Londoño-Vallejo, 2013; Sfeir, 2012). Telomeres span a wide range of total lengths, from as short as 300 base pairs in yeasts, to between two and 15 kilobases in humans, and as long as 150 kilobases in tobacco (Fulcher et al., 2014).

Not all organisms follow the theme of compact, regular repeats; for instance, *Saccharomyces cerevisiae* telomeres are usually designated 5'-(C1-3A/TG1-3)-3' to indicate a consensus core telomere sequence (Wellinger and Zakian, 2012). Budding yeast telomere repeats in fact range from eight to 26 base pairs in length, and are on average less G-rich than typical telomere repeats (McEachern et al., 2000). Even human telomeres show slight variability, generally having perfect repeats in the centromere-distal majority of the telomere with variant repeats occurring in the centromere-proximal end of the telomere (McEachern et al., 2000). Small variations like those found in human telomeres are likely the consequence of errors by the DNA replication machinery. Since the centromere-proximal end of the telomere is more likely exclusively produced by the DNA replication machinery rather than telomerase, sequence polymorphisms can manifest and remain (McEachern et al., 2000).
Telomere secondary structure

Because of the repetitive sequence and ssDNA overhang, telomeres can form secondary structures more complex than a linear dsDNA stretch with an ssDNA terminus. The most documented of these is the telomeric loop, or t-loop. T-loops form by a strand invasion event in which the 3’ ssDNA overhang invades the dsDNA portion of the telomere. This event produces a small (approx. 150 nucleotide) ssDNA displacement loop (D-loop) of G-rich sequence at the site of invasion, as well as a large dsDNA loop (t-loop) (de Lange, 2004). T-loops were first identified in electron micrographs of telomeric DNA, and have since been observed using super-resolution fluorescence microscopy (Doksani et al., 2013; de Lange, 2004). At face value, t-loops would seem to be an effective means to prevent end-to-end chromosome fusions; by sequestering the 3’ overhang, t-loops inhibit ATM signaling and non-homologous end joining. However, t-loops themselves resemble strand invasion intermediate structures produced during homologous recombination; it is unclear if such a structure elicits a DNA damage response if persistent, and how telomeres might avoid this response. It is likely that telomeric proteins play a role in both the formation and stabilization of t-loops; the telomere protein TRF2 in particular is sufficient for t-loop formation in vitro, and is necessary for t-loop formation and maintenance in mammalian cells (Doksani et al., 2013; de Lange, 2004).

In addition to t-loops, telomeres are also capable of forming G-quadruplexes. G-quadruplexes form by an association of four single strands of DNA or RNA (monomeric, dimeric, or tetrameric in origin) in a helical structure, where the strands assemble such that four guanines align in a cyclic Hoogsteen hydrogen-bonded tetrad (Paeschke et al.,
G-quadruplexes are characterized by the stacking of multiple tetrad cores that are connected by linker “loops”, and can form in multiple orientations (Phan, 2010). Both the ssDNA overhang, and ssDNA portions of the G-strand that form during replication or repair can presumably form into G-quadruplexes. The ability of the telomere to form G-quadruplexes is a significant phenomenon, as G-quadruplexes inhibit both semi-conservative DNA replication and telomerase-mediated telomere lengthening (Paeschke et al., 2011). Data from ciliates demonstrating that G-quadruplexes form at telomeres in a cell cycle-specific manner, and the observation that treatment of mammalian cells with G-quadruplex-stabilizing small molecules triggers telomere dysfunction, suggest that G-quadruplexes at the telomere must be actively regulated by the cell (Lipps and Rhodes, 2009). Among the strongest candidates for G-quadruplex regulation in the cell are helicases—in particular, the Fanconi anemia group helicase FANCJ and RecQ helicase BLM are known to unwind G-quadruplexes, and are known to contribute to telomere stability (Lipps and Rhodes, 2009).

**Proteins associated with the telomere**

In addition to the DNA itself, telomeres are host to a number of proteins important for telomere maintenance and function. In mammals, the network of proteins present at the telomere are coordinated by six telomere-specific proteins: TRF1 (telomeric repeat-binding factor 1, also TERF2), TRF2 (telomeric repeat-binding factor 2, also TERF2), POT1 (protection of telomeres protein 1), Rap1 (telomeric repeat-binding factor 2-interacting protein 1, also TERF2IP), TPP1 (adrenocortical dysplasia protein homolog, also ACD), and TIN2 (TERF1-interacting factor 2, also TINF2) (de Lange, 2005) (Figure 1.1). These six proteins together form the shelterin complex, which exclusively binds
telomeres due to the DNA binding specificities of TRF1 and TRF2 for telomeric dsDNA, and POT1 for telomeric ssDNA.

TRF1 and TRF2 share homology in the form of a central TRFH dimerization domain and C-terminal Myb DNA binding domain, though TRF1 possesses an acidic N-terminus, while TRF2’s N-terminus is basic. TRF1 is also substantially more divergent (65% identity between human and mouse) in mammalian evolution than TRF2 (82% identity) (Broccoli et al., 1997). While both proteins bind telomeric dsDNA and are abundant at telomeres, their functions in telomere maintenance and stability are very different. TRF1 is required for semi-conservative DNA replication through the telomere, and prevents a phenotype known as telomere fragility by recruiting helicases to the telomere to facilitate replication fork progression (Sfeir, 2012). TRF1 is also capable of looping, bending, and pairing arrays of telomere repeats, which may be involved in positioning or folding of telomeres, though TRF1 is dispensable for t-loop formation (Doksani et al., 2013; de Lange, 2005). TRF2 suppresses ATM kinase activation at the telomere, thus preventing a DNA damage response (H2AX phosphorylation and 53BP1 accumulation) and p53 activation (Sfeir, 2012). In the absence of TRF2, ligase IV- and Ku-mediated non-homologous end joining occurs, resulting in aberrant end-to-end chromosome fusions and early embryonic lethality in mice. The protective activities of TRF2 likely originate from its ability to facilitate t-loop formation, as well as its role (along with TRF1) in recruiting TPP1 to the telomere (Doksani et al., 2013; de Lange, 2004, 2005). Putative TRF1/2 homologs have been identified in Schizosaccharomyces pombe, trypanosomes, and plants (Sfeir, 2012).
The other mammalian shelterin protein with telomere sequence binding specificity, POT1, binds to the ssDNA overhang. POT1 is responsible for suppression of ATR kinase activation at the telomere, which is achieved by exclusion of the ssDNA binding protein RPA from the ssDNA overhang (Baumann and Price, 2010). POT1 also restricts the length of the ssDNA overhang and regulates the activity of telomerase by competing for binding at the overhang, which is the substrate for telomerase elongation (Longhese et al., 2012; Sfeir, 2012). POT1 is largely conserved among eukaryotes, with homologs in *S. pombe*, *Tetrahymena*, nematodes, and plants (Baumann and Price, 2010). In mouse and *Tetrahymena*, there are two POT1 gene homologs, *Pot1a* and *Pot1b*, with each playing a subset of the roles attributed to the single POT1 gene observed in human and yeast.

The three other shelterin proteins (Rap1, TPP1, and TIN2) lack the ability to bind telomeric sequence directly in vertebrates, but are telomere-specific by virtue of their direct or indirect binding to TRF1 and TRF2. Indeed, simultaneous deletion of TRF1 and TRF2 from cells results in telomeres completely devoid of shelterin (Sfeir and de Lange, 2012). Rap1 is the most conserved of all the shelterin proteins and the only shelterin protein with well-defined extra-telomeric functions – it is a transcriptional regulator and can modulate nuclear factor kappa B (NF-κB) signaling. At the mammalian telomere, Rap1 is a negative regulator of telomere length. It also acts with POT1 to repress aberrant homology-directed repair through an unclear mechanism (Sfeir, 2012). In *S. cerevisiae*, which lacks a TRF homolog, Rap1 is highly diverged from its orthologs and directly binds telomeric dsDNA; it effectively serves as the shelterin core in this yeast (de Lange, 2005). TIN2 is a “bridging” protein that connects TRF1 and TRF2 to one
another, and recruits TPP1 to the telomere. TPP1 in turn is required for efficient recruitment of POT1 to the telomere, as POT1’s DNA binding affinity is insufficient to keep it tethered to the ssDNA overhang (Sfeir, 2012). Both TIN2 and TPP1 are exclusive to vertebrate telomeres, and their emergence may coincide with the appearance of two TRF genes (de Lange, 2005).

In addition to the telomere-specific shelterin proteins, a growing number of other proteins localize to telomeres and are important for telomere function. The majority of these proteins are associated with DNA metabolism. The protein complex CST, consisting of Cdc13, Stn1, and Ten1 in *S. cerevisiae* and CTC1, STN1, and TEN1 in mammals, associates with the ssDNA telomere overhang and due to structural similarity to the heterotrimeric RPA complex, has been proposed to function as a “telomere specific RPA” (Longhese et al., 2012). The RecQ helicases WRN and BLM interact with shelterin and play roles in lagging strand telomere synthesis and replication fork progression (de Lange, 2005). These are but a few of the numerous proteins that localize to the telomere and contribute to telomere synthesis and maintenance.

**Telomere replication and length maintenance**

Telomeres, like the rest of the genome, must be replicated during S phase to ensure genome continuity during cell division. Telomeres can be replicated primarily in two ways: first, the semi-conservative DNA replication machinery replicates telomeres along with the rest of the genome, and second, the ribonucleoprotein (RNP) enzyme telomerase adds telomere repeats to extend telomeres. In addition to these two main mechanisms for telomere replication and maintenance, there are two additional
identified means of telomere length maintenance: recombination-based telomere maintenance and the alternative lengthening of telomeres (ALT) mechanism. Additionally, some insects maintain their telomeres by a unique mechanism involving retrotransposition, but this will not be discussed here (de Lange, 2004).

**Semi-conservative telomere replication**

Telomere replication poses unique challenges for the semi-conservative DNA replication machinery. The first of these challenges is the consequence of the telomere’s location at chromosome termini. Unlike the rest of the genome, which can be replicated by one or more DNA replication origins with replication forks approaching from either direction, the most centromere-distal origin is thought to be the sole origin responsible for the replication of a given telomere. Additionally, because there are no known replication origins within the telomere, the “last” origin to fire must replicate the entire length of the telomere; in the event of fork collapse, telomere replication may remain incomplete (Gilson and Géli, 2007). This potential problem is exacerbated by the second main challenge in telomere replication: the repetitive, G-rich sequence, which leads to increased replication fork stalling compared to the rest of the genome (Cesare and Karlseder, 2012). The secondary structures that telomeric DNA forms, in particular G-quadruplexes and t-loops, must be resolved into linear dsDNA for replication to occur.

The combination of structures that induce replication fork stalling and the lack of a replication fork approaching from the opposite direction to rescue a stalled fork necessitates robust mechanisms to ensure that telomere replication is completed. These mechanisms are coordinated by the shelterin complex, which recruits DNA
replication and repair proteins to the telomere. In *S. pombe*, the TRF1/2 homolog Taz1 prevents replication fork arrest and telomere loss in a telomerase-negative background (Gilson and Géli, 2007). Subsequent work in murine cells has shown that TRF1 is required to facilitate replication fork progression through the telomere and prevent telomere fragility, and that this activity depends upon the helicases BLM and RTEL1 (Sfeir et al., 2009). TRF2 also plays critical roles in telomere replication – both by recruiting the RecQ helicases BLM and WRN to the telomere, and by acting in a pathway with Apollo and topoisomerase 2α to relieve topological stress during replication (Ye et al., 2010). The recruitment of two RecQ helicases, BLM and WRN, by shelterin is not coincidental; both proteins are able to unwind G-quadruplexes and are theorized to have overlapping functions in resolving G-quadruplexes formed on the lagging strand during replication.

The cooperation between shelterin and its binding partners described above ensures that replication forks can progress through the telomere with as little stalling as possible. Nevertheless, replication forks do stall in telomeric sequence, and additional mechanisms are in place to ensure successful fork restart. The DNA replication and repair protein flap endonuclease 1 (FEN1) localizes to telomeres during and after replication, and ensures that replication forks are reinitiated on the lagging strand template following stalling (Saharia et al., 2010). The mammalian CST complex rescues stalled replication forks that occur as a result of replication stress by facilitating dormant origin firing (Stewart et al., 2012a). Stalled replication forks at the telomere appear to require ATR, which is recruited to telomeres during S-phase, for restart (Verdun and Karlseder, 2006). These are unlikely to be the only contributing mechanisms for
replication fork restart at the telomere. The preponderance of DNA replication and repair proteins that are recruited to the telomere to facilitate replication fork progression and fork restart following stalling illustrates the difficulty of telomere replication by the semi-conservative DNA replication machinery, as well as the robust series of mechanisms in place to ensure that replication is completed in spite of the challenges.

The fact that both leading and lagging strand-replicated telomeres possess similar 3' ssDNA overhangs implies that at least the leading strand, which would be expected to produce a blunt end, has to be processed to produce an overhang. In fact, leading and lagging strand-replicated telomere ends are significantly resected and processed following the completion of semi-conservative DNA replication (Gilson and Géli, 2007). This process must be highly regulated by the cell, as too little resection would generate a short overhang which precludes POT1 binding and t-loop formation, while too much resection would accelerate the end replication problem. In S. cerevisiae, resection of the leading strand-replicated telomere to produce the G-rich overhang is facilitated by the same DNA repair proteins involved in 5' resection at DNA double strand breaks prior to homologous recombination, notably Cdk1 and the Mre11-Rad50-Xrs2 (MRX) complex. Indeed, yeast strains with deficient Mre11 exhibit shorter 3' ssDNA overhangs (Gilson and Géli, 2007). Following recognition of the end by MRX, the nucleases Exo1 and/or Dna2 are recruited to the 5' end, and in concert with the helicase Sgs1, cleave the 5' end to produce the 3' ssDNA overhang (Stewart et al., 2012b). In mammalian cells, the Apollo nuclease is recruited to telomere ends by its interaction with TRF2. At the leading strand-replicated telomere, Apollo initiates resection of the 5' end. At the lagging strand-replicated telomere, where an overhang already exists following replication, POT1b
binds the ssDNA and inhibits the resection activity of Apollo; similarly, after a sufficient ssDNA overhang is generated at the leading strand-replicated telomere, POT1b binds and inhibits further resection by Apollo (Wu et al., 2012). Exo1 then further and transiently resects the 5' ends at both strands to produce long overhangs. Lastly, POT1b recruits CST and polymerase α to shorten the extended overhangs, presumably by fill-in C-strand synthesis (Wu et al., 2012).

**Telomerase**

Semi-conservative replication and telomere end processing cause the end replication problem, which manifests as progressive telomere shortening with each cell division cycle. The solution to the end replication problem is telomerase, a holoenzyme minimally composed of the telomerase reverse transcriptase protein (TERT) and the telomerase RNA component (TERC or TR). TERC binds accessory proteins in addition to TERT, which contribute to telomerase localization and processing (Martínez and Blasco, 2011). Telomerase adds telomeric DNA repeats to the terminal end of an existing telomere, thus lengthening telomeres and offsetting the end replication problem.

Telomerase is unique among known reverse transcriptase enzymes in being an RNP. The TERT protein is the catalytic portion of the holoenzyme, and consists of four conserved structural domains. Two of these, the reverse transcriptase and C-terminal extension domains, are conserved between TERT and other reverse transcriptases; the telomerase essential N-terminal domain and telomerase RNA binding domain are unique to TERT (Podlevsky and Chen, 2012). The RNA portion of the telomerase RNP,
TERC, is required for telomerase activity not only because it provides the telomere repeat sequence template, but also because two conserved regions, the template/pseudoknot domain and the CR4/5 domain, contribute to template positioning in the active site and provide important protein–nucleic acid contacts (Podlevsky and Chen, 2012). In addition to these two regions, which are conserved among all known species, vertebrate TERCs contain a conserved H/ACA domain. Each of two stems in the H/ACA domain binds a protein complex consisting of dyskerin, NOP10, NHP2, and GAR1. These proteins are required for TERC maturation and processing, RNP biogenesis, and Cajal body localization of TERC (Podlevsky and Chen, 2012).

TERT protein production occurs via the canonical processes of mRNA transcription and cytoplasmic translation, after which TERT is first recruited to nucleoli, and then Cajal bodies. TERC is transcribed by RNA polymerase II, after which the ends are processed; TERC binds the dyskerin-anchored complex of accessory proteins, which facilitate maturation and localization to Cajal bodies. The chaperone proteins HSP90 and p23 facilitate the assembly of the TERC/accessory protein complex with TERT into the active telomerase RNP holoenzyme, after which it localizes to telomeres (Podlevsky and Chen, 2012).

At the telomere, the catalytic process of telomere elongation occurs in two steps. In the first step, the 3' end of the telomere base pairs with the 5' region of TERC; the TERT active site then uses the 3' end of the telomere as a primer to reverse transcribe a telomere repeat (in human, the six nucleotides 5'-GGTTAG-3') using TERC as a template. In the second step, TERC dissociates from the telomeric DNA, translocates
5’-to-3’ along the DNA, and re-anneals for a new round of nucleotide addition (Podlevsky and Chen, 2012). By repeating the synthesis and translocation steps, telomerase can processively add repeats to a single telomere end without ever completely dissociating from the DNA. Here, shelterin plays a role in telomere synthesis, as the POT1–TPP1 complex has been shown to hold the telomeric DNA primer close to telomerase, inhibiting primer release and enhancing processivity (Podlevsky and Chen, 2012). Telomerase activity is also restricted in a shelterin-dependent manner; POT1 bound to the ssDNA overhang is thought to limit the initial binding of telomerase to the telomere, and the t-loop structure prevents telomerase activity (de Lange, 2005). Once telomerase dissociates from its template, polymerase α synthesizes the complementary C-rich strand. It is thought that the CST complex, which is known to interact with the polymerase α complex, facilitates this event (Gilson and Géli, 2007). In yeast, telomere repeat-containing RNA (TERRA), the noncoding RNA product of telomere transcription, may act as a seed to nucleate clusters of telomerase prior to telomere recruitment; TERRA transcription is induced by telomere shortening (Cusanelli et al., 2013).

Recombination at telomeres and alternative lengthening of telomeres

In addition to lengthening by telomerase activity, cells have evolved other mechanisms to elongate or maintain telomeres. In *S. cerevisiae*, deletion of the telomerase reverse transcriptase EST1 produces colonies of survivor cells. Genetic analysis revealed the requirement for recombination in virtually all est1Δ survivor cells, as est1Δ rad52Δ strains produce virtually no survivors (Wellinger and Zakian, 2012). In addition to RAD52, all survivor cells require the Pol32p replication protein. Survivors are
categorized into one of two types (I and II), which appear to have different mechanisms but are not mutually exclusive. Type I survivors have telomeres with multiple, repeated Y' subtelomere elements and short, terminal telomere repeats; they also possess extrachromosomal circular Y' elements thought to serve as recombination substrates. These cells grow relatively slowly, easily convert to type II survivors, and require RAD51, RAD54, and RAD57 in addition to RAD52 and POL32 (Wellinger and Zakian, 2012). Type II survivors have telomeres with few subtelomere repeats but extensive amplification in telomere repeats. The telomeres in these cells may depend on rolling circle amplification as an initiating event to lengthen their telomeres. Type II survivors require MRX, RAD59, and SGS1 (Wellinger and Zakian, 2012). Interestingly, the recombination-mediated mechanisms of telomere maintenance in yeast appear to be promoted by RNA:DNA hybrid formation between telomeric DNA and TERRA (Balk et al., 2013). This observation suggests the possibility that TERRA may be required for both telomerase-mediated and recombination-mediated telomere elongation pathways.

In mammalian cells lacking telomerase activity, the alternative lengthening of telomeres (ALT) mechanism provides a means to maintain telomere length. Found in 10-15% of human cancers, ALT appears to be recombination-based and is characterized by several striking phenotypes. ALT cells possess an abundance of extrachromosomal telomeric DNA, much of which is in the form of double stranded telomeric circles (t-circles) and C-rich single stranded telomeric circles (C-circles) (Cesare and Reddel, 2010). ALT cells also exhibit telomere localization to promyelocytic leukemia (PML) nuclear bodies, elevated levels of telomere sister chromatid exchange (T-SCE) events, and heterogeneous chromosomal telomere lengths. The molecular mechanism(s)
responsible for ALT have remained elusive, and two models have emerged to explain it. First, the unequal T-SCE model proposes that T-SCE events produce long and short telomere lengths in the chromosomes experiencing T-SCE; if the chromosomes with longer telomeres were able to segregate into one of the two daughter cells, the enhanced proliferative capacity of one daughter over the other could produce selection at the population level for theoretically unlimited proliferation (Cesare and Reddel, 2010). The second model, which is not mutually exclusive from the unequal T-SCE model, proposes that ALT is the result of homologous recombination-dependent DNA replication. In this model, shorter telomeres extend by recombination-based DNA synthesis using an existing telomere sequence substrate in the cell. The telomere substrate for the elongating telomere could be a telomere on another chromosome, the same telomere via t-loop formation, linear extrachromosomal telomeric DNA, t-circles, and/or C-circles (Cesare and Reddel, 2010). Both telomere elongation and length maintenance in ALT cells appear to depend on a striking number of DNA replication and repair proteins, which suggests that ALT results from deregulation of normal DNA metabolic processes. Indeed, the shelterin proteins TRF2 and POT1 have been suggested as suppressors of ALT due to both proteins’ ability to inhibit recombination at the telomere; ALT cells’ vast expansion of telomeric DNA content may dilute shelterin saturation at telomeres and contribute to the phenotype (Cesare and Reddel, 2010). Like in recombination-mediated telomere elongation in yeast, TERRA may play a role in ALT. Recent data suggests that RNA:DNA hybrid levels between TERRA and the telomere are “fine-tuned” by RNase H1 expression in ALT cells (Arora et al., 2014).
**Telomere Physiology**

**Senescence and telomere crisis**

One of the most significant consequences of telomere erosion at the cell biological level is the induction of cellular senescence. Indeed, the fact that telomere length is the primary determinant of cellular lifespan is well-established (Deng et al., 2008). Telomere shortening beyond a critical length causes deprotection of the telomere via a loss of TRF2. Deprotection induced by replicative shortening or other telomere dysfunction induces a DNA damage response (DDR). The DDR induced by telomere dysfunction causes phosphorylation of H2AX and recruitment of 53BP1 and other DNA repair proteins to the telomere. Like the classical DDR, the DDR induced by telomere attrition activates both the ATM and ATR checkpoints, which in turn activate p53 via the CHK1 and CHK2 kinases (Deng et al., 2008). p53 activation by telomere shortening induces one of two physiological consequences: either entry into cellular senescence by p21 and RB signaling, or apoptosis (Frias et al., 2012) (Figure 1.2). Both senescence and apoptosis serve as potent anti-proliferative mechanisms in cells with eroded telomeres.

Absence of a functional p53 and Rb checkpoint, as often occurs in cancer cells, abrogates both senescence and apoptosis as responses to telomere dysfunction and attrition. Because this causes checkpoint bypass and continued proliferation, telomeres in p53-deficient cells will continue to shorten, ultimately reaching a condition called crisis (Figure 1.2). Crisis inherently acts as a second checkpoint to continued proliferation; critically short telomeres induce aberrant end-to-end fusions between chromosomes followed by chromosome breakage and additional fusion events in what is referred to as the breakage–fusion–bridge cycle (Deng et al., 2008). The breakage–fusion–bridge
cycle causes massive genomic instability and aneuploidy, both of which lead to rapid cell death in a majority of cells. However, a small fraction of cells are able to escape crisis by activating either telomerase or the ALT pathway (Figure 1.2); in these cells, the chromosome fusions resulting from crisis can produce oncogene amplification, tumor suppressor loss of function, and gene fusions that contribute to rapid proliferation and tumorigenesis (Deng et al., 2008).

**Telomeres in cancer cells**

Telomere biology contributes to cancer phenotypes via two distinct but related mechanisms. First, telomere shortening induces senescence and/or apoptosis, acting as a significant anti-proliferative barrier to the incipient tumor cell; these cells must activate either telomerase or ALT to continue proliferating. Second, telomere dysfunction, whether induced by DNA damage, genetic mutation, or avoidance of replicative senescence, causes genomic instability that can drive tumorigenesis.

The observation that telomerase is aberrantly activated in as many as 85-90% human cancers, with ALT mechanisms active in the remainder, demonstrates the absolute barrier that telomere shortening imposes on incipient tumor cells’ ability to survive (Gomez et al., 2012). Despite the universal requirement for a telomere maintenance mechanism for unlimited proliferation, the significance of a tumor cell’s use of telomerase vs. ALT remains unclear. Several studies have identified an ability of cells to switch between the two mechanisms; of particular note is the ability of telomerase-positive cancer cells to become ALT-positive upon genetic or pharmacologic inactivation of telomerase (Hu et al., 2012; Queisser et al., 2013). In tumors that are telomerase-
positive, studies conflict on the relevance of telomerase expression levels or activity as a prognostic marker. In some late-stage non-small cell lung cancers, colorectal cancers, and soft tissue sarcomas, telomerase expression correlates with poor prognosis, but many of these studies remain controversial (Chen and Chen, 2011). Tumors that are ALT-positive (commonly, glioblastoma multiforme and sarcomas) frequently have poor prognosis (Cesare and Reddel, 2010).

While aberrant telomere elongation by telomerase or ALT contributes directly to the proliferative capacity of cancer cells, telomere-related genomic instability is perhaps just as significant a contributor to tumorigenesis. As described above, the breakage–fusion–bridge cycle can arise from critically short or lost telomeres. In cancer cells, critically short telomeres are likely to induce the breakage–fusion–bridge cycle, resulting in genomic instability and generating tumor-promoting conditions. Indeed, mice with a TERC deletion and p53 deletion experience a wide range of carcinomas consistent with human cancers; when examined for cytogenetic abnormalities, the tumor cells in these mice show chromosome rearrangements with inverted repeats that are consistent with the breakage–fusion–bridge model (Murnane, 2010). Human cancer cells frequently contain amplified regions in the genome with inverted repeats (Murnane, 2012). Analysis of tumor samples by chromosome painting indicates that the breakage–fusion–bridge cycle frequently involves a single chromosome involved in multiple sister chromatid fusions, continuing over multiple generations until telomere stability can be achieved; this allows the cell to survive, and facilitates substantial amplification of loci on the involved chromosome(s) (Murnane, 2012). In addition to amplification events generated by the breakage–fusion–bridge cycle, telomere loss can trigger end-to-end
chromosome fusions that ultimately produce gross chromosomal rearrangements. These rearrangements are capable of producing fusion genes that encode oncogenic proteins in specific human cancers (Jones et al., 2012).

