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Flap Endonuclease 1 ensures telomere replication and stability

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ABSTRACT OF THE DISSERTATION

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Telomeres, protein-DNA structures that distinguish the end of a chromosome from a *bona fide* DNA double strand break, are integral to genomic stability. High fidelity replication of telomeres is indispensable for their stability. Telomere replication is a challenging task that is completed through the coordinated actions of telomere binding proteins and DNA replication and repair proteins in ways that are not well understood. This work focuses on delineating the function of one DNA replication and repair protein, Flap Endonulcease 1 (FEN1), in telomere replication and maintenance. I demonstrate that FEN1 is essential for the efficient replication of telomeres through its ability to re-initiate stalled replication forks. FEN1 depletion leads to telomere dysfunction characterized by the recognition of the telomeres as DNA double strand breaks and the specific loss of telomeres replicated by the lagging strand machinery. Expression of catalytically active telomerase, the reverse transcriptase that adds telomeric repeats to chromosome ends, was sufficient to rescue telomere dysfunction upon FEN1

depletion. Genetic rescue experiments revealed that the nuclease activity of FEN1, its interaction with the RecQ helicases and its ability to process DNA bubble structures are essential to prevent telomere loss, whereas its ability to process Okazaki fragments is dispensable. However, FEN1 depletion did not affect cell cycle progression or *in vitro* DNA replication through non-telomeric substrates and in the absence of telomere dysfunction, FEN1 depletion did not affect overall genomic stability. Further analysis revealed that FEN1 is important for the efficient re-initiation of stalled replication forks and that this function ensures telomere stability. As with telomere loss, FEN1's ability to process bubble DNA structures and its ability to interact with the RecQ helicases are vital for the re-initiation of stalled replication forks. Finally, FEN1 depletion in transformed telomerase-negative ALT-positive cells leads to telomere end-to-end fusions. I propose that FEN1 maintains stable telomeres through the efficient re-initiation of stalled replication forks that occur in the G-rich lagging strand telomere, ensuring high fidelity telomere replication. This model suggests that mutations that arise in FEN1 are detrimental to telomere stability, leading to genomic instability and driving the transformation process.

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CHAPTER 1:

BACKGROUND AND SIGNIFICANCE

1.1 Significance and Overview

Normal human cells possess a limited replicative lifespan. These cells enter a state of permanent cell cycle arrest termed senescence [1]. Cells possess an internal mitotic clock, identified as the telomere, capable of limiting their potential for cell division [1-3]. Work since then has demonstrated that telomeres can function as tumor suppressors in the face of intact checkpoints, promote tumorigenesis through the induction of genomic instability and are essential for cellular immortality, a defining characteristic of tumor cells [4]. The realization that the telomere plays a pivotal role in the transformation process led researchers to focus on the biochemical mechanisms that govern its stability. What has emerged from this work is an understanding that telomere maintenance is the result of the coordinated actions of telomere binding proteins and the DNA replication and repair machinery. Delineating how these mechanisms interact and how the DNA repair machinery differentiates between the telomere and a *bona fide* DNA double strand break is a current challenge.

1.2 Telomeres and Cancer: The Telomere Hypothesis

In 1961, Leonard Hayflick observed that fibroblasts isolated from an individual underwent a limited number of cell divisions (later referred to as the 'The Hayflick limit'), at which point they underwent a checkpoint-dependent permanent growth arrest termed senescence [5, 6]. These observations led to the hypothesis that the cell contained an internal clocking mechanism that counted the number of cellular divisions. The realization that the telomere was the long sought after internal clocking mechanism came

much later in 1990s. In the 1970s, James Watson and Alexey Olovnikov had proposed the "end replication problem", suggesting that due to the inherent nature of lagging strand DNA replication, the termini of linear chromosomes would not be completely replicated leading to the loss of genetic material with every cell division [7, 8]. Harley and Greider eventually demonstrated the connection between the end replication problem and the Hayflick limit, postulating that the internal clocking mechanism was the telomere [2]. They demonstrated that the mean telomere length of normal human cells shortened progressively with each cell division and that telomere length predicted the replicative capacity of cells [2, 3]. Importantly, when cells were isolated from the same individual on multiple occasions, they entered senescence with similar kinetics and with approximately the same telomere length, suggesting that telomere length was responsible for triggering senescence [3]. These observations gave rise to the "telomere hypothesis", which suggested that telomeres represent the internal clocking mechanisms originally described by Hayflick [2, 9]. Indeed, it is now appreciated that senescence can be triggered by loss of telomere structural integrity and that telomere length is one component that contributes to this integrity [9-11]. Additionally, senescence can also be induced through other telomere-independent mechanisms such as environmental and oncogenic stress and DNA damage [12].

The telomere hypothesis, describing the mitotic clock, postulates that the loss of telomeric DNA due to incomplete end replication leads to cellular senescence, the first proliferative barrier (**Figure 1.1**). Telomere-induced senescence is a critical tumor

suppressor mechanism *in vivo*, forming a barrier to cellular immortalization [13-19]. Inactivation of both the p53 and Rb tumor suppressor pathways allows the bypass of senescence in human cells, leading to continued cell division and further telomere shortening [9, 20]. Cells that bypass senescence eventually reach a second proliferative barrier referred to as crisis (**Figure 1.1**). At crisis, telomere lengths are critically short leading to structural destabilization, chromosomal end-to-end fusions, and subsequent cell death [9]. Cells that continue to divide despite the loss of telomere integrity undergo chromosome breakage-fusion-bridge cycles, develop chromosomal aberrations and genomic instability, a driving force in the evolution of a human tumor cell [21, 22].

Approximately 1 in 10^7 cells entering crisis escape death. Those that do escape crisis activate a telomere maintenance mechanism and thus acquire immortality (**Figure 1.1**), a defining characteristic of human tumors [23]. The majority of cells activate telomerase (hTERT) – a reverse transcriptase that utilizes an RNA template to add *de novo* telomeric sequences to the ends of telomeres [24-26]– which lengthens the telomeres and allows the formation of a stable telomere structure [27, 28]. The ability of telomerase to immortalize cells and its necessity in the transformation process suggested that inhibition of telomerase would diminish the proliferative capacity of cancer cells. Indeed, telomerase inhibition in telomerase-positive immortal tumor cells led to progressive telomere loss and the induction of apoptosis [29] (**Figure 1.1**). It also led to loss of tumorigenic potential in xenograft models indicating the necessity of cellular immortality for tumorigenesis [29].

Alternatively, cells can utilize a non-telomerase based method for stabilizing and lengthening their telomeres, known as 'ALT' (Alternative Lengthening of Telomeres) [30, 31]. Unlike telomerase which utilizes an RNA component to add telomeric repeats to chromosomes ends, the ALT mechanism is postulated to utilize homologous recombination (HR) to lengthen telomeres [31-34], though the molecular details of this mechanism still require elucidation. Together, these studies underscore the importance of telomeres at different stages in the transformation process and signify the importance of understanding mechanisms that govern its stability.

1.3 Telomere structure and function

Since Barbara McClintock and Herman Muller first identified them in the 1930s, telomeres have been recognized as capping structures that play an essential role in distinguishing natural chromosome ends from *bona fide* DNA DNA double strand breaks (DSBs) [35, 36]. When telomeres become critically short their protective structure is compromised, triggering a DNA damage response (DDR) [37]. These unprotected telomere ends are recognized as DSBs, established by the presence of several DNA DSB response factors such as phosphorylated-ATM (ataxia-telangiectasia mutated), γ-H2AX, MDC1, NBS1 and 53BP1 [37]. Such DNA damage foci at the telomeres were named telomere dysfunction-induced foci (TIFs) [38]. Recent work has clearly demonstrated that the ability to maintain telomere structure is dependent on the length of the telomere, presence of the ssOH and a plethora of proteins that bind and/or modify the telomere [39,

40]. While it was originally believed that the telomere must exclude DDR proteins to maintain its stability, it is now clear that such machinery plays an integral role in telomere maintenance [41]. The current challenge is to define the mechanisms that govern the actions of the DDR machinery that leads to the formation of a stable capped telomere versus those that act upon uncapped (i.e. dysfunctional) telomeres.

The telomere is a dynamic nucleoprotein structure (**Figure 1.2**). It forms a complex displacement loop (D-loop) structure, referred to as the "T-loop", where the single strand overhang loops back around and inserts itself into the double strand region of the telomere (**Figure 1.2**). The T-loop structure was visualized both *in vitro* and *in vivo* through electron microscopy [42, 43]. It is suggested that this 'capped' telomere hides the end of the chromosome and prevents its recognition by the DNA damage pathway. The stability of the T-loop is dependent on the presence of the ssOH as well as TRF2, an essential telomere binding protein [40, 44]. It is therefore interesting to note that the length of the ssOH is significantly shorter in senescent cells compared to younger cells [45], suggesting that the telomere structure is disrupted in senescent cells.

In addition to TRF2, other proteins are important for telomere maintenance. Many of these proteins have been characterized, including a core complex of six proteins referred to as Shelterin [40, 46], consisting of TRF1, TRF2, TIN2, TPP1, POT1 and RAP1 (**Figure 1.2**). TRF1 and TRF2 coat the length of the telomere as homodimers with high specificity to telomere double-strand DNA [47-51]. TIN2 binds to both TRF1

and TRF2 forming a protein 'bridge' between these two proteins [52-54]. POT1 binds to single strand telomeric DNA via an OB (oligonucleotide binding)-fold domain, while TPP1 has been shown to recruit POT1 to the telomere [55, 56]. TPP1 also links POT1 to TIN2, and therefore forms a bridge to the TRF1 and TRF2 proteins. RAP1 is recruited to the telomere by TRF2 [57, 58]. All the above-mentioned proteins are required for the maintenance of a functional telomere. Disturbing the equilibrium of these proteins by either overexpression or depletion results in telomere dysfunction [40]. For example, depletion of TRF2 or introduction of a TRF2 dominant-negative allele results in telomere destabilization or uncapping, recognition as a DNA DSB, and formation of TIFs, characterized by the presence of 53BP1 and γH2AX at the telomere [37, 38, 59]. The cell attempts to repair these lesions via non-homologous end joining (NHEJ) or single-strand annealing, leading to chromosome end-to-end fusions with telomeric DNA at the junctions [60, 61].

The Shelterin components perform several essential tasks that maintain stable telomeres [40]. In addition to maintaining telomere structure, TRF2 is an inhibitor of ATM and Chk2 at the telomere [62, 63], suggesting that the ATM pathway is spatially controlled at the telomere. The other Shelterin components also influence telomere dynamics when their levels are modulated. For example, overexpression of TRF1 results in telomere shortening both in telomerase-positive human and mouse cells, suggesting that TRF1 controls telomerase access to chromosome ends [49]. Recent work has also demonstrated that TRF1 is essential for telomere replication and that its absence leads to

the formation of stalled replication forks at telomeres [64]. POT1 functions to maintain telomere length by modulating the action of telomerase and protects their stability by inhibiting the action of the ataxia-telangiectasia and Rad3-related (ATR) pathway [62, 65, 66]. TIN2 and TPP1 do not bind telomeric DNA directly, but influence telomere dynamics by forming a protein bridge between the double strand DNA binding proteins TRF1 and TRF2 and the single strand DNA binding protein POT1 [54, 67]. The loss of either protein leads to the activation of DDR at telomeres and the formation of TIFs [40]. Recently, a novel form of TIN2 has also been identified at the nuclear matrix, suggesting a unique role for it in anchoring telomeres [68]. TPP1 interacts directly with POT1 and is involved in the recruitment of telomerase to the telomeres [67]. Finally, RAP1 interacts directly with TRF2 and this complex inhibits nonhomologous end-joining repair proteins from recognizing the telomere as a DSB [69].

In addition to the core Shelterin complex, a number of DDR proteins have been associated with the telomere (**Figure 1.2**). The functional importance of the DDR proteins at the telomere was first observed in *Saccharomyces cereviseae (S. cereviseae)*, where cells with mutations in TEL1, an Ataxia Telangiectasia Mutated (ATM) homolog, resulted in shortened telomeres [70, 71]. This was further confirmed by studies in *Schizosaccharomyces pombe (S. pombe)*, mice and human cells [72]. Similarly, several proteins involved in DNA replication, DNA damage signaling and DNA repair play critical roles in telomere maintenance. These include DNA damage signaling phosphoinositide 3-kinase related kinases (PIKKs) such as ATM, ATR and DNA-PK [73,

74]; homologous recombination machinery such as RAD54, RAD51D, the MRN complex (comprising the Mre11, Rad50 and NBS1 proteins) and BRCA1 [75-77]; proteins involved in non-homologous end joining such as KU70/KU86 [73, 78]. Several base excision repair proteins also play an important role in telomere biology such as the XPF/ERCC1 complex, PARP1 and PARP2 proteins [79-84]. A recent study also identified Apollo, a nuclease that plays a specific role in telomere biology [85, 86]. Furthermore, the Werner (WRN) protein, a RecQ helicase that functions in recombination and repair pathways, is essential to ensure high fidelity replication of the lagging strand at the telomere [87, 88]. Finally, the Origin recognition complex (Orc2) localizes to the telomere where it prevents telomere loss and the formation of telomeric circles – a hallmark of telomere replication stress [89]. All the above examples illustrate the intricate relationship between the DNA replication and repair factors and telomere maintenance; however, a comprehensive understanding of this interplay has yet to be elucidated. The work discussed in this thesis takes our understanding further by characterizing the function of Flap Endonuclease 1 (FEN1), a DDR factor, at human telomeres.

1.4 Telomere replication

Continued cellular proliferation requires high fidelity duplication of the genome and proper maintenance of the telomeres. Loss of genomic integrity contributes to the transformation process, therefore several compensatory mechanisms have evolved to ensure high fidelity replication of the genome. Highly repetitive sequences, such as the

telomere, present unique challenges to the DNA replication machinery making them exquisitely sensitive to the loss of repair and replication mechanisms [90, 91]. As detailed above, several DNA replication and repair proteins bind telomeres, interact with Shelterin proteins and are critical for telomere function [40]. Depletion of these proteins or abrogation of their telomere binding capability leads to telomere dysfunction, indicating that these proteins play a specific role in maintaining telomere stability. Based on these observations, I hypothesize that replication of this specialized structure – the telomere – requires a coordinated effort by the telomere binding proteins and the DNA replication and repair proteins. Several questions arise from this hypothesis – Which are the telomere binding proteins involved? Which are the DNA replication and repair proteins involved? Are the telomere-binding proteins actively involved in telomere replication and repair or do they simply provide a scaffold? Do the telomere binding proteins modulate the activity of the DNA replication and repair machinery? How is this process regulated and coordinated? Several lines of evidence have recently emerged supporting the stated hypothesis [64, 89-92]. Indeed, Shelterin components, TRF1 and TRF2 interact with and modulate the activities of several DNA repair and replication proteins [40]. These findings have led to a new model of telomere function: once thought to exclude the DNA repair machinery it is now appreciated that telomere replication and stability is the result of an intricate interplay between the telomere binding proteins and the DNA replication and repair mechanisms (**Figure 1.3**).