**Non-cancer telomere-related diseases**

Diseases with telomeric origin or contribution can largely be grouped into two categories: those that are caused by mutation to specific genes with telomeric functions, and those that are associated with changes in telomere length. The first category, often referred to as telomeropathies or telomere syndromes, constitutes a diverse group of diseases that are monogenic in origin, with mutations typically occurring in one of several telomere-associated genes. The most well known telomeropathy is dyskeratosis congenita (DC), a disease initially presenting with nail dystrophy, leukoplakia, and abnormal skin pigmentation; DC patients frequently progress to experience aplastic anemia and increased cancer incidence (Savage and Bertuch, 2010). Among the most frequently mutated genes in DC are **TERC** and **TERT**; DC is also associated with mutations in **DKC1** (common), **TINF2** (common), **NHP2** (rare), and **NOP10** (rare) (Holohan et al., 2014). In addition, three other disorders presenting with overlapping symptoms–Hoyeraal-Hreidarsson, Revesz, and Coats plus syndromes–are associated with mutations in the same group of genes, and may constitute a group of diseases including DC with common origins that exhibit distinct phenotypes due to different mutations or penetrance (Armanios and Blackburn, 2012). Most of the mutations identified in DC and the DC-related syndromes cause decreased telomerase function; in affected cells, telomere length is frequently below the first percentile in the population for the age of the patient (Savage and Bertuch, 2010). Mutations in **TERC** and **TERT**
have also been identified in studies as either contributors to aplastic anemia, idiopathic pulmonary fibrosis, and unexplained severe liver disease (Savage and Bertuch, 2010). The symptoms of these conditions are all observed in DC patients as either complications or comorbidities, suggesting that abrogation of telomerase function by mutation of TERC, TERT, or another protein can produce a wide spectrum of disease presentations that are all caused by similar molecular mechanisms. Two mutated genes identified in diseases of the DC spectrum, TINF2 (common) and CTC1 (rare), produce proteins not known to directly affect telomerase function. TIN2, a shelterin complex member, could indirectly affect telomerase recruitment by modulating the binding of other shelterin proteins to the telomere, but any mechanism remains unclear. CTC1, associated only with Coats plus syndrome, is a member of the mammalian CST complex and thus facilitates telomere replication, but there is little evidence about how it might contribute to the disease (Armanios and Blackburn, 2012).

In addition to the monogenic telomeropathies described above, several age-related human diseases show association with telomere length. A wide number of risk factors for atherosclerosis (smoking, hypertension, and obesity), as well as atherosclerosis itself, have been studied with regard to telomere length. Reports indicate that tobacco use, hypertension with sclerotic plaques, and weight gain correlate with shorter telomere lengths; multiple studies have demonstrated that shorter telomeres or increased telomere attrition are present in atherosclerotic patients (Khan et al., 2012). Some of these studies, particularly those examining atherosclerosis risk factors as opposed to the disease itself, have weaknesses: several studies were unable to control all the confounding factors, while others showed statistically significant effects only in
certain subgroups (i.e., males or females). Nonetheless, the overall picture of data certainly indicates that shorter telomere length is associated with atherosclerosis (Khan et al., 2012). Age-related musculoskeletal diseases have also been associated with telomere length. Chondrocytes from patients with osteoarthritis exhibit shorter telomere lengths than control patients; given that chondrocyte senescence causes changes known to accelerate osteoarthritis, it is possible that accelerated telomere loss might contribute to osteoarthritis (Li et al., 2012). Some studies have indicated that shorter telomeres are associated with lower bone mineral density, a characteristic of osteoporosis (Li et al., 2012). Other studies, however, have found no such association, and so associations between telomere length and age-related musculoskeletal diseases are challenging to identify and interpret. In mouse models, a number of other diseases appear to be telomere-dependent. Mice with short telomeres display a wide variety of phenotypes—these include: immune problems (opportunistic infections, impaired vaccine responses, altered T-cell ratios), gastrointestinal problems (enterocolitis, villous blunting), defects in fibrosis following injury, and altered β-cell function leading to insulin resistance (Armanios and Blackburn, 2012). Many of the phenotypes observed in mice appear to be related to telomere-induced senescence. While most of the non-cancer diseases with known telomere dependence in humans fall into the DC spectrum of disorders and phenotypes, the evidence in mice suggests that telomere-dependent senescence contributes to age-related diseases.
Figure 1.1. A diagrammatic representation of telomere structure and telomere-binding proteins.

TRF1 (green) and TRF2 (red) form homodimers and directly bind telomere dsDNA. TIN2 (blue) acts as a bridge between the homodimers and interacts with TPP1 (yellow). TPP1 also interacts with POT1 (orange), which binds telomere ssDNA at the 3' overhang. RAP1 (purple) interacts with TRF2. The telomere likely exists in a dynamic
equilibrium as a linear structure with a free 3' overhang and the t-loop structure, where the G-rich 3' end loops and hybridizes with the C-rich strand, forming a displacement loop.
Germline cells and embryonic stem cells (green) possess high telomerase activity and maintain long telomeres. Adult stem cells and multipotent progenitor cells (purple) possess detectable telomerase activity, but do not express telomerase sufficiently to entirely prevent telomere shortening; the telomeres in these cells shorten gradually over time. Somatic cells have no detectable telomerase activity, and the telomeres in these cells shorten with each cell division. When telomeres shorten to a particular length (M1), most cells enter senescence and cease dividing. In cells without functional p53/Rb checkpoints, senescence is bypassed; cell division and telomere shortening continue until telomere length becomes short enough to trigger crisis (M2). The majority of cells that reach crisis die; however, a very small subset of incipient cancer cells that reach crisis activate either telomerase or the ALT pathway to stabilize their short telomeres, thus becoming immortal.
References


Chapter 2:

FEN1 ensures telomere stability by facilitating replication fork re-initiation

Abhishek Saharia, Daniel C. Teasley, Julien P. Duxin, Benjamin Dao,
Katherine B. Chiappinelli, and Sheila A. Stewart

Daniel C. Teasley was a contributing author to this work.

This chapter was originally published in The Journal of Biological Chemistry, 2010; 285:
27057–27066. © the American Society for Biochemistry and Molecular Biology
Introduction

High-fidelity DNA replication is critical for genome stability and continued cellular proliferation. Given the importance of high-fidelity DNA replication to genomic stability, it is not surprising that numerous redundant mechanisms of DNA replication exist. Inherited syndromes in which DNA replication/repair proteins are mutated or lost but overall DNA replication continues relatively unabated (Sidorova, 2008; Singh et al., 2009; Wu and Hickson, 2002) best illustrate the compensatory nature of these mechanisms. However, in some cases, this compensation is incomplete, and thus patients with these mutations manifest replication defects and genomic instability (Sidorova, 2008).

Deficiencies in various DNA replication/repair mechanisms become particularly detrimental in highly repetitive DNA sequences that present unique challenges to the DNA replication machinery (Gilson and Geli, 2007; Verdun and Karlseder, 2007). For example, triplet repeats can lead to replication fork slippage, resulting in deleterious expansions and deletions (Kovtun and McMurray, 2008). Similarly, replication fork pausing and stalling occur within telomeric repeats (Khadaroo et al., 2009; Makovets et al., 2004; Ohki and Ishikawa, 2004; Verdun and Karlseder, 2006), and telomeres were recently identified as fragile sites (Martinez et al., 2009; Sfeir et al., 2009). Because fragile sites are thought to arise in response to replication stress, these data support the hypothesis that telomeric DNA presents a challenging template for the DNA replication machinery that requires the actions of specialized replication complexes, including a replication fork re-initiation complex (Gilson and Geli, 2007; Maizels, 2006; Parkinson et
al., 2002). Recent work has shown that telomeres are highly sensitive to the loss of DNA replication/repair proteins shown to localize to stalled replication forks, including the Werner helicase (WRN) and flap endonuclease 1 (FEN1) proteins (Sharma et al., 2004). Indeed, cells from WRN patients display overt telomere dysfunction while only minor defects in genomic replication are observed (Crabbe et al., 2007; Crabbe et al., 2004; Sidorova, 2008), suggesting that other proteins compensate for WRN throughout the genome but are insufficient at the telomere.

DNA replication mechanisms at the telomere are coordinated by the six-protein Shelterin complex (including: TRF1, TRF2, TIN2, POT1, RAP1, and TPP1) (de Lange, 2005; Gilson and Geli, 2007; Verdun and Karlseder, 2007). For example, TRF2 interacts with and modulates the activities of numerous DNA replication and repair proteins (de Lange, 2005). These interactions include TRF2 binding to the WRN and Bloom syndrome (BLM) helicases, which stimulates their activity in vitro, suggesting that TRF2 recruits them to replicate or repair telomeric DNA (Opresko et al., 2002). In *Schizosaccharomyces pombe*, the TRF1/2 homolog Taz1 is essential for DNA replication through the telomeres (Miller et al., 2006). Upon Taz1 deletion, replication forks stall within telomeric repeats and telomeres are rapidly lost (Miller et al., 2006). TRF1 plays a similar role in mammalian cells (Martinez et al., 2009; Sfeir et al., 2009). After deletion of TRF1, stalled replication forks accumulate within the telomeric repeats, resulting in a replication stress response characterized by an ATR (ataxia telangiectasia mutated (ATM)- and Rad3-related)-dependent DNA damage response and expression of fragile sites within telomeric DNA (Martinez et al., 2009; Sfeir et al., 2009). Together,
these data underscore the importance of the coordinated actions of the Shelterin components and the DNA replication and repair machinery to efficiently complete telomere replication.

FEN1 is a structure-specific endonuclease that plays an important role in DNA metabolism. FEN1 participates in Okazaki fragment processing during lagging strand DNA replication (Li et al., 1995) and is important for several DNA repair processes (Liu et al., 2004; Shen et al., 2005). FEN1 co-localizes to stalled replication forks where it interacts with the RecQ helicase, WRN, and is postulated to re-initiate stalled DNA replication forks (Sharma et al., 2004; Zheng et al., 2005). Recently, we demonstrated that FEN1 is vital for telomere stability (Saharia et al., 2008). Indeed, FEN1 depletion in telomerase-deficient cells leads to a DNA damage response at telomeres and telomere dysfunction characterized by loss of lagging strand-replicated sister telomeres (Saharia et al., 2008; Saharia and Stewart, 2009). Furthermore, genetic rescue experiments demonstrate that the nuclease activity and the C-terminal WRN-interacting domain of FEN1 are important for telomere stability (Saharia et al., 2008).

The above findings prompted us to investigate how FEN1 contributes to telomere stability. Here, for the first time, we demonstrate that FEN1 promotes efficient re-initiation of stalled replication forks. The C-terminal domain of FEN1 and its gap endonuclease activity (GEN) are critical for its ability to re-initiate stalled replication forks. However, FEN1 depletion does not affect progression through S phase or SV40 large T antigen-dependent in vitro DNA replication of non-repetitive sequences. Instead,
FEN1 depletion leads to replicative stress within telomeric sequences as evidenced by expression of fragile sites. Finally, we demonstrate that the PCNA-interacting domain of FEN1 is dispensable for its telomere function and that the GEN activity is critical for its ability to prevent sister telomere loss (STL). We propose that FEN1 maintains stable telomeres through efficient re-initiation of stalled replication forks that occur in the G-rich telomere, ensuring high fidelity telomere replication.

**Experimental Procedures**

**Cell culture**

All cells were grown as reported (Saharia et al., 2008; Saharia and Stewart, 2009; Stewart et al., 2003a; Stewart et al., 2002). Briefly, cells were grown at 37 °C in 5% carbon dioxide. HeLa and 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS) and 1% penicillin/streptomycin. BJ fibroblasts were cultured in DMEM with 15% Medium 199 (Sigma, St. Louis, MO), 15% heat-inactivated FCS and 1% penicillin/streptomycin.

**Virus production and infection**

Lentiviral production and cell infections were carried out as described (Saharia et al., 2008; Saharia and Stewart, 2009; Stewart et al., 2003b). Briefly, 293T cells were transfected with TransIT-LT1 (Mirius, Madison, WI). Virus was harvested 48 hours post transfection, and infections were carried out overnight in the presence of 10 µg/ml of protamine sulfate. Following infection, transduced cells were selected with 1 µg/ml puromycin.
For adenovirus production, FEN1 cDNAs were cloned into the pShuttle vector (Stratagene, La Jolla, CA) at the EcoRV site. The hWT, DA and ΔC cDNAs were previously described (Saharia et al., 2008); the ΔP cDNA was previously described (Stucki et al., 2001a); the ΔPΔC cDNA was constructed using a forward primer complementary to the FLAG epitope 5'-GGT ACC ATG GAC TAC AAA GAC CAT GAC GG-3' and the following reverse primer, 5'-CTC GAG TTA TTA GGT GCT GCC TTG GCG GCT CTT AC-3', and was cloned into the pShuttle plasmid; the mWT and mED cDNAs were previously described (Zheng et al., 2005). Following subcloning, the FEN1 cDNAs were recombined into the pAdEasy-1 plasmid (Stratagene, La Jolla, CA) and the resultant DNA was transfected into HEK293 cells to produce infectious adenovirus. Adenovirus production and concentration were carried out according to the manufacturer’s protocol using the AdEasy XL Adenoviral Vector System (Stratagene, La Jolla, CA). Adenovirus was titered prior to use with the AdEasy Viral Titer kit (Strategene, La Jolla, CA) according to the manufacturer’s protocol.

**Western blot analyses**

All western blots were conducted as described (Saharia et al., 2008). Antibodies used: rabbit polyclonal anti-FEN1 (#586, Bethyl Labs, Montgomery, TX), mouse monoclonal anti-Actin (ABCAM, Cambridge, MA), rabbit polyclonal anti-TRF2 (H-300; Santa Cruz Biotech, Santa Cruz, CA), mouse monoclonal anti-FLAG M2 (Sigma St. Louis, MO), rabbit polyclonal anti-Cyclophilin A (Cell Signaling Technology, Danvers, MA).
S phase progression assay

HeLa cells were cultured for 1 hour in the presence of 50 µM 5-bromo-2-deoxyuridine (BrdU) in the dark. The cells were then washed in phosphate buffered saline (PBS), replaced in culture medium and harvested at the indicated times. The harvested cells were washed with PBS and fixed in 4% paraformaldehyde and 0.1% Triton X-100 in PBS for 20 minutes at room temperature. Cells were further permeabilized with 0.1% Triton X-100 for 10 minutes on ice and fixed for an additional 5 minutes in 4% paraformaldehyde and 0.1% Triton X-100 in PBS. The DNA was denatured with 30 µg of DNase I (Sigma, St. Louis, MO) at 37 °C for one hour. BrdU was detected with an Alexa Fluor 488-conjugated anti-BrdU antibody (A21303, Invitrogen, Carlsbad, CA) and the DNA content of the cells was determined by 7-amino-actinomycin D (7-AAD; BD Biosciences, San Jose, CA) staining. The stained cells were analyzed on a FACSCalibur machine (BD Biosciences, San Jose, CA).

SV-40 Large-T antigen-dependent in vitro DNA replication assay

The crude cell extracts for this assay were prepared using HeLa cells as described (Brush et al., 1995). Briefly, HeLa cells were harvested, washed in cold isotonic buffer [20 mM HEPES, pH 7.8, 1.5 mM magnesium chloride, 5 mM potassium chloride, 250 mM sucrose, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF)] and then with cold hypotonic buffer (isotonic buffer without sucrose). The cells were then swollen on ice for 15 minutes in hypotonic buffer and lysed with 10 strokes of the Dounce homogenizer (pestle B). The cell lysate suspension was incubated on ice for another 60 minutes. Following this incubation, the lysate was centrifuged at 1700 g at 4
°C for 10 minutes to remove the nuclei and then centrifuged again at 12,000 g for 10 minutes at 4 °C to clarify the lysate. The resulting lysate was flash frozen in liquid nitrogen and stored at −80 °C. Linear plasmid DNA (pSVO.11-2K; (Ohki and Ishikawa, 2004)) used in the replication reactions was prepared by equilibrium centrifugation in cesium chloride–ethidium bromide gradients and then digested with BbsI (New England Biolabs, Ipswich, MA). The in vitro replication reactions were carried out as described (Brush et al., 1995). Briefly, each 25 µL reaction contained 30 mM HEPES/hydrochloric acid pH 7.8, 7 mM magnesium chloride, 4 mM ATP, 200 µM each of CTP, UTP, GTP, 100 µM each of dATP, dGTP, dTTP, 0.5 mM DTT, 40 mM creatine phosphate, 0.625 units creatine phosphokinase, 50 µM (2.5 µCi) [α-³²P]dCTP (Perkin-Elmer, Waltham, MA), 50 ng linearized plasmid DNA, 1 µg large T antigen (Chimerx, Madison, WI) and 100 µg cytoplasmic extract. The reaction was incubated for 10 minutes on ice and then at 37 °C for the indicated time. To stop the reaction, an equal volume of stop solution (2% SDS, 50 mM EDTA, 1 mg/ml Proteinase K) was added and the reaction was incubated for an additional 30 minutes at 37 °C. The reactions were subject to a phenol/chloroform/isoamyl alcohol extraction and the DNA was precipitated with isopropanol, followed by a 70% ethanol wash. To verify that the products were generated by semi-conservative replication, additional samples were digested following precipitation with 10 units of DpnI (New England Biolabs, Ipswich, MA) for 5 minutes at 37 °C, which completely degraded the methylated plasmid template. The isolated DNA was separated on an agarose electrophoresis gel to determine replication products that were quantified using a Phosphor Imager (Amersham, Piscataway, NJ).
**Replication re-initiation assay**

The protocol was adapted from (Kennedy et al., 2000; Sengupta et al., 2003). Briefly, cells were cultured with 1.5 mM hydroxyurea (HU) for 16 hours. The cells were then released from HU inhibition into medium containing 150 µM BrdU for 10 minutes in the dark. The cells were fixed immediately, permeabilized with 0.5% Triton X-100, and treated with 10 units of DNase I at 37°C for 1 hour to denature the DNA. The antibodies used for staining were mouse anti-BrdU (BD Biosciences, San Jose, CA); rabbit anti-FLAG M2 (Sigma, St. Louis, MO); Alexa Fluor 488 goat anti-mouse and Alexa Fluor 546 goat anti-rabbit (Invitrogen, Carlsbad, CA).

**Chromatin immunoprecipitation (ChIP)**

ChIP was conducted as described (Saharia et al., 2008).

**Metaphase preparation, FISH, and chromosome orientation FISH (CO-FISH)**

Metaphase preparation, FISH and CO-FISH were conducted as described (Saharia et al., 2008). Aphidicolin treatments were conducted as described (Sfeir et al., 2009).

**Statistical Analysis**

The Student’s T-test (two-tailed distribution with equal variance) was used for BrdU foci, CO-FISH, and fragile telomere analyses.
Results

FEN1 depletion does not impact S phase progression

Previously, we demonstrated that FEN1 depletion in telomerase-negative cells resulted in telomere dysfunction (Saharia et al., 2008). However, in telomerase-positive cells neither telomere dysfunction nor cytogenetic abnormalities were observed upon FEN1 depletion (Saharia et al., 2008). Although this observation suggested that FEN1 is dispensable for genomic replication at large, we wished to more directly assess the impact of FEN1 depletion on genomic replication by measuring S phase progression. Because telomere dysfunction might impact S phase progression and this defect is rescued in telomerase-positive cells (Saharia and Stewart, 2009), telomerase-positive HeLa cells were transduced with a lentiviral construct encoding a short hairpin RNA (shRNA) targeting the FEN1 3'-UTR (shFEN3) or a control hairpin (shSCR). Expression of shFEN3 led to a significant reduction in FEN1 protein compared with control cells expressing shSCR. To follow cells through the cell cycle, cells were pulsed with 5-bromo-2-deoxyuridine (BrdU) for one hour to label the S phase population, and cells were followed as they continued through the cell cycle. As expected from our previous work, in the absence of telomere dysfunction, there was no difference in S phase progression when cells were transduced with shFEN3 or shSCR. As shown in Figure 2.1A, regardless of the status of FEN1, approximately 35% of the cells were in S phase after a one-hour BrdU pulse. Both control and FEN1-depleted cells exited S phase and progressed through the cell cycle with similar kinetics (Figure 2.1B). These data indicates that FEN1 depletion does not significantly impact cell cycle progression and suggests that it is not essential for DNA replication in vivo.
FEN1 depletion does not impact DNA replication kinetics in vitro

Above, we showed that FEN1 depletion does not impact S phase progression, suggesting that other nucleases compensate for FEN1 loss during Okazaki fragment processing. However, because minor effects on DNA replication might be missed by the S phase progression assay, we next examined the impact of FEN1 depletion on DNA replication kinetics through non-telomeric DNA sequences. To measure replication kinetics in the presence or absence of FEN1, we conducted an SV40 Large-T antigen-dependent in vitro DNA replication assay (Brush et al., 1995) using cell lysates isolated from control or FEN1-depleted HeLa cells (Figure 2.1C). The DNA replication reaction was reconstituted with lysates from control or FEN1-depleted cells and carried out for 0, 15, 30, 60, and 120 minutes using a linearized plasmid containing an SV40 origin of replication. We compared the kinetics of replication by measuring the formation of newly synthesized full length linear DNA. As shown in Figures 2.1D and 2.1E, there was no difference in DNA replication efficiency when lysates from control versus FEN1-depleted cells were used. In addition, we found that the product was insensitive to DpnI treatment (data not shown), indicating that DNA replication was semi-conservative and proceeded with the same efficiency in control and FEN1-depleted cells. Previous work (Brush et al., 1995) utilizing a defined, reconstituted system indicated that FEN1 is required for SV40-dependent DNA replication. However, our data clearly show that replication continues unabated upon FEN1 depletion. These results are in agreement with our S phase progression data and suggest that other nucleases (e.g. Dna2 and/or ExoI) present in the cell lysate compensate for FEN1 function during DNA replication in non-telomeric sequences.
FEN1 depletion leads to inefficient replication fork restart

Recently, we demonstrated that in human cells, FEN1 depletion results in telomere dysfunction while having little impact on total genome stability (Saharia et al., 2008). Above, we showed that FEN1 depletion has no impact on S phase progression or DNA replication kinetics in vitro. These results were intriguing as they suggested that other proteins compensate for FEN1 depletion during genomic replication and/or repair, but these same proteins are ineffective within telomeric sequences. Interestingly, the FEN1ΔC mutant that does not interact with WRN is unable to rescue telomere dysfunction upon depletion of endogenous FEN1 (Saharia et al., 2008; Sharma et al., 2005). Given the data implicating FEN1 and WRN in replication fork re-initiation (Sharma et al., 2004; Zheng et al., 2005) and the perceived need for this complex for efficient telomere replication, we hypothesized that FEN1 is required for the re-initiation of stalled replication forks in telomeric sequences. Therefore, we first addressed how FEN1 depletion impacts DNA replication fork re-initiation after hydroxyurea treatment.

We have hypothesized that failure to rescue stalled replication forks results in STLs. Therefore, we created an experimental paradigm that allowed us to examine the impact of FEN1 depletion on the efficiency of re-initiation of stalled replication forks. Because telomerase rescues the STL phenotype (Saharia et al., 2008) and we wished to first examine the impact of FEN1 loss on re-initiation of stalled replication forks in the absence of telomere dysfunction, we carried out our analysis in telomerase-positive HeLa cells.
Hydroxyurea treatment causes nucleotide depletion, resulting in DNA replication fork stalling. Upon removal of hydroxyurea, nucleotide pools recover and stalled DNA replication forks re-initiate, allowing S phase to proceed. To investigate whether FEN1 contributes to DNA replication fork re-initiation, we induced stalled DNA replication forks in HeLa cells by treating them with hydroxyurea for 16 hours and then releasing them in the presence of BrdU for ten minutes. Because BrdU is only incorporated where DNA replication forks re-initiate, the efficiency of fork re-initiation can be determined by quantifying BrdU foci (Figure 2.2A) (Kennedy et al., 2000). We hypothesized that if FEN1 participates in the stabilization or restart of stalled DNA replication forks, its depletion would result in fewer re-initiation events, and thus, fewer BrdU foci would be observed.

HeLa cells were transduced with a lentiviral construct encoding shFEN3 or shSCR. Expression of shFEN3 led to a significant reduction in FEN1 protein compared to cells expressing a control hairpin (shSCR) (Figure 2.2B). Control cells and FEN1-depleted cells were cultured for 16 hours in the presence of hydroxyurea and then released from hydroxyurea inhibition in the presence of BrdU for 10 minutes (Figure 2.2A). BrdU foci were observed by immunofluorescence. As expected, FEN1 depletion resulted in a striking decrease in the number of BrdU foci, indicating that FEN1 is important for efficient re-initiation of stalled DNA replication forks in vivo (Figure 2.2C). In cells expressing the control hairpin, there was an average of 15 BrdU foci per cell. In contrast, FEN1 depletion led to an average of 6.5 BrdU foci per cell, a greater than 50% decrease ($p < 0.0001$; Figure 2.2D). Importantly, upon FEN1 depletion, cells retained
the ability to re-initiate stalled DNA replication forks, albeit less efficiently. Together these results demonstrate that FEN1 is important for efficient restart and/or stabilization of stalled DNA replication forks.

To date, the biochemical properties of FEN1 critical to the restart of stalled DNA replication forks have not been determined. Therefore, we carried out the re-initiation assay described above in cells depleted of endogenous FEN1 and expressing various FEN1 mutants as outlined in Figure 2.3A. The different FEN1 alleles used in this study included 1) human wild-type (hWT), which is competent for both replication and repair functions, 2) D181A (DA), which lacks nuclease activity (Shen et al., 1996), thus representing a loss-of-function allele, 3) delta C (ΔC; 20 amino acid deletion of the C-terminus), which retains near wild-type ability to process flap structures together with the replication clamp (Stucki et al., 2001a; Stucki et al., 2001b), PCNA, and is, therefore, competent for Okazaki fragment processing but is unable to bind the BLM and WRN helicases and participate in FEN1’s DNA repair functions (Sharma et al., 2005; Stucki et al., 2001a), 4) delta P (ΔP; an eight-amino acid deletion), which retains the ability to interact with the RecQ helicases, BLM and WRN, but is unable to interact with PCNA (Stucki et al., 2001a; Stucki et al., 2001b), thus rendering it replication-incompetent yet repair-competent, and 5) delta P–delta C (ΔPΔC; 44-amino acid deletion of the C-terminus), which deletes FEN1’s nuclear localization signal and abrogates its ability to interact with PCNA, BLM, and WRN, thus creating a second loss-of-function allele that retains the nuclease domain. Finally, we expressed a mouse E160D (mED) mutant FEN1, which retains near wild-type levels of FEN activity and the
ability to participate in DNA replication but is devoid of a GEN activity (Zheng et al., 2007). The GEN activity has been shown to process DNA bubble structures reminiscent of stalled replication forks and is hypothesized to participate in re-initiation of stalled replication forks (Liu et al., 2006; Zheng et al., 2005).

To facilitate our analysis, we depleted cells of endogenous FEN1. After depletion of FEN1, cells were infected with adenoviral constructs expressing a wild-type or mutant FEN1 allele. Transduced cells were allowed to grow for 4 days and then treated with hydroxyurea for 16 hours followed by a 10-minute BrdU pulse to label re-initiated DNA replication forks (Figure 2.3B). To facilitate identification of successfully transduced cells, each of the FEN1 constructs was tagged with a FLAG epitope. Therefore, after the BrdU pulse, cells were fixed and stained with anti-BrdU and anti-FLAG antibodies, and BrdU foci were only quantified in FLAG-positive cells that expressed the transduced cDNAs. As expected, expression of hWT FEN1 recovered the number of BrdU foci lost in FEN1-depleted cells to numbers slightly higher than that observed in control cells. Indeed, expression of wild-type FEN1 led to an average of 18 BrdU foci per nucleus compared to 6.5 foci in FEN1-depleted cells, demonstrating that the phenotype observed was specific to FEN1 loss (Figures 2.3C and 2.3D). The significance of this slight increase in re-initiated replication forks is unclear but may be related to the level of FEN1 expression. In contrast, expression of the nuclease-deficient FEN1 mutant (also devoid of GEN activity), DA, did not rescue FEN1 depletion and resulted in an average of 5.5 foci per nucleus, indicating that the nuclease activity of FEN1 is critical for its function in the re-initiation of stalled DNA replication forks (Figures 2.3C and
Similarly, expression of the \( \Delta P \Delta C \) mutant, a functionally null allele, was unable to rescue the reduction in BrdU foci observed upon FEN1 depletion (Figures 2.3C and 2.3D). Expression of FEN1\( \Delta C \) also failed to rescue the decreased number of BrdU foci observed in FEN1-depleted cells. Because this mutant is able to interact with PCNA and is competent for Okazaki fragment processing, this result suggests that the interactions between FEN1 and the RecQ helicases, BLM and WRN, are important for FEN1’s role in the re-initiation of stalled DNA replication forks (Figures 2.3C and 2.3D). Finally, we found that expression of the \( \Delta P \) mutant resulted in an average of 15.6 BrdU foci (Figures 2.3C and 2.3D), demonstrating that the FEN1 interaction with PCNA is not critical for its role in the re-initiation of stalled DNA replication forks.

Analysis of our FEN1 mutants indicates that FEN1 activity distinct from its ability to participate in Okazaki fragment processing is critical for the restart of stalled DNA replication forks. This result and the existence of the FEN1 GEN activity, which is stimulated by WRN (Liu et al., 2006) to cleave DNA bubble structures that resemble stalled replication forks, suggests that this activity is functionally important at stalled replication forks. To establish whether the GEN function is important for the restart of stalled replication forks, we next tested the impact of expression of a GEN-deficient FEN1 mED allele. Expression of the mED mutant failed to rescue the phenotype observed in FEN1-depleted cells, which displayed an average of 5.1 BrdU foci per nucleus (Figures 2.3C and 2.3D). As expected, the mouse wild-type protein, mWT, completely recovered the number of BrdU foci observed upon FEN1 depletion with an average of 17 foci per nucleus (Figures 2.3C and 2.3D). Because the mED mutant
processes Okazaki fragments near wild-type levels, these data indicate that FEN1 GEN activity is required to restart stalled DNA replication forks.

**FEN1 localizes to the telomere**

Our previous work supports the hypothesis that FEN1 activity at the telomere is critical for high fidelity DNA replication and that other nucleases compensate for FEN1 loss across the genome but fail to do so at the telomere (Saharia et al., 2008). Given these results, we next wished to characterize the impact of the FEN1 mutants described above at the telomere. Because recent work demonstrates that FEN1 localizes to the mammalian telomere (Muftuoglu et al., 2006; Saharia et al., 2008; Verdun and Karlseder, 2006), we first examined the ability of the FEN1 mutants to localize to the telomere.

To determine whether the various FEN1 mutants retain the ability to localize to the telomere, we carried out chromatin immunoprecipitation (ChIP) experiments. As expected, the hWT FEN1 and FEN1ΔP mutant localized to the telomere (Figures 2.4A and 2.4B). In contrast, the FEN1ΔPΔC mutant was unable to precipitate telomeric DNA (Figures 2.4A and 2.4B). The latter result was expected because the ΔPΔC mutant lacks the nuclear localization domain and is unable to localize to the nucleus (Figure 2.3A). Finally, both the mWT and mED proteins localized to the telomere. These data indicate that FEN1 mutants that retain the ability to participate in replication fork re-initiation also localize to the telomere.
FEN1 depletion leads to the induction of fragile telomeres

Telomeres are chromosome fragile sites as evidenced by the appearance of multiple telomeric signals after aphidicolin treatment (Martinez et al., 2009; Sfeir et al., 2009). Interestingly, the presence of these multiple telomere signals also arises upon depletion of Apollo, ATM, ATR, BLM, and TRF1, suggesting that several protein components repress telomere fragility (Martinez et al., 2009; Sfeir et al., 2009; Undarmaa et al., 2004; van Overbeek and de Lange, 2006). Because fragile sites are thought to result from replication stress and an inability to resolve stalled replication forks (Durkin and Glover, 2007), this observation raised the possibility that the STL observed upon FEN1 depletion (Saharia et al., 2008) is the result of unresolved stalled replication forks and expression of fragile sites within telomeric sequences. Given our results above demonstrating that FEN1 facilitates re-initiation of stalled replication forks, we postulated that FEN1 depletion would lead to fragile telomere expression. Analysis of metaphase spreads prepared from aphidicolin-treated or FEN1-depleted BJ fibroblasts revealed an increase in fragile telomeres (data not shown and Figure 2.5A). Indeed, 16% of the chromosomes from BJ fibroblasts depleted of FEN1 demonstrated the fragile telomere phenotype, significantly up from the control cells (8.2%; \( p < 0.0001 \)). Surprisingly, this increase in fragile telomere expression was also observed upon FEN1 depletion in BJ fibroblasts expressing SV40 Large T antigen and telomerase (BJLT). FEN1 depletion in BJLT cells resulted in 15.1% of chromosomes exhibiting multiple telomere signals, significantly higher than the 9.3% observed in the control samples (Figure 2.5B). These results indicate that FEN1 plays a role in the repression of fragile site expression at mammalian telomeres. Furthermore, because telomerase expression
rescues the STL phenotype (Saharia et al., 2008) but not expression of telomeric fragile sites, these results suggest that fragile telomere expression is either upstream of STLs or represents a second form of telomere dysfunction independent of STLs.

**FEN1’s DNA replication fork re-initiation activity is critical to telomere stability**

The telomere consists of G-rich repetitive DNA that has the propensity to form secondary structures, including G-quadruplexes that can impede the movement of the DNA replication fork (Gilson and Geli, 2007; Maizels, 2006; Miller et al., 2006; Ohki and Ishikawa, 2004; Verdun and Karlseder, 2006). Indeed, it has been hypothesized that stalled DNA replication forks frequently occur at the telomere (Gilson and Geli, 2007; Verdun and Karlseder, 2006). Failure to resolve a stalled DNA replication fork within the telomere would lead to fork collapse, formation of a double strand DNA break, and telomere loss (Branzei and Foiani, 2005). In support of this, recent studies suggest that collapsed replication forks at telomeres lead to the formation of very short telomeres (Crabbe et al., 2004; Khadaroo et al., 2009; Xu and Blackburn, 2007), and, as discussed above, the expression of fragile telomeres (Martinez et al., 2009; Sfeir et al., 2009). We recently demonstrated that FEN1 depletion results in telomere dysfunction characterized by STLs (Saharia et al., 2008), indicating that FEN1 functions in telomere maintenance through DNA replication or repair. Given our observation that FEN1 contributes to efficient re-initiation of stalled DNA replication forks, we next wished to determine whether it was the role of FEN1 in Okazaki fragment processing or the restart of stalled DNA replication forks that contributes to telomere stability. Because telomerase expression compensates for FEN1 loss at the telomere, thus masking the STL phenotype (Saharia et al., 2008; Saharia and Stewart, 2009), we utilized BJ
fibroblasts, which express insufficient telomerase to maintain telomere lengths (Masutomi et al., 2003) for these studies.

To determine the impact of FEN1 mutant expression on telomere stability, endogenous FEN1 was depleted from BJ fibroblasts (Figure 2.6A). After shRNA-mediated FEN1 depletion, cells were infected with an adenovirus expressing a wild-type or mutant FEN1 allele (Figure 2.6B, greater than 85% of the cells were infected; data not shown). Because FEN1 depletion leads to lagging strand-specific STL, we analyzed the strand-specific loss of telomeres in cells expressing different FEN1 alleles (Saharia et al., 2008). To carry out this analysis, we utilized a technique referred to as chromosome orientation fluorescent in situ hybridization (CO-FISH), which takes advantage of the fact that the G- and C-rich strands of the telomere are exclusively replicated by lagging and leading strand DNA synthesis, respectively (Figure 2.6C). In agreement with our previous results (Saharia et al., 2008), FEN1 depletion led to specific loss of lagging strand-replicated telomeres (9.8% in shFEN3 cells compared to 3.1% in the control shSCR cells; \( p < 0.0001 \)) while having no impact on telomeres replicated by the leading strand machinery (Figures 2.6D and 2.6E). Expression of wild-type FEN1 rescued the lagging strand STL phenotype (3.2% lagging strand STLs were observed, similar to that observed in shSCR control cells), indicating that the phenotype was specific to FEN1 depletion. Similarly, expression of the FEN1\(\Delta P\) mutant resulted in 3.6% lagging strand STLs (\( p < 0.0001 \) compared with shFEN3), indicating that the FEN1 interaction with PCNA, and, hence, its ability to participate in Okazaki fragment processing is not important for its function at the telomere. In contrast, expression of the \(\Delta P\Delta C\) null allele
led to 8% lagging strand STLs, indicating that it failed to rescue telomere dysfunction upon FEN1 depletion. Intriguingly, in contrast to the mWT protein, which rescued the lagging strand STL defect upon FEN1 depletion, the mED mutant failed to rescue FEN1 depletion at the telomere. Indeed, expression of mWT significantly decreased the number of lagging strand STLs upon FEN1 depletion to 2.8%, whereas expression of the mED mutant resulted in lagging strand STLs (9.7%, \( p < 0.0001 \)) similar to those observed in ΔPΔC-expressing cells (Figure 2.6) and in ΔC-expressing cells (Saharia et al., 2008). However, the FEN1ΔC protein demonstrated reduced telomere localization, raising the possibility that the STL phenotype observed upon FEN1ΔC expression was not because of reduced FEN1 re-initiation function, but, rather, its reduced telomere localization. Because the mED mutant retains the ability to participate in Okazaki fragment processing and localizes to the telomere at the same efficiency as the wild-type protein (Figure 2.4), the failure of mED to rescue the STL phenotype indicates that the FEN1 gap endonuclease activity is critical for its role at the mammalian telomere. Furthermore, these data demonstrate that the FEN1 interaction with PCNA is dispensable for its role at the telomere.

**Discussion**

Telomeres perform a critical cellular function by distinguishing the chromosome end from a bona fide double-stranded DNA break. As such, mechanisms that modify the activities of DNA repair and replication proteins, presumably through interaction with the Shelterin complex, have evolved to protect the telomere and ensure its faithful replication. The need for telomere-specific replication mechanisms is likely due to the
nature of the telomeric DNA sequence, which presents a number of challenges to the DNA replication machinery (Gilson and Geli, 2007; Verdun and Karlseder, 2007). G-rich, repetitive, telomeric sequences have a high propensity to form secondary structures such as G-quadruplexes (G4) that impede the progressing replication fork, leading to the formation of stalled forks (Gilson and Geli, 2007; Maizels, 2006; Parkinson et al., 2002). Indeed, telomeres were recently identified as fragile sites (Sfeir et al., 2009), and several reports have indicated pausing or stalling of replication forks within telomeres (Ivessa et al., 2002; Khadaroo et al., 2009; Makovets et al., 2004; Verdun and Karlseder, 2006). Additionally, telomere replication is primarily initiated by the most centromere-distal origin of replication and continues unidirectionally toward the end of the telomere (Sfeir et al., 2009). If a replication fork stalls within the telomere and is not re-initiated, the absence of a converging replication fork would result in telomere loss. Therefore, mechanisms that facilitate replication fork movement through the telomere are critical to high fidelity telomere replication.

The importance of the Shelterin complex to telomere replication is underscored by several studies. For example, Taz1 in S. pombe and TRF1 in mice are required for efficient telomere replication. Loss of Taz1 results in replication fork stalling throughout telomeric sequences (Miller et al., 2006), whereas loss of TRF1 leads to expression of fragile telomeres (Sfeir et al., 2009). The ability of telomere-binding proteins to facilitate replication fork progression through the telomere is postulated to require recruitment of specialized proteins (Gilson and Geli, 2007; Sfeir et al., 2009). For example, TRF1 and TRF2 interact with and stimulate the RecQ helicases, BLM and WRN (Lillard-Wetherell
et al., 2004; Opresko et al., 2004; Sfeir et al., 2009), suggesting that they recruit these proteins to enhance DNA replication and/or repair at the telomeres. Interestingly, a recent study demonstrated that TRF2 increases branch migration of Holliday junction intermediates, suggesting that this promotes the formation of chicken foot structures in the context of a stalled replication fork at telomeres (Poulet et al., 2009). FEN1 also interacts with TRF2 (Muftuoglu et al., 2006; Saharia et al., 2008), and because FEN1 GEN activity is postulated to process chicken foot structures (Liu et al., 2006; Zheng et al., 2005), this raises the possibility that TRF2 engages the RecQ helicase–FEN1 complex coordinately at the telomere to resolve stalled replication forks and enable their efficient restart.

WRN participates in the re-initiation of stalled replication forks in vivo (Dhillon et al., 2007; Sidorova et al., 2008). Interestingly, FEN1 was shown to localize with WRN, raising the possibility that it contributes to replication fork restart (Sharma et al., 2004). Furthermore, FEN1 and WRN process branch migrating structures that resemble regressed replication forks in vitro (Sharma et al., 2004). The present study demonstrates for the first time that FEN1 functionally participates in the re-initiation of stalled replication forks in vivo. Together with previous work (Nikolova et al., 2009), this indicates that FEN1’s role in S phase is two-fold: first, in Okazaki fragment processing during DNA replication and, second, in the re-initiation of stalled replication forks. FEN1 localizes to mammalian telomeres during S phase (Saharia et al., 2008; Verdun and Karlseder, 2006), so it could be involved in one or both of the functions outlined above. However, given that the proliferating cell nuclear antigen (PCNA)-interacting domain of
FEN1 is dispensable for telomere stability, our data indicate that the role of FEN1 in Okazaki fragment processing is non-essential for telomere stability. This result indicates that either sufficient FEN1 remains in FEN1-depleted cells to support continued replication or that other nucleases such as DNA2 or EXO1, which can also process Okazaki fragments (Ayyagari et al., 2003; Bae and Seo, 2000; Kang et al., 2000; Kao et al., 2004; Moreau et al., 2001), compensate for FEN1 loss during lagging strand DNA replication. However, these same nucleases are insufficient when replication forks stall within telomeric sequences. Indeed, we find that in the absence of the ability of FEN1 to re-initiate stalled replication forks, sister telomeres are lost despite the presence of other nucleases. Interestingly, other proteins involved in the re-initiation of stalled replication forks such as PARP1 and PARP2 have also been implicated in telomere maintenance (Bryant et al., 2009; Dantzer et al., 2004; Ye and de Lange, 2004), further indicating the importance of the re-initiation process for efficient telomere replication. An alternate hypothesis is that FEN1 is important for fork stabilization after hydroxyurea treatment. The assay we have conducted cannot differentiate between FEN1-dependent fork stabilization and fork re-initiation.

Intriguingly, the C-terminal region of FEN1 is essential for its function at the telomere and also mediates its interaction with another RecQ helicase, BLM (Sharma et al., 2005). Similar to WRN, BLM is able to unwind G4 DNA, is critical for the re-initiation of stalled replication forks, and has recently been suggested to be important for efficient telomere replication (Davies et al., 2007; Sengupta et al., 2003; Sfeir et al., 2009; Sun et al., 1998). This suggests that there is complicated interplay between WRN, BLM, and
FEN1 at mammalian telomeres. Although the function of BLM at telomeres has not been well characterized, recent work suggests that it is important for repression of fragile telomeres (Sfeir et al., 2009). Interestingly, FEN1 depletion also leads to an increase in fragile telomere expression, raising the possibility that these proteins work as a complex to repress telomere fragility. Together, these data are consistent with the hypothesis that FEN1 and the RecQ helicases play an important role in the maintenance of stable telomeres through re-initiation of stalled replication forks.

Here we demonstrate that FEN1 is important for efficient re-initiation of stalled replication forks in vivo. This function of FEN1 is dependent on its C-terminal domain and its GEN activity. However, despite the importance of FEN1 in re-initiation of stalled replication forks, FEN1 depletion in telomerase-positive cells did not affect S phase progression or SV40 Large T antigen-dependent in vitro DNA replication, suggesting that other nucleases compensate for FEN1-dependent replication function throughout the genome. However, these same proteins fail to compensate for FEN1 at the telomere. Indeed, FEN1 depletion leads to increased telomere fragility and lagging strand STLs. As with the re-initiation of stalled replication forks, both the FEN1 C-terminus and GEN activity are essential for its function at telomeres, whereas its ability to interact with PCNA is dispensable. Collectively, these data demonstrate that FEN1 is necessary for efficient replication of telomeres, and we propose that FEN1 promotes replication fork re-initiation within telomeric sequences.
Acknowledgments

We are grateful to Dr. Ulrich Hübscher for providing the FEN1ΔP cDNA, Dr. Binghui Shen for providing the mWT and mED FEN1 cDNAs, and Dr. Fuyuki Ishikawa for providing pSVO.11-2K plasmid. We are also grateful to Dr. Marc Wold, Dr. Peter Burgers, and members of the Stewart Laboratory for valuable discussions, and to Ying Jie Lock, Ermira Pazolli, and Dr. Susana Gonzalo for critical reviews. This work was supported in part by the Children’s Discovery Institute (S.A.S.) and Training Program in Cellular Biology, T32GM007067-35 (D.C.T.).
Figure 2.1. FEN1 depletion does not affect S phase progression or in vitro DNA replication.

(A) Cell cycle progression of HeLa cells expressing shSCR or shFEN3 is shown. HeLa cells were labeled with BrdU for 1 h and analyzed at the indicated times using an anti-BrdU antibody (FITC-conjugated) and 7-amino-actinomycin D (7-AAD) to label DNA content. BrdU-positive cells are displayed on the y axis and represent cells that transit
through S phase during BrdU labeling. The x axis displays the DNA content of the cells as indicated by incorporation of 7-amino-actinomycin D (G1 and G2/M cells have a 2n and 4n content of DNA, respectively).

(B) Quantification of the percent of BrdU-positive cells in S phase after BrdU pulse (representative experiment is shown) is shown. The cells present in the inset boxes in (A) are BrdU-positive and consist of cells in G1, S, and G2/M phases of the cell cycle. Only the S phase cells (those that are present between G1 and G2 (2n and 4n DNA content, respectively) within the BrdU-positive population are plotted on the graph. Error bars represent S.E.

(C) Shown are Western blots of S100 lysates from control and FEN1-depleted HeLa cells. Cyclophilin A (CycA, lower panel) is shown as a loading control.

(D) An SV40 Large T antigen-dependent in vitro DNA replication assay was conducted using lysates from control (shSCR) and FEN1-depleted (shFEN3) HeLa cells as described under Experimental Procedures. The assay was stopped at the indicated times, and the replication products were separated via gel electrophoresis. The replication products were detected via autoradiography (Autorad), and the input DNA was observed via ethidium bromide (EtBr) staining.

(E) Shown is quantification of the replication products at the indicated times in (D). Two independent experiments were conducted in duplicate, and the average of the four experiments is shown. The error bars represent S.E.
Figure 2.2. FEN1 depletion decreases re-initiation of stalled replication forks.

(A) Shown is a schematic of the stalled replication fork re-initiation assay and the expected results. HU, hydroxyurea.

(B) A western blot analysis shows FEN1 depletion. Short hairpins against FEN1 (shFEN3) or a scrambled sequence (shSCR) were expressed in HeLa cells. FEN1
(upper panel) and β-actin (lower panel) protein levels were assessed by western blot analysis.

(C) Representative images show that FEN1 depletion decreases BrdU incorporation in hydroxyurea-treated cells. Immunofluorescence was conducted using an anti-BrdU antibody (green) and 4',6-diamidino-2-phenylindole (DAPI, blue).

(D) Quantification of the number of BrdU foci per cell in HeLa cells transduced with the indicated shRNA is shown. BrdU foci in no fewer than 100 cells were counted for each condition, and the experiment was conducted twice (a representative experiment is presented). Error bars represent S.E. (*, p < 0.0001).
Figure 2.3. The gap endonuclease activity and C terminus of FEN1 are essential to re-initiate stalled replication forks.

(A) The schematic shows the different FEN1 alleles used in the study. Inferences on whether the different FEN1 alleles are replication competent or repair competent are shown on the right of the schematic with their associated references. These inferences were made based on nuclease activity and ability to interact with the WRN and PCNA proteins. The mutant proteins are as follows: ΔC (amino acids 360–380 deleted), ΔP (amino acids 337–344 deleted), and ΔPΔC (amino acids 337–380 deleted).

(B) The timeline of the experimental procedure is given in days.

(C) Representative images show BrdU incorporation after hydroxyurea treatment in FEN1-depleted cells expressing wild-type or FEN1 mutants. Immunofluorescence was conducted using an anti-BrdU (green) antibody, anti-FLAG (red) antibody, and DAPI (blue).

(D) Quantification of the number of BrdU foci per cell in FEN1-depleted HeLa cells with the indicated ectopic FEN1 expression (wild-type or mutant) is shown. Only cells expressing FLAG-tagged FEN1 (marked by red in (C)) was quantified. No fewer than 75 cells were counted for each condition, and the experiment was conducted twice (a representative experiment is presented). The error bars represent S.E. (*, p < 0.0001 compared with shSCR; Δ, p < 0.0001 compared with hWT; #, p < 0.0001 compared with mWT).
Figure 2.4. FEN1 mutants localize to the telomere.

(A) FEN1 alleles localize to the telomere. Representative ChIP analysis of 293T cells (Ctrl) or 293T cells transfected with wild-type FEN1 (hWT or mWT) or FEN1 mutants (ΔP, ΔPΔC, or mED), subjected to immunoprecipitation with the FLAG (M2) antibody. Precipitated DNA was probed for the presence of telomeric sequences as described under Experimental Procedures. The inputs indicate 0.1% and 0.2% of total.

(B) Quantification of the representative ChIP assay is shown. Percent of telomeric DNA immunoprecipitated with the FLAG antibody was calculated using input DNA, and the control pulldown percentage was set to 1.
Figure 2.5. FEN1 depletion results in fragile site expression at telomeres.

(A) Representative FISH of metaphases obtained from BJ fibroblasts (BJ) or BJ fibroblasts expressing SV40 Large T antigen and telomerase (BJLT). Cells expressing a control hairpin (shSCR) or depleted of FEN1 (shFEN3) are indicated. Chromosomes were hybridized with the PNA telomere probe Cy3-(CCCTAA)₃ (red) and stained with DAPI (blue). Magnified images show representative fragile telomeres (arrowheads).

(B) Quantification of telomere fragility, depicted as the number of fragile telomeres observed per chromosome. No fewer than 60 metaphases from two independent experiments were analyzed for each condition, and an average of the two experiments is shown (*, p < 0.0001; Δ, p < 0.001). Error bars represent S.E.
Figure 2.6. The gap endonuclease activity of FEN1 is essential for its function at the telomere.

(A) Western blot analysis of FEN1 (upper panel) from BJ fibroblasts expressing a control hairpin (shSCR) or depleted of FEN1 (shFEN3) is shown. β-Actin (lower panel) is shown as a loading control.

(B) Shown is a timeline of experimental procedure given in days.

(C) A CO-FISH schematic is shown. Newly synthesized DNA strands incorporate BrdU and BrdC. UV and ExoIII treatment resulted in degradation of newly synthesized DNA containing BrdU and BrdC, and the template strands were hybridized with Cy3-(CCCTAA)$_3$ (red, lagging strand) and fluorescein-(TTAGGG)$_3$ (green, leading strand) PNA probes.

(D) Representative CO-FISH of chromosomes from BJ fibroblasts expressing shSCR or shFEN3 and the indicated FEN1 alleles as shown. Ctrl refers to cells that do not express exogenous FEN1 protein. Color schemes are as described in (C). DNA was stained with DAPI (blue). The arrowheads indicate missing telomeres.

(E) Shown is quantification of STLs on metaphase chromosomes after depletion of endogenous FEN1 and expression of the indicated FEN1 allele, depicted as percentage of chromosomes with missing leading (green) and lagging (red) strand telomeres. A minimum of 60 metaphases from two independent experiments was analyzed per treatment in a blinded fashion, and an average of the two experiments is shown (*, p < 0.0001). The error bars represent S.E.
References


Kao, H.I., Campbell, J.L., and Bambara, R.A. (2004). Dna2p helicase/nuclease is a tracking protein, like FEN1, for flap cleavage during Okazaki fragment maturation. J. Biol. Chem. 279, 50840-50849.