1.5 Challenges to telomere replication

Replication of telomeres poses several challenges to the moving fork (**Figure 1.4**). Telomeric repetitive G-rich sequences have a high propensity to form G-quadruplexes (G4) that impede the progressing replication fork, leading to the formation of stalled forks [90, 93, 94]. Indeed, several reports have indicated pausing and/or stalling of replication forks moving through telomeres [95-97]. The presence of the T-loop also presents a topological barrier for the replication fork. Additionally, telomere replication is primarily initiated by the most distal origin of replication from the centromere and continues unidirectionally towards the end of the telomere (**Figure 1.4**) [64]. If this fork stalls and is not re-initiated efficiently, it will collapse leading to the formation of a DSB [98] resulting in telomere deletion. Therefore it is not surprising that telomeres have been recently identified as fragile sites that are highly sensitive to replication stress [64]. As a result, successful telomere replication requires the coordinated action of telomere binding proteins and their recruitment and/or modification of traditional DNA replication and repair factors [90, 91].

Recent work indicates that stalled forks are formed within replicating human telomeres [97]. During S-phase the ATR-dependent DDR machinery (ATR and RPA) is recruited together with the DNA replication machinery (Pol α, PCNA, FEN1) [97] to the telomere. The ATR-dependent repair machinery is postulated to be present at the telomere in response to stalled replication forks, resolving them and leading to efficient telomere replication. In addition, the T-loop has to be resolved to allow the passage of

the replication fork and reformed post duplication, to form a stable telomere structure. In late S/early G2 phase of the cell cycle, phosphorylated ATM and the HR machinery are recruited to the telomere [97, 99], suggesting that these proteins are involved in the re-formation of the T-loop. Together, these observations indicate the presence of stalled replication forks at human telomeres and reveal a critical role for the DNA replication and repair machinery to efficiently duplicate telomeres.

Robust mechanisms evolved to ensure high fidelity replication and repair of the telomere appear to be coordinated by telomere binding proteins [90, 91]. Work done in *S. pombe* demonstrated that Taz1 (the TRF1 and TRF2 homolog) is required for efficient replication fork progression through the telomere [92]. Taz1 loss causes progressing replication forks to stall upon encountering telomere sequences, resulting in a lack of telomere replication and an abrupt, complete loss of telomeres. Importantly, Taz1 requirement for telomere replication occurs due to the sequence of the telomere itself, independent of its location in the genome [92]. This was the first evidence suggesting that telomere replication is dependent on telomere-binding proteins.

Similar work in mammalian cells demonstrated that TRF1 is critical for telomere replication [64]. Deletion of TRF1 resulted in an increase in stalled replication forks at telomeres, decreased telomere replication and activation of the ATR-dependent DDR [64]. The report concluded that telomeres resemble fragile sites and that TRF1 is required for efficient replication primarily through its recruitment of the BLM and

RTEL1 helicases [64]. The requirement of TRF1 for efficient telomere replication is analogous to Taz1.

Recent work also supports the putative role of TRF2 in coordinating the activities of DDR proteins at the telomere. TRF2 binds and initiates the replication of the oriP sequence in the Epstein Barr virus DNA [100]. In addition, TRF2 directly interacts with ORC2, a central component of the origin of replication complex that binds replication origins and initiates bidirectional DNA replication [100]. Interestingly, loss of ORC2 leads to telomere dysfunction, characterized by signal free ends and the appearance of circular telomeric DNA (T-circles), suggesting inefficient telomere replication [89]. Further work demonstrated that deletion of the region of TRF2 that interacts with ORC2 also leads to the formation of T-circles suggesting that this interaction is important for efficient telomere replication, and that TRF2 indeed coordinates telomere replication [101].

In addition to the G-rich sequence, the T-loop also presents a barrier to telomere replication (**Figure 1.4**). Indeed, as a replication fork approaches the distal end of the telomere, the T-loop is likely to inhibit free rotation of the DNA, thus posing a topological barrier to the replication machinery. To allow passage of the replication fork, the T-loop needs to be resolved by the actions of specialized helicases. Post duplication of the DNA, the T-loop needs to be reformed by the HR machinery to ensure a stable telomere. Several lines of evidence suggest that the resolution and reformation of the T-

loop are also coordinated by telomere binding proteins such as TRF1 and TRF2. Both TRF1 and TRF2 interact with and stimulate the activities of the WRN and BLM RecQ helicases that are able to assist in the resolution of D-loop structures [102, 103]. In addition, TRF1 and TRF2 are integral to T-loop re-formation as well [97]. Indeed, it was recently described that TRF2 can directly enhance strand invasion required for the formation of the T-loop (**Figure 1.2**) [104]. Together these results demonstrate that telomere replication is unique, and requires a coordinated effort from telomere binding proteins and the DNA replication and repair machinery to efficiently complete replication.

1.6 Flap Endonuclease 1 and Telomere Replication

The inherent nature of the telomeric DNA sequence causes strand specific DNA replication – the C-rich strand always provides the template for leading strand synthesis while the G-rich strand is the template for lagging strand replication (**Figure 1.4**). The repetitive nature of the telomeric DNA and the tendency of G-rich DNA to form secondary structures create a situation in which lagging strand replication at the telomere is particularly challenging. Therefore, several studies have focused on understanding the role of lagging strand replication proteins in telomere maintenance [105-107].

Lagging strand DNA synthesis requires the formation and processing of Okazaki fragments, which need to be cleaved and ligated (known as Okazaki fragment maturation) [108, 109]. One model of Okazaki fragment maturation suggests that

maturation requires the coordinated activity of two nucleases, FEN1 and Dna2 [109]. This model proposes that upon encountering the RNA/DNA strand from the adjacent Okazaki fragment, polymerase δ displaces the strand producing a flap structure. If the flap is long or forms a secondary structure it is coated by RPA initiating its cleavage by Dna2 into a shorter flap, which is then processed by FEN1 to generate a nick sealed by DNA ligase I [110].

FEN1 is a structure-specific metallonuclease that plays an important role in DNA metabolism. In addition to Okazaki fragment processing, FEN1 is also a critical DNA repair enzyme. Work done in *S. cerevisiae*, *S. pombe* and human cells demonstrate that FEN1 and its homologs are also involved in long-patch base-excision repair (LP-BER) [111, 112]. Indeed, deletion of FEN1 homologs in *S. cerevisiae* and *S. pombe* leads to UV sensitivity indicating a role for FEN1 in BER [113-115]. Yeast *rad27Δ* (the FEN1 homolog) mutants and human carcinoma cells expressing a dominant negative FEN1 protein are also sensitive to the DNA alkylating agents such as methylmethane sulfonate (MMS) [114, 116]. Additionally, genetic work in yeast and chicken cells has suggested that FEN1 is involved in NHEJ and homologous recombination pathways [117-119]. Defects and deletions of FEN1 in yeast also lead to an increase in spontaneous mutations [113, 114]. Besides its role in DNA replication and repair of genomic DNA, recent work indicates that FEN1 activity is important in mitochondrial DNA repair [120].

FEN1 also prevents trinucleotide repeat (TNR) expansion and contraction [111, 118, 121]. Work in yeast shows that deletion or haploinsufficiency of *RAD27* leads to expansion of triplet repeats presumably due to strand slippage and/or formation of secondary structures that cannot be processed by FEN1 [122]. Similarly, in mice haploinsufficiency of FEN1 leads to the expansion of a Huntington's disease locus CAG repeat [118]. However, recent studies have shown that TNR expansion upon FEN1 loss is a species-specific phenomenon. Mutation of FEN1 in Drosophila did not affect the CAG repeat locus [123]. Similarly, FEN1 depletion in human cells that were cultured over 27 successive passages did not affect the CAG Huntington's locus stability [124]. Despite clear species differences, these results demonstrate the importance of FEN1 in DNA replication, DNA repair and maintenance of genome stability.

FEN1 has multiple nuclease activities [111, 125]. It has a 5' flap endonuclease (FEN) activity and a 5' to 3' exonuclease (EXO) activity, both of which have been studied extensively [115, 126]. These two activities are considered crucial for Okazaki fragment maturation and long patch–base excision repair. Recently, human FEN1 was also found to cleave gapped DNA forks or bubble structures that resemble stalled replication forks [127, 128]. Mutational analysis enabled the separation of this gap endonuclease (GEN) activity from the FEN and EXO activities. Interestingly, the GEN activity did not require a free 5' flap like the FEN and EXO activities and it has since been implicated in apoptotic DNA fragmentation and reinitiation of stalled replication forks [127, 129, 130].

The multiple FEN1 nuclease functions and DNA replication and repair activities suggests differential regulation of these through post-translational modification. Indeed, FEN1 is phosphorylated by Cdk1-Cyclin A in late S phase [131], which reduces its FEN and EXO activities without affecting its DNA binding capabilities. In addition, phosphorylation abolishes PCNA binding, suggesting that it is able to regulate FEN1 function in DNA replication and repair [131]. FEN1 is also acetylated by p300 histone acetyl transferase (HAT) at four lysine residues on the C-terminus [132]. Acetylation of FEN1 is induced by UV treatment and reduces its DNA binding capability as well as its FEN and EXO nuclease activities [132], indicating a role for it in DNA repair. Although these post-translational modifications suggest a functional modulation of FEN1 activity, their biological significance has yet to be elucidated.

Given FEN1's role in genome maintenance, it is not surprising that it is an important tumor suppressor [133]. Homozygous deletion of FEN1 in mice is inviable, whereas the heterozygous animals are viable and disease-free [134, 135]. However, in a heterozygous adenomatous polyposis coli (APC) background, the haploinsufficiency of FEN1 leads to adenocarcinomas in the gastrointestinal tract and decreased survival of the mice [134, 136]. The biological relevance of FEN1 is further underscored by the discovery of mutations in human FEN1 that have been implicated in increased tumorigenesis [130, 137]. Analysis of several tumor types from lung, breast and melonoma revealed point mutants that abrogated two of the three known FEN1 functions

[130]. Interestingly, these mutations affected the GEN activity of FEN1, leaving the FEN activity intact. Knocking in one of these FEN1 mutations, E160D, in mice led to the development of autoimmunity and neoplasias in the lung [130]. This was attributed to incomplete DNA fragmentation during apoptosis due to decreased GEN activity. Another study conducted on a different strain of mice with the same point mutation in FEN1 revealed a different spectrum of cancers with early B-cell lymphomas [138]. Comparative genomic hybridization analyses of these lymphomas showed genomic instability with changes in chromosome copy number, chromosomal rearrangements, gains and losses [138].

Finally, Rad27, the FEN1 homolog in *S.cerevisease*, plays an important role in telomere function [105, 106]. Indeed, deletion of *RAD27* led to heterogeneous telomere lengths and an increase in single strand telomeric overhang (ssOH). The increase in ssOH is attributed to incomplete or defective lagging strand synthesis at the telomeres [105, 106]. Similarly, deletion of Rad2, the FEN1 homolog in *S. pombe*, led to telomere shortening [139]. Together these results demonstrate that FEN1 is important for telomere maintenance in lower eukaryotes, suggesting a role for FEN1 at mammalian telomeres.

Indeed, work on mammalian FEN1 also supports a role for this protein in telomere maintenance. FEN1 physically interacts with WRN [140], a RecQ helicase that plays an important role in lagging strand DNA replication at the telomere [87]. This interaction stimulates both the FEN and the GEN activities of FEN1 [127, 140],

suggesting a role for this complex in DNA repair and replication. Interestingly, FEN1 and WRN form a complex upon encountering stalled replication forks and processes branch migrating structures resembling regressed forks *in vitro* [141]. Importantly, the Shelterin component, TRF2 also interacts with FEN1 [142], suggesting that it recruits FEN1 to the telomere for accurate processing of telomeric DNA. Finally, recent work demonstrated that FEN1 associates with mammalian telomeres in a biphasic manner, localizing to the telomere during the S and G2 phases of the cell cycle [97], suggesting a role for FEN1 in mammalian telomere replication and repair.

1.7 Summary

Elucidating the different components involved in the efficient replication and maintenance of stable telomeres is paramount to our understanding of these complicated structures that play a pivotal role in tumorigenesis. The complex nature of the telomere presents a uniquely challenging template for the DNA replication machinery, making coordination between telomere binding proteins and DNA replication and repair machinery vital to successful telomere replication. Several lines of evidence suggest that FEN1, an important nuclease in DNA replication and repair, is a *bona fide* telomere binding protein that has a role in mammalian telomere biology. The following sections of this thesis will elucidate the role of FEN1 in maintaining and preserving telomere stability. Chapter 2 focuses on the role of FEN1 in replicating mammalian telomeres in primary human fibroblasts that do not have an active telomere maintenance mechanism. Chapter 3 concentrates on elucidating the mechanism through which FEN1 is able to

efficiently replicate human telomeres in primary human fibroblasts. Chapter 4 examines the necessity of FEN1 in the maintenance of stable telomeres in human tumor cells that have two different methods of telomere maintenance. In Chapter 5 I highlight the significance of this work in advancing our understanding of telomere replication and draw attention to questions that remain to be addressed from this work.