Chapter 3:

Flap endonuclease 1 limits telomere fragility
on the leading strand

Daniel C. Teasley, Shankar Parajuli, Mai Nguyen, Hayley R. Moore,
Elise Alspach, Ying Jie Lock, Yuchi Honaker, Abhishek Saharia,
Helen Piwnica-Worms, and Sheila A. Stewart

This chapter was originally published in The Journal of Biological Chemistry, 2015; 290:
15133–15145. © the American Society for Biochemistry and Molecular Biology
Introduction

DNA replication and repair are high-fidelity processes that maintain genome stability. Due to the importance of these processes, robust mechanisms have evolved to ensure they are completed even when components of the replication and repair pathways are compromised or absent due to mutation. In some instances, this compensation is inadequate. Indeed, mutations in specific replication or repair proteins give rise to genetic disorders such as ataxia telangiectasia, Bloom syndrome, and Fanconi anemia. Cells from these patients reveal that while gross DNA metabolism continues largely unabated, mild replication defects and sensitivity to DNA damaging agents or ionizing radiation contribute to genomic instability and increased cancer incidence (Sidorova, 2008; Singh et al., 2009).

While the redundancy of replication and repair mechanisms ensures faithful replication of the bulk genome, regions with repetitive sequence or an ability to form secondary structures are problematic and thus particularly sensitive to mutations in DNA replication and repair proteins (Gilson and Géli, 2007). This is best illustrated at common fragile sites, where replication stressors lead to replication defects and genomic instability. Why particular regions of the genome manifest as fragile sites remains obscure, but insufficient replication origins, repetitive sequences, and replication–transcription interference have all been implicated (Burrow et al., 2010; Helmrich et al., 2011; Letessier et al., 2011).

Recently, telomeres have also been described as fragile sites because treatment with aphidicolin, a potent inducer of replication stress, results in reduced replication fork
progression and abnormal telomere structures (Martínez et al., 2009; Sfeir et al., 2009).

In checkpoint-competent backgrounds, aphidicolin treatment increases telomere fragility by 1.5 to 4.5-fold (Martínez et al., 2009; McNees et al., 2010; Sfeir et al., 2009), while suppression of the ataxia and telangiectasia and Rad3-related (ATR) kinase is sufficient to induce a 1.7-fold increase in telomere fragility in murine Seckel cells (McNees et al., 2010). Telomere fragility is also induced in the absence of telomere-binding proteins that participate in telomere replication. Indeed, knockout of the Shelterin complex member TRF1, which is required for replication fork progression through the telomere, increases the rate of telomere fragility in murine cells by 3.0-to 4.5-fold (Martínez et al., 2009; Sfeir et al., 2009; Vannier et al., 2012); similarly, depletion of the CST complex members CTC1 or STN1, which are important for replication fork restart at the telomere, causes between a 2.0- and 3.0-fold increase in telomere fragility in human cells (Stewart et al., 2012).

DNA replication and repair proteins are also important in maintaining telomere stability by preventing or suppressing telomere fragility. We previously reported that depletion of flap endonuclease 1 (FEN1) results in a 2.0-fold increase in telomere fragility (Saharia et al., 2010). Loss of the DNA glycosylase Nth1, which participates in the repair of oxidative stress-induced lesions, causes a 1.8-fold increase in telomere fragility (Vallabhaneni et al., 2013). Helicases and topoisomerases also play roles in reducing telomere fragility. Depletion of Topollα causes up to an approximately 7-fold increase in telomere fragility, and depletion of the RecQ helicase BLM induces a 1.9-fold increase in telomere fragility (D'Alcontres et al., 2014; Sfeir et al., 2009). Similarly, RTEL1 depletion or deletion induces 2.3-fold and 4.0-fold increases in telomere fragility,
respectively (Sfeir et al., 2009; Vannier et al., 2012). These studies demonstrate the wide range of genetic manipulations that can induce telomere fragility with varying levels of severity.

The mechanism(s) by which telomere fragility occurs is not clear, but the large number of proteins implicated in the phenotype suggests that multiple mechanisms exist. G-quadruplexes may play a role, as telomere fragility induced by RTEL1 deletion is exacerbated by treatment with the G-quadruplex-stabilizing drug TMPyP4 (Vannier et al., 2012). Given these data, if the molecular event inducing telomere fragility occurs after the replication fork has passed, RTEL1-induced telomere fragility would be expected to exhibit lagging strand specificity; however, few studies have examined strand-specific telomere fragility. Sfeir et al. examined TRF1 knockout mouse cells using chromosome-orientation fluorescent in situ hybridization (CO-FISH), which is capable of distinguishing telomeres replicated by the leading versus lagging strand DNA replication machinery; they found that telomere fragility induced by loss of TRF1 did not exhibit strand specificity (Sfeir et al., 2009). Similarly, Chawla et al. identified UPF1, an ATPase and helicase associated with cytoplasmic RNA quality control, as a telomere binding protein; in UPF1-depleted cells, telomere fragility increased at both the leading and lagging strands, with a slightly larger increase observed at the leading strand (Chawla et al., 2011). Most recently, Arora et al. demonstrated that ectopic expression of ribonuclease H1 (RNase H1) reduced fragile telomere formation on the leading strand in alternative lengthening of telomeres (ALT)-positive cells (Arora et al., 2014).
Among the stressors the replisome encounters, transcription has a significant impact on DNA replication. Indeed, head-on collisions between the replisome and RNA polymerase (RNAP) are extremely damaging to the replication process (Rudolph et al., 2007). In contrast to head-on collisions, co-directional replisome–RNAP collisions in bacteria are more common and better tolerated by the cell (Liu et al., 1993; Prado and Aguilera, 2005). This may be due to a mechanism recently elucidated in viral and prokaryotic polymerases: following a co-directional collision with RNAP on the leading strand-replicated DNA, DNA polymerase III is removed from the template, moves forward to the 3′ end of the nascent transcript, displaces RNAP, and restarts DNA synthesis using the transcript as a primer (Pomerantz and O'Donnell, 2008). Despite this mechanism, which would seem to permit damage-free replication across a region being transcribed, co-directional collisions between the replisome and RNAP can lead to unresolved RNA:DNA hybrids. If such collisions occur in mammalian cells, the RNA:DNA hybrids left behind would likely lead to DNA double strand breaks, an ataxia telangiectasia mutated (ATM)-mediated DNA damage response (DDR), and genomic instability (Skourti-Stathaki and Proudfoot, 2014; Sordet et al., 2010). Thus, robust mechanisms would need to evolve to remove the RNA:DNA hybrids produced by a collision event.

The known role of FEN1 in limiting telomere fragility (Saharia et al., 2010), as well as the idea that telomere fragility might be the result of replication stress or interference with transcription, led us to explore the mechanism by which FEN1 reduces telomere fragility. We show that treatment with α-amanitin, which reduces the rate of RNAP elongation and thus may increase the rate of stochastic co-directional replisome–RNAP
collisions, exacerbates the telomere fragility induced upon FEN1 depletion. Additionally, we find that the telomere fragility phenotype induced by FEN1 depletion and collision induction is RNA:DNA hybrid-dependent by rescuing telomere fragility with ectopic expression of RNase H1. FEN1’s role in limiting telomere fragility is distinct from its role in limiting sister telomere loss, as FEN1 depletion-induced telomere fragility is restricted to the leading strand. Neither FEN1’s classical replication role as mediated by its interaction with proliferating cell nuclear antigen (PCNA), nor FEN1’s DNA repair function mediated by its C-terminal interactions with numerous repair proteins are required for its activity in limiting telomere fragility. We find that FEN1’s gap endonuclease and exonuclease activities are also dispensable for limiting telomere fragility, but that FEN1’s flap endonuclease activity is required. Our data support a model in which co-directional replisome–RNAP collisions on the leading strand-replicated telomere produce RNA:DNA hybrid/flap structures that accumulate in the absence of FEN1. We propose that FEN1, a classical lagging strand replication protein, acts on the leading strand during telomere replication to resolve RNA:DNA hybrid/flap structures resembling Okazaki fragment substrates; in the absence of this activity, the subsequent replication stress and DNA damage manifests as telomere fragility. We believe this to be the first report placing an Okazaki fragment-processing protein explicitly on the leading strand during DNA replication.

**Experimental Procedures**

**Cell culture**

Cells were cultured at 37 °C in 5% carbon dioxide and atmospheric oxygen, as reported previously (Saharia et al., 2008, 2010; Stewart et al., 2003a). 293T cells and HEK 293
cells were cultured in high-glucose Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal bovine serum (ΔFBS) and 1% penicillin/streptomycin (P/S) (Sigma-Aldrich, St. Louis, MO). BJ fibroblasts and BJ fibroblasts expressing Large T Antigen (BJL) were cultured in high-glucose Dulbecco’s modified Eagle’s medium containing 15% Medium 199 (HEPES modification), 15% ΔFBS, and 1% P/S. RPE1 cells were cultured in Dulbecco’s modified Eagle’s medium (F12 modification) containing 7.5% ΔFBS and 1% P/S (Sigma-Aldrich, St. Louis, MO). Treatment with α-amanitin (Sigma-Aldrich, St. Louis, MO) was performed at 10 µg/mL for 12 hours prior to collection. All cell cultures were verified free of Mycoplasma contamination by PCR analysis. RPE1 cells were obtained from ATCC; all other cells were obtained from Dr. Robert Weinberg (Massachusetts Institute of Technology).

**Virus production and infections**

Lentiviral production and transductions were carried out as reported previously (Stewart et al., 2003b). Briefly, 293T cells were transfected with an 8:1 ratio of pHR’-CMV-8.2ΔR packaging plasmid and pCMV-VSV-G, and a pLKO.1-puro plasmid carrying an shRNA using TransIT-LT1 (Mirus Bio, Madison, WI). Supernatant-containing virus was collected 48 hours post-transfection and 72 hours post-transfection and filtered through a 0.45-µm PVDF membrane. Target cells were infected for four hours each on two consecutive days in the presence of 8 µg/mL protamine sulfate (Sigma-Aldrich, St. Louis, MO). Following infection, transduced BJ and BJL cells were selected with 1 µg/mL puromycin sulfate (Sigma-Aldrich, St. Louis, MO); transduced RPE1 cells were selected with 15 µg/mL puromycin sulfate.
Production of recombinant adenovirus type 5 was carried out using the AdEasy adenoviral vector system (Agilent Technologies, La Jolla, CA) according to the manufacturer’s protocol. Following collection of primary adenoviral stock, secondary and tertiary viral stocks were prepared by sequential infection of HEK 293 cells and purification from a cesium gradient. Briefly, infected cells were lysed in 0.5% Nonidet P-40 and cell debris was cleared by centrifugation. Viral particles were precipitated from the lysate with 6.7% PEG 8000, 0.83 M sodium chloride, collected by centrifugation, and washed in PBS. Viral particles were suspended in 1.32 g/mL cesium chloride and centrifuged at 33,000 rpm for 18 hours at 4 °C in a swinging-bucket rotor. Intact viral particles were collected from the cesium gradient, dialyzed in PBS, suspended in 33% glycerol, and frozen. Viral stocks were quantified using the AdEasy viral titer kit (Agilent Technologies, La Jolla, CA) according to the manufacturer’s instructions.

Adenoviral transduction was carried out following lentiviral transduction. Cells were lifted, combined with concentrated adenovirus, and re-plated in media containing puromycin to select for lentiviral integration. Adenovirus was used at a multiplicity of infection of 20 on RPE1 cells. Following 48 hours of simultaneous selection and adenoviral infection, the media was replaced.

**Western blot analysis**

Western blots were conducted as described previously (Honaker and Piwnica-Worms, 2010). Briefly, cells were washed with PBS and lysed in mammalian cell lysis buffer (100 mM sodium chloride, 50 mM tris-HCl pH 8, 5 mM EDTA, 0.5% Nonidet P40) supplemented with 2 mM dithiothreitol, 1 mM Microcystin-LR, 2 mM
phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, 1 mM sodium orthovanadate, protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), and phosphatase inhibitor cocktail set I (EMD Millipore, Billerica, MA). Following centrifugation, clarified lysate was quantified using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Lysates were resolved by SDS-PAGE and transferred to PVDF membranes for western blotting. The following antibodies were used: mouse monoclonal anti-Chk1 (sc8408, Santa Cruz Biotechnology, Santa Cruz, CA); rabbit monoclonal anti-Chk1, phospho-S345 (2348, Cell Signaling Technology, Danvers, MA); rabbit polyclonal anti-FEN1 (A300-255A, Bethyl Laboratories, Montgomery, TX); mouse monoclonal anti-RNase H1 (H00246243-M01, Novus Biologicals, Littleton, CO); rat monoclonal anti-α-tubulin (ab6160, Abcam, Cambridge, UK); mouse monoclonal anti-β-catenin (610154, BD Biosciences, San Jose, CA); rabbit polyclonal anti-γH2AX (07-164, Millipore, Billerica, MA).

**Metaphase chromosome preparation**

Metaphase chromosome spreads were prepared as described previously (Lansdorp et al., 1996). Briefly, BJ and BJL fibroblasts were cultured in the presence of 0.1 µg/mL colcemid (Sigma-Aldrich, St. Louis, MO) for five hours; RPE1 cells were cultured in the presence of 0.3 µg/mL colcemid for four hours. Following arrest, metaphase cells were collected by mitotic shake-off, swollen in 75 mM potassium chloride, and fixed in 3:1 methanol:acetic acid. Chromosomes were spread by dropping onto glass slides and aged for 18 hours at 65 °C. When metaphases were to be analyzed by CO-FISH, 0.3 µg/mL of 5-bromo-2'-deoxyuridine (Sigma-Aldrich, St. Louis, MO) and 0.1 µg/mL of 5-bromo-2'-deoxycytidine (MP Biomedicals, Santa Ana, CA) were added to the culture media 18 hours prior to collection of the cells.
Fluorescent in situ hybridization (FISH)

FISH was performed as described previously (Lansdorp et al., 1996). Metaphase chromosomes were probed with a Cy3-(CCCTAA)$_3$ (telomere) peptide nucleic acid (PNA) probe at 0.03 µg/mL and a FAM-CENPB (centromere) PNA probe at 0.03 µg/mL (PNA Bio, Thousand Oaks, CA) and mounted using ProLong Gold (Life Technologies, Grand Island, NY) with 125 ng/mL DAPI (Sigma-Aldrich, St. Louis, MO).

Chromosome-orientation FISH (CO-FISH)

CO-FISH was conducted as described previously (Bailey et al., 2001) with modifications. Briefly, metaphase chromosomes were rehydrated and treated with 100 µg/mL RNase for 10 minutes at 37 °C, rinsed, and re-fixed in 4% paraformaldehyde for 10 minutes at room temperature. Chromosomes were UV sensitized in 0.5 µg/mL Hoechst 33258 (Sigma-Aldrich, St. Louis, MO) in 2x SSC for 15 minutes and exposed to 365 nm UV light for 60 minutes using a UV crosslinker (Vilber-Lourmat, Marne-la-Vallée, France). Chromosomes were then digested with 3 U/µL exonuclease III (Promega, Madison, WI) for 15 minutes at room temperature, denatured in 70% formamide in 2x SSC at 72 °C for 90 seconds, and immediately dehydrated in cold ethanol before hybridization. Metaphase chromosomes were probed first with a FAM-(TTAGGG)$_3$ (leading strand telomere) PNA probe at 0.03 µg/mL, then probed with a Cy3-(CCCTAA)$_3$ (lagging strand telomere) PNA probe at 0.03 µg/mL (PNA Bio, Thousand Oaks, CA) and mounted as described for FISH.
Immunofluorescence (IF) and IF–FISH

IF was carried out as described (Duxin et al., 2009). For IF-FISH, following the completion of IF, the cells were probed as described for chromosomes above using a Cy3-(CCCTAA)$_3$ (telomere) PNA probe at 0.03 µg/mL (PNA Bio, Thousand Oaks, CA). Antibodies used were: rabbit polyclonal anti-γH2AX (07-164, Millipore, Billerica, MA) and goat anti-rabbit IgG–Alexa Fluor 488 (Life Technologies, Grand Island, NY).

Fluorescence imaging

Chromosomes were imaged on a Nikon 90i epifluorescence microscope using a 100x 1.40 NA Plan Apo VC objective (Nikon Instruments, Melville, NY) with Cargille Type FF or Cargille Type LDF immersion oil (Cargille-Sacher Laboratories, Cedar Grove, NJ) at room temperature. Cells were imaged using a 40x 1.0 NA Plan Apo objective (Nikon Instruments, Melville, NY) under the same conditions as those for chromosomes. Filter cube sets used were: DAPI-1160B-000-ZERO, FITC-2024B-000-ZERO, and CY3-4040C-000-ZERO (Semrock, Inc., Rochester, NY). Images were captured using a CoolSnap HQ2 CCD camera (Photometrics, Tucson, AZ). Individual channel lookup tables were auto-adjusted non-destructively and linearly, and images were deconvolved with a blind algorithm using NISElements AR (Nikon Instruments, Melville, NY) prior to quantification.

RNA preparation and northern hybridization

RNA was prepared using TRI Reagent (Life Technologies, Grand Island, NY). RNA was serially diluted, denatured as previously described (Sambrook et al., 1989), and spotted onto a Hybond-XL charged nylon membrane (GE Healthcare, Waukesha, WI) using a
Bio-Dot Microfiltration apparatus (Bio-Rad, Hercules, CA) according to the manufacturers’ instructions. Samples were also treated with ribonuclease A (Roche Applied Science, Penzberg, Germany) and spotted to identify any DNA contamination in the RNA preparation. Following UV crosslinking, the membrane was prehybridized in northern hybridization buffer (15% formamide, 1% BSA, 100 mM sodium phosphate pH 7.7, 1 mM EDTA, 7% SDS) for one hour at 65 °C. A purified 1.6 kb fragment consisting exclusively of vertebrate telomere repeats was random prime-labeled with [α-32P]dCTP (3000 Ci/mmol) using the High Prime DNA Labeling Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer’s instructions to produce a telomere-specific DNA probe. Similarly, a purified cDNA of the human 5S ribosomal RNA was random prime-labeled to produce a 5S rRNA-specific DNA probe. Probes were purified using Illustra ProbeQuant G-50 Micro Columns (GE Healthcare, Waukesha, WI) and diluted to 1.2×10^6 dpm/mL in 10 mL of northern hybridization buffer. Probes were hybridized to the membrane overnight at 65 °C, after which the membrane was washed and imaged using either autoradiography or a storage phosphor screen and imager. Quantitation was performed in Fiji by first background subtracting the image and then computing the integrated density for each spot.

**Quantitative reverse transcription PCR (qRT-PCR)**

For qRT-PCR, cDNA was synthesized using Superscript III reverse transcriptase (Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. qRT-PCR was conducted using TaqMan Gene Expression Assays (Life Technologies, Grand Island, NY) according to the manufacturer protocol. Target genes used for verification of α-amanitin efficacy were MYC (Hs00153408_m1) and SIAH1 (Hs02339360_m1).
Statistical analysis

Telomere fragility events were defined as chromatid arms with telomere FISH signal observed as either multiple telomere signals or elongated smears as previously described (Sfeir et al., 2009). Fragility was counted in metaphase chromosome spreads; for each experimental condition, a minimum total of 600 chromosomes was counted. The minimum sample size was chosen based on its ability to consistently detect aphidicolin-induced and FEN1 depletion-induced telomere fragility. Chromosomes completely lacking telomere FISH signal or exhibiting no strand specificity in CO-FISH (indicating the technical issue of incomplete brominated strand digestion) were excluded and not quantified. Image groups were blinded prior to quantification. Two or more independent biological replicates were carried out for each experiment.

Where data are shown as representative, the telomere fragility rate was computed for each metaphase chromosome spread (% fragile telomeres), and each experiment was statistically analyzed. Where data are shown as combined, telomere fragility rates were computed for each metaphase chromosome spread, and a normalized value was computed for each metaphase chromosome spread by dividing the raw value by the mean of the control values. The mean of the normalized values from each sample in two independent experiments was computed and graphed with error bars representing the standard error of the mean. For statistical analysis, raw values were centered by computing a t-statistic for each data point: the centered value for each chromosome spread was calculated by dividing the residual of each raw value relative to the control sample’s mean by the median absolute deviation of the control values. Centered values from two independent experiments were then combined for statistical analysis. Data are
represented either by scatter plots with mean and standard error of the mean marked by a line and error bars, or by a bar graph with bars indicating the mean and error bars indicating standard error of the mean marked.

For IF, γH2AX foci were counted in each nucleus. A minimum of 30 nuclei was counted for each condition in an experiment, and two independent biological replicates were combined for data quantification. Data are represented by a box and whiskers plot with the box marking 25th and 75th percentiles, line marking the median, whiskers marking the 5th and 95th percentiles, and dots marking data points outside the 5–95 percentile range.

For all data, p-values were computed using a two-tailed Mann-Whitney U test with a 95% confidence interval in Prism 5 (GraphPad Software, La Jolla, CA). The Mann-Whitney U test was chosen because not all samples exhibited normal distributions. All figures except the box and whiskers plot include standard error of the mean as an indicator of variance, and in all cases the variance within samples was similar.

Results

FEN1 depletion and transcription inhibition induce replication stress and a DNA damage response

Because telomeres are transcribed to produce telomeric repeat-containing RNA (TERRA) (Azzalin et al., 2007; Schoeftner and Blasco, 2008), and because interference between replication and transcription is a known cause of genomic instability (Azvolinsky et al., 2009; Helmrich et al., 2011; Sabouri et al., 2012), we turned our
attention to the impact that putative collisions between the replication and transcription machinery would have on telomere stability. Unlike in *Schizosaccharomyces pombe*, where transcription of telomeres and subtelomeres occurs using both strands as templates (Bah et al., 2012), mammalian telomeres are transcribed exclusively using the C-rich leading strand as a template (Azzalin et al., 2007; Schoeftner and Blasco, 2008); as such, co-directional collisions are the only type that can occur at the telomere. In bacteria, co-directional collisions are resolved by a mechanism that leaves behind an RNA:DNA hybrid/flap structure (Pomerantz and O’Donnell, 2008) which would need to be resolved in a eukaryotic cell to avoid a DDR and genomic instability (Skourti-Stathaki and Proudfoot, 2014; Sordet et al., 2010). FEN1 has been previously shown to reduce telomere fragility (Saharia et al., 2010), and the yeast FEN1 homolog Rad27p can hydrolyze RNA flaps (Stewart et al., 2006). We hypothesized that co-directional collisions are a molecular origin of telomere fragility, and that FEN1 can prevent post-collision RNA:DNA hybrid/flap structures from accumulating, causing damage, and ultimately leading to fragile telomere formation.

To address this hypothesis, we first examined whether increasing the rate of stochastic collisions between the replisome and RNAP would increase replication stress and trigger a DDR in the context of FEN1 depletion. We treated BJ fibroblasts with the RNA polymerase II (Pol II) elongation inhibitor α-amanitin, a cyclic peptide toxin that reduces the rate of Pol II transcription approximately 100-fold, allowing chain elongation to continue without triggering transcript cleavage (Bushnell et al., 2002; Rudd and Luse, 1996). We expected α-amanitin treatment to increase the frequency of stochastic collisions between the replisome and RNAP and thus increase replication stress and
telomere fragility. Following transduction with a validated shRNA targeting the 3′-untranslated region of the FEN1 mRNA (shFEN1) (Saharia et al., 2008) or a control hairpin (shCtrl), we treated BJ fibroblasts with either vehicle or 10 µg/mL α-amanitin for 12 hours and collected both RNA and protein lysates from the cells. qRT-PCR analysis confirmed the efficacy of α-amanitin treatment by quantitation of two short-lived transcripts, c-Myc and SIAH1. α-amanitin-treated control cells retained 2% and 6% of the c-Myc and SIAH1 mRNAs, respectively, compared to the levels observed in vehicle-treated control cells. Similarly, in FEN1-depleted cells, α-amanitin treatment resulted in 4% and 10% of the levels of c-Myc and SIAH1 mRNAs, respectively, compared to vehicle-treated cells (Fig. 3.1A). Since transcription inhibition by α-amanitin might reduce steady-state TERRA levels and produce telomere phenotypes as a result of decreased TERRA, we carried out a northern blot analysis of total RNA to detect TERRA. Because TERRA are expressed at low levels in BJ fibroblasts, we utilized a dot blot rather than a gel to maximize signal intensity and hybridized the membrane to a telomere repeat-specific probe; treatment with ribonuclease A was used to show the absence of contaminating DNA, and a 5S rRNA-specific probe was used as a loading control. Northern analysis of vehicle- and α-amanitin-treated cells demonstrated that the α-amanitin treatment conditions subsequently used for western and metaphase analysis did not impact steady state levels of TERRA in our system, demonstrating that the phenotypes resulting from the treatment were not due to a loss of TERRA, which are known to impact telomere stability (Balk et al., 2013; Deng et al., 2009) (Fig. 3.1B,C).

To determine if Pol II inhibition induces replication stress and a DDR in the context of FEN1 depletion, we performed western blot analysis to examine phosphorylation of
Chk1 at S345 and phosphorylation of histone H2AX at S139 (γH2AX), classical markers for the replication stress response and DDR, respectively. BJ fibroblasts transduced with the control hairpin and treated with vehicle displayed neither Chk1 phosphorylation nor H2AX phosphorylation (Fig. 3.2A). Treatment with α-amanitin induced a small but detectable increase in Chk1 phosphorylation, but did not induce γH2AX, indicating that α-amanitin treatment can induce limited replication stress, but is not sufficient to induce a DDR (Fig. 3.2A). Similarly, BJ fibroblasts depleted of FEN1 and treated with vehicle also displayed a small level of Chk1 phosphorylation and no detectable γH2AX (Fig. 3.2A). Strikingly, upon treatment with α-amanitin, FEN1-depleted cells showed a robust phosphorylation of Chk1 and strong induction of γH2AX (Fig. 3.2A).

We also used IF to examine the formation of γH2AX foci in asynchronous BJ fibroblasts, and IF-FISH to assess the formation of telomere dysfunction-induced foci. Quantification of γH2AX foci demonstrated that while FEN1 depletion induced foci formation (2.14-fold in shFEN1+vehicle vs. shCtrl+vehicle, \( p < 0.0001 \)), there was no change in γH2AX foci upon treatment with α-amanitin (Fig. 3.2B,C). Furthermore, we did not observe an increase in telomere dysfunction-induced foci in response to FEN1 depletion or α-amanitin treatment (data not shown). These results indicate first that the amount of DNA damage induced in conditions that increase collision events causes a response only robust enough to be detected by the more sensitive western analysis. Second, they indicate that FEN1 depletion- and Pol II inhibition-induced replication stress and DNA damage is not restricted to telomeres; rather, DNA damage likely occurs throughout the genome wherever collisions occur. Thus, Pol II inhibition alone
induces mild replication stress, and the depletion of FEN1 combined with Pol II inhibition results in a DDR that is not observed when FEN1 is depleted alone.

Inhibition of transcription exacerbates the telomere fragility observed upon FEN1 depletion

We next examined whether the replication stress and DDR phenotypes observed in response to Pol II inhibition and FEN1 depletion manifest as telomere fragility. If failure by FEN1 to resolve the structures induced by collision events between the replisome and RNAP results in fragility, then we anticipated the rate of telomere fragility in α-amanitin-treated and FEN1-depleted cells to mirror the replication stress phenotype. As before, we transduced BJ fibroblasts with either shCtrl (control) or shFEN1 and treated the cells with vehicle or α-amanitin for 12 hours prior to collecting metaphase chromosomes. Consistent with our model, cells expressing shCtrl exhibited an increased rate of telomere fragility upon α-amanitin treatment (1.55-fold in shCtrl+α-amanitin vs. shCtrl+vehicle, \( p = 0.0079 \)) (Fig. 3.2D,E). When examining only the vehicle-treated cells, we found that as previously demonstrated, FEN1 depletion causes a significant increase in telomere fragility (2.15-fold in shFEN1+vehicle vs. shCtrl+vehicle, \( p < 0.0001 \)) (Fig. 3.2D,E). Strikingly, FEN1-depleted cells treated with α-amanitin displayed a significant 2.76-fold increase in telomere fragility when compared to control, vehicle-treated cells (shFEN1+α-amanitin vs. shCtrl+vehicle, \( p < 0.0001 \)), and a significant 1.28-fold increase compared to FEN1-depleted, vehicle-treated cells (shFEN1+ α-amanitin vs. shFEN1+vehicle, \( p = 0.0017 \)) (Fig. 3.2D,E). These fragility data mirror the Chk1 phosphorylation phenotype and support a model in which α-amanitin treatment increases co-directional replisome–RNAP collision events that result
in structures requiring FEN1 for resolution; without FEN1, the collision events generate replication stress, a DDR, and fragile telomere formation. These experiments suggest that FEN1’s role in limiting telomere fragility is dependent upon its ability to resolve structures produced by telomere transcription during DNA replication.

**Leading strand-specific telomere fragility is caused by RNA:DNA hybrids**

Our data above suggest a role for telomere transcription in telomere fragility induced by FEN1 depletion. Based on findings in prokaryotes, if co-directional collisions occur between the replisome and an RNAP, a structure resembling an Okazaki fragment with a segment of RNA:DNA hybrid would result (Pomerantz and O’Donnell, 2008); we postulate that if not resolved, this structure could give rise to fragile telomeres. Indeed, post-collision structures resemble R-loops, which are semi-stable displacement loops in which a nascent mRNA remains hybridized to its DNA template, while the coding strand DNA remains single-stranded, resulting in replication stress and common fragile site expression (Helmrich et al., 2011). At common fragile sites, the enzyme RNase H1 suppresses replication stress phenotypes induced by R-loop formation by hydrolyzing the RNA in RNA:DNA hybrids and thus resolving displacement loops (Helmrich et al., 2011). We reasoned that since the post-co-directional collision structure resembles an R-loop, RNA:DNA hybrids might be responsible for telomere fragility, and thus ectopic expression of RNase H1 should resolve the structure and telomere phenotype. Additionally, because our model predicts that the causative structure for fragile telomere formation occurs after the replication fork has passed the locus in question, we wondered if the telomere fragility observed upon FEN1 depletion manifests only on the leading strand, where collisions could occur. This question was especially prescient
given that FEN1 is canonically a lagging strand replication protein, and has a previously established role in limiting sister telomere loss at the lagging strand (Saharia et al., 2010).

Following lentiviral transduction with a control hairpin (shCtrl) or FEN1-depleting hairpin (shFEN1), we transduced RPE1 cells with RNase H1 (Ad-RH1) (Fig. 3.3C) and collected cells for protein analysis and metaphase chromosome preparation. To identify if telomere fragility exhibited strand specificity, we used CO-FISH, a technique which exploits the fact that the C-rich and G-rich strands of the mammalian telomere are replicated exclusively by the leading and lagging strand machinery, respectively, allowing the use of strand-specific probes to identify which machinery replicated a given telomere on a metaphase chromosome (Bailey et al., 2001). Strikingly, FEN1 depletion significantly increased leading strand-specific telomere fragility (2.30-fold in shFEN1 vs. shCtrl, \( p = 0.0021 \)) (Fig. 3.3A,B) with no change observed on lagging strand-replicated telomeres (1.26-fold in shFEN1 vs. shCtrl) (Fig. 3.3A,B). Additionally, ectopic expression of RNase H1 rescued fragility on the leading strand-replicated telomere, returning fragility levels to those observed in control cells (1.19-fold in shFEN1+Ad-RH1 vs. shCtrl) (Fig. 3.3A,B). Given the specificity of RNase H1 for RNA:DNA hybrids, these data indicate that RNA:DNA hybrids lead to telomere fragility and suggest that the hybrid/flap structures that arise from co-directional collisions on the leading strand are responsible for the telomere fragility observed upon FEN1 depletion. Furthermore, given that RPE1 cells are telomerase-positive and telomerase expression rescues the sister telomere loss observed upon FEN1 depletion, these data indicate that FEN1’s role in
limiting telomere fragility at the leading strand is distinct from its known role in limiting sister telomere loss at the lagging strand (Saharia et al., 2008, 2010).

α-amanitin is known to slow but not disengage the RNAP from the template strand (Bushnell et al., 2002; Rudd and Luse, 1996), and its use would be expected to increase replisome–RNAP collisions and RNA:DNA hybrids. Thus, we next wanted to determine if the fragility we observed upon α-amanitin treatment was also RNA:DNA hybrid-dependent. To address this question, we transduced RPE1 cells with Ad-RH1 (Fig. 3.4C) and treated the transduced cells with α-amanitin for 12 hours prior to metaphase collection. As before, α-amanitin treatment induced an increase in telomere fragility (1.79-fold in α-amanitin vs. vehicle, \( p = 0.0008 \)) (Fig. 3.4A,B). As in the case of telomere fragility following FEN1 depletion, ectopic RNase H1 expression protected α-amanitin-treated cells from telomere fragility, resulting in levels similar to those observed in cells treated with vehicle (1.05-fold in Ad-RH1+α-amanitin vs. vehicle) (Fig. 3.4A,B). Because α-amanitin treatment exacerbates telomere fragility in the absence of FEN1 (Fig. 3.2D,E), the ability of RNase H1 to rescue fragility in both α-amanitin-treated (Fig. 3.4A,B) and FEN1-depleted cells (Fig. 3.3A,B) suggests that FEN1’s role in limiting telomere fragility is to resolve RNA:DNA hybrid/flap structures that are produced following replisome–RNAP collisions.

**FEN1 flap endonuclease activity is required for limiting telomere fragility**

Given the unprecedented finding that FEN1 limits leading strand-specific telomere fragility, we sought to identify which of FEN1’s known functions were necessary for this activity. FEN1 possesses three unique enzymatic activities: an endonuclease activity on
unannealed 5’ flaps consisting of either DNA or RNA, a weak exonuclease activity that cleaves nicks, gaps, or recessed 5’ ends of double-stranded DNA, and a gap endonuclease activity that cleaves double-stranded DNA at the 3’ end of a short single-stranded gap (Liu et al., 2004; Parrish et al., 2003; Zheng et al., 2005). FEN1 is also known to interact with PCNA via a PCNA interacting peptide (PIP) box, directly pertaining to its role in DNA replication, and a number of DNA repair proteins via its C-terminus, pertaining to its role in base excision repair (Guo et al., 2008; Li et al., 1995). We utilized a series of previously described FEN1 mutants that impact FEN1’s different roles in replication (D181A, ΔP, ΔPΔC) versus repair (ΔC, D181A, ΔPΔC, E160D) in genetic knockdown–rescue experiments (Saharia et al., 2008, 2010) (Fig. 3.5A). To test whether the reduction in telomere fragility mediated by FEN1 requires its DNA repair functions, we used a lentiviral vector to express shCtrl (control) alone, shFEN1 alone, or shFEN1 simultaneously with the wild type (WT), ΔC, or D181A allele of FEN1 (Fig. 3.5A); following transduction we prepared metaphase chromosomes. As before, FEN1 depletion induced leading strand-specific telomere fragility (2.05-fold in shFEN3 vs. shLuc, p < 0.0001) (Fig. 3.5B,D). Expression of the WT allele of FEN1 rescued the leading strand-specific induction of telomere fragility upon endogenous FEN1 knockdown, indicating that the phenotype is specific to FEN1 knockdown (1.18-fold in shFEN1+WT vs. shCtrl) (Fig. 3.5B,D). Unexpectedly, expression of the ΔC allele also rescued FEN1 depletion-induced telomere fragility on the leading strand (1.02-fold in shFEN1+ΔC vs. shCtrl) (Fig. 3.5B,D). In contrast to the WT and ΔC alleles, the D181A nuclease-dead allele, which is deficient in all known nuclease activities (Shen et al., 1996; Tsutakawa et al., 2011), failed to rescue the phenotype, instead resulting in an increase in leading strand-specific telomere fragility comparable to the expression of
shFEN1 alone (1.83-fold in shFEN1+D181A vs. shCtrl, \( p < 0.0001 \)) (Fig. 3.5B,D).

Neither knockdown of FEN1 nor expression of any of the mutant alleles of FEN1 altered the level of telomere fragility on the lagging strand, confirming that FEN1 does not play a role in the phenotype on lagging strand-replicated telomeres (Fig. 3.5B,D). These data indicate that FEN1’s flap endonuclease activity is required to limit leading strand-specific telomere fragility, but its interactions with several DNA repair proteins including WRN and BLM (deficient in the \( \Delta C \) allele), and thus its DNA repair activities, are dispensable for this role. Consequently, FEN1’s ability to limit leading strand-specific telomere fragility is distinct from its previously described role in telomere stability, which depends upon FEN1’s C-terminally mediated DNA repair activity to suppress sister telomere loss on the lagging strand-replicated telomere (Saharia et al., 2008, 2010).

Given that FEN1’s repair activity is dispensable for its ability to limit telomere fragility, and telomere fragility is associated with replication stress, we next investigated whether FEN1’s interaction with PCNA, and thus its replication activity, might be important in this role. To test this possibility, BJ fibroblasts depleted of FEN1 were transduced with the WT, \( \Delta P \), \( \Delta P \Delta C \), or E160D cDNA of FEN1 (Fig. 3.5A). Analysis of telomere fragility on metaphase chromosomes revealed that as before, expression of the WT allele rescued the leading strand-specific induction of telomere fragility following FEN1 depletion (1.58-fold in shFEN1 vs. shCtrl, \( p < 0.0001 \); 0.88-fold in shFEN1+WT vs. shCtrl) (Fig. 3.5C,D). Surprisingly, expression of both the \( \Delta P \) and E160D constructs also rescued the fragility defect (0.77-fold in shFEN1+\( \Delta P \) vs. shCtrl; 1.20-fold in BJ shFEN1+E160D vs. shCtrl) (Fig. 3.5C,D). Only the \( \Delta P \Delta C \) allele, a functionally null allele due to its lack of nuclear localization, failed to rescue the leading strand telomere fragility observed upon FEN1
depletion, resulting in an increase similar to that observed upon FEN1 depletion alone (1.61-fold in shFEN1+ΔPΔC vs. shCtrl, \( p < 0.0001 \)) (Fig. 3.5C,D). As in the previous experiment, none of the FEN1 alleles induced lagging strand-specific telomere fragility (Fig. 3.5C,D). These data indicate that FEN1 requires neither its interaction with PCNA (deficient in the ΔP allele), nor its gap endonuclease and exonuclease activity (deficient in the E160D allele) to limit leading strand-specific fragility. In combination with the data from expression of the ΔC and D181A mutants, our experiments identify FEN1 flap endonuclease activity as necessary for its role in limiting telomere fragility. These data are consistent with FEN1’s known activities, as it has previously been shown to cleave flap structures with numerous modifications, including flaps composed of RNA (Bornarth et al., 1999; Liu et al., 2004; Stewart et al., 2006). As such, our data and the literature support a model in which FEN1’s flap endonuclease activity could cleave the RNA:DNA hybrid/flap structures produced following a replisome–RNAP collision event (Fig. 3.6).

**Discussion**

The role of FEN1 described here provides new insights into the breadth of its functions in maintaining genome stability. In addition to known roles in lagging strand DNA replication, base excision repair, and lagging strand telomere stability, we illustrate for the first time a role for FEN1 in leading strand replication. Furthermore, we have identified transcription as an important contributor to telomere fragility, and we have shown that FEN1 may resolve the RNA:DNA hybrid/flap structures resulting from collisions between the transcription and replication machinery. The strand specificity of telomere fragility observed in the absence of FEN1 shows that it has two independent molecular roles for promoting telomere stability: (1) FEN1 limits sister telomere loss at
the lagging strand-replicated telomere by facilitating replication fork reinitiation (Saharia et al., 2010), and (2) FEN1 limits telomere fragility at the leading strand-replicated telomere by resolving RNA:DNA hybrid/flap structures produced by co-directional replisome–RNAP collisions (Fig. 3.6).

Though co-directional collisions between the replisome and RNAP are postulated to be less deleterious to DNA replication than head-on collisions, they still necessitate mechanisms to ensure replication fidelity. In bacteria, the primary replicative helicase, DnaB, translocates along the lagging strand template as it unwinds DNA ahead of the replication fork; as such, the helicase can move past an RNAP transcribing from the leading strand, which would result in an inevitable collision between the two polymerases (Pomerantz and O'Donnell, 2008). While accessory helicases such as Rep move along the leading strand template, this activity alone cannot prevent co-directional collisions (Atkinson et al., 2011; Pomerantz and O'Donnell, 2008). Bacteria thus can use a mechanism in which replication restarts on the leading strand template following a co-directional collision using the 3’ end of the nascent mRNA as a primer (Pomerantz and O'Donnell, 2008). Collisions between the replisome and RNAP also present a problem to the eukaryotic cell, where highly-transcribed Pol II and Pol III genes are known to impede replication fork progression (Azvolinsky et al., 2009; Sabouri et al., 2012). Extremely long genes that require more than a single cell cycle to transcribe are also known to induce collision events; these collisions induce common fragile site expression (Helmrich et al., 2011). Observations suggest that even though the eukaryotic replicative helicase, a complex of Cdc45, Mcm2-7, and GINS (CMG), translocates along the leading strand (Fu et al., 2011), its activity is insufficient to
prevent collisions from occurring. Indeed, CMG is unable to bypass both biotin-
streptavidin and Qdot (20 nm) roadblocks on the leading strand (Fu et al., 2011). Even though the eukaryotic replicative helicase translocates along the leading strand, our data suggest that it is unable to bypass an RNAP and/or RNA:DNA hybrid on this strand. Together, these observations suggest that eukaryotes require a similar mechanism to that used by bacteria for the resolution of co-directional replisome–RNAP collisions on the leading strand.

Although FEN1 has no known existing roles in leading strand DNA replication, our results provide an explanation consistent with the enzyme’s known substrates and activity. The putative RNA:DNA hybrid/flap structure produced following a co-directional replisome–RNAP collision is similar to the Okazaki fragment flaps FEN1 cleaves during lagging strand replication—differing only in that the flap is composed entirely of ribonucleotides. Thus, our model suggests that human FEN1 acts at the leading strand because co-directional collisions at the telomere only happen on the leading strand template. Because FEN1’s ability to limit telomere fragility does not require its C-terminal domain, which interacts with the shelterin protein TRF2 to recruit FEN1 to telomeres during S and G2 phases of the cell cycle (Muftuoglu et al., 2006; Saharia et al., 2008), it is unlikely that FEN1’s ability to process post-collision structures is limited to the telomere. However, in other portions of the genome where replication begins from origins to either side of a particular locus, transcription could be more coordinated with replication to prevent head-on collisions from occurring. Wherever co-directional collisions occur, FEN1 is likely able to process the structures produced.
Because the replication fork replicates the telomere in the centromere-to-telomere direction only, and because mammalian telomeres are only transcribed from the C-rich leading-strand template in the same direction (Azzalin et al., 2007; Schoeftner and Blasco, 2008), replisome–RNAP collisions at the telomere can only occur co-directionally. Our work here, as well as the fact that TERRA depletion induces telomere fragility (Deng et al., 2009), underscores the role of telomere transcription in fragile telomere formation. Indeed, work in yeast has shown that RNA:DNA hybrids produced by TERRA transcription promote recombination-mediated telomere elongation (Balk et al., 2013). In ALT-positive cells, RNase H1 has recently been shown to regulate the levels of RNA:DNA hybrids between TERRA and telomeric DNA (Arora et al., 2014). Like in yeast, TERRA RNA:DNA hybrids are hypothesized to promote recombination between ALT telomeres. In the absence of RNase H1, hybrids accumulate and promote excessive replication stress that causes fragile telomere formation and telomere loss; conversely, overexpression of RNase H1 reduces TERRA hybrids such that they cannot promote recombination, leading to progressive telomere shortening (Arora et al., 2014). Strikingly, the telomere loss that occurs following RNase H1 depletion in ALT cells is leading strand-specific (Arora et al., 2014). This work, when combined with ours, strongly implicates transcription-associated RNA:DNA hybrid formation at the telomere as a contributor to telomere fragility.

Despite the recency of telomere fragility as a defined phenotype, it has been identified in reports manipulating the expression of many proteins involved in DNA replication and telomere stability. ATR deficiency or depletion, BRCA2 deletion, RAD51 depletion, and RECQL1 depletion all induce elevated rates of telomere fragility (Badie et al., 2010;
Martinez et al., 2009; McNees et al., 2010; Popuri et al., 2014; Sfeir et al., 2009). In addition, CTC1 and STN1, both members of the mammalian CST complex, limit telomere fragility (Stewart et al., 2012). Like FEN1, these proteins participate in replication fork progression, replication fork reinitiation, and telomere stability. To our knowledge, no report has identified any perturbation that induces telomere fragility exclusive to the leading or lagging strand, though RNase H1 overexpression has been shown to reduce telomere fragility at the leading strand (Arora et al., 2014). Indeed, the lack of strand specificity in the telomere fragility produced by TRF1 deletion (Sfeir et al., 2009), as well as the involvement of G-quadruplexes (which form exclusively on the lagging strand) in RTEL1 deletion-induced telomere fragility (Vannier et al., 2012), suggests that there are multiple mechanisms leading to fragile telomere formation. Our work underscores the complexity of DNA replication, and in placing the canonical Okazaki fragment-processing protein FEN1 at the leading strand, reveals the first molecular mechanism for fragile telomere formation on the leading strand.

**Acknowledgments**

We thank Dr. Peter Burgers, Dr. Susana Gonzalo, Dr. Barry Sleckman, Kevin Flanagan, and Megan Ruhland for critical reading. We thank Jingqin Luo for statistical advice. This project was supported, in whole or in part, by National Institutes of Health Grant GM95924 (S.A.S.) and by Training Grant GM007067 from NRSA (D.C.T. and E.A.). This work was also supported by the Siteman Cancer Center at Barnes-Jewish Hospital and Washington University School of Medicine (S.P. and H.R.M.).
Figure 3.1. α-amanitin treatment abrogates expression of mRNAs with short half lives but does not alter steady-state TERRA levels. (A) qPCR analysis of c-Myc and SIAH1 mRNA expression in cells expressing a control hairpin (shCtrl) or FEN1-depleted cells (shFEN1), treated with either vehicle or α-amanitin (α-aman). mRNA levels in α-amanitin-treated cells are shown as a fold change relative to the vehicle-treated cells. Fold changes were calculated using the ΔΔCt method; fold changes from two biological replicates were averaged to produce the graph. Error bars represent standard error of
the mean. (B) Northern dot blot to detect TERRA. RNA was isolated from cells expressing a control hairpin (shCtrl) or FEN1-depleted cells (shFEN1) that were treated with either vehicle or α-amanitin (α-aman). Serial dilutions of RNA were loaded onto a membrane. Samples treated with RNase A to control for genomic DNA contamination were also loaded (+RNase A). A telomere repeat DNA probe was hybridized to the membrane (telomere probe) to detect TERRA; the membrane was stripped and re-probed with a 5S rRNA DNA probe (5S) as a loading control. The membrane was visualized with autoradiography. (C) Quantification of TERRA in cells treated with α-amanitin. The northern dot blot in (B) was imaged with a phosphor imager and analyzed by densitometry using Fiji; TERRA levels in α-amanitin-treated cells are shown as a fold change relative to vehicle-treated cells. Two independent experiments were averaged to produce the graph; error bars represent standard error of the mean.
**Figure 3.2.** FEN1 depletion and transcription inhibition induce replication stress, a DNA damage response, and telomere fragility. (A) Western analysis of FEN1 expression, Chk1 phosphorylation (pS345), and H2AX phosphorylation (γH2AX) in control (shCtrl) or FEN1-depleted (shFEN1) cells treated with vehicle or α-amanitin (α-aman). β-catenin is shown as a loading control. (B) Quantification of γH2AX foci per cell. Two independent biological replicates were combined. The box marks the 25th to 75th percentile with the median marked by a horizontal line, whiskers mark the 5th and 95th percentiles, and dots represent values outside the 5–95 percentile range. *p*-values were computed using a two-tailed Mann-Whitney *U* test (***, *p* < 0.001 relative to shCtrl). (C) Representative immunofluorescence images stained with a γH2AX antibody (green) and DAPI (blue) from BJ fibroblasts expressing a control hairpin (shCtrl) or depleted of FEN1 (shFEN1). Cells were treated with vehicle or α-amanitin (α-aman) as indicated. The scale bar (white) represents 25 µm. (D) Representative quantification of the rate of telomere fragility. *p*-values were computed using a two-tailed Mann-Whitney *U* test (**, *p* < 0.01; ***, *p* < 0.001). Error bars represent standard error of the mean. (E) Representative metaphase chromosomes processed with FISH from BJ fibroblasts expressing a control hairpin (shCtrl) or depleted of FEN1 (shFEN1). Cells were treated with vehicle or α-amanitin (α-aman) as indicated. Centromeres are green and telomeres are red. Arrowheads mark fragile telomeres in the magnified images.
Figure 3.3. RNA:DNA hybrids are responsible for FEN1 depletion-induced leading strand-specific telomere fragility. (A) Representative metaphase chromosomes processed with CO-FISH from RPE1 cells expressing a control hairpin (shCtrl) or depleted of FEN1 (shFEN1), with or without ectopically expressed RNase H1 (Ad-RH1). Leading strand-replicated telomeres are green and lagging strand-replicated telomeres are red. Arrowheads mark fragile telomeres in the magnified images. (B) Representative quantification of the rate of strand-specific telomere fragility, with leading strand-specific telomere fragility shown in green and lagging strand-specific telomere fragility shown in...
$p$-values were computed using a two-tailed Mann-Whitney $U$ test (*, $p < 0.05$; **, $p < 0.01$). Error bars represent standard error of the mean. (C) Western analysis of FEN1 and RNase H1 expression in control (shCtrl) or FEN1-depleted (shFEN1) cells, with or without ectopically expressed RNase H1 (Ad-RH1). Two exposures of the same RNase H1 blot are shown. $\alpha$-tubulin is shown as a loading control.
**Figure 3.4.** RNA:DNA hybrids are responsible for α-amanitin-induced telomere fragility. (A) Representative metaphase chromosomes processed with FISH from RPE1 cells with or without ectopically expressed RNase H1 (Ad-RH1) and treated with either vehicle or α-amanitin (α-aman). Centromeres are green and telomeres are red. Arrowheads mark fragile telomeres in the magnified images. (B) Representative quantification of the rate of telomere fragility. *p*-values were computed using a two-tailed Mann-Whitney *U* test (***, *p* < 0.001). Error bars represent standard error of the mean. (C) Western analysis of RNase H1 expression in cells with or without ectopically expressed RNase H1 (Ad-RH1) treated with vehicle or α-amanitin (α-aman). Two
exposures of the same RNase H1 blot are shown. α-tubulin is shown as a loading control.
### A. Allele Characteristics

<table>
<thead>
<tr>
<th>Allele</th>
<th>NLS</th>
<th>Replication</th>
<th>Repair</th>
<th>Rescues TF</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ΔC</td>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>D181A</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ΔP</td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ΔPΔC</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E160D</td>
<td></td>
<td>+</td>
<td>+/-</td>
<td>+</td>
</tr>
</tbody>
</table>

### B. shFEN1 Effect on Metaphase Chromosomes

![Images of metaphase chromosomes with shFEN1 expression]

### C. shFEN1 Effect on Metaphase Chromosomes

![Images of metaphase chromosomes with shFEN1 expression]

### D. Fold Change in Fragile Telomeres per Chromosome

<table>
<thead>
<tr>
<th>shFEN1</th>
<th>cDNA</th>
<th>Leading strand</th>
<th>Lagging strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>shCtrl</td>
<td>+</td>
<td>1.5 ± 0.2</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>shFEN1</td>
<td>-</td>
<td>2.0 ± 0.3</td>
<td>1.6 ± 0.2</td>
</tr>
</tbody>
</table>

**Annotations:**
- **Leading strand:** Fold change in fragile telomeres per chromosome.
- **Lagging strand:** Fold change in fragile telomeres per chromosome.
- *****:** Significant difference compared to control.
Figure 3.5. FEN1 flap endonuclease activity is required to limit leading strand-specific telomere fragility. (A) Schematic showing FEN1 alleles used in this study. Features indicated include a PIP box (PIP), nuclear localization signal (NLS), C-terminal region (C), and point mutations. The replication competency, repair competency, and ability to rescue telomere fragility (this study) of each allele are shown to the right. (B) Representative metaphase chromosomes processed with CO-FISH from BJL fibroblasts expressing a control hairpin (shCtrl) or depleted of FEN1 (shFEN1). Leading strand-replicated telomeres are green and lagging strand-replicated telomeres are red. FEN1 alleles were ectopically expressed where indicated. Arrowheads mark fragile telomeres in the magnified images. (C) Representative metaphase chromosomes processed with CO-FISH from BJ fibroblasts expressing a control hairpin (shCtrl) or depleted of FEN1 (shFEN1). Leading strand-replicated telomeres are green and lagging strand-replicated telomeres are red. FEN1 alleles were ectopically expressed where indicated. Arrowheads mark fragile telomeres in the magnified images. (D) Quantification of strand-specific telomere fragility per chromosome, with leading strand-specific telomere fragility shown in green and lagging strand-specific telomere fragility shown in red. Two independent biological replicates were analyzed, normalized with shCtrl set to 1 for each mutant group, and combined. \( p \)-values were computed using a two-tailed Mann-Whitney \( U \) test (***, \( p < 0.001 \) relative to shCtrl). Error bars represent standard error of the mean.
Figure 3.6. A model of FEN1’s role following co-directional replisome–RNAP collisions.