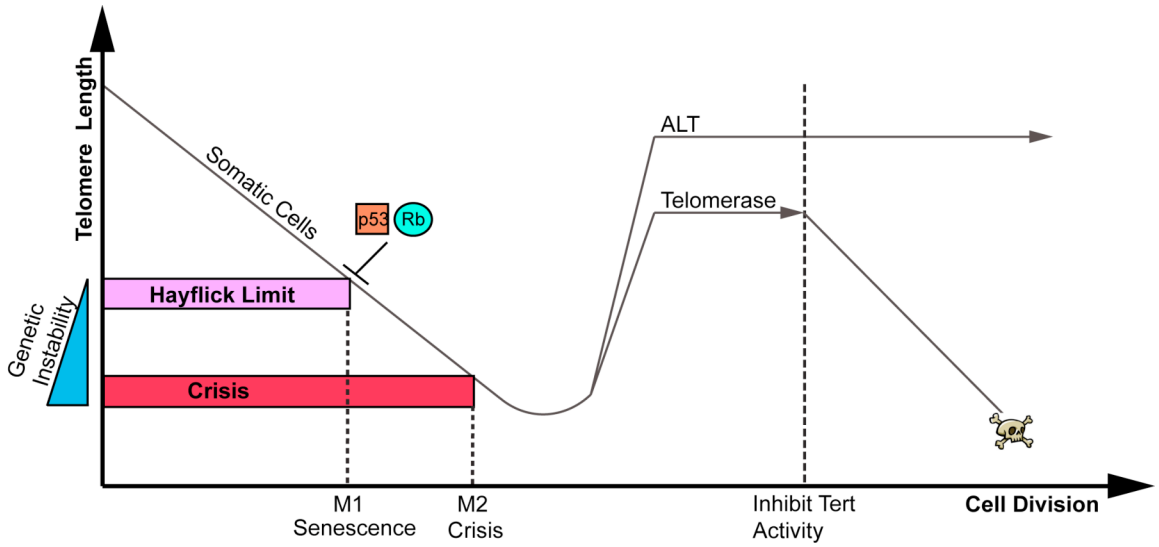

Figure 1.1 The telomere hypothesis
Telomere length is progressively lost during successive rounds of cellular division, eventually leading to p53- and Rb-dependent permanent growth arrest, referred to as senescence. Inactivation of p53 and Rb pathways allows continued cellular division and further telomere shortening. Continued telomere erosion leads the cells into their second proliferative barrier, crisis, where the telomeres are unable to protect the chromosome ends, resulting in chromosome fusions and cell death. Rare clones escape crisis by activating a telomere maintenance mechanism, either through the expression of telomerase or alternative lengthening of telomeres (ALT). Inhibition of telomerase leads to cell death in telomerase-positive cells.

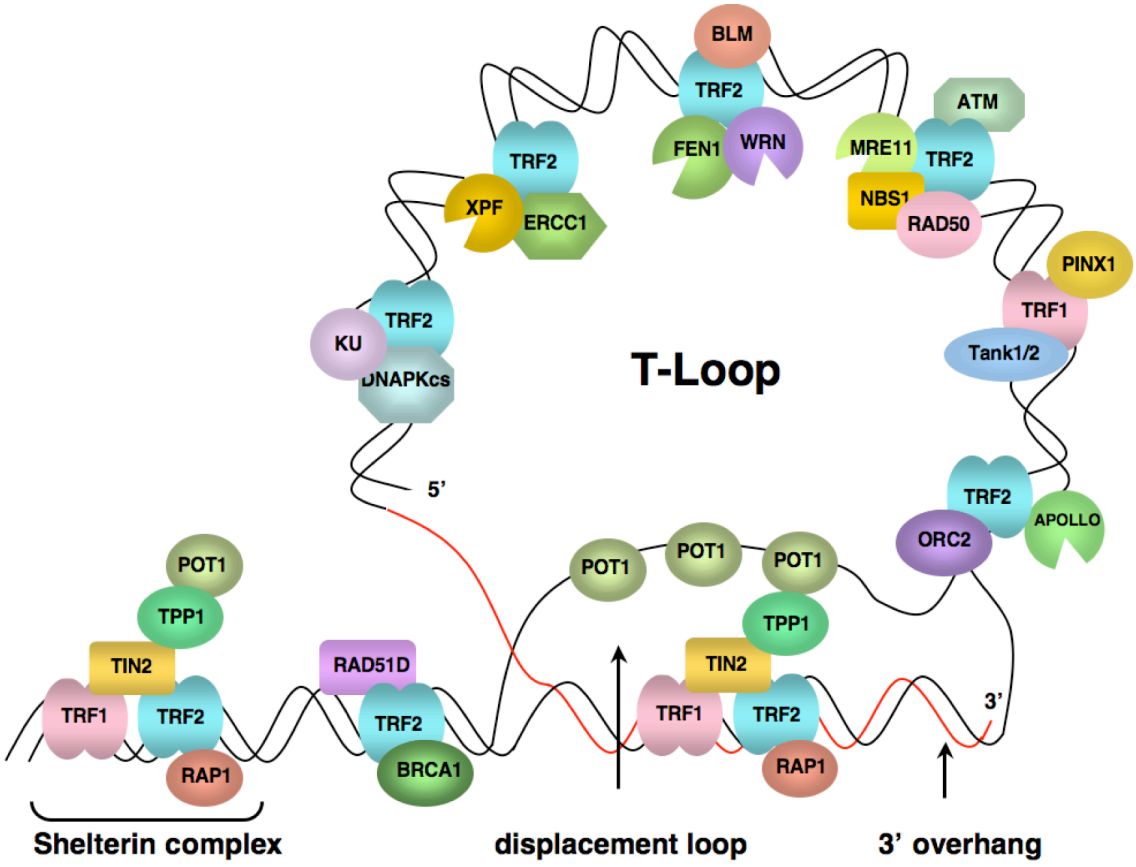

Figure 1.2 Schematic of the T-loop and telomere structure
Representation of the T-loop, in which the single-stranded overhang inserts into the double-stranded telomeric DNA, creating a displacement loop. The DNA and associated telomeric proteins create a capped, or functional, telomere.

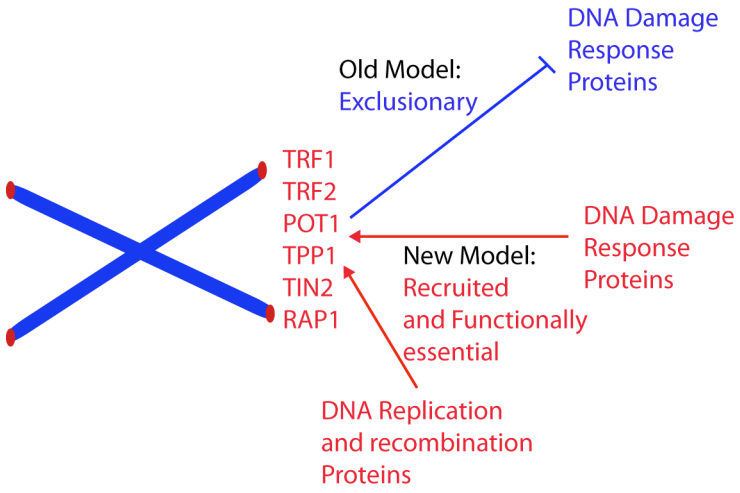

Figure 1.3 Telomere stability requires DNA damage response proteins
Telomeres were once thought to exclude DNA repair and replication machinery but are now known to recruit and modulate the activities of this machinery. Failure to recruit this machinery leads to telomere dysfunction and subsequent genomic instability.

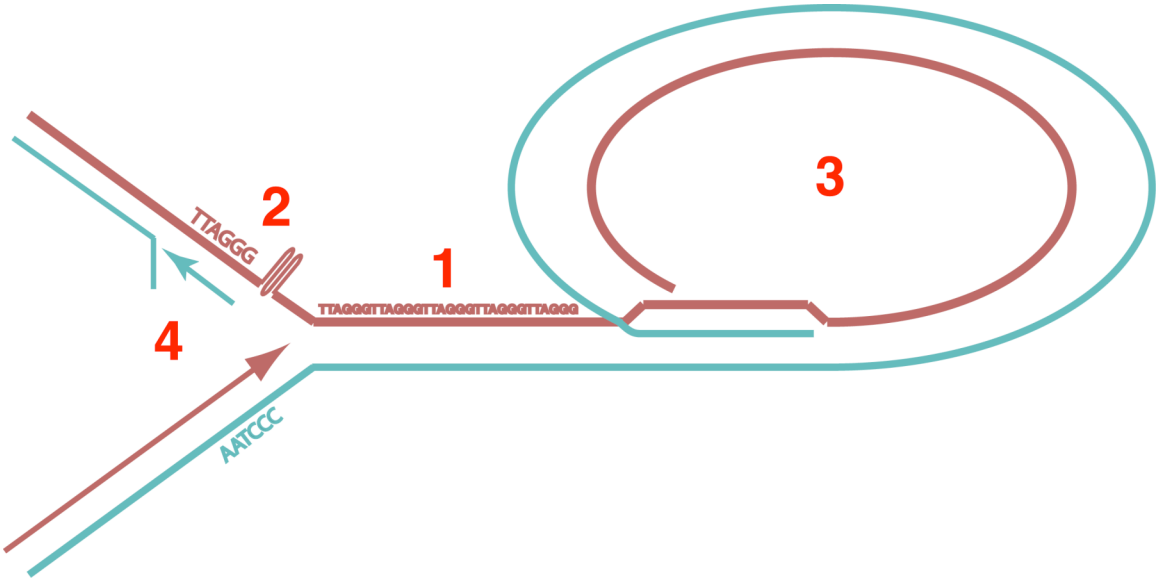

Figure 1.4 Telomere replication is a challenging task
Replication of telomeres inherently poses several challenges. The telomeric DNA consists of a highly repetitive G-rich sequence (1) that has a greater probability of forming secondary structures such as G-quadruplexes (2). The T-loop also presents a topological barrier to telomere replication (3). Telomeres replicate from a single unidirectional fork (4) and remain unreplicated if this fork collapses.

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CHAPTER 2: FLAP ENDONUCLEASE 1 CONTRIBUTES TO TELOMERE STABILITY

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Abstract

Telomere stability plays an important role in the preservation of genomic stability and is maintained through the coordinated actions of telomere specific proteins and DNA repair and replication proteins [1, 2]. Flap Endonuclease 1 (FEN1) is a protein that plays a role in lagging strand DNA replication, base excision repair, homologous recombination, and re-initiation of stalled replication forks [3, 4]. Here, we demonstrate that FEN1 depletion leads to telomere dysfunction characterized by the presence of γH2AX and sister telomere loss. Expression of catalytically active telomerase, the reverse transcriptase that adds telomeric repeats to chromosome ends, was sufficient to rescue telomere dysfunction upon FEN1 depletion. Strikingly, FEN1 depletion exclusively abrogates telomeres replicated by lagging strand DNA replication. Genetic rescue experiments utilizing FEN1 mutant proteins that retained the ability to localize to telomeric repeats revealed that FEN1's nuclease activity and ability to interact with the Werner protein (WRN) and telomere binding protein, TRF2 were required for FEN1 activity at the telomere. Given FEN1's role in lagging strand DNA replication and re-initiation of stalled replication forks, we propose that FEN1 contributes to telomere stability by ensuring efficient telomere replication.

Results and Discussion

High fidelity replication of telomeres is critical to maintain telomere stability, and is confounded by both the end replication problem and repetitive G-rich nature of

telomeric DNA [5]. Repetitive DNA sequences such as those found in the telomere present a challenging template for the replication machinery due to a propensity to form secondary structures that can lead to stalled replication forks [6, 7]. Due to the importance and difficulty of high fidelity replication through the telomere, recent studies have focused on the role DNA replication/repair proteins play in telomere stability [8-11]. Rad27, the FEN1 homolog is one such replication and repair protein that plays a role at *Sachharomyces cerevisiae* telomeres [8, 12]. Here, we demonstrate that FEN1 plays a critical role in mammalian telomere stability.

Previous work demonstrated that FEN1 localized to the telomere in a cell cycle dependent manner [13]. We confirmed this observation by chromatin immunoprecipitation (ChIP) from cells 1) synchronized with thymidine and aphidicolin (**Figure 2.S1**) and 2) enriched in different phases of the cell cycle by centrifugal elutriation (**Figure 2.S2**). In agreement with previous work, we found that FEN1 localized to the telomere in the S and G2/M phases of the cell cycle. Purified FEN1 has been shown to interact directly with TRF2 through both the basic and myb domains of TRF2 [14]. Utilizing antibodies specific for endogenous FEN1 and TRF2, we demonstrate that these proteins interact *in vivo* (**Figure 2.S3**).

FEN1's presence at the telomere and its interaction with TRF2 raised the intriguing possibility that it played a role in telomere biology. To address this directly, lentiviral expressed RNA interference (RNAi) hairpins targeting FEN1 (shFEN) or a scrambled hairpin (negative control, shSCR) were introduced into BJ fibroblasts (**Figure 2.1A**). Upon transduction, FEN1 protein expression was virtually undetectable compared

to control cells (**Figure 2.1B**). To determine whether FEN1 depletion resulted in telomere dysfunction, we analyzed telomeres for the presence of γH2AX (an indicator of DNA damage) by ChIP. Lysates from cells expressing shSCR or shFEN were subject to immunoprecipitation using an antibody to γH2AX, followed by quantitation of isolated telomeric and genomic DNA (ALU). We found that upon FEN1 depletion, immunoprecipitation of γH2AX resulted in a significant increase in the amount of isolated telomeric DNA compared to control cells (1.39 fold greater than control; $P<0.05$; **Figure 2.1C and 2.1D**). In contrast, no significant increase was observed in γH2AX associated with ALU DNA (1.09 fold; $P=0.59$), indicating that there is increased DNA damage upon FEN1 depletion at telomeric sequences compared to the genome at large. A similar increase in γH2AX associated telomeric and genomic DNA was observed when cells were treated with the ribonucleotide reductase inhibitor, hydroxyurea (data not shown). Together these results indicate that FEN1 depletion results in telomere dysfunction similar to that observed upon replication stress following hydroxyurea treatment.

We next assessed the telomeres directly upon FEN1 depletion. FEN1 was depleted in BJ fibroblasts expressing the SV40 early region (BJL) (the presence of the early region facilitated isolation of metaphase chromosomes) (**Figure 2.2A**). Following FEN1 depletion, we utilized fluorescence *in situ* hybridization (FISH) to visualize telomeres. We found that FEN1 depletion resulted in increased sister telomere loss (STL) (**Figure 2.2B and 2.2C**). On average, 9.4% of the chromosomes isolated from control cells displayed STLs (**Figure 2.2C**). Upon FEN1 depletion, the percentage of

chromosomes displaying STLs increased nearly two-fold (16.8%, P<0.0001; **Figure 2.2C**), indicating that FEN1 depletion impacted telomere stability.

Depletion of FEN1 leads to sister telomere loss (**Figure 2.2**) resulting in recognition of telomeres by the DNA damage machinery (**Figure 2.1**). Several papers have demonstrated that telomerase is preferentially recruited to the shortest telomeres [15-18], raising the possibility that telomerase may compensate for FEN1 depletion at the telomere. Therefore, we expressed shSCR or shFEN in BJL cells expressing telomerase (BJLT; **Figure 2.2A**). We found that in the presence of telomerase, STLs were significantly reduced upon FEN1 depletion. Indeed, in BJLT cells only 2.6% of chromosomes displayed STLs upon FEN1 depletion (P<0.05; **Figure 2.2B and C**), which was significantly lower than the 16.8% STLs observed in BJL cells devoid of telomerase activity. Together, these results demonstrate that telomerase compensates for FEN1 depletion at the telomere.