(A) RNA Pol II (RNAP) transcribes TERRA from the C-rich leading strand. The
replisome approaches the transcription complex and a co-directional collision occurs. Pol II dissociates from the nascent TERRA. (B) The replisome moves to the 3’ end of the TERRA, leaving a 5’ RNA flap and RNA:DNA hybrid. (C) The replisome resumes replication of the leading strand using the 3’ end of the nascent TERRA as a primer. (D) FEN1 cleaves the 5’ RNA flap left behind by the collision. (E) FEN1’s cleavage leaves behind a gap and a stretch of RNA:DNA hybrid that can be repaired. (F) In the absence of FEN1, RNA:DNA hybrid/flap structures accumulate and lead to telomere fragility.
References


Chapter 4:

FEN1 interacts with TRF1

Daniel C. Teasley, Ying Jie Lock*, Adrián Rivera*, and Sheila A. Stewart

*Y.J.L. and A.R. contributed equally to this work
Introduction

Faithful DNA replication is a prerequisite for the maintenance of genome stability and cellular proliferation. Because of the continual challenges presented to genome stability by environmental and endogenous forms of DNA damage, robust mechanisms have evolved to ensure that replication is completed even in the absence of some components of the DNA replication and repair machinery. However, some regions of the genome with repetitive sequences or secondary structures, such as telomeres, are sensitive to mutations or deletions of replication and repair proteins (Gilson and Géli, 2007). Telomere replication and stability are essential for genome stability, and as such, unique mechanisms are present to ensure that telomeres can be replicated despite the challenges presented by their repetitive sequence and secondary structure. Central to these mechanisms are the six telomere-specific proteins making up the shelterin complex: TRF1, TRF2, POT1, Rap1, TPP1, and TIN2 (de Lange, 2005). Once thought to act as a protective cap that “hides” telomeres from the DNA repair machinery to prevent aberrant chromosome end-to-end fusions, an emerging model for shelterin function instead proposes that shelterin coordinates the activities of specific replication and repair proteins to ensure telomeres are replicated and maintained. Indeed, a host of DNA replication and repair proteins can be found at the telomere: the protein complex CST (CDC13, STN1, TEN1), RecQ helicases WRN and BLM, and lagging strand replication/base excision repair protein flap endonuclease 1 (FEN1) all have known roles in facilitating replication fork progression through the telomere and preventing telomere loss (de Lange, 2005; Longhese et al., 2012; Saharia et al., 2010).
The loss of replication fork progression induces replication stress; if unresolved, stalled forks ultimately produce DNA damage and genome instability (Ozeri-Galai et al., 2011). Regions of the genome that frequently become unstable following treatment with the replication stress-inducing drug aphidicolin (or other forms of replication stress) are known as fragile sites. Why particular loci in the genome manifest as fragile sites is unclear, and three mechanisms have been proposed to explain what causes a particular locus to be fragile: repetitive, AT-rich sequence that causes replication fork stalling; a paucity of sufficient replication origins to rescue stalled forks; and transcription of the locus that requires longer than a single cell cycle to complete, thus forcing collisions between the replisome and transcription machinery (Burrow et al., 2010; Helmrich et al., 2011; Letessier et al., 2011). These models are not mutually exclusive, and all may contribute to fragile site expression under conditions of replication stress.

Recent work has described telomeres as fragile sites because treatment with aphidicolin causes a reduction in replication fork progression through the telomere and the formation of abnormal telomere structures (Martínez et al., 2009; Sfeir et al., 2009). Additionally, deletion of the shelterin protein TRF1 in mouse cells induces telomere fragility; TRF1 prevents telomere fragility by facilitating replication fork progression through the telomere (Sfeir et al., 2009). Recently, we demonstrated that FEN1 also prevents telomere fragility (Saharia et al., 2010); however, FEN1 depletion-induced telomere fragility is restricted to the leading strand-replicated telomere, while TRF1 deletion induces non-strand-specific telomere fragility (Sfeir et al., 2009). This role for FEN1 is distinct from the previously described role in preventing sister telomere loss (STL), which is restricted to the lagging strand-replicated telomere (Saharia et al.,
Moreover, the STL phenotype can be rescued by a FEN1 allele deficient in Okazaki fragment processing (ΔP), but is not rescued by alleles deficient in DNA repair (ΔC, E160D) (Saharia et al., 2008, 2010). Both repair-deficient alleles (ΔC, E160D), as well as the Okazaki fragment processing-deficient allele (ΔP), rescue telomere fragility induced upon FEN1 depletion, indicating that FEN1’s roles in preventing STL and telomere fragility depend upon distinct biochemical activities of FEN1. We previously demonstrated that the C-terminally truncated (30 amino acids), repair-deficient allele of FEN1 (ΔC) fails to interact with the shelterin protein TRF2, yet still localizes to the telomere. Together, these findings suggest that FEN1 is recruited differentially to the telomere for its roles in telomere replication and stability: first, FEN1 is recruited to the telomere by TRF2 (via the FEN1 C-terminus) to prevent STL on the lagging strand, and second, FEN1 is recruited to the telomere independently of its interaction with TRF2 to protect against telomere fragility on the leading strand.

**Experimental Procedures**

**Plasmids and protein expression**

pProFEN1 and pProFEN1ΔC were produced by PCR amplifying the FEN1 cDNA (primers: KasI+FEN1-aa2 and 3′-FEN1-HindIII). PCR product was digested with KasI and HindIII (New England Biolabs, Ipswich, MA) and ligated into the KasI and HindIII restriction sites in pProEX-HTb (Life Technologies, Grand Island, NY).

For protein production, *E. coli* transformed with pProFEN1 or pProFEN1ΔC were grown in 5 mL of TB (12 g/L tryptone, 24 g/L yeast extract, 0.4% v/v glycerol, 17 mM monobasic potassium phosphate, 72 mM dibasic potassium phosphate) supplemented
with ampicillin (Sigma-Aldrich, St. Louis, MO) overnight at 37 °C. Saturated culture was used to inoculate 2 L of TB supplemented with ampicillin to an OD600 of 0.15, and the culture was grown at 37 °C to an OD600 of 0.75. Protein expression was then induced by addition of isopropyl β-D-1-thiogalactopyranoside to a concentration of 0.4 mM; cells were grown for 2 hours at 37 °C and collected by centrifugation.

**Cell culture and baculovirus amplification**

Sf9 insect cells (*Spodoptera frugiperda*) were obtained from Orbigen (San Diego, CA) and propagated at 27 °C as adherent cultures in TNM-FH medium (Grace’s insect medium, 3.3 g/L lactalbumin hydrolysate, 3.3 g/L yeastolate ultrafiltrate, 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, and 50 µg/mL gentamicin). Suspension cultures were seeded from adherent culture at 1.0×10⁶ cells/mL and grown in TNM-FH supplemented with 0.1% v/v Pluronic F-68.

*Autographa californica* multicapsid nucleopolyhedrovirus (baculovirus) encoding human his-TRF1 was generously provided by Dr. Titia de Lange (Rockefeller University). Baculovirus was amplified for protein production by infecting adherent Sf9 cultures at an MOI of 0.1 for 5 days; cells were lifted and pelleted, and supernatant (baculovirus) was recovered and stored at 4 °C. For protein expression, 1.2×10⁸ cells were collected from suspension culture, pelleted, and resuspended in a conical tube in a volume of amplified baculovirus stock corresponding to an MOI of 15. The cells were rocked at room temperature for 1 hour, transferred to a spinner flask, volume increased to 100 mL with TNM-FH with Pluronic F-68, and cultured at 27 °C for 48 hours.
Protein purification

Hexahistidine-tagged proteins were purified using immobilized metal ion affinity chromatography (IMAC). Nickel-charged Sepharose beads (Ni Sepharose 6 Fast Flow, GE Life Sciences, Waukesha, WI) were prepared by centrifuging 12 mL of slurry for 5 minutes at 500 × g. The supernatant was removed and replaced with 5 mL of water. The beads were washed by gently rocking the beads for 5 minutes at 4 °C. One additional wash with water was conducted and two washes with Buffer A20 (20 mM imidazole, 0.5 M sodium chloride, 20 mM tris-HCl pH 7.9) were conducted. The beads were sedimented by centrifuging for 5 minutes at 500 × g. 5 mL of Buffer A20 was added to make a 50% slurry, and the beads were stored at 4 °C.

For purification of recombinant FEN1, 30 mL of sonication buffer (100 mM sorbitol, 50 mM tris-HCl pH 7.9, 2X bacterial protease inhibitors [100X: 0.5 mM bestatin, 0.4 mM leupeptin, 0.2 mM pepstatin A, 0.2 mM E-64]) was added to each pellet of cells and incubated at 4 °C for 30 minutes; the cells were subsequently resuspended. The cells were sonicated using a Misonix 3000 sonicator and a micro-tip probe (4 pulses: 10 seconds on, 10 seconds off, power 6). The lysates were transferred to a 30 mL Oak Ridge tube centrifuged at 15,000 rpm in an SA-600 rotor for 30 minutes at 4 °C. The supernatant was transferred to a new tube, sodium chloride was added to a concentration of 0.5 M, imidazole was added a concentration of 20 mM, and 5 mL of prepared Ni Sepharose slurry was added. The slurry samples were incubated at 4 °C on a rotator for 1.5 hours. The slurries were then poured into a column (Glass econo column, 2.5 cm ID x 5 cm, Bio-Rad, Hercules, CA), allowed to settle, and drained of flow through. The beads were washed with 40 column volumes (CVs) of Buffer A20, then
with 10 CVs of Buffer A40 (40 mM imidazole, 0.5 M sodium chloride, 20 mM tris-HCl pH 7.9) to elute weakly-bound proteins. A stepwise elution was then carried out by adding 2 CVs of elution buffers of increasing imidazole concentrations (50 mM, 100 mM, 150 mM, 200 mM, 300 mM, 500 mM). All remaining protein was removed by adding 3 CVs of Buffer A1000 (1 M imidazole, 0.6 M sodium chloride, 20 mM tris-HCl pH 7.9). Separate fractions were collected for each elution. Samples from each wash step and fractions were denatured, separated on a 10% polyacrylamide-SDS gel, and visualized with colloidal Coomassie (LabSafe GEL Blue, G-Biosciences, St. Louis, MO).

To remove imidazole and concentrate the protein, fractions were dialyzed in purified protein buffer (30 mM HEPES pH 7.4, 10% v/v glycerol, 137 mM sodium chloride, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.125x bacterial protease inhibitors, 1 mM dithiothreitol, 0.01% NP40). Each fraction was put into a length (approximately 12 cm) of dialysis tubing (Fisherbrand nominal MWCO 12k-14k, 25 mm flat width regenerated cellulose, Thermo Fisher Scientific, Waltham, MA) and was stirred in purified protein buffer at 4 °C for two days with a change of dialysis buffer at the end of the first day. The dialysate was recovered from the tubing and stored at −80 °C.

For purification of recombinant TRF1, baculovirus-infected Sf9 cells were washed twice with PBS. The cells were resuspended in Buffer A20 and lysed by sonication were using a Misonix 3000 sonicator and a micro-tip probe (4 pulses: 30 seconds on, 30 seconds off, power 6). The lysate was centrifuged at 16,000 rpm in an SS-34 rotor for 30 minutes at 4 °C to pellet cell debris, and the supernatant was collected and filtered through a 0.45 um PVDF low protein-binding syringe filter. 1 mL of prepared 50% Ni Sepharose
slurry was added to the supernatant and rotated at 4 °C for 2 hours. The slurry was poured into a column, allowed to settle, and drained of flow through (Glass econo column, 2.5 cm ID x 5 cm, Bio-Rad, Hercules, CA). The beads were washed twice with 250 CVs of Buffer A20 with protease and phosphatase inhibitors and once with 20 CVs of Buffer A60 (60 mM imidazole, 0.5 M NaCl, 20 mM tris-HCl pH 8) to elute weakly-bound proteins. The remaining protein was eluted in four 3 CV fractions with Buffer A1000. Samples from each wash step and fraction were denatured, separated on a 10% polyacrylamide-SDS gel, and visualized with colloidal Coomassie (LabSafe GEL Blue, G-Biosciences, St. Louis, MO).

The eluates were then dialyzed to remove imidazole and concentrate the protein. The eluates were transferred into dialysis cassettes (Pierce Slide-A-Lyzer, 10,000 MWCO; 0.5-3 mL capacity, Thermo Fisher Scientific, Waltham, MA) and stirred in 1.5 liters of Buffer D (300 mM potassium chloride, 20 mM HEPES, 3 mM magnesium chloride, 1 mM dithiothreitol, 2% v/v glycerol, 0.1% v/v NP40, 0.5 mM phenylmethylsulfonyl fluoride) at 4 °C overnight, after which the cassettes were transferred to fresh Buffer D and allowed to dialyze for 6 hours. The dialysate of purified TRF1 was collected from the cassettes and stored at −80 °C.

Recombinant human his-TRF2 was generously provided by Dr. Judith Campbell (California Institute of Technology, Pasadena, CA).
Mass spectrometry

Purified human his-TRF1 and his-TRF2 were trypsinized and peptide mass fingerprinting was performed using tandem mass spectrometry. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version Mascot). Mascot was set up to search the uniprot_sprot_20100305 database (unknown version, 515203 entries). Mascot was searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 50 PPM. Scaffold (version Scaffold_3_00_08, Proteome Software Inc, Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications with a probability of 95% or greater were accepted while protein identifications established at greater than 95% or greater and contained at least 1 identified peptide were accepted.

Electrophoretic mobility shift assay

The duplex telomeric substrate was hybridized by combining complementary ssDNA telomere oligonucleotides in a 95 °C, 1 L water bath and allowing to cool below 37 °C. 20 pmoles of duplex substrate was radiolabeled at the 5’ termini with $[\gamma^{-32P}]$ATP (3000 Ci/mmol) using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA), recovered using Illustra MicroSpin G25 columns (GE Healthcare, Waukesha, WI), and stored at −20 °C.

25 nM, 50 nM, or 100 nM of recombinant TRF1 was added to labeled telomeric duplex (2.5 nM), in 1X TEL buffer (20 mM HEPES pH 7.9, 150 mM potassium chloride, 1 mM magnesium chloride, 0.1 mM EDTA, 0.5 mM dithiothreitol, 5% v/v glycerol, 0.1% v/v NP-40), and incubated for 20 minutes at 4 °C. For competitor reactions, 1:1, 10:1, or
100:1 cold telomeric duplex was added. The reactions were then run out on a native 5% polyacrylamide-TAE gel at 300 V at 4 °C for 2 hours. The gel was dried on a vacuum gel dryer at 80 °C for 1 hour, then imaged on film for four hours.

**Western blot analysis**

Proteins were quantified by running dilutions on a 10% polyacrylamide-SDS gel alongside bovine serum albumin (BSA) as standards. For western analysis, proteins were separated on a 10% polyacrylamide-SDS gel and transferred to a PVDF membrane and processed as previously reported (Saharia et al., 2010). Antibodies used were as follows: rabbit polyclonal anti-FEN1 (A300-255A, Bethyl Labs, Montgomery, TX), rabbit polyclonal anti-TRF2 (H-300, Santa Cruz Biotech, Santa Cruz, CA).

**Flap endonuclease activity assay**

Downstream/flap, upstream, and template oligonucleotides were ordered from and HPLC-purified by Integrated DNA Technologies (Coralville, IA). Downstream/flap oligonucleotide was radiolabeled at the 5′-terminus with [γ-32P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) and recovered using Illustra MicroSpin G-25 columns (GE Healthcare, Waukesha, WI). Downstream oligonucleotide was annealed with the upstream and template oligonucleotides at a 1:3:3 molar ratio by incubating in 1 L of 95 °C water allowed to cool to room temperature.
Ladder oligonucleotide was labeled as described above. To make a single base-pair ladder, 150 fmoles of labeled ladder were incubated in 1X nuclease assay reaction buffer (20 mM HEPES/potassium hydroxide pH 7.4, 1 mM dithiothreitol, 5 mM magnesium acetate/5 mM manganese acetate, 10 mM sodium chloride, 100 µg/mL BSA) with 0.14 units of snake venom phosphodiesterase, and samples at 5, 15, 30, 45 seconds, and 3 minutes were collected in 95% formamide containing bromophenol blue and xylene cyanol to terminate the reaction.

Labeled substrate and recombinant human FEN1 (MyBioSource, San Diego, CA) or Rad27p (Dr. Peter Burgers, Washington University School of Medicine) were incubated in reaction buffer (20 mM HEPES / potassium hydroxide pH 7.4, 1 mM dithiothreitol, 5 mM magnesium acetate, 10 mM sodium chloride, 100 µg/mL BSA) for 5 minutes at 37 ºC. Reactions were terminated by addition of 95% formamide containing bromophenol blue and xylene cyanol. Reaction products were separated on a 20% polyacrylamide, 7 M urea, tris-borate-EDTA gel and imaged using a storage phosphor screen.

**Enzyme-linked immunosorbent assay (ELISA)**

ELISA was conducted as reported with modifications (Muftuoglu et al., 2006). 10 nM his-TRF1 or his-TRF2 were prepared in 50 µL of ELISA carbonate buffer (16 mM sodium carbonate, 34 mM sodium bicarbonate, pH 9.6). The proteins were adsorbed to each well on a 96-well immunoassay microplate (BRANDplates, immunoGrade) by incubation at 37 ºC for 2 hours. The plate was washed three times with 100 µL of ELISA wash buffer (PBS + 0.5% v/v Tween-20), and then blocked with 200 µL ELISA blocking/binding buffer (Pierce Blocker Casein (Thermo Fisher Scientific, Waltham, MA)

138
+ 0.1% v/v Tween-20) in each well, incubating at 37 °C for 2 hours. After blocking, the plate was washed three times with 200 μL ELISA blocking/binding buffer. A range of concentrations (0-40 nM) of his-FEN1 or his-FEN1ΔC were prepared in 50 μL of ELISA carbonate buffer, added in triplicate to the wells, and incubated at 37 °C for 2 hours. The plate was then washed three times with 200 μL ELISA conjugate buffer (50 mM tris-HCl pH 8.0, 150 mM sodium chloride, 0.05% Tween-20, 1% BSA). 50 μL of primary antibody (1:5000 in ELISA blocking/binding buffer: polyclonal rabbit anti-FEN1, A300-255A, Bethyl Labs, Montgomery, TX) was added to the wells, and incubated at 37 °C for one hour. The plate was then washed three times with 200 μL ELISA blocking/binding buffer and one time with 200 μL ELISA conjugate buffer. 50 μL of HRP-conjugated secondary antibody (1:10,000 in ELISA conjugate buffer: donkey anti-rabbit IgG, Jackson ImmunoResearch, West Grove, PA) was added to each well and incubated at 37 °C for 1 hour. The plate was washed five times with 200 μL ELISA conjugate buffer. 100 μL of OPD solution (0.1 M citric acid-phosphate buffer pH 5.0, 1 mg/mL o-phenylenediamine dihydrochloride, 1 μL/mL 30% hydrogen peroxide) was added to each well and incubated at room temperature in the dark for 20 minutes. The reactions were terminated by adding 50 μL of 3 M sulfuric acid to each well and absorbances were read at 490 nm. Values for wells coated with TRF1 or TRF2 were corrected for the background signals obtained with addition of 0 nM FEN1.

**Oligonucleotides**

Oligonucleotides used were as follows. All oligonucleotides were ordered from Integrated DNA Technologies (Coralville, IA). Oligonucleotides used for PCR were
ordered with standard desalting; oligonucleotides for the flap endonuclease activity assay and EMSA were HPLC purified.

KasI+FEN1-aa2: 5′-(ACT GGG CGC CGG AAT TCA AGG CCT GGC CAA)-3′

3' FEN1-HindIII: 5′-(GAC TAA GCT TTC ATT ATT TTC CCC TTT TAA ACT TCC C)-3′

Flap template oligonucleotide 5′-(GCC CAG TCA CGT TGT AAA ACG GGT CGT GAC TGG GAA AAC CCT GGC G)-3′

Downstream/flap oligonucleotide: 5′-(TCG CGC GTT TCA CGC CTG TTA CTT AAT TCA CTG GCC GTC GTT TTA CAA CGT GAC TGG G)-3′

Upstream flap oligonucleotide: 5′-(CGC CAG GGT TTT CCC AGT CAC GAC C)-3′

Ladder oligonucleotide: 5′-(GTG CCG TAG AAC GCT TTT TTT TTC CGA TCG AGA CCT G)-3′

Telomere (G): 5′-(TTA GGG TTA GGG TTA GGG TTA GGG)-3′

Telomere (C): 5′-(CCC TAA CCC TAA CCC TAA CCC TAA)-3′

**Results**

**FEN1 interacts with TRF1 in vitro**

FEN1 is known to interact with TRF2 via the TRF2 myb domain (Muftuoglu et al., 2006), and TRF1 and TRF2 share substantial homology between their myb domains (Broccoli et al., 1997). Because human FEN1 depletion and murine TRF1 deletion both induce telomere fragility, and FEN1’s ability to prevent fragility does not depend on its interaction with TRF2, we hypothesized that FEN1 is recruited to the telomere by TRF1 to prevent telomere fragility. While such an interaction could be indirect, as TRF1 and TRF2 are both part of the shelterin complex, our hypothesis was predicated on the possibility that FEN1’s interaction with TRF1 would occur independently of its interaction
with TRF2. We therefore used an in vitro approach with purified proteins to determine if a direct interaction exists between FEN1 and TRF1. We expressed and purified recombinant, hexahistidine-tagged (N-terminal) human FEN1 from *Escherichia coli* using immobilized metal ion affinity chromatography (IMAC), and verified its identity by western blot. An N-terminal tag was selected because the C-terminal domain of FEN1 is known to interact with at least 20 proteins (Guo et al., 2008). To determine if the recombinant protein was folded correctly, we carried out an in vitro flap endonuclease activity. However, because crystallography studies of human FEN1 have shown that the N-terminus localizes into the enzyme active site, we first cleaved the hexahistidine tag from the protein using AcTEV protease. Following cleavage and IMAC to remove the tag, the protein was incubated with a DNA flap structure in vitro (Figure S4.1A); as expected, FEN1 was able to cleave the 5' flap (Figure S4.1B). We also expressed and purified recombinant, hexahistidine-tagged human TRF1 from Sf9 cells using IMAC, verified the identity of the protein by peptide mass fingerprinting (Figure S4.2), and confirmed its conformational integrity using an electrophoretic mobility shift assay (EMSA) to determine if the protein bound telomeric dsDNA (Figure S4.3).

To determine if FEN1 interacts directly with TRF1, we used the purified proteins to perform an enzyme-linked immunosorbent assay (ELISA). Recombinant TRF1 or TRF2 (positive control) were adsorbed to an ELISA plate. Following a block for nonspecific interactions, recombinant FEN1 was incubated on the plate in a range of concentrations to allow binding interactions to occur. After extensive washing, FEN1 was detected with an anti-FEN1 antibody, conjugated secondary antibody, and colorimetric reaction. As previously reported (Muftuoglu et al., 2006), increasing concentrations of FEN1
produced increasing signal when incubated with TRF2 (Figure 4.1). Similarly, increasing concentrations of FEN1 produced increasing signal when incubated with TRF1, demonstrating that a direct interaction exists between FEN1 and TRF1 (Figure 4.1).

**FEN1 interacts with TRF1 via its C-terminal domain**

The observation that FEN1 and TRF1 interact directly supported our model that FEN1 could be recruited by TRF1 to the telomere to prevent telomere fragility. To better characterize the interaction, we sought to determine if TRF1 interacted with FEN1 via the same domain that TRF2 interacts with: the C-terminus. Because the FEN1ΔC allele cannot rescue STL but does rescue telomere fragility, we anticipated that if the TRF1–FEN1 interaction is important for preventing telomere fragility, TRF1 should bind to FEN1 at a domain other than its C-terminus. To address this possibility, we expressed and purified recombinant human FEN1ΔC in *E. coli*. We again carried out an ELISA, adsorbing TRF1 or TRF2 to the plate, and incubating either full-length FEN1 or FEN1ΔC on the plate. As before, full-length FEN1 exhibited concentration-dependent binding to both TRF1 and TRF2, and FEN1ΔC failed to interact with TRF2 as we previously showed using co-immunoprecipitation (Figure 4.2A) (Saharia et al., 2008). Strikingly, FEN1ΔC also failed to interact with TRF1, indicating that the FEN1–TRF1 direct interaction occurs via the FEN1 C-terminal domain (Figure 4.2B). Combined with the data that the FEN1ΔC allele rescues telomere fragility, these data demonstrate that in contrast to our original hypothesis, the direct interaction between FEN1 and TRF1 does not contribute to FEN1’s ability to prevent telomere fragility.
Discussion

Our previous data demonstrate two distinct roles for FEN1 in maintaining telomere stability. First, FEN1 facilitates replication fork reinitiation in a mechanism dependent on its DNA repair activity and/or TRF2 interaction, and this activity prevents sister telomere loss on the lagging strand-replicated telomere (Saharia et al., 2008, 2010). Second, FEN1 resolves RNA:DNA hybrid/flap structures in a mechanism dependent on its nuclease activity, but independent of its replication and repair activities and interaction with TRF2. This activity of resolving RNA:DNA hybrid/flap structures is responsible for preventing telomere fragility on the leading strand-replicated telomere. Here, we investigate if a potential interaction between TRF1, which is known to prevent telomere fragility in a non-strand-specific manner (Sfeir et al., 2009), and FEN1 might contribute to the latter’s ability to prevent telomere fragility. We find that FEN1 does indeed directly interact with TRF1. However, because this interaction requires the C-terminus of FEN1, and that domain is dispensable for preventing telomere fragility on the leading strand, we conclude that the FEN1–TRF1 interaction is dispensable for FEN1’s ability to prevent telomere fragility.