The above observation was reminiscent of a report demonstrating that mutations in WRN, a known FEN1 binding protein, led to STLs that were limited to telomeres replicated by lagging strand DNA synthesis [19]. Given FEN1's known role in lagging strand DNA replication and its interaction with the WRN protein [20, 21], we investigated whether FEN1 depletion compromised lagging strand DNA synthesis of the telomere. To carry out these studies, we employed chromosome orientation fluorescent *in situ* hybridization (CO-FISH), which is capable of distinguishing between telomeres replicated by leading versus lagging strand DNA synthesis (**Figure 2.3A**). CO-FISH analysis revealed that reduction in FEN1 protein levels led to a specific loss of the

lagging strand telomere (**Figure 2.3B and 3C**). BJL cells expressing the control hairpin (shSCR) had similar levels of telomere loss of both leading and lagging strands (4.4% and 3.8%, **Figure 2.3C**). Strikingly, cells expressing shFEN exhibited a significant 2-fold increase in loss of the lagging strand sister telomeres (9.5% versus 3.8%, $P<0.0001$; **Figure 2.3C**), with no change in the number of leading strand STLs observed. Together, these data demonstrate that FEN1 depletion exclusively compromises lagging strand DNA replication at the telomere.

Several biochemical functions have been ascribed to FEN1 [3, 4]. To determine whether FEN1 nuclease activity or its interaction with the WRN protein is necessary for telomere stability we created a novel vector, pResQ, capable of expressing both an shRNA and a cDNA (**Figure 2.S4**), and conducted genetic rescue experiments. We also designed a second shRNA targeted to the FEN1 3' UTR (shFEN3), which facilitated our analysis by allowing us to deplete endogenous protein, while having no effect on mRNA produced from a cDNA devoid of the 3'UTR sequence. The FEN1 mutants utilized were D181A (DA), which completely lacks nuclease activity [22] and delta C (ΔC; 20 amino acid deletion on the C-terminus), which retains partial ability to process flap structures with the replication clamp, PCNA, but is unable to bind the WRN protein [23, 24]. Cells transduced with the indicated vector confirmed that endogenous FEN1 protein was significantly reduced and the wild-type and mutant proteins were expressed, albeit to varying levels (**Figure 2.4A**).

We next determined whether the wildtype or mutant FEN1 proteins could rescue the telomere dysfunction observed upon FEN1 depletion. No significant change in

leading strand STLs was observed following expression of any of the FEN1 proteins (**Figure 2.S5** and **Figure 2.4B**). Reduction of FEN1 protein with a second independent hairpin (shFEN3) led to a 2-fold increase in lagging strand STLs (**Figure 2.4B**). Importantly, expression of wild-type FEN1 (WT) rescued the lagging strand STL phenotype, indicating that the observed phenotype was specific to the depletion of FEN1 (**Figure 2.4B**). In contrast, ectopic expression of either the DA nuclease deficient mutant or the ΔC mutant was unable to rescue the lagging strand STL phenotype upon FEN1 depletion (**Figure 2.S5** and **Figure 2.4B**). These observations suggest that both the nuclease activity and FEN1's interaction with WRN is critical for its role at the telomere.

To rule out that failure of the mutants to rescue FEN1 depletion resulted from an inability of the mutants to interact with TRF2 or localize to the telomere, we conducted immunoprecipitation and ChIP experiments. As shown in **Figure 2.4C**, the DA mutant, but not the ΔC mutant retained the ability to interact with TRF2, indicating that the C-terminal 20 amino acids are critical for FEN1 binding to TRF2. This also suggests that the phenotype of the ΔC mutant may be compounded by the combined loss of TRF2 and WRN interactions. To determine whether the mutant FEN1 proteins retained the ability to localize to the telomere, we also carried out ChIP analysis on lysates from 293T cells ectopically expressing epitope-tagged proteins. We found that all three FEN1 proteins associated with telomeric DNA (**Figure 2.4D**). These results, together with the functional data presented above, demonstrate that failure to rescue sister telomere loss was not due to an inability of the mutants to localize to the telomere.

FEN1 is a DNA replication and repair protein [4, 25]. To explore the possible impact of FEN1 depletion on the genome at large, we carried out karyotypic analysis of BJL and BJLT cells. Upon FEN1 depletion, BJL cells displayed a mild increase in genomic instability as evidenced by a modest increase in the number of chromatid breaks and chromosome gaps observed (**Table 2.S1**). Because telomerase rescues the telomere phenotype observed upon FEN1 depletion (**Figure 2.2**), any chromosomal abnormalities observed in BJLT cells depleted of FEN1 would be attributed to a non-telomeric effect. Karyotypic analysis of BJLT cells revealed no significant differences between cells expressing shSCR or shFEN (**Table 2.S1**), indicating that the impact of FEN1 depletion on the genome is the result of telomere dysfunction.

FEN1 is a structure specific endonuclease that acts in DNA replication and repair. Here, we assessed FEN1's role in telomere stability. In agreement with previous work [13, 14], we found that FEN1 is present at mammalian telomeres in a cell cycle dependent manner, and that it interacts with TRF2. This interaction requires the C-terminal region of FEN1. FEN1 depletion led to telomere dysfunction characterized by an increase in γH2AX at telomeres and sister telomere loss (STL). The latter was repressed by telomerase expression. CO-FISH analysis revealed that STLs were limited to telomeres replicated by lagging strand DNA synthesis. We further demonstrated that FEN1 nuclease activity and its C-terminal region are critical for its function at the telomere. FEN1 depletion revealed only a mild increase in genomic instability that was completely abolished in the presence of telomerase. Collectively, these data demonstrate

that FEN1 is important for telomere stability and suggest that FEN1 is required for proficient replication and/or repair of telomeres.

Telomere repeat binding proteins interact with DNA replication and repair proteins to maintain telomere stability [1, 5]. Abrogation of these protein-protein interactions in both yeast and mammalian systems can have profound effects on telomere stability [2]. These observations raise the possibility that the telomere represents a specialized structure whose replication and stability is ensured by the coordinated efforts of numerous redundant systems [5]. Highly repetitive sequences such as those present in the telomere can adopt complex secondary structures that are challenging to replicate and have the potential to lead to stalled replication forks [5, 7]. If left unresolved, these can result in double strand breaks [26]. Given FEN1's potential role in the reinitiation of stalled replication forks [27, 28], its absence is likely to compound the ability of the replication machinery to successfully transit the G-rich TTAGGG tracks. In support of this, our data demonstrate that FEN1 depletion results in specific loss of lagging strand-replicating sister telomeres. We propose that FEN1 is recruited to the telomere to facilitate replication and in its absence the replication machinery has a propensity to stall and/or inefficiently re-initiate stalled replication forks within the telomeric repeats. This hypothesis is particularly attractive in light of work demonstrating that loss of the Werner protein, which localizes with FEN1 at stalled replication forks thereby facilitating processing of branch migrating structures [28], phenocopies FEN1 depletion at the telomere [19]. In both the case of FEN1 depletion (this report) and WRN loss [19], telomerase rescues the telomere phenotype. Because telomerase is recruited to, and

extends the shortest telomeres [15-18], its presence would be expected to rescue STLs by lengthening shortened telomeres created after a stalled-fork-induced break. Interestingly, the ΔC FEN1 mutant that does not interact with the WRN [24] or TRF2 protein is unable to rescue the telomere defect observed upon FEN1 depletion despite its ability to localize to the telomere. Because the ΔC mutant retains a partial ability to interact with PCNA [23, 24], this result suggests that it is FEN1's repair function that is critical for its activity at the telomere.

Materials and Methods

Cell Culture and Synchronization. All cells were grown as reported [29-31]. Briefly, cells were grown at 37°C in 5% CO_2. HeLa and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum and 1% penicillin/streptomycin. BJ fibroblasts were cultured in DMEM with 15% Medium 199 (Sigma, St. Louis, MO), 15% fetal calf serum and 1% penicillin/streptomycin. HeLa cells were synchronized using a sequential Thymidine (2mM)-Aphidicolin (1μg/ml) block (16 hours) with a 10-hour release between them. Centrifugal elutriation was carried out on 293 cells using a Beckman JE6B elutriation rotor (Beckman). Small fractions of cells were collected at the indicated time points, stained with propidium iodide, and subjected to FACS analysis to determine the cell cycle phase before subsequent analyses.

Virus Production and Infection. Viral production and infections were carried out as described [32]. Briefly, 293T cells were transfected using TransIT-LT1 (Mirius, Madison, WI). Virus was harvested 48 hours post transfection, and infections were carried out in the presence of 10 μg/ml of protamine sulphate for 4-6 hours. Following infection, target cells were selected with 1 μg/ml puromycin. The pLKO.1shSCRp, pLKO.1shFENp and pResQ lentiviruses were produced by cotransfection with pCMVΔR8.2 and pCMV-VSV-G (8:1 ratio). The sequences used for the hairpins were for shFEN 5'-GGAGATCGTGCGGCGACTTGA-3' shFEN3 5'-TTAAGAGCTACAGCTAGAGAA-3' and shSCR [33]. The pResQ was constructed by inserting the U6 promoter into pFIRu (Unpublished data, Dr. Greg Longmore). The U6 promoter was amplified using 5'-TCTAGATGAGGGCCTATTTCCCATGATTCC-3' and 5'-CTCGAGTACGTAACCGGTGTTTCGTCCTTTCCAC-3' primers, and inserted with the XbaI and XhoI endonucleases.

Western Blot Analysis. Cells were washed with PBS and lysed in RIPA buffer (150mM NaCl, 1% Triton X-100, 0.5% Sodium Deoxycholate, 0.1% SDS, 50mM Tris pH8.0, aprotonin, leupeptin, pepstatin, and PMSF). Protein concentration was quantified using the Bio-Rad Protein Assay (500-0006, Bio-Rad, CA) Proteins were separated on an SDS-polyacrylamide gel and transferred to a PVDF membrane. Antibodies used: rabbit polyclonal anti-FEN1 (#586, Bethyl Labs, Montgomery, TX), mouse monoclonal anti-Actin (ABCAM, Cambridge, MA), rabbit polyclonal anti-TRF2 (Santa Cruz Biotech, H-300), mouse monoclonal anti-Flag M2 (Sigma St. Louis, MO).

Co-Immunoprecipitation. 293T nuclear extracts were prepared as described [34]. Nuclear extracts were precleared with 150µl of Protein A beads (Amersham) for 1 hr at 4°C and incubated with 5µg TRF2 antibody (Santa Cruz Biotech, H-300) or IgG (Sigma) and 40µl of Protein A beads at 4°C, overnight. Bound proteins were eluted and analyzed by SDS-PAGE and western blot analysis.

Chromatin Immunoprecipitation (ChIP). ChIP was conducted as previously described with the following modifications [13]. Cells were crosslinked on the plate with 1% formaldehyde for 60 minutes at room temperature, washed with PBS and lysed in 1% SDS, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA plus protease inhibitors at a density of $2x10^7$ cells/ml. Lysates were sonicated with a Missonix 3000 sonicator (Missonix, NY) at a setting of 5 (6 cycles of 30 seconds), producing chromatin fragments ranging from 100 and 500 bases. Equal quantities of protein lysate were diluted and precleared with 40µl of Protein A beads (GE Healthcare, Sweden) for 4 hours at 4°C. Immunoprecipitations were performed overnight with 8µg of antibodies and 30µl of Protein A/G-PLUS Sepharose beads (Santa Cruz, sc-2003) that had been pre-blocked with 5µg of sheared *E.coli* DNA and 30µg BSA. Antibodies used were: anti-TRF2 (05-521, Upstate, Charlottesville, VA), anti-phospho-H2AX (JBW301, Upstate, Charlottesville, VA), rIgG (Sigma, St. Louis, MO), and anti-Fen1 (#587, Bethyl Labs, Montgomery, TX). The beads were washed and eluted in 70mM Tris (pH8.0), 1mM EDTA and 1.5% SDS at 65°C. Crosslinks were reversed by incubation at 65°C for 4-6

hours. The eluate was treated with 40μg of Proteinase K treatment for an hour at 37°C. Samples were phenol-chloroform extracted and precipitated overnight at -20°C. The precipitates were resuspended in 0.4N NaOH and blotted onto a Hybond-XL membrane (Amersham Biosciences, UK) (70% loaded for telomere signal and 20% loaded for ALU signal). The telomere probe, TEL $(TTAGGG)_n$ and Alu probe (ALU), were produced by random priming using the Amersham Ready-To-Go beads, -dCTP. Following hybridization, membranes were washed and analyzed by a Phosphor Imager (Amersham, Piscataway, NJ).

Fluorescence *in situ* Hybridization (FISH). FISH was carried out as described [35]. A subconfluent population of cells was incubated with 0.1μg/ml of colcemid for 2-6 hours to allow mitotic cells to accumulate. After hypotonic swelling in 75 mM KCl (10 min, 37°C), cells were fixed in methanol/acetic acid (3:1) and then dropped onto clean glass slides and aged overnight at 65°C. Slides were rehydrated in PBS, and fixed with 4% paraformaldehyde (in PBS) for 2 minutes. After washing, the slides were dehydrated with a cold ethanol series, and hybridized with 0.3μg/ml PNA probes targeted to the telomere, Cy3-$(CCCTAA)_3$ and the centromere (FFLU-OO-CTTCGTTGGAAACGGGA), in 70% formamide, 10mM Tris (pH 7.2) and blocking agent (Sigma, St. Louis, MO). DNA was denatured for 3 minutes at 80°C and hybridization was carried out at 37°C for 2-4 hours in a moist chamber. The slides were washed in 70% formamide, 10mM Tris (pH 7.2) and 0.1% BSA, and subsequently washed with TBS-T (0.1M Tris pH 7.2, 0.15M NaCl and 0.08% Tween-20). The slides

were dehydrated in a cold ethanol series and mounted using VectaShield (Vector Labs, Burlingame, CA) containing 0.1μg/ml DAPI. Images were taken using a Nikon 90i Microscope and analyzed on the ISIS FISH imaging software (Metasystems, Altlussheim, Germany).

Choromosome Orientation-FISH (CO-FISH). CO-FISH was conducted as previously described [36] with the following modifications. The cells were incubated with BrdU and BrdC, at the ratio of 3:1 (3μg/ml:1μg/ml) for one cell cycle. Metaphases were prepared as described above, and processed as described [36]. The metaphases were probed with Fluorescein-$(TTAGGG)_3$ at 0.06μg/ml and Cy3-$(CCCTAA)_3$ at 0.3μg/ml. The Flu probe was also boiled for 5 minutes at 95°C in the hybridization buffer, and then crashed on ice before hybridization.

Statistical Analysis. The Wilcoxon Two-Sample test (two-tailed distribution) was used for all the FISH analyses. Student's T-test was used to analyze the ChIP data.

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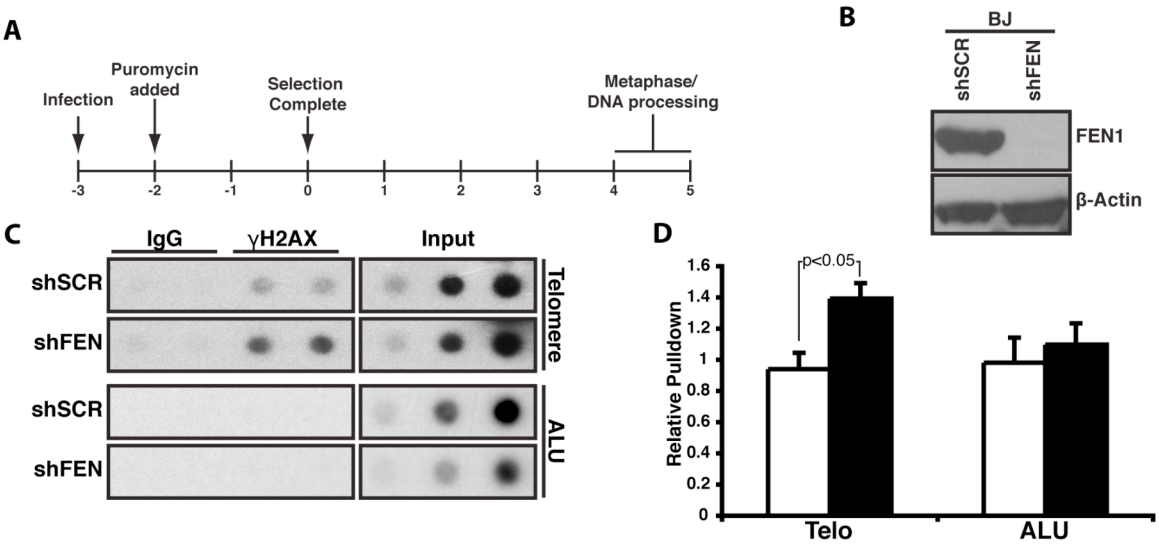

Figure 2.1. FEN1 depletion leads to telomere dysfunction.
(A) Timeline of experimental procedure given in days. (B) Short hairpins against FEN1 (shFEN) or a scrambled sequence (shSCR) were expressed in BJ fibroblasts. FEN1 (upper panel) and β-Actin (lower panel) protein levels were assessed by Western blot analysis. (C) Representative ChIP assay of cells expressing shSCR or shFEN. ChIPs were conducted as described in the supplemental materials. The inputs indicate 0.2%, 0.1% and 0.04% of the total protein extract. (D) Quantification of six independent ChIP assays. The graph indicates the relative amount of telomere (Telo) or ALU repeat (ALU) DNA isolated from cells expressing shSCR (white) or shFEN (black). The error bars represent standard error of the mean (SEM).

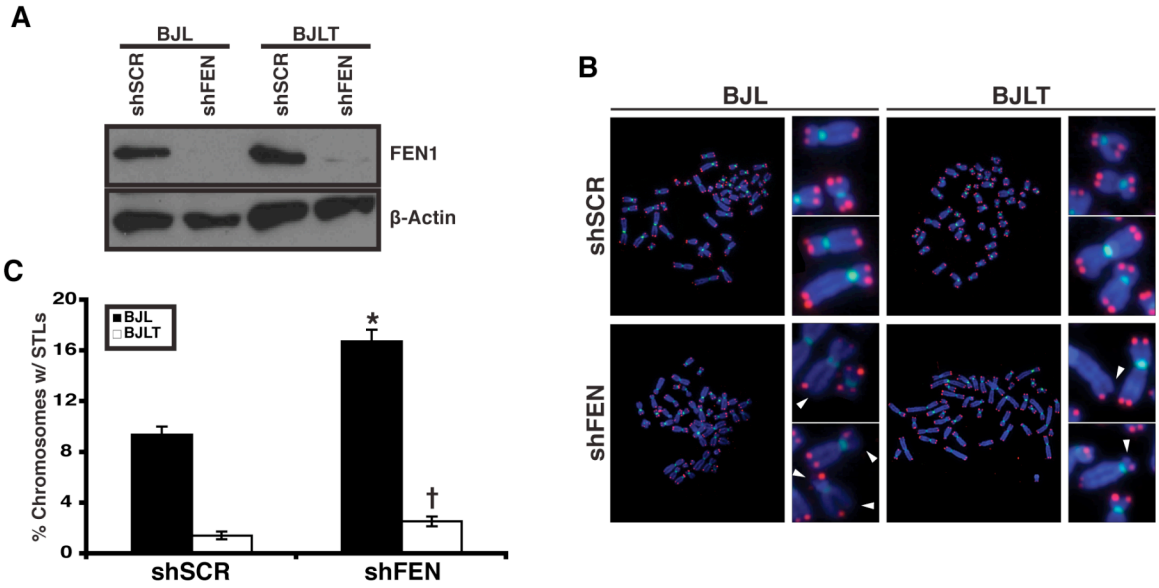

Figure 2.2. Increased sister telomere loss (STL) upon FEN1 depletion.
(A) Short hairpins against a scrambled sequence (shSCR) or FEN1 (shFEN) were expressed in BJ fibroblasts expressing SV40 early region, in the absence (BJL) or presence of telomerase (BJLT). FEN1 (upper panel) and β-Actin (lower panel) protein levels were assessed by Western blot analysis. (B) Representative metaphases from BJL and BJLT cells following shRNA expression. FISH analysis was conducted using Cy3-$[CCCTAA]_3$ (red) and FLU-labeled centromere probes (green). DNA was stained with 4',6-diamidino-2-phenylindole (DAPI; blue). Arrowheads indicate missing sister telomeres. The side panels show higher magnification images of the metaphase chromosomes. (C) Quantification of chromosomes displaying STLs following shRNA expression in BJL (black bars) and BJLT (white bars) cells. A minimum of 60 metaphases, from two independent experiments, was analyzed per treatment in a blinded fashion (* $P<0.0001$; †, $P<0.05$). The error bars represent SEM.

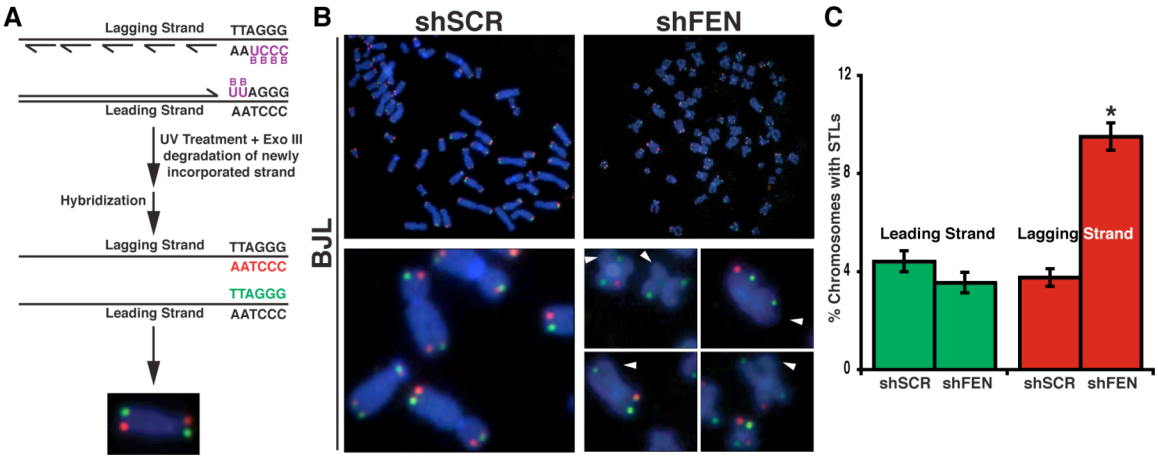

Figure 2.3. FEN1 depletion leads to loss of telomeres replicated by lagging strand DNA synthesis.

(A) CO-FISH schematic. Newly synthesized strands incorporate BrdU and BrdC. UV and ExoIII treatment results in degradation of newly synthesized DNA containing BrdU and BrdC, and the template strands are hybridized with Cy3-$[CCCTAA]_3$ (red, lagging strand) and FLU-$[TTAGGG]_3$ (green, leading strand) probes. (B) Representative CO-FISH of metaphases from BJL cells expressing the indicated hairpins. Color schemes are as described in (A). The arrowheads indicate missing telomeres. (C) Quantification of (B). A minimum of 60 metaphases from two independent experiments was analyzed per treatment in a blinded fashion (*P<0.0001). The error bars represent SEM.

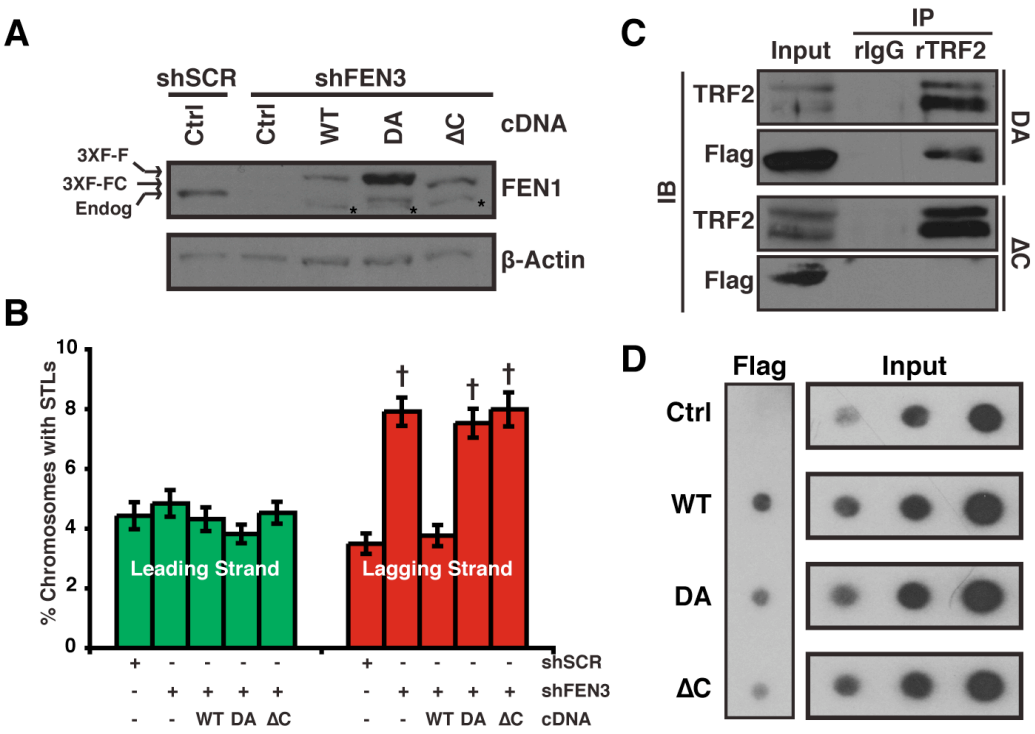

Figure 2.4. The nuclease activity and C-terminal region of FEN1 are essential for its role at the telomere.

(A) Western blot analysis of endogenous and ectopically expressed FEN1 proteins following transduction of BJL cells (upper panel). The ectopically expressed FEN1 proteins carry a triple-flag tag (3XF), which produces a larger protein. Abbreviations are as follows: Ctrl indicates control cells in which GFP was ectopically expressed, 3XF-F indicates the ectopically expressed wildtype and DA mutant, 3XF-FC indicates the ΔC mutant, and Endog indicates the endogenous FEN1 protein (*, Non-specific band). β-Actin (lower panel) is shown as a loading control. (B) Quantification of STLs after CO-FISH on metaphase chromosomes following depletion of the endogenous protein and expression of the indicated FEN1 protein, depicted as percentage of chromosomes with missing leading and lagging strand telomeres. A minimum of 60 metaphases from two independent experiments was analyzed per treatment in a blinded fashion (†, $P<0.0001$). The error bars represent SEM. (C) 293T cells transduced with flag-tagged FEN1 mutants, DA and ΔC and subjected to immunoprecipitation (IP) with an anti-TRF2 antibody as described in the supplemental. The presence of TRF2 and the FEN1 mutants were detected by immunoblot (IB) using anti-TRF2 and anti-Flag antibodies, respectively. The input lane indicates 10% of total protein used per immunoprecipitation. (D) FEN1 mutants localize to the telomere. Representative ChIP analysis of 293T cells (Ctrl) or 293T cells transduced with wildtype (WT) or a FEN1 mutant (DA and ΔC), subjected to immunoprecipitation with the M2 flag antibody. Precipitated DNA was probed for the presence of telomeric sequences as described in the supplemental information. The inputs indicate 0.2%, 0.1% and 0.04% of the total protein extract.