The fact that FEN1 does not require an interaction with either TRF1 or TRF2 to prevent telomere fragility merits investigation into whether FEN1’s ability to prevent telomere fragility is truly a telomere-specific role, or whether the proposed RNA:DNA hybrid/flap resolution activity contributes to genomic stability at non-telomeric loci as well. If FEN1’s biochemical activity in preventing telomere fragility occurs throughout the genome as part of a mechanism to respond to structures produced by replisome–RNAP collisions, then perhaps telomere fragility is simply a visible manifestation of the consequences of
a loss of this activity, rather than an indication of a telomere-specific role. Such a phenomenon would be consistent with the concept that chromosomal fragile sites are an inherent “reporter” of replication stress due to their enhanced sensitivity to conditions generating replication stress genome-wide (Ozeri-Galai et al., 2011); telomere fragility might simply result from an enhanced sensitivity to replisome–RNAP collisions that occur genome-wide and remain unrepaired in the absence of FEN1. On the other hand, should FEN1’s molecular activity responsible for preventing telomere fragility occur only at the telomere, the necessity of FEN1’s localization to the telomere for this role comes into question. If FEN1 does not require an interaction with TRF1 or TRF2 to prevent telomere fragility, how might it localize to the telomere for this role? Perhaps the presence of FEN1 at the replisome is sufficient for preventing telomere fragility. Alternatively, FEN1 might localize to the telomere for this role by an interaction independent of its C-terminus, whether an interaction with another shelterin protein or an indirect interaction. In addition to interacting with TRF1 and TRF2, FEN1 is known to interact with POT1, which stimulates FEN1’s flap endonuclease activity, though the domain of FEN1 that POT1 interacts with has not been reported (Miller et al., 2012).

The observation here that TRF1 interacts with the same domain of FEN1 as TRF2 raises additional questions. Previously, FEN1 has been presumed to localize to the telomere and prevent sister telomere loss on the lagging strand in a manner dependent on its interaction with TRF2, if not also its interactions with base excision repair proteins (Saharia et al., 2008, 2010). At the very least, these new data indicate that because the FEN1 C-terminal domain mediates interactions with TRF1 and TRF2, FEN1’s activity in preventing STL may be dependent on an interaction with either protein, rather than only
TRF2. Since TRF2 interacts with FEN1 via both the TRF2 basic domain (divergent from TRF1) and the TRF2 myb domain (conserved with TRF1) (Muftuoglu et al., 2006), it seems most likely that the myb domains of both TRF1 and TRF2 are capable of interacting with the FEN1 C-terminus, making separation-of-function experiments difficult to conduct due to the low probability of identifying a FEN1 mutant that interacts differentially with TRF1 and TRF2. However, given that FEN1’s role in preventing telomere fragility does not require an interaction with TRF1 or TRF2, it is possible that FEN1’s role in preventing STL is actually dependent on another FEN1 C-terminally-mediated interaction, whether that interaction facilitates FEN1 recruitment to the telomere or simply enhances FEN1 activity, such as the FEN1–WRN interaction (Sharma et al., 2005).

Though the data here were unable to identify different roles for TRF1 and TRF2 in FEN1’s activities at the telomere, they do not speak to the physiological roles that TRF1 and TRF2 might play with FEN1 in vivo. Interestingly, in an unrepeated experiment, we found that TRF1 was able to immunoprecipitate FEN1 from cell lysates, suggesting that the interaction observed in vitro might indeed occur in physiological settings. Dissecting whether either interaction is more important for FEN1’s telomere roles in vivo would require the production of mutants of TRF1 and TRF2 that are competent and incompetent to interact with FEN1, either using a knock-in or dominant-negative approach. Despite the unanswered questions, this work provides new insight into the complexity of DNA replication and repair at the telomere, and proves that FEN1’s multiple activities at the telomere cannot be accounted for simply by interactions with two different telomere proteins.
Acknowledgments

The authors thank Dr. Heather True and Dr. Tejas Kalastavadi for assistance with protein purification from *E. coli*; Dr. Helen Piwnica-Worms and Christine Ryan for assistance with Sf9 culture and baculovirus infections; Dr. Titia de Lange for advice on recombinant TRF1 purification; and Dr. Peter Burgers and Dr. Justin Sparks for assistance with protein purification and the flap endonuclease activity assay. This project was supported by National Institutes of Health Grant GM95924 (S.A.S.), NRSA Training Grant GM007067 (D.C.T.), and BioMedRAP at Washington University (A.R.).
Figure 4.1. FEN1 interacts with TRF1.

ELISA showing the interaction between recombinant FEN1 and either TRF1 (green) or TRF2 (orange). TRF1 or TRF2 were adsorbed to an ELISA plate, after which varying concentrations of FEN1 were incubated on the adsorbed surface. Following extensive washing, bound FEN1 was detected with an antibody/horseradish peroxidase-based detection system. Error bars represent standard error of the mean; lines are linear regressions of each data set.
Figure 4.2. FEN1’s interactions with TRF1 and TRF2 are mediated by its C-terminal domain.

(A) ELISA showing the interaction between two FEN1 alleles and TRF2. TRF2 was adsorbed to an ELISA plate, after which varying concentrations of FEN1 (red) or FEN1ΔC (blue) were incubated on the adsorbed surface. Following extensive washing,
bound FEN1 was detected with an antibody/horseradish peroxidase-based detection system.

(B) ELISA as in (A), with TRF1 rather than TRF2. In both graphs, error bars represent standard error of the mean; lines are exponential plateau regressions of each data set.
Figure S4.1. Flap endonuclease activity of recombinant FEN1

(A) The substrate utilized to assess endonuclease activity. The red strand was 5' labeled and hybridized with the green and blue strands to produce the flap structure.

(B) Untagged FEN1, his-FEN1, or Rad27p (positive control) were incubated with the labeled substrate and separated by denaturing polyacrylamide gel electrophoresis. The
presence of a 58-nt band indicates substrate that was not cut; cleaved products are observed as 38- and 39-nt bands.
Figure S4.2. Peptide mass fingerprinting of purified TRF1.

Purified TRF1 (TERF1) was analyzed by peptide mass fingerprinting; the unique spectra corresponding to peptides within TRF1 and corresponding to 18% of the protein sequence are highlighted on the TRF1 protein sequence.
Figure S4.3. Electrophoretic mobility shift assay of purified TRF1.

Purified TRF1 was incubated with a radioactive telomeric dsDNA substrate, with cold competitor sequence included as indicated. Binding of TRF1 to telomeric DNA is indicated by the upward shift of labeled substrate.
References


Sfeir, A.J., Kosiyatrakul, S.T., Hockemeyer, D., MacRae, S.L., Karlseder, J.,

Chapter 5:

Conclusions and future directions

Daniel C. Teasley
Conclusions

Telomeres must be replicated with high fidelity to maintain their stability and ensure genome stability. When telomeres are not completely replicated, the consequential telomere dysfunction causes a host of cellular defects including activation of a DNA damage response, end-to-end chromosome fusions, breakage-fusion-bridge cycles, senescence, and cell death (Frias et al., 2012). The genome instability induced by telomere dysfunction can even promote carcinogenesis (Begus-Nahrmann et al., 2012). These potential problems necessitate mechanisms to ensure telomere replication completes in spite of the difficulties presented by the repetitive sequence and secondary structures found at telomeres. In this dissertation, I describe distinct roles for a DNA replication and repair protein, flap endonuclease 1 (FEN1). FEN1, a structure-specific endonuclease, has well-established roles in Okazaki fragment processing during lagging strand synthesis, long-patch base excision repair, and the prevention of trinucleotide repeat expansion (Balakrishnan and Bambara, 2013). More recently, FEN1 has been identified as a contributor to telomere stability. Our lab recently demonstrated that FEN1 prevents sister telomere loss (STL) on the lagging strand-replicated telomere (Saharia et al., 2008). Additionally, FEN1 associates with TRF2 and localizes to the telomere during S and G2 phases of the cell cycle, maintains telomere length and stability in Saccharomyces cerevisiae and Schizosaccharomyces pombe, and can process flaps that form on substrates that fold into G-quadruplexes (Balakrishnan and Bambara, 2013).

In light of these data, we chose to examine the roles that FEN1 plays in human telomere maintenance. In Chapter 2, we show that FEN1’s flap endonuclease activity,
gap endonuclease activity, and C-terminal interactions are required to facilitate replication fork restart, and in turn are also required to prevent STL on lagging strand-replicated telomeres (Saharia et al., 2010). We propose a model in which the G-rich sequence encountered by the lagging strand machinery causes spontaneous replication fork stalling, which FEN1 is required to resolve. In the absence of FEN1, failure to restart the replication fork ultimately leads to STL (Saharia et al., 2010). In Chapter 3, we show that FEN1’s nuclease activity, but none of its previously identified activities or domains, is required to resolve RNA:DNA hybrid-dependent, leading strand-specific telomere fragility. We propose that FEN1 resolves RNA:DNA hybrid/flap structures that form following co-directional collisions between the replisome and transcribing RNA polymerases (RNAPs). Without FEN1, stochastic collision events cause an accumulation of RNA:DNA hybrid/flap structures that ultimately cause telomere fragility. Lastly, in Chapter 4, we show that FEN1 interacts directly with the shelterin protein TRF1 via the FEN1 C-terminus. We anticipated that the FEN1–TRF1 interaction might be required for preventing telomere fragility, as TRF1 knockout mice display increases in telomere fragility; however, because the FEN1–TRF1 interaction occurs via the FEN1 C-terminus, and FEN1’s C-terminus is dispensable for its role in preventing telomere fragility, this hypothesis was incorrect. Nevertheless, the knowledge that FEN1 and TRF1 can directly interact provides a second means by which FEN1 can localize to telomeres, in addition to its established interaction with TRF2 (Muftuoglu et al., 2006).

These data are most intriguing because FEN1’s roles in mammalian telomere maintenance appear independent of one another: the STL and telomere fragility phenotypes occurring in the absence of FEN1 are exclusively found on lagging and
leading strand-replicated telomeres, respectively. These two roles for FEN1 are also biochemically and genetically distinct from one another, as FEN1’s ability to reinitiate stalled forks and prevent STL is dependent on its C-terminal protein–protein interactions, gap endonuclease activity, and flap endonuclease activity. In contrast, FEN1’s putative ability to resolve RNA:DNA hybrid/flaps and prevent telomere fragility is only dependent on its flap endonuclease activity. Lastly, the work described here provides the first molecular mechanism for strand-specific telomere fragility, and the first known role for FEN1 in leading strand DNA replication.

**FEN1 facilitates replication fork restart and prevents STL on the lagging strand**

Our lab previously demonstrated that FEN1 is required for telomere stability on the lagging strand, where FEN1’s nuclease activity and C-terminally-mediated protein–protein interactions are required to prevent lagging strand-specific STL (Saharia et al., 2008). Additionally, our lab has shown that FEN1 is required for telomere stability in ALT-positive cells, but not telomerase-positive cells (Saharia and Stewart, 2009). While these data established a clear role for FEN1 in telomere maintenance, the molecular mechanism by which FEN1 prevented STL remained elusive. In Chapter 2, we identify the molecular role FEN1 plays at the lagging strand-replicated telomere: FEN1 facilitates replication fork reinitiation following stalling in a manner dependent on its gap endonuclease activity.

We show that while human FEN1 is not required for overt DNA replication in human cells, it is required for human cells to reinitiate replication forks stalled by treatment with hydroxyurea. A mutant analysis identified that this ability is dependent on FEN1’s
nuclease activity, C-terminally-mediated protein–protein interactions, and gap endonuclease activity, but is not dependent on FEN1’s interaction with PCNA. Strikingly, these requirements precisely mirror those for FEN1’s ability to prevent STL, strongly implicating FEN1’s activity at stalled replication forks as the means by which it prevents lagging strand-specific STL. Given the lagging strand specificity of the STL phenotype, and the fact that FEN1 interacts with the WRN and BLM helicases via its C-terminus (Sharma et al., 2005), these data suggest an intriguing model for FEN1 function in fork reinitiation. During DNA replication at the telomere, unwinding of the telomere duplex exposes ssDNA, which on the lagging strand template is composed of G-rich sequence. This sequence stochastically forms G-quadruplexes, which stall the replication fork and prevent further replication. Given that WRN and BLM are known to unwind G-quadruplexes and have established roles in telomere stability (Paeschke et al., 2010), we propose that FEN1, which is enriched at telomeres in the S and G2 phases of the cell cycle (Saharia et al., 2008), recruits WRN or BLM to the stalled fork to resolve G-quadruplexes, thus facilitating fork reinitiation.

**FEN1 resolves replisome–RNAP collision intermediates and suppresses telomere fragility on the leading strand**

As we report in Chapter 2, in addition to suppressing lagging strand-specific STL, FEN1 suppresses telomere fragility. Unlike the STL phenotype, FEN1 depletion-induced telomere fragility is not rescued by telomerase expression. Given this distinction, we sought in Chapter 3 to explore how FEN1 limits telomere fragility. We propose that collisions between the replication fork and transcription complexes at the telomere might drive the replication-dependent telomere fragility phenotype. We treated cells with α-
amanitin to increase the number of stochastic collisions occurring between the replication fork and transcribing Pol II by slowing the rate of translocation of Pol II along its template. Strikingly, α-amanitin treatment increased the rate of telomere fragility in control cells and FEN1-depleted cells, suggesting that replisome–RNAP collisions are indeed responsible for telomere fragility. Because the telomere is only transcribed using the C-rich leading strand as a template, we surmised that if FEN1 helps resolve collision-induced replication stress to prevent telomere fragility, FEN1 depletion should induce telomere fragility only on the leading strand. CO-FISH analysis confirmed this hypothesis, showing that FEN1-depletion induced telomere fragility is restricted entirely to the leading strand-replicated telomere. Based upon work in prokaryotes characterizing co-directional replisome–RNAP collisions, we also predicted that collisions on the leading strand-replicated telomere should result in an RNA:DNA hybrid structure with a 5′ RNA flap. In support of this model, we found that ectopic expression of RNase H1, an RNA:DNA hybrid-specific endoribonuclease, rescued cells from the telomere fragility phenotype, whether induced by FEN1 depletion or α-amanitin treatment. We also showed by mutant knockdown/rescue experiments that FEN1’s flap endonuclease activity is required to limit telomere fragility, while its gap endonuclease activity, exonuclease activity, and ability to interact with PCNA or WRN are dispensable. Given the previous reports demonstrating that FEN1 is capable of cleaving RNA and DNA flaps (Bornarth et al., 1999; Liu et al., 2004; Stewart et al., 2006), the requirement for FEN1’s flap endonuclease activity is not surprising. Together these data support a model in which stochastic co-directional replisome–RNAP collisions occurring at the telomere produce an RNA:DNA hybrid/flap intermediate structure; FEN1 cleaves the
flaps from these structures and in doing so prevents their accumulation and suppresses subsequent telomere fragility.

**FEN1 and TRF1 interact via the FEN1 C-terminus**

In Chapter 3, we found that FEN1’s ability to prevent telomere fragility is independent of its C-terminally-mediated protein–protein interactions, unlike its ability to prevent STL. Notably, the FEN1 C-terminus is required for FEN1’s interaction with the shelterin protein TRF2 (Saharia et al., 2008). These data indicate that FEN1’s role in preventing telomere fragility does not require an interaction with TRF2, and raise the question of how FEN1 might localize to the telomere specifically to prevent telomere fragility. Given that the shelterin protein TRF1, which shares significant sequence and structural homology to TRF2 (Broccoli et al., 1997), is known to prevent telomere fragility by facilitating replication fork progression through the telomere (Sfeir et al., 2009), we wondered if a direct FEN1–TRF1 interaction existed, and if such an interaction could contribute to the ability of either protein to prevent telomere fragility. Because TRF1 and TRF2 are both part of the six-protein shelterin complex, we chose to assess whether FEN1 and TRF1 interact directly in vitro, rather than use immunoprecipitation, to avoid the possibility of detecting an indirect FEN1–TRF1 interaction that was mediated by TRF2. Using purified proteins, we found that FEN1 and TRF1 do interact. However, the FEN1–TRF1 interaction, like the FEN1–TRF2 interaction, depends on FEN1’s C-terminus: a FEN1 mutant lacking its final 20 amino acids (FEN1ΔC) failed to interact with TRF1. Since the FEN1ΔC allele is competent to prevent leading strand-specific telomere fragility, but fails to interact with TRF1, we conclude that while FEN1 and
TRF1 do directly interact, this interaction is dispensable for FEN1’s ability to prevent telomere fragility.

**FEN1 in human cancers**

Several studies have reported mutations in FEN1 in human cancers. Two *FEN1* germline mutations have been identified that cause decreased FEN1 expression and correlate with significantly increased risk for the development of breast cancer (Lv et al., 2014). A recent genome-wide association study of colorectal cancer in East Asians identified a polymorphism in the *FEN1* 3’-UTR associated with colorectal cancer risk (Zhang et al., 2014). Other work has focused on the expression of FEN1 in cancers as a potential biomarker. FEN1 is overexpressed in breast, uterine, colon, gastric, lung, and renal cancers, and in breast cancer has a hypomethylated promoter associated with this increased expression (Singh et al., 2008). In breast and ovarian cancers, higher FEN1 mRNA and protein expression is associated with higher grade and poor survival, indicating its potential as a biomarker in multiple cancer types (Abdel-Fatah et al., 2014). Perhaps the most persuasive work that suggests that FEN1 plays a role in cancer is a study that identified mutations in FEN1’s gap endonuclease and exonuclease activities. A mouse carrying the E160D mutation (gap endonuclease- and exonuclease-deficient) exhibited chronic inflammation and increased cancer susceptibility, especially in the lungs (Zheng et al., 2007). In another study, a *FEN1* germline mutation (E359K) was identified in a family with a history of breast cancer; following characterization showing that FEN1 E359K fails to interact with WRN, a mouse model carrying the E359K mutation was produced. Cells from E359K mice exhibit increased telomere fragility, spontaneous chromosomal anomalies, and
transformation. Most significantly, E359K mutants develop spontaneous cancers at a rate over four-fold greater than wild-type mice; more than half of E359K mice develop lung tumors (Chung et al., 2014). Given that a loss of FEN1 function leads to a DDR and replication stress at the telomere, the tumor models and human studies raise the possibility that telomere instability (STL and fragility) induced by FEN1 loss of function contributes to tumorigenesis.

**Future directions**

**The molecular structure, formation, and consequences of telomere fragility**

The telomere fragility phenotype has been described in a wide variety of manipulations in both mouse and human cells (D’Alcontres et al., 2014; McNees et al., 2010; Saharia et al., 2010; Sfeir et al., 2009; Stewart et al., 2012; Vallabhaneni et al., 2013; Vannier et al., 2012). Despite these observations, a great deal remains unknown about the phenotype. The molecular structure(s) that leads to the observation of multiple or smeared telomere signals is unknown; given the three-dimensional structure of metaphase chromosomes, it is difficult to predict what primary structure would produce such signals, though telomere signal interrupted by non-telomere signal seems a likely candidate. Additionally, little is known about the mechanisms by which replication stress produces a fragile telomere, and especially whether a fragile telomere is a “lesser of evils” repair product that maintains genome stability (as compared to telomere loss), or a product of aberrant repair. Lastly, the consequences of fragile telomere formation are poorly understood – are these structures pathological or benign, and do they have long-term consequences for the cell?
Several avenues may better elucidate the primary structure of a fragile telomere. One way to address this question would be to combine telomere PNA FISH and subtelomere DNA FISH on metaphase chromosomes. If the presumed primary structure of a fragile telomere is telomere sequence interrupted by non-telomere sequence, the most likely origin of the intervening sequence might be the subtelomere. The microhomology existing between subtelomere sequences, which is composed of degenerate telomere repeats, and telomere sequence, may be sufficient to allow insertion of subtelomere sequence into the telomere, or vice versa. Intriguingly, because TRF1 and TRF2 have a low tolerance for substitutions in the telomere repeat sequence for binding (Broccoli et al., 1997), FEN1 might be unable to localize to these insertions; the absence of TRF1, TRF2, or FEN1 in part of a telomere could theoretically destabilize the telomere. These types of insertion events could be detected as adjacent or interspersed FISH signals at chromosome termini using a combination of differentially labeled telomere PNA FISH and subtelomere DNA FISH. Because subtelomere probes are chromosome-specific, this approach holds the potential to reveal the source of intervening subtelomere sequence as an intra-chromosomal rearrangement or inter-chromosomal translocation/amplification. Additional information might also be gained by employing super-resolution microscopy to examine metaphase chromosomes. Recent work has employed stochastic optical reconstruction microscopy (STORM) to observe t-loops in interphase nuclei by PNA FISH (Doksani et al., 2013); while a chromosome is more compacted than an interphase nucleus, the application of similar techniques to metaphase chromosomes may yield additional clarity on the structure of a fragile telomere. Multiple telomere signals and telomere signal smears are both quantified as fragile telomeres, but it is unknown if they represent a similar structure due largely to the
diffraction limit in conventional fluorescence microscopy; the application of super-resolution microscopy may allow for the classification of different “fragile” abnormalities to determine if they are of a common molecular origin. A complementary approach with the resolution to show the true linear structure of a fragile telomere would be to utilize FISH with DNA combing to examine individual, linearly stretched telomeres. However, the current microfluidic technology used in this technique is limiting for such an analysis, as the track lengths of DNA obtained are often shorter than the multiple kilobases that would be required to visualize a single telomere, much less a fragile telomere with additional sequence. Another route to identifying the sequence composition of a fragile telomere would be high-throughput sequencing; unfortunately, the read lengths of current sequencing technologies and short repeat composition of telomere sequence result in the inability to map non-telomere sequence that interrupts a telomere to a reference sequence. While the combing and sequencing approaches to examining fragile telomeres appear less practical due to technical limitations, the combination FISH approach on metaphase chromosomes is feasible, if labor intensive; super-resolution imaging of metaphase chromosomes, on the other hand, could be as easily completed as a typical FISH experiment, with hardware availability being the only limitation.

Addressing the mechanism of formation of a fragile telomere presents its own challenges. While assumptions have been made that fragile telomere formation likely requires the DNA repair machinery, elucidating which DNA repair pathways play a role remains an important goal in understanding telomere fragility. Normally, the best approach to such a question would be a genetic one, knocking down or knocking out
genes involved in various DNA replication and repair pathways to identify which are required for fragile telomere formation. However, because telomere fragility appears to be induced by replication stress, and knockdown of many DNA replication and repair proteins induces replication stress, this approach may be counter-productive. Nonetheless, even though knockdown of repair proteins such as Rad51 (required for homologous recombination) induces telomere fragility (Badie et al., 2010), the telomere fragility produced by the general replication stress induced upon Rad51 knockdown may be outweighed by a reduction in aphidicolin-induced telomere fragility upon Rad51 knockdown if homologous recombination is required for fragile telomere formation – that is to say, a specific role of a particular DNA replication or repair protein in fragile telomere formation may have a greater influence on that protein’s impact on telomere fragility than the replication stress induced upon depletion of that protein. It is difficult to predict the outcomes of such experiments without conducting them. Another approach to this issue would be to use chemical inhibitors of replication and repair proteins, which would allow for titration of the inhibitor dose to achieve an impact on fragile telomere formation without necessarily inducing broad replication stress. Such experiments may need to be applied to different means of inducing telomere fragility, as the literature and our work suggest that multiple mechanisms for fragile telomere formation exist. Notably, telomere fragility induced by RTEL1 is antagonized by treatment with a G-quadruplex stabilizing compound; given that G-quadruplexes only form on the G-rich lagging strand telomere, RTEL1 loss may induce lagging strand-specific telomere fragility, though this question was not explored (Vannier et al., 2012). In contrast, we show that FEN1 depletion induces leading strand-specific telomere fragility in an RNA:DNA hybrid-dependent manner. Lastly, TRF1 deletion-induced fragility exhibits no strand specificity
(Sfeir et al., 2009). The different strand specificities of telomere fragility in different conditions, in addition to the broad range of manipulations that induce telomere fragility, suggest that multiple mechanisms exist for the formation of fragile telomeres, and any work attempting to identify replication or repair pathways involved in the phenotype should include multiple means of inducing telomere fragility.

Lastly, the consequences of fragile telomere formation are poorly elucidated. While the manipulations that induce telomere fragility have known phenotypes, it is not clear if specific cellular functions are altered by higher rates of telomere fragility, and if there are any organismal consequences to the induction of telomere fragility. One of the key aspects of telomere fragility that has largely remained unexplored is the kinetics of the phenotype. Work in our lab has shown that telomere fragility induced by low doses of aphidicolin in BJT fibroblasts declines to control levels 48 hours following removal of the drug, and that this decline cannot be explained by the death of the cells in the population exhibiting fragility (Nguyen et al., unpublished). These data indicate that cells possess a mechanism to resolve telomere fragility, requiring at most two cell cycles to return fragility to background levels. These experiments did not address if different doses of aphidicolin or other means of inducing telomere fragility produce irreparable telomere fragility, or if the time required to repair telomere fragility is proportional to the initial increase in telomere fragility. However, the mere fact that fragile telomeres are resolved raises the possibility that persistent fragile telomeres are a pathological structure. Future experiments examining the physiological consequences of telomere fragility may need to first identify a method of inducing telomere fragility that does not cause a genome-wide, persistent DNA damage response, as higher doses and
extended treatment times with aphidicolin might. Given that in certain conditions, telomere DNA damage is irreparable yet damage in other parts of the genome is repaired, conditions likely exist in which telomere fragility might persist without genome-wide replication stress (Fumagalli et al., 2012). If such conditions can be identified, then examining cells for phenotypes present during persistent telomere fragility may reveal the reasons why cells repair fragile telomeres when they are able.