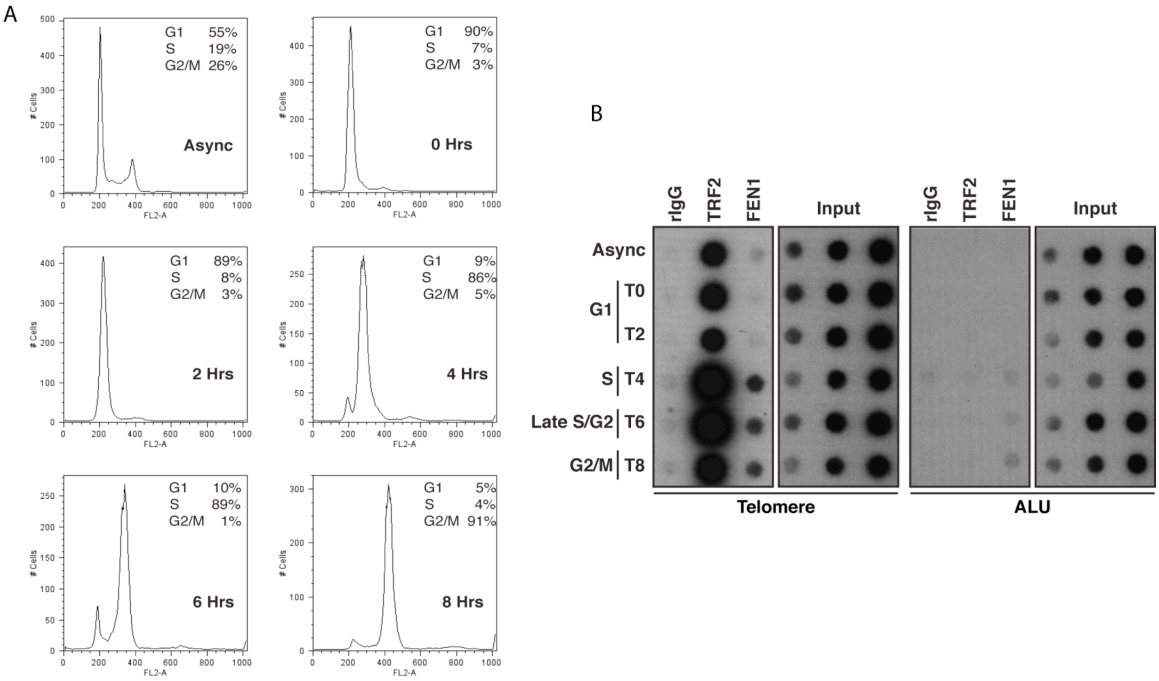

Figure 2.S1 FEN1 localizes to the telomere.
(A) Cell Cycle analysis of the HeLa cells used for chromatin immunoprecipitation (ChIP) assays. FACS analysis of propidium iodide stained synchronized HeLa cells taken at the indicated times post-release. The percentage of cells in each phase of the cell cycle is indicated. (B) ChIP with the indicated antibodies on synchronized lysates. Isolated DNA was probed with a probe against the telomere and Alu DNA. The inputs indicate 0.2%, 0.1% and 0.04% of the total protein extract.

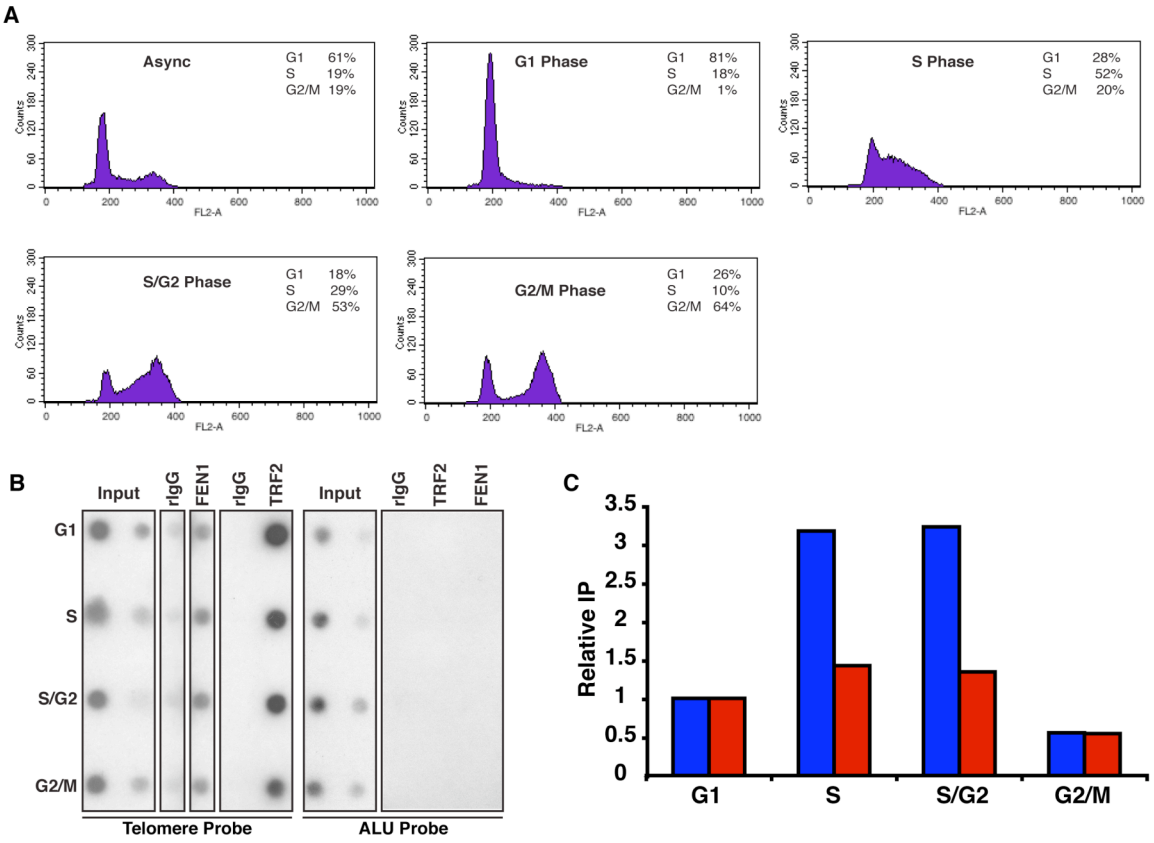

Figure 2.S2 FEN1 localizes to the telomere.
(A) Cell Cycle analysis of the 293 cells used for ChIP assays. FACS analysis of propidium iodide stained synchronized 293 cells post elutriation. The percentage of cells in each phase of the cell cycle is indicated. (B) Representative ChIP assay from the 293 cells separated via centrifugal elutriation using the indicated antibodies. Assays were conducted using equal amounts of protein extracts. rIgG was used as a negative control. The input for each of the lysates indicates 0.1% and 0.04% of the total protein extract. The isolated DNA samples were subjected to southern blot analysis using telomere and ALU probes. A lighter exposure is provided for the TRF2 telomere panel relative to the FEN1 panel. (C) Quantification of the relative FEN1 immunoprecipitation in the ChIP assays (n=2) following normalization for input and efficiency of DNA extraction. The graph shows relative pulldown compared to the G1 phase. Blue bars indicate the telomere signal and the red bars indicate the ALU signal.

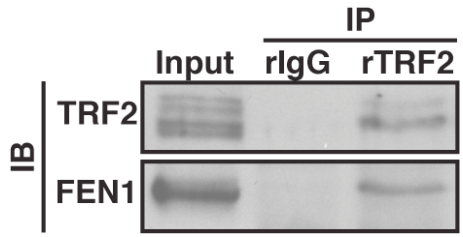

Figure 2.S3 FEN1 interacts with TRF2.
Co-immunoprecipitation experiments were conducted with nuclear lysates from HeLa cells to detect interaction between endogenous proteins. TRF2 was immunoprecipitated (IP) with anti-TRF2. The presence of FEN1 and TRF2 in the IP was confirmed by immunoblotting (IB) with antibodies against TRF2 and FEN1 as indicated.

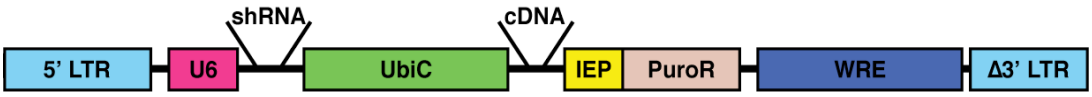

Figure 2.S4. Schematic representation of the pResQ vector.
A novel retroviral vector capable of: 1) utilizing RNAi to knock down a gene of interest (shRNA), 2) overexpressing a cDNA, and 3) expressing the puromycin resistance gene, facilitating isolation of transduced populations.

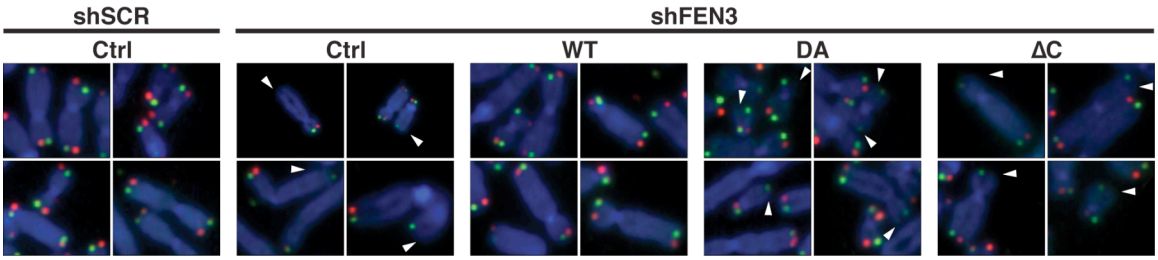

Figure 2.S5. The nuclease activity and C-terminal region of FEN1 are essential for its role at the telomere.

Representative images of metaphases isolated from BJL cells with the indicated treatments. CO-FISH was conducted on these metaphases and the color schematic is the same as in Figure 2.3. The white arrowheads indicate missing sister telomeres.

Table 2.S1. Cytogenetic analysis upon FEN1 depletion in the absence (BJL) and presence of telomerase (BJLT). Karyotypic analysis was conducted on blinded samples.

Chromosomal Aberrations	BJL		BJLT	
	shSCR	shFEN	shSCR	shFEN
Chromosome Gap	1	5	0	0
Minute	0	0	0	0
Chromatid Break	9	24	6	1
Chromatid Gap	0	0	0	0
Chromatid Exchange	10	10	0	0
Tri-Radial	1	1	0	0
Quadra-Radial	0	0	0	0
Dicentric	0	0	0	0
Ring Chromosome	0	0	0	0
Metaphase Number	68	118	41	33

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CHAPTER 3: FEN1 ENSURES TELOMERE STABILITY BY FACILITATING DNA REPLICATION FORK RE-INITIATION

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and Sheila A. Stewart

A. Saharia was the principal contributor to this work.

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Abstract

High fidelity telomere replication is critical to telomere stability and genomic stability. Telomeres present a challenging template for DNA replication and are duplicated by the coordinated actions of telomere binding proteins and DNA replication and repair proteins. Recently, we demonstrated that Flap Endonuclease 1 (FEN1), a nuclease important for DNA replication and repair, is critical for telomere stability and its depletion led to a loss of lagging strand-replicated telomeres. Here, we show that FEN1 contributes to telomere stability by ensuring efficient duplication of telomeres. FEN1 depletion does not affect cell cycle progression nor *in vitro* DNA replication through non-telomeric substrates, suggesting other nucleases compensate for FEN1 loss throughout the genome during DNA replication. However, FEN1 depletion leads to a decrease in replication fork re-initiation events following hydroxyurea challenge, indicating that FEN1 is important for efficient re-initiation of stalled replication forks. Genetic rescue experiments revealed that a novel gap endonuclease (GEN) activity involved in processing DNA bubble structures and FEN1's ability to interact with the RecQ helicases are vital for FEN1-dependent re-initiation of stalled replication forks. We further demonstrate that FEN1's ability to process Okazaki fragments is not required for efficient telomere replication. In contrast, FEN1's GEN activity, which is critical for re-initiation of stalled forks, prevents replication-dependent loss of lagging strand telomeres. We propose that FEN1 maintains stable telomeres through the efficient re-initiation of stalled

replication forks that occur in the G-rich lagging strand telomere, ensuring high fidelity telomere replication.

Introduction

High fidelity DNA replication is critical for genome stability and continued cellular proliferation, and is thus ensured by redundant mechanisms. The existence of inherited syndromes in which DNA replication/repair proteins are mutated or lost and overall DNA replication is able to continue best illustrate the robustness of these redundant systems (58). For example, Werner (WRN) helicase function is lost in Werner's syndrome, yet these patients replicate their DNA, suggesting that other RecQ helicases compensate for its function in DNA replication (49, 50). However, this compensation is incomplete and patients with mutations in WRN manifest progeria syndromes characterized by genomic instability (48).

Deficiencies in various DNA replication/repair mechanisms become particularly detrimental in highly repetitive DNA sequences that present unique challenges to the DNA replication machinery (13, 56). For example, triplicate repeats can cause replication fork slippage, resulting in deleterious expansions and deletions (19). Similarly, telomeric DNA, which consists of repetitive G-rich sequences capable of forming secondary structures such as G-quadruplexes (G4) can impede the replication fork (13, 27, 37). Thus, telomeres are particularly sensitive to the loss of DNA

replication/repair proteins such as WRN. Indeed, cells from WRN patients display overt telomere dysfunction while displaying only minor defects in overall genomic replication (6, 7, 48), suggesting that other proteins partially compensate for WRN throughout the genome but are unable to fully compensate for WRN function at the telomere.

Given the difficulties encountered during telomere replication, robust mechanisms have evolved to ensure high fidelity replication and repair of the telomere. These mechanisms appear to be coordinated by the Shelterin complex, a six-protein complex of telomere binding proteins (10, 13, 56). Indeed, TRF2, an essential component of the Shelterin complex, interacts with and modulates the activities of numerous DNA replication and repair proteins (10). For example, TRF2 binds WRN and BLM helicases and stimulates their activity *in vitro*, suggesting that it recruits them to replicate/repair telomeric DNA (36). In *Schizosaccharomyces pombe* (*S. pombe*), the TRF1/2 homolog, Taz1 is essential for DNA replication through the telomeres (30). Upon Taz1 deletion, replication forks stall and telomeres are rapidly lost (30). TRF1 plays a similar role in mammalian cells (43). Following deletion of TRF1, stalled replication forks accumulate within the telomeric repeats and an ATR-dependent DNA damage response (DDR) is detected, indicating a replication defect (43). Together, these data underscore the importance of the coordinated action between Shelterin components and the DNA replication and repair machinery to ensure efficient telomere replication.

We recently demonstrated that FEN1, a structure-specific metallonuclease that participates in DNA replication and repair, is vital for maintaining telomere stability (39). FEN1 directly participates in processing of Okazaki fragments during lagging strand replication (23). FEN1 also participates in several DNA repair pathways including base excision repair, homologous recombination, and re-initiation of stalled replication forks (26, 47). In addition, FEN1 directly interacts with TRF2 and localizes to mammalian telomeres during S-phase (32, 39, 57). Recently, we demonstrated that FEN1 depletion in telomerase-deficient cells results in a DNA damage response (DDR) at telomeres and telomere dysfunction characterized by loss of sister telomeres (STL) replicated by the lagging strand machinery (39, 40). Furthermore, genetic rescue experiments demonstrated that the nuclease activity and the C-terminus, WRN-interacting domain were important for FEN1 function at the telomere (39).

Here we demonstrate that FEN1 promotes efficient re-initiation of stalled replication forks. The C-terminal domain of FEN1 and its novel 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 nor SV40 Large-T-dependent *in vitro* DNA replication. Finally, we demonstrate that the PCNA-interacting domain of FEN1 is dispensable for its telomere function, and that the GEN activity of FEN1 is critical for its ability to prevent STLs upon FEN1 depletion. We propose that FEN1 maintains stable telomeres through the efficient re-initation of stalled replication forks that occur in the G-rich telomere, ensuring high fidelity telomere replication.

Materials and Methods

Cell Culture. All cells were grown as reported (39, 40, 51, 53). Briefly, cells were grown at 37°C in 5% CO_2. HeLa and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum and 1% penicillin/streptomycin. BJ fibroblasts were cultured in DMEM with 15% Medium 199 (Sigma, St. Louis, MO), 15% heat-inactivated fetal calf serum and 1% penicillin/streptomycin.

Virus Production and Infection. Lentiviral production and cell infections were carried out as described (39, 40, 52). 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 (39); the ΔP cDNA was previously described (45); the ΔPΔC cDNA was constructed using a forward primer complementary to the flag epitope 5'-GGTACCATGGACTACAAAGACCATGACGG-3' and the following reverse primer, 5'-CTCGAGTTATTAGGTGCTGCCTTGGCGGCTCTTAC-3', and cloned into the pShuttle plasmid; the mWT and mED cDNAs were previously described (62). 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. The adenovirus production and concentration was carried out according to manufacturer's protocol using the AdEasy XL Adenoviral Vector System (Stratagene, La Jolla, CA). The titer of the concentrated adenovirus was determined using AdEasy Viral Titer kit (Strategene, La Jolla, CA) according to the manufacturer's protocol.

Western Blot Analysis. All western blots were conducted as described (39). 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 50μM 5-bromo-2-deoxyuridine (BrdU). 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 then fixed in 4% paraformaldehyde and 0.1% TritonX-100 in PBS for 20 minutes at room temperature. Cells were further permeabilized with 0.1% TritonX-100 for 10 minutes on ice and fixed again for 5 minutes as previously described. The DNA was denatured with 30μg of DNaseI (Sigma, St. Louis, MO) at 37°C for an hour. The BrdU was detected with a FITC-conjugated anti-BrdU antibody (A21303, Invitrogen, Carlsbad, CA) and the DNA content of the cells

was determined by 7-amino-actinomysin 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 in (4). Briefly, HeLa cells were harvested, washed in cold isotonic buffer [20mM HEPES, pH 7.8, 1.5mM $MgCl_2$, 5mM KCl, 250mM sucrose, 1mM dithiothreitol (DTT), 0.1mM 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 kept on ice for another 60 minutes. Following this incubation, the lysate was centrifuged at 1700g at 4°C for 10 minutes to remove the nuclei and then centrifuged again at 12,000g 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; (34)) used in the replication reactions was prepared by equilibrium centrifugation in cesium chloride-ethidium bromide gradients and then digested with BbsI. The *in vitro* replication reactions were carried out according to the protocol in (4). Briefly, each 25μL reaction contained 30mM HEPES, pH 7.5, 7mM $MgCl_2$, 4mM rATP, 50μM each of rCTP, rUTP, rGTP, 100μM each of dATP, dGTP, dTTP, dCTP, 0.5mM DTT, 40mM creatine phosphate, 0.625 units creatine phosphokinase, 20μM [α-^{32}P]dCTP (1μCi), 50ng linearized plasmid DNA, 1μg Large-T antigen (Chimerx, Madison, WI) and 100μg

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, 50mM EDTA, 1mg/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 then the DNA was precipitated. The precipitated 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 (17, 42). Briefly, cells were cultured with 1.5mM hydroxyurea (HU) for 16 hours. The cells were then released from HU inhibition into medium containing 150μM BrdU for 10 minutes. The cells were fixed immediately, permeabilized with 0.5% TritonX-100, and treated with 10units of DNaseI 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 (Sigma, St. Louis, MO); Alexa Fluor 488 goat anti-mouse and Alexa Fluor 546 goat anti-rabbit (Invitrogen, Carlsbad, CA).

Co-Immunoprecipitation (Co-IP). Co-IP was conducted as described (39). Briefly, 293T cells were transfected with the indicated cDNA constructs and then nuclear extracts were prepared as described (12). Nuclear extracts were precleared with 150μl of Protein A beads (GE Healthcare, Piscataway, NJ) for 1 hr at 4°C and incubated with 5μg TRF2

antibody (H-300; Santa Cruz Biotech, Santa Cruz, CA) or IgG (Sigma, St. Louis, MO) and 40μl of Protein A beads at 4°C, overnight. Bound proteins were eluted and analyzed by SDS-PAGE and western blot analysis.

Chromatin Immunoprecipitation (ChIP). ChIP was conducted as described (39).

Metaphase preparation and Chromosome Orientation-FISH (CO-FISH). Metaphase preparation and CO-FISH was conducted as described (39).

Statistical Analysis. The Student's T-test (two-tailed distribution with equal variance) was used for all the BrdU foci and CO-FISH analyses.

Results

FEN1 depletion leads to inefficient replication fork restart

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 and is important for several DNA repair processes (26, 47). Indeed, FEN1 localizes to stalled replication forks where it interacts with the RecQ helicase, Werner (WRN), and is postulated to participate in DNA replication fork re-initiation (44, 62). The telomeric DNA sequence presents a challenging template for the DNA

replication machinery. As such, telomeres resemble fragile sites that are revealed upon aphidicolin treatment (43), providing evidence that telomeric replication is challenging. Furthermore, replication fork pausing and stalling have been observed within telomeric repeats both *in vitro* and *in vivo* (18, 28, 34, 57). This suggests that high fidelity telomere replication requires the actions of a replication fork re-initiation complex.

Recently, we demonstrated that in human cells FEN1 depletion results in telomere dysfunction while having little impact on total genome stability (39). 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 the Werner protein is unable to rescue telomere dysfunction upon depletion of endogenous FEN1, although it is able to bind the telomere (39, 45). It is therefore interesting to speculate that FEN1 is required for the re-initiation of stalled replication forks at telomeres. Given the data implicating FEN1 in replication fork re-initiation we first addressed how FEN1 depletion impacted DNA replication fork re-initiation following forced fork stalling induced by hydroxyurea treatment.

Hydroxyurea treatment causes nucleotide depletion, resulting in DNA replication fork stalling and S-phase arrest. 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 the DNA replication forks re-initiate, the efficiency of repair can be determined by quantitating BrdU foci (**Figure 3.1A**) (17). If FEN1 participates in the repair and restart of stalled DNA replication forks, its depletion would cause fewer re-initiation events and thus fewer BrdU foci would be observed.

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) (39). Expression of shFEN3 led to a significant reduction in FEN1 protein compared to cells expressing shSCR (**Figure 3.1B**). Control cells and FEN1-depleted cells were cultured for 16 hours in the presence of hydroxyurea (HU) and then released from HU inhibition in the presence of BrdU for ten minutes (**Figure 3.1A**). BrdU foci were observed through immunostaining. As expected, we observed a striking decrease in the number of BrdU foci upon FEN1 depletion, indicating that FEN1 is important for the re-initiation of stalled DNA replication forks *in vivo* (**Figure 3.1C and 3.1D**). In cells expressing the control hairpin, there were 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 3.1D**). Importantly, even in the absence of FEN1, cells were able to re-initiate stalled DNA replication forks, albeit less efficiently. Together these results demonstrate that FEN1 is important for efficient repair/restart 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 next carried out the re-initiation assay described above in cells depleted of endogenous FEN1 and expressing various FEN1 mutants as outlined in **Figure 3.2B**. The different FEN1 proteins used in this study included 1) human wildtype (hWT), which is competent for both replication and repair functions; 2) D181A (DA), which lacks nuclease activity (46) and thus represents a loss-of-function allele; 3) delta C (ΔC; 20 amino acid deletion on the C-terminus), which retains near wildtype ability to process flap structures with the replication clamp, PCNA, but is unable to bind the BLM and WRN helicases and participate in FEN1's DNA repair functions (45, 54); 4) delta P (ΔP; 9 amino acid deletion in the gene), which retains the ability to interact with the RecQ helicases, BLM and WRN, but is unable to interact with the replication clamp, PCNA (45), thus rendering it replication incompetent yet repair competent; and 5) deltaP-deltaC (ΔPΔC; 44 amino acid deletion on the C-terminus), which deletes FEN1's nuclear localization signal and abrogates its ability to interact with PCNA, BLM and WRN (45), thus creating a second loss-of-function allele that retains the nuclease domain. In addition, we also expressed a murine E160D (mED) mutant, which retains near wild-type levels of flap endonuclease (FEN) activity and the ability to participate in DNA replication, but is devoid of the novel gap endonuclease (GEN) activity, involved in processing DNA bubble structures and hypothesized to function in DNA repair including the re-initiation of stalled replication

forks (25, 62). Finally, we also expressed a murine wildtype (mWT) protein as a control for the mED mutant.

To facilitate our analysis, we utilized an shRNA that targeted the FEN1 3' untranslated region (shFEN3), which allowed depletion of the endogenous protein while having no effect on expression of the various FEN1 cDNAs (39, 40). Following depletion of FEN1, cells were subsequently infected with an adenovirus expressing wildtype or a mutant FEN1 protein. Transduced cells were allowed to grow for 4 days and then treated with HU for 16 hours followed by a 10 minute BrdU pulse to label re-initiated DNA replication forks (**Figure 3.2A**). To facilitate identification of successfully transduced cells, each of the FEN1 constructs was tagged with a Flag epitope. Therefore, following the BrdU pulse, cells were fixed and stained with anti-BrdU and anti-Flag antibodies and BrdU foci were quantitated in Flag-positive cells. As expected, expression of hWT FEN1 recovered the number of BrdU foci in FEN1-depleted cells to that observed in control cells. Indeed, expression of wildtype 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 (**Figure 3.2C & 3.2D**). In contrast, expression of the nuclease deficient FEN1 mutant, 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 repair of stalled DNA replication forks (**Figure 3.2C & 3.2D**). Similarly, expression of FEN1ΔC 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 interaction between FEN1 and the RecQ helicases, BLM and WRN is important for FEN1's role in the repair of stalled DNA replication forks (**Figure 3.2C & 3.2D**). In agreement, we found that expression of the ΔP mutant resulted in an average of 15.6 BrdU foci (**Figure 3.2C & 3.2D**), demonstrating that FEN1's interaction with PCNA is not critical for its role in the repair of stalled DNA replication forks. As expected, expression of the ΔPΔC mutant, a functionally null allele, was unable to rescue the reduction in BrdU foci observed upon FEN1 depletion (**Figure 3.2C & 3.2D**).

Analysis of our FEN1 mutants indicated that FEN1's DNA repair function and not its ability to participate in Okazaki fragment processing is critical for the restart of stalled DNA replication forks. Recently, a novel activity referred to as GAP endonuclease (GEN) was ascribed to FEN1 (62). The existence of this activity was intriguing because it has the ability to cleave DNA bubble structures, which resemble stalled replication forks. Furthermore, WRN stimulates FEN1's GEN activity, (25), suggesting that this activity is functionally important at stalled replication forks. To establish whether the GEN function was important for the restart of stalled replication forks, we next tested the impact of expression of a GEN-deficient FEN1 mutant (mED). 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 (**Figure 3.2C & 3.2D**). As expected, the wildtype protein, mWT completely recovered the number of BrdU foci observed upon FEN1 depletion with an average of 13.7 foci per nucleus (**Figure 3.2C & 3.2D**). These

data indicate that the GEN activity of FEN1 plays an important role in the repair/restart of stalled DNA replication forks. Taken together, these data support the hypothesis that FEN1 is important for the re-initiation of stalled replication forks. Moreover, FEN1's gap endonuclease activity and ability to interact with the RecQ helicases, BLM and WRN, are critical for this function.

FEN1 depletion does not impact S phase progression

Previously we showed that FEN1 depletion in telomerase-positive cells led to mild telomere dysfunction in the absence of cytogenic abnormalities (39). Furthermore, we showed that FEN1's ability to interact with the WRN protein was important in its function at telomeres and therefore ascribed the telomere dysfunction to FEN1's DNA repair activities (39). Above, we demonstrate that FEN1 directly contributes to the restart of stalled DNA replication forks and that this does not depend on FEN1's activity in Okazaki fragment processing but rather its DNA repair activities. Because the BrdU foci assay described above recognizes replication fork re-initiation across the genome, we wished to further examine how FEN1 depletion impacted genomic replication. Therefore, to explore the effect of FEN1 depletion on DNA replication, we utilized shRNA technology to deplete FEN1 and determine its impact on S-phase progression. Because telomere dysfunction might impact S-phase progression and this defect is rescued in telomerase positive cells (40), telomerase-positive HeLa cells were transduced with a lentiviral construct encoding shFEN3 or a control hairpin (shSCR). Expression of shFEN3 led to a significant reduction in FEN1 protein compared to cells expressing

shSCR (**Figure 3.1B**). To follow cells through the cell cycle, cells were pulsed with BrdU for one hour to label the S-phase population and then cells were followed as they continued through the cell cycle (**Figure 3.3A**). As expected from our previous work, in the absence of telomere dysfunction, we did not observe a significant difference in S-phase progression when cells were transduced with shFEN or shSCR (**Figure 3.3A & 3.3B**). As shown in **Figure 3.3**, in both cell lines approximately 35% of the cells were in S-phase following the one hour BrdU pulse. Both the control and FEN1-depleted cells exited S-phase and progressed throughout the cell cycle with similar kinetics (**Figure 3.3B**). This data suggests that FEN1 is not essential for DNA replication *in vivo* and its depletion does not significantly impact cell cycle progression.

FEN1 depletion does not impact DNA replication kinetics *in vitro*

Our initial studies indicated that FEN1 depletion has no detectable impact on S phase progression. Because minor effects on DNA replication might be missed by this 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 SV-40 Large-T antigen-dependent *in vitro* DNA replication assay (4) using cell lysates isolated from control or FEN1-depleted HeLa cells (**Figure 3.3C**). 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. As shown in **Figure 3.3D and 3.3E**, there was no difference in DNA replication efficiency when lysates from control versus FEN1-depleted cells were used (**Figure 3.3E**), indicating that

DNA replication proceeded in FEN1-depleted lysates with the same efficiency as lysates obtained from control cells. These results are in agreement with our S-phase progression data and suggest that another nuclease compensates for FEN1 function during DNA replication in non-telomeric sequences.