**RNA:DNA hybrids and the telomere**

Our model for FEN1 activity at the leading strand-replicated telomere proposes that upon FEN1 depletion, RNA:DNA hybrid/flap structures accumulate, and that they are ultimately responsible for telomere fragility. This model is especially interesting in light of recent work in ALT cells demonstrating that RNase H1 regulates RNA:DNA hybrids. In the absence of RNase H1, RNA:DNA hybrids accumulate, RPA is recruited to telomeres, and telomeres are rapidly lost; in contrast, upon RNase H1 overexpression in ALT cells, telomeres shorten and become less recombinogenic (Arora et al., 2014). These data indicate that the presence of RNA:DNA hybrids at telomeres has dramatic effects on telomere stability, which would necessitate RNA:DNA hybrid regulation at the telomere. Our data suggest that FEN1 may also be an important regulator of RNA:DNA hybrids at the telomere. While the ability of ectopic RNase H1 expression to rescue telomere fragility induced upon FEN1 depletion and α-amanitin treatment supports our model that FEN1 processes post-collision RNA:DNA hybrid/flaps, it does not provide concrete evidence that hybrids actually accumulate in the absence of FEN1.
In Appendix 2, we sought to obtain physical evidence for RNA:DNA hybrid accumulation using DNA immunoprecipitation (DIP) with an antibody specific for RNA:DNA hybrids (monoclonal antibody S9.6). We ultimately found that in contrast to our expectation, RNA:DNA hybrids decreased at a portion of the β-actin locus known to form RNA:DNA hybrids and at the telomere upon shRNA-mediated FEN1 depletion. One explanation for this observation comes from the finding described in Appendix 1: because FEN1 depletion induces rapid loss of RNase H1 at the protein level, it is possible that the long time-course of the shRNA-mediated FEN1 depletion in the DIP experiment leaves sufficient time and applies selective pressure for the cells to upregulate other proteins capable of processing RNA:DNA hybrids. Another potential explanation for the decrease in RNA:DNA hybrids observed upon FEN1 depletion may be rooted in the sequence length requirements of the S9.6 antibody, which likely requires at least 20–25 nucleotides of hybrid sequence to efficiently bind an RNA:DNA hybrid (Hu et al., 2006). This latter issue in particular means that for detection of short RNA:DNA hybrids, which would be expected to be shorter than 20 nucleotides given the proposed model of a co-directional collision event, the S9.6 antibody may be inadequate. Recently, a single chain variable fragment (scFv) of the S9.6 antibody was produced that is capable of binding an RNA:DNA hybrid as short as 6 nucleotides (Phillips et al., 2013). It is possible that the S9.6 scFv could be used in a DIP experiment, either by capturing using protein L (which binds kappa light chains) or by producing a tagged S9.6 scFv for capture with FLAG or streptavidin beads. Another potential means to detect RNA:DNA hybrids would be use of a fusion protein of the DNA–RNA hybrid-binding domain of RNase H1 and GFP (HB-GFP), which has recently been used to identify RNA:DNA hybrids in vivo (Bhatia et al., 2014). Regardless of how they are identified
experimentally, RNA:DNA hybrids contribute substantially to telomere biology. In light of our work demonstrating that RNA:DNA hybrids contribute to telomere fragility (Chapter 3), and work in both yeast and human ALT cells finding that telomeric RNA:DNA hybrids alter recombination at the telomere, RNA:DNA hybrids constitute an important new avenue of research in telomere biology.

**The telomere as a canary for genome stability**

One of the most intriguing questions surrounding DNA replication and repair proteins acting at the telomere is whether the proteins’ roles at the telomere are relevant at other non-telomeric loci in the genome. Our work suggests that FEN1’s role at the lagging strand telomere – preventing STL – is to recruit RecQ helicases that resolve G-quadruplexes forming on the lagging strand during replication. There is little reason to believe that FEN1 could not perform this role elsewhere in the genome, though G-quadruplexes are more likely to form at the telomere than most other portions of the genome. Along similar lines, we find that FEN1 prevents leading strand-specific telomere fragility, and that the fragility formed upon FEN1 depletion is transcription- and RNA:DNA hybrid-dependent, suggesting that co-directional replisome–RNAP collision events lead to telomere fragility in the absence of FEN1. We suspect that FEN1 resolves the structures produced following these collisions regardless of the genomic locus at which they occur. If fragile telomeres form by a mechanism using recombination with subtelomeric DNA, it could simply be that telomeres serve as a “reporter” for collision events by producing a readily visible phenotype on metaphase chromosomes, while other loci do not produce substantial rearrangements. As such, the telomere may continue to prove an attractive target when exploring the role of various
proteins in maintaining genome stability, thanks to its increased sensitivity to replication stress and readily quantifiable phenotypes (notably, telomere fragility) under such conditions.
References


Appendix 1:

FEN1 loss triggers downregulation of RNase H1

Daniel C. Teasley, Mai Nguyen, and Sheila A. Stewart
**Introduction**

As described in Chapter 3, we demonstrated that ectopic expression of ribonuclease H1 was sufficient to rescue telomere fragility induced by FEN1 depletion or α-amanitin treatment. In the course of these experiments, we first attempted to ectopically express human RNase H1 using a Moloney murine leukemia virus (MMLV) vector. Strikingly, while RNase H1 was successfully overexpressed approximately four-fold over endogenous levels and persisted in cells through one freeze-thaw cycle, expression of the ectopic and endogenous RNase H1 was abrogated by subsequent flap endonuclease 1 (FEN1) depletion. Given the necessity of successful RNase H1 expression to demonstrate the involvement of RNA:DNA hybrids in the telomere fragility phenotype, we proceeded to clone human RNase H1 into an adenoviral vector and produced recombinant adenovirus. Even in the setting of the large overexpression produced by adenoviral transduction, we found again that RNase H1 expression appeared to be FEN1-dependent. We repeated these results and carried out additional experiments to identify if our observation of RNase H1 expression dependence on FEN1 was a technical issue or reproducible phenomenon, as described herein.

**Experimental Procedures**

**Cell culture and virus production**

All cell culture was carried out as described in Chapter 3. Moloney murine leukemia virus (MMLV) was produced as described for lentivirus in Chapter 3 using the pUMVC3 packaging vector and pCMV-VSV-G envelope vector (8:1). Lentivirus was produced as described in Chapter 3. Production of recombinant adenovirus type 5 was carried out using the AdEasy adenoviral vector system (Agilent Technologies, La Jolla, CA).
according to the manufacturer’s protocol, and virus was amplified as described in Chapter 3.

**Plasmids**

The human RNase H1 cDNA (cloned into pCMV6-AC) was purchased from Origene Technologies (Rockville, MD). For MMLV, the RNase H1 cDNA was cloned into pBABE-hygro by excising the cDNA from pCMV6-AC using *Eco*RI and *Xho*I (New England Biolabs, Ipswich, MA) and ligating into the *Eco*RI and *Sal*I sites in pBABE-hygro using the Rapid Ligation Kit according to the manufacturer’s instructions (Roche Applied Science, Penzberg, Germany). For adenoviral production, the RNase H1 cDNA was PCR amplified using Phusion polymerase according to the manufacturer’s instructions (New England Biolabs, Ipswich, MA) and gel purified using a QIAquick gel extraction kit (Qiagen, Venlo, Netherlands). 3′-A overhangs were added using *Taq* polymerase and the fragment was cloned into pCR2.1-TOPO (Life Technologies, Grand Island, NY). Finally, the RNase H1 cDNA was subcloned from pCR2.1-TOPO by digestion with *Hind*III and *Xho*I (New England Biolabs, Ipswich, MA) and ligation into the corresponding sites in pShuttle-CMV (Agilent Technologies, La Jolla, CA) using the Rapid Ligation Kit according to the manufacturer’s instructions (Roche Applied Science, Penzberg, Germany).

**Western blot analysis**

Western blot analysis was carried out as described (Honaker and Piwnica-Worms, 2010). The following antibodies were used: rabbit polyclonal anti-FEN1 (A300-255A, Bethyl Laboratories, Montgomery, TX); mouse monoclonal anti-RNase H1 (H00246243-
M01, Novus Biologicals, Littleton, CO); rat monoclonal anti-α-tubulin (ab6160, Abcam, Cambridge, UK).

RNA preparation and quantitative reverse transcription PCR (qRT-PCR)
RNA was prepared using TRI Reagent (Life Technologies, Grand Island, NY). cDNA was synthesized using Superscript III reverse transcriptase (Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. qRT-PCR was conducted using TaqMan Gene Expression Assays (Life Technologies, Grand Island, NY) according to the manufacturer protocol. Assays used were RNASEH1 (Hs00268000_m1) and GAPDH (Hs02758991_g1). Fold changes were computed using the ΔΔCt method.

Results

Endogenous and ectopic RNaseH1 expression is reduced upon FEN1 depletion
We first expressed RNase H1 in BJT fibroblasts by transducing them with an MMLV carrying the RNase H1 cDNA. Following selection, we observed a modest but significant overexpression of RNase H1 compared to empty vector control cells by western blot using a monoclonal RNase H1 antibody that detects both endogenous and ectopic RNase H1 (Fig. A1.1A). Ectopic expression was maintained under drug selection through a single cell freeze-thaw cycle (data not shown). Next, we depleted cells of FEN1 using a lentivirus expressing an shRNA targeting the 3′-UTR of FEN1 (shFEN1) or a control hairpin (shCtrl) (Saharia et al., 2008). Following selection, we observed a marked reduction in RNase H1 expression both in control (empty vector-expressing) and RNase H1-overexpressing BJT fibroblasts (Fig. A1.1A), indicating that
both endogenous and ectopically expressed RNase H1 were reduced following FEN1 depletion.

Given our need to overexpress RNase H1 even in the absence of FEN1, we next produced a recombinant adenovirus encoding the human RNase H1 cDNA (Ad-RH1). We first depleted FEN1 from RPE1 cells using a lentivirus expressing shFEN1. During drug selection for shFEN1 expression, we infected the cells with Ad-RH1 to overexpress RNase H1 and collected the cells immediately following the completion of selection and transduction. As in BJT fibroblasts, FEN1-depleted RPE1 cells showed a marked decrease in endogenous RNase H1 levels compared to control cells (Fig. A1.1B). RPE1 cells transduced with Ad-RH1 displayed strong overexpression of RNase H1, but strikingly, even this level of ectopic expression was reduced in FEN1-depleted cells (Fig. A1.1B). These data indicate that even when expressed at extremely high levels, RNase H1 protein levels are decreased by FEN1 depletion.

**FEN1-dependent RNase H1 expression is controlled post-transcriptionally**

Given the rapid reduction of both endogenous and ectopic RNase H1 expression following FEN1 depletion, we wondered if the loss of RNase H1 was controlled at the transcriptional or post-transcriptional level. First, we used the BLAST algorithm to identify if the shFEN1 targeting sequence was present in RNase H1 mRNA, and found that no sequence homology existed between the two (data not shown). Next, we isolated RNA from control and FEN1-depleted RPE1 cells and produced cDNA for qRT-PCR analysis. Surprisingly, FEN1 depletion induced no change in the expression levels of the RNase H1 mRNA (Fig. A1.1C). The lack of a change in RNase H1 mRNA at a
time when the protein level observed by western blot analysis was so markedly reduced indicates that the RNase H1 reduction occurring following FEN1 depletion occurs post-transcriptionally.

**Discussion**

Our studies of RNase H1 protein and mRNA expression following FEN1 depletion do not strongly support one mechanism explaining how RNase H1 is regulated upon a loss of FEN1. Nonetheless, the rapidity of the RNase H1 loss (we assayed as soon as selection for shFEN1-expressing cells was complete) better supports the possibility that the RNase H1 protein is destabilized in the absence of FEN1 than it supports the possibility that RNase H1 translation is downregulated. Future work to address the nature of this phenomenon might first include treatments with the translation inhibitor cycloheximide and proteasome inhibitor MG132 to identify if RNase H1 loss following FEN1 loss is mediated by either translation or proteasomal degradation. Additionally, protein levels could be assessed sooner after FEN1 depletion by the use of a transfected siRNA instead of a lentivirally-delivered shRNA. Lastly, it is unclear if the cell cycle plays a role in the regulation of RNase H1. Though FEN1 depletion only produces mild cell cycle aberrations and does not impact S-phase progression (Chapter 2), we did not assess cell cycle distribution in these experiments, and such an analysis might yield additional information on RNase H1 regulation.

It is also interesting to surmise the functional implications of the possibility that FEN1 and RNase H1 are post-transcriptionally co-regulated. Do FEN1 and RNase H1 physically interact, with such an interaction stabilizing RNase H1? Does the loss of
RNase H1 provide evidence that FEN1 loss results in the production of substrates upon which RNase H1 activity is ultimately toxic for the cell? Answers to these and other questions may lead to a better understanding of the relationship between FEN1 and RNase H1, which has long been restricted to Okazaki fragment processing.
Figure A1.1. RNase H1 protein expression decreases upon FEN1 depletion. (A) Western analysis of FEN1 and RNase H1 expression in BJT fibroblasts transduced with empty vector (EV) or RNase H1 (pB-RH1), and expressing a control shRNA (shCtrl) or FEN1-depleting shRNA (shFEN1). α-tubulin is shown as a loading control. (B) Western analysis of FEN1 and RNase H1 expression in control (shCtrl) or FEN1-depleted (shFEN1) RPE1 cells, with or without ectopically expressed RNase H1 (Ad-RH1). Two exposures of the same RNase H1 blot are shown. α-tubulin is shown as a loading control. (C) qPCR analysis of RNase H1 mRNA expression in cells expressing a control hairpin (shCtrl) or FEN1-depleted cells (shFEN1). mRNA levels in FEN1-depleted cells...
are shown as a fold change relative to the control cells. Fold changes were calculated using the ΔΔCt method; fold changes from two biological replicates were averaged to produce the graph. The error bar represents standard error of the mean.
References


Appendix 2:

FEN1 loss decreases long RNA:DNA hybrids

at the telomere and β-actin locus

Daniel C. Teasley, Mai Nguyen, and Sheila A. Stewart
Introduction

As discussed in Chapter 3, we propose that co-directional collisions between the replisome and transcribing RNA polymerases produce RNA:DNA hybrid/flap structures, which FEN1 processes to prevent telomere fragility. In our attempts to build support for this model, we wondered if we could show physical evidence that RNA:DNA hybrids accumulate upon FEN1 depletion, as would be predicted if FEN1 were required for their resolution. We sought to employ DNA immunoprecipitation (DIP), a technique similar to chromatin immunoprecipitation, differing in the absence of a crosslinking step and purification of protein-free genomic DNA rather than chromatin (Skourti-Stathaki et al., 2011). Using an RNA:DNA hybrid-specific antibody (monoclonal antibody S9.6), this technique can be used to unambiguously identify the presence of RNA:DNA hybrids longer than approximately 25 nucleotides in length (Hu et al., 2006). Here, we sought to identify whether RNA:DNA hybrid levels change following FEN1 depletion at two loci: the telomere and the β-actin locus.

Experimental Procedures

Cell culture and virus production

Cell culture of BJT fibroblasts was carried out as described in Chapter 3. Lentivirus expressing control (shCtrl) and FEN1-depleting shRNA (shFEN1) (Saharia et al., 2008) was produced as described in Chapter 3.

Western blot analysis

Western blot analysis was carried out as described (Honaker and Piwnica-Worms, 2010). The following antibodies were used: rabbit polyclonal anti-FEN1 (A300-255A,
Bethyl Laboratories, Montgomery, TX); rat monoclonal anti-α-tubulin (ab6160, Abcam, Cambridge, UK).

**DNA immunoprecipitation (DIP)**

DIP was carried out as described with modifications (Skourti-Stathaki et al., 2011). Subconfluent BJT fibroblasts were collected from two 15 cm dishes by scraping. Cells were lysed in DIP lysis buffer (0.5% NP40, 85 mM potassium chloride, 5 mM PIPES), after which nuclei were pelleted. Nuclei were lysed in DIP nuclear lysis buffer (1% sodium dodecyl sulfate, 25 mM tris-HCl pH 8, 5 mM EDTA), sheared, and treated with two sequential, 1.5 hour, 55 °C, 100 µg proteinase K digests. Following phenol:chloroform extraction and ethanol precipitation (Sambrook et al., 1989), samples were split and digested overnight with recombinant ribonuclease H (Roche Applied Science, Penzberg, Germany) as a negative control or mock digested (no enzyme). Samples were diluted in DIP dilution buffer (0.01% sodium dodecyl sulfate, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM tris-HCl pH 8, 166.5 mM sodium chloride) and sonicated to produce fragments approximately 200 bp in length. Nucleic acid content was quantified using the PicoGreen assay according to the manufacturer's instructions (Life Technologies, Grand Island, NY).

10 µg of lysate (measured by nucleic acid content) was immunoprecipitated overnight with 10 µg of S9.6 antibody or normal mouse IgG. Protein A magnetic beads (Life Technologies, Grand Island, NY) were equilibrated in DIP dilution buffer and used to capture antibody–DNA complexes. After extensive washing, antibody–DNA complexes
were eluted from the beads and treated with proteinase K; DNA was recovered using PCR cleanup columns (Qiagen, Venlo, Netherlands).

**Quantitative PCR (qPCR)**

Genomic qPCR was conducted using Power SYBR Green Master Mix (Life Technologies, Grand Island, NY) according to the manufacturer’s protocol. A fragment immediately 5’ to the β-actin pause element (known to form RNA:DNA hybrids; “5’-pause”) was amplified to assess hybrid formation at the β-actin locus; a non-transcribed region (“C”) downstream of β-actin was used as a non-transcribed negative control locus. Thermocycling was performed per the master mix manufacturer’s instructions, using 58.7 °C as the annealing temperature. Primers used were: 5’-pause F: 5’-TTA CCC AGA GTG CAG GTG TG-3’; 5’-pause R: 5’-CCC CAA TAA GCA GGA ACA GA-3’; C F: 5’-TGG GCC ACT TAA TCA TTC AAC-3’; C R: 5’-CCT CAC TTC CAG ACT GAC AGC-3’.

**Southern hybridization**

Southern hybridization was carried out as previously described (Sambrook et al., 1989) with modifications. Briefly, DNA was serially diluted, denatured in 0.4 M sodium hydroxide at 95 °C, and spotted onto a Hybond-XL charged nylon membrane (GE Healthcare, Waukesha, WI) using a Bio-Dot Microfiltration apparatus (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Following UV crosslinking, the membrane was prehybridized in Southern hybridization buffer (6x SSC, 5x Denhardt's solution, 0.2% sodium pyrophosphate, 0.2% SDS) containing 100 μg/mL denatured salmon sperm DNA for four hours at 65 °C. A purified 1.6 kb fragment
consisting exclusively of vertebrate telomere repeats was random prime labeled with \([\alpha-^{32}\text{P}]\text{dCTP}\) (3000 Ci/mmol) using the High Prime DNA Labeling Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer’s instructions to produce a telomere-specific DNA probe; the probe was purified using an Illustra ProbeQuant G-50 Micro Column (GE Healthcare, Waukesha, WI) and diluted to \(1.2 \times 10^6\) dpm/mL in 10 mL of Southern hybridization buffer. Probe was hybridized to the membrane overnight at 65 °C, after which the membrane was washed and imaged using a phosphor screen and imager or by autoradiography.

**Results**

In order to obtain direct evidence that FEN1 depletion could alter RNA:DNA hybrid levels in the genome, we carried out a DNA immunoprecipitation (DIP) on BJT cells transduced with a control shRNA (shCtrl) or depleted of FEN1 using a previously validated shRNA (shFEN1) (Saharia et al., 2008). Following confirmation of FEN1 knockdown by western blot analysis, cells were isolated and processed for DIP. Given that ectopic RNase H1 expression rescues telomere fragility induced by FEN1 depletion (Chapter 3), we surmised that RNA:DNA hybrids might increase at the telomere in the absence of FEN1, and examined S9.6 immunoprecipitated DNA by Southern hybridization with a telomere-specific probe. We also assayed two specific portions of the \(\beta\)-actin locus: one known to form RNA:DNA hybrids in unperturbed conditions (5′-pause region) and one downstream of the transcribed portion of the locus that should be unable to form transcription-associated RNA:DNA hybrids (C-region). As expected, we were unable to detect a significant presence of RNA:DNA hybrids at the C-region of the \(\beta\)-actin locus (data not shown). We observed RNA:DNA hybrids at both the 5′-pause
site of the β-actin locus (Fig. A2.1A) and at the telomere (A2.1B) in control cells; these were verified as specific by treatment of lysates with recombinant RNase H prior to immunoprecipitation, which abrogated precipitation of detectable β-actin 5′-pause site DNA (Fig. A2.1A). Surprisingly, following FEN1 depletion, RNA:DNA hybrids at the 5′-pause site of the β-actin locus decreased dramatically following FEN1 depletion (Fig A2.1A). Similarly, RNA:DNA hybrids decreased substantially at the telomere (Fig. A2.1B). These data demonstrate that at multiple genomic loci, RNA:DNA hybrids are decreased or eliminated following lentiviral knockdown of FEN1.

Discussion

Our model proposed that replication stress and telomere fragility arise following co-directional collisions that produce RNA:DNA hybrid/flap structures; FEN1 resolves these structures to prevent replication stress and telomere fragility (Chapter 3). Our model also suggested that in the absence of FEN1, RNase H1 is sufficient to process the RNA:DNA hybrid/flap structures and prevent telomere fragility. As such, we expected that upon FEN1 depletion, cells would exhibit an increase in RNA:DNA hybrids at the telomere and potentially at other transcribed loci, such as the β-actin locus. Our results found the opposite of this expectation. Nonetheless, the RNase H1 overexpression experiments described in Chapter 3 provide strong evidence that RNA:DNA hybrids are responsible for the telomere fragility induced upon FEN1 depletion and α-amanitin treatment: the only known function of RNase H1 is endoribonucleolytic cleavage of oligomeric RNA in RNA:DNA hybrids.
In attempting to reconcile these seemingly opposite findings, we found multiple possible explanations that could account for the discrepancy from expectations of the DIP data. First, we note that in all of the experiments reported herein, FEN1 depletion was achieved by lentiviral transduction, in which the cells undergo 48 hours of puromycin selection and at least 24 additional hours of culture before collection for DIP analysis; as such, the cells in question are collected between 72 and 96 hours following infection. As described in Appendix 1, FEN1 knockdown by this method substantially decreases RNase H1 levels as well. Given the amount of time the cells survive with diminished levels of FEN1 and RNase H1 in this experimental setting, we propose that over time the cells may achieve a compensatory upregulation of other proteins able to process the accumulating RNA:DNA hybrid/flap structures. Many proteins are known to cleave or unwind RNA:DNA hybrids – key suspects would be RNase H2, which possesses similar activity to RNase H1; senataxin, a helicase known to unwind RNA:DNA hybrids with known roles in telomere stability (De Amicis et al., 2011); and Dhx9, a helicase capable of unwinding RNA:DNA hybrids that has been identified as a telomere-associated protein (Nittis et al., 2010). Activation of these or other proteins to compensate for FEN1 and/or RNase H1 loss might cause a subsequent dramatic loss of RNA:DNA hybrids. This idea is supported by preliminary data indicating that knockdown of FEN1 by siRNA (with collection occurring after only 36-48 hours) shows no change in RNA:DNA hybrids at the β-actin locus; the more rapid collection of cells following siRNA transfection may not leave enough time for the cell to upregulate compensatory proteins enough that RNA:DNA hybrids are eliminated.
Another complication of the DIP data comes from the length specificity of the S9.6 antibody. Recent work has shown that the S9.6 antibody’s binding affinity to RNA:DNA hybrids is length dependent, with hybrids less than 20 nucleotides in length exhibiting no detectable binding, and the signal for a 25-nucleotide hybrid only 10% of that for a 50-nucleotide hybrid (Hu et al., 2006). Crystallographic evidence indicates that if the RNA polymerases involved in co-directional collisions are normally progressing through their templates (i.e., not backtracking), no more than 9 nucleotides of hybrid should be present at the moment of the collision during active transcription (Gnatt et al., 2001); disengagement of the RNA polymerase and subsequent elongation by the replisome would not be expected to alter the length of hybrid at the site of the collision, meaning that the RNA:DNA hybrid/flap structures may possess as few as 9 nucleotides of RNA:DNA hybrid. In such an instance, the majority of the hybrids produced by co-directional collisions would be undetectable by DIP with the S9.6 antibody.

Especially in light of the data showing length dependence of the S9.6 antibody, we are forced to conclude that the DIP assay may be insensitive to the RNA:DNA hybrid/flap structures produced following a co-directional collision at the telomere. As such, the RNA:DNA hybrids observed in shCtrl cells (and eliminated in shFEN1 cells) likely correspond to long (>20 nucleotides) RNA:DNA hybrids, such as those that might be associated with an R-loop or backtracked Pol II elongation complex. It is unclear if long hybrids are more or less prevalent than collision-derived RNA:DNA hybrid/flaps, if they are impacted directly by FEN1, or if they play any role in telomere fragility.
Figure A2.1. RNA:DNA hybrids decrease at the β-actin 5′-pause site and telomere upon FEN1 depletion. (A) Representative qPCR of the β-actin 5′-pause site in DNA immunoprecipitated with the S9.6 antibody from control cells (shCtrl) or cells depleted of FEN1 (shFEN1). Lysates were untreated or treated with recombinant RNase H prior to immunoprecipitation (negative control). Pull down is shown as percent input. (B) Representative Southern analysis to detect telomere DNA in DNA immunoprecipitated with non-specific IgG or S9.6 antibody from control cells (shCtrl) or cells depleted of FEN1 (shFEN1). Serial dilutions of DNA were loaded onto a membrane, and a telomere repeat DNA probe was hybridized to the membrane. The membrane was visualized with autoradiography.
References