FEN1 interacts with the Shelterin component, TRF2

Our previous work (39) and the data in this paper support the hypothesis that FEN1 activity at the telomere is critical for high fidelity replication. The data further suggest that other nucleases are capable of compensating for FEN1 across the genome but fail to do so at the telomere. Given these results, we next wished to characterize the impact of the FEN1 mutants described above at the telomere. Because recent work demonstrated that FEN1 localizes to the mammalian telomere and interacts with the Shelterin component, TRF2 (32, 39, 57), we first examined the ability of the FEN1 mutants to interact with TRF2 and localize to the telomere.

To assess the interaction between TRF2 and the various FEN1 mutants, 293T cells were transfected with constructs expressing Flag-tagged wildtype or mutant FEN1 proteins. Nuclear lysates were obtained from the transfected cells and subject to immunoprecipitation with an anti-TRF2 antibody followed by western blot analysis. To restrict our analysis to the ectopically expressed FEN1 proteins, western blot analysis was carried out using an anti-Flag antibody. As shown in **Figure 3.4A**, TRF2 immunoprecipitated the ΔP FEN1 mutant, indicating that deletion of the PCNA-

interacting domain did not impair FEN1's ability to interact with TRF2. Given that our previous work indicated that the ΔC FEN1 mutant was unable to interact with TRF2 (39), it was not surprising to find that the ΔPΔC FEN1 mutant also failed to interact with TRF2 (**Figure 3.4A**). Analysis of the mWT FEN1 protein revealed that it, as well as the mED mutant, interacted with TRF2 (**Figure 3.4A**).

We next carried out chromatin immunoprecipitation (ChIP) experiments to determine whether the various FEN1 mutants retained the ability to localize to the telomere. As expected the hWT and ΔP mutant FEN1 localized to the telomere (**Figure 3.4B and 3.4C**). In contrast, the ΔPΔC FEN1 mutant was unable to precipitate telomeric DNA (**Figure 3.4B and 3.4C**). As above, the latter result was expected because the ΔPΔC mutant lacks the nuclear localization domain and is unable to localize to the nucleus (**Figure 3.2B and 3.2C**). Finally, both the mWT and mED proteins localized to the telomere. Together, these data indicate that the ability of FEN1 to interact with TRF2 and telomeric DNA correlate, suggesting that TRF2 might recruit FEN1 to the telomere for specialized processing and/or repair of telomeric DNA.

FEN promotes telomere stability by facilitating DNA replication fork re-initiation

The telomere consists of G-rich repetitive DNA that has the propensity to form G-quadruplex structures that can impede the movement of the DNA replication fork (13, 27, 30, 34, 57). Indeed, it has been hypothesized that stalled DNA replication forks frequently occur in the telomere (13, 57)**.** 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 (3). In support of this, recent studies have suggested that collapsed replication forks exist at telomeres leading to the formation of very short telomeres (7, 18, 59). We recently demonstrated that FEN1 depletion results in telomere dysfunction characterized by STLs (39), indicating that FEN1 functions in telomere maintenance, either through DNA replication or through DNA repair. Given these results and our observations indicating that FEN1 contributes to the efficient re-initiation of stalled DNA replication forks (**Figure 3.1**), we next wished to determine whether it was FEN1's role in Okazaki fragment processing or the restart of stalled DNA replication forks that contributed to telomere stability. Because telomerase expression compensates for FEN1 loss at the telomere thus masking the STL phenotype (39, 40), we utilized primary BJ fibroblasts, which express insufficient telomerase to maintain telomere lengths (29) for these studies.

Primary BJ fibroblasts were transduced with an shRNA construct targeting the 3'UTR (shFEN3) to deplete the cells of endogenous FEN1 (**Figure 3.5B and 3.5C**). Following depletion of FEN1, cells were infected with an adenovirus expressing wildtype or mutant FEN1 (greater than 85% of the cells were infected; data not shown). Because FEN1 depletion leads to lagging strand specific sister telomere loss (STL), we analyzed the strand specific loss of telomeres in cells expressing the different FEN1 mutants (39). 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 replicated by lagging and leading strand DNA synthesis, respectively (**Figure 3.5A**). In agreement with our previous results (39), the CO-FISH analysis demonstrated that FEN1 depletion led to a specific loss of 9.8% of lagging strand replicated telomeres (up from 3.1% in the control shSCR cells; $P<0.0001$) while having no impact on telomeres replicated by the leading strand machinery (**Figure 3.5**). 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 observed phenotype was specific to FEN1 depletion (**Figure 3.5**). Similarly, expression of the ΔP FEN1 mutant resulted in 3.6% lagging strand STLs ($P<0.0001$ compared to shFEN3), indicating that FEN1's interaction with PCNA was not important for its function at the telomere (**Figure 3.5**). In contrast, expression of the ΔPΔC protein led to 8% lagging strand STLs, indicating that it failed to rescue telomere dysfunction upon FEN1 depletion (**Figure 3.5**). Intriguingly, in contrast to the mWT protein, which was able to rescue the lagging strand STL defect upon FEN1 depletion, the mED mutant failed to rescue the depletion of FEN1 at the telomere (**Figure 3.5**). Indeed, expression of mWT significantly decreased the number of lagging strand STLs observed upon FEN1 depletion to 2.8%, while expression of the mED mutant resulted in lagging strand STLs (9.7%, $P<0.0001$) similar to that observed in ΔPΔC expressing cells. Because the mED mutant retains the ability to interact with TRF2 (**Figure 3.4A**) and localize to the telomere (**Figure 3.4B and 3.4C**) this result indicates that the gap endonuclease activity is critical for FEN1's role at the mammalian telomere. Taken together, these data demonstrate that FEN1's interaction with PCNA is dispensable for its

role at the telomere and that FEN1's gap endonuclease function is critical for telomere stability.

Discussion

Telomeres perform a critical cellular function by distinguishing the chromosome end from a *bona fide* double stranded DNA break. As such, numerous mechanisms have evolved to protect the telomere including components of the Shelterin complex that bind to the unique six base pair repeat sequence. The nature of the telomeric DNA sequence offers a number of challenges to the cell during DNA replication. G-rich, telomeric repetitive 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 (13, 27, 37). Indeed, several reports have indicated pausing and/or stalling of replication forks moving through telomeres (14, 18, 28, 57). Additionally, telomere replication is primarily initiated by the most distal origin of replication from the centromere and continues unidirectionally towards the end of the telomere (43). Given that telomeres are particularly susceptible to replication fork stalling (14, 18, 28, 57) and if the stalled fork cannot be resolved, it will lead to the formation of a double strand break (3) and telomere deletion will result. In support of this, telomeres have been recently identified as fragile sites that are highly sensitive to replication stress (43). Therefore successful telomere replication requires specialized machinery such as the coordinated

action of telomere binding proteins and their recruitment and/or modification of traditional DNA replication and repair factors (13, 56).

Recent reports demonstrate that Taz1 in *S. pombe* and TRF1 in mice are required for efficient telomere replication (30, 43). Telomere-binding proteins have evolved mechanisms to facilitate replication fork progression through the telomere possibly by the recruitment of proteins involved in DNA replication and repair (43). TRF1 and TRF2 interact with and stimulate the RecQ helicases, BLM and WRN (24, 35, 43), suggesting that they recruit these proteins to enhance DNA repair at the telomeres. FEN1 also interacts with TRF2 (32, 39). This raises the possibility that TRF2 engages the RecQ helicase-FEN1 complex coordinately at the telomere. Interestingly, a recent study demonstrated that TRF2 increases branch migration of Holliday Junction (HJ) intermediates suggesting that this promotes the formation of chickenfoot structures in the context of a stalled replication fork at telomeres (38). TRF1 and TRF2 may then recruit the WRN-FEN1 complex to resolve this structure and enable efficient restart of the stalled replication fork (44).

FEN1 localizes to stalled replication forks with the WRN helicase and together they process branch migrating structures that resemble regressed replication forks (44). In addition, WRN was shown to be essential for the re-initiation of stalled replication forks, *in vivo* (11, 49). The present study demonstrates for the first time that FEN1 is important for the re-initiation of stalled replication forks *in vivo* (**Figure 3.1**). Together with

previous work (33), this indicates that FEN1's role in S-phase is two-fold 1) in DNA replication through processing of Okazaki fragments and 2) in DNA repair through the re-initiation of stalled replication forks. FEN1 localizes to mammalian telomeres during S-phase (39, 57) so it could be involved in one or both of the functions outlined above. However, given that the PCNA-interacting domain of FEN1 is dispensable for telomere stability, our data indicate that FEN1's role in Okazaki fragment processing is non-essential for telomere stability. This result indicates that other nucleases such as Dna2 or Exo1, which can also process Okazaki fragments (1, 2, 15, 16, 31), compensate for FEN1 loss during lagging strand DNA replication. However, these same nucleases fall short when replication forks stall within the telomeric sequences. Indeed, we find that in the absence of FEN1's ability to re-initiate stalled replication forks, sister telomeres are still lost despite the presence of Dna2 and Exo1. Interestingly, other proteins involved in the re-initiation of stalled replication forks, PARP1 and PARP2, have also been implicated in telomere maintenance (5, 8, 60), further indicating the importance of the re-initiation process for the efficient replication of telomeres.

A novel activity of FEN1, the gap endonuclease (GEN) activity, which is essential for FEN1's ability to re-initiate stalled replication forks (**Figure 3.2**) is also essential for FEN1 function at telomeres (**Figure 3.5**). In support of our data, a recent report demonstrates telomere dysfunction in mouse embryonic fibroblasts (MEFs) that have a knock-in of the E160D (mED) mutation (41). Strikingly, mice with the mED knock-in spontaneously develop lung cancer (61) and lymphomas (22). Importantly, mED knock-

in mice displayed a modest increase in mutation rates and limited genomic instability (22), suggesting that telomere dysfunction induces genomic instability and drives this transformation process. Additionally, several reports have indicated a role for FEN1 as an important tumor suppressor gene (20-22, 33), suggesting that its role in preserving telomere stability may affect tumorigenesis.

Intriguingly, the C-terminal region of FEN1, essential for its function at the telomere, is also essential for its interaction with another RecQ helicase, BLM (45). Similar to WRN, BLM is also able to unwind G4 DNA, is critical for the re-initiation of stalled replication forks and has recently been suggested to be important for the efficient replication of telomeres (9, 42, 43, 55). This suggests that there is complicated interplay between the RecQ helicases, WRN and BLM, and FEN1 at mammalian telomeres, although the effect of BLM depletion on normal human telomeres has not been well characterized. Together, these data indicate that FEN1, together with the RecQ helicases (WRN and BLM), plays an important role in the re-initation of stalled replication forks at mammalian telomeres.

Here we demonstrate that FEN1 is important for efficient re-initiation of stalled replication forks *in vivo*. This repair function of FEN1 is dependent on its C-terminal domain and its novel 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 activity throughout the genome. However, these same proteins fail to compensate for FEN1 at the telomere. Indeed, FEN1 depletion led to an increase in lagging strand STLs. As with the re-initiation of stalled replication forks, both FEN1's C-terminus and GEN activity were essential for its function at telomeres while its ability to interact with PCNA was dispensable. Collectively, these data demonstrate that FEN1 is necessary for efficient replication of telomeres.

Acknowledgments

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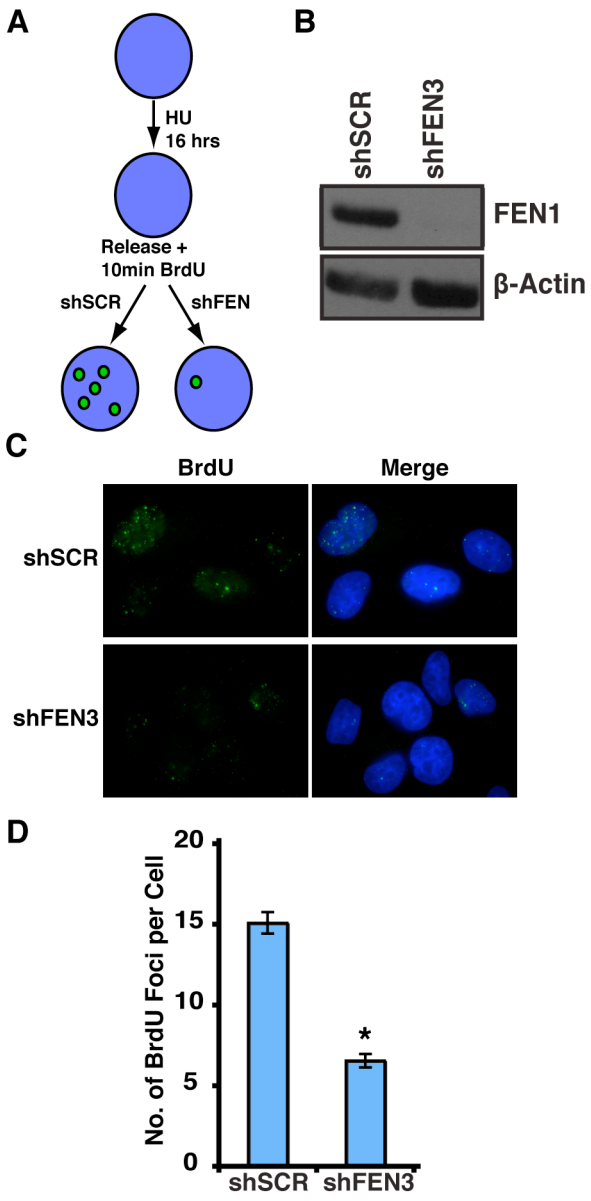

Figure 3.1. FEN1 depletion decreases re-initiation of stalled replication forks.
(A) Schematic of the stalled replication fork re-initiation assay. HU – Hydroxyurea. (B) Western blot showing FEN1 depletion. Short hairpins against FEN1 (shFEN3) or a scrambled sequence (shSCR) were expressed in HeLa cells. FEN1 (upper panel) and β-Actin (lower panel) protein levels were assessed by Western blot analysis. (C) Representative images showing FEN1 depletion decreases BrdU incorporation in HU treated cells. Immunofluorescence was conducted using anti-BrdU (green) and DAPI (blue). (D) Quantification of the number of BrdU foci per cell in HeLa cells with the indicated shRNA. BrdU foci in minimum of a 100 cells were counted for each condition

and the experiment was conducted twice (a representative experiment is presented). The error bars show standard error of the mean (SEM) (*P<0.0001).

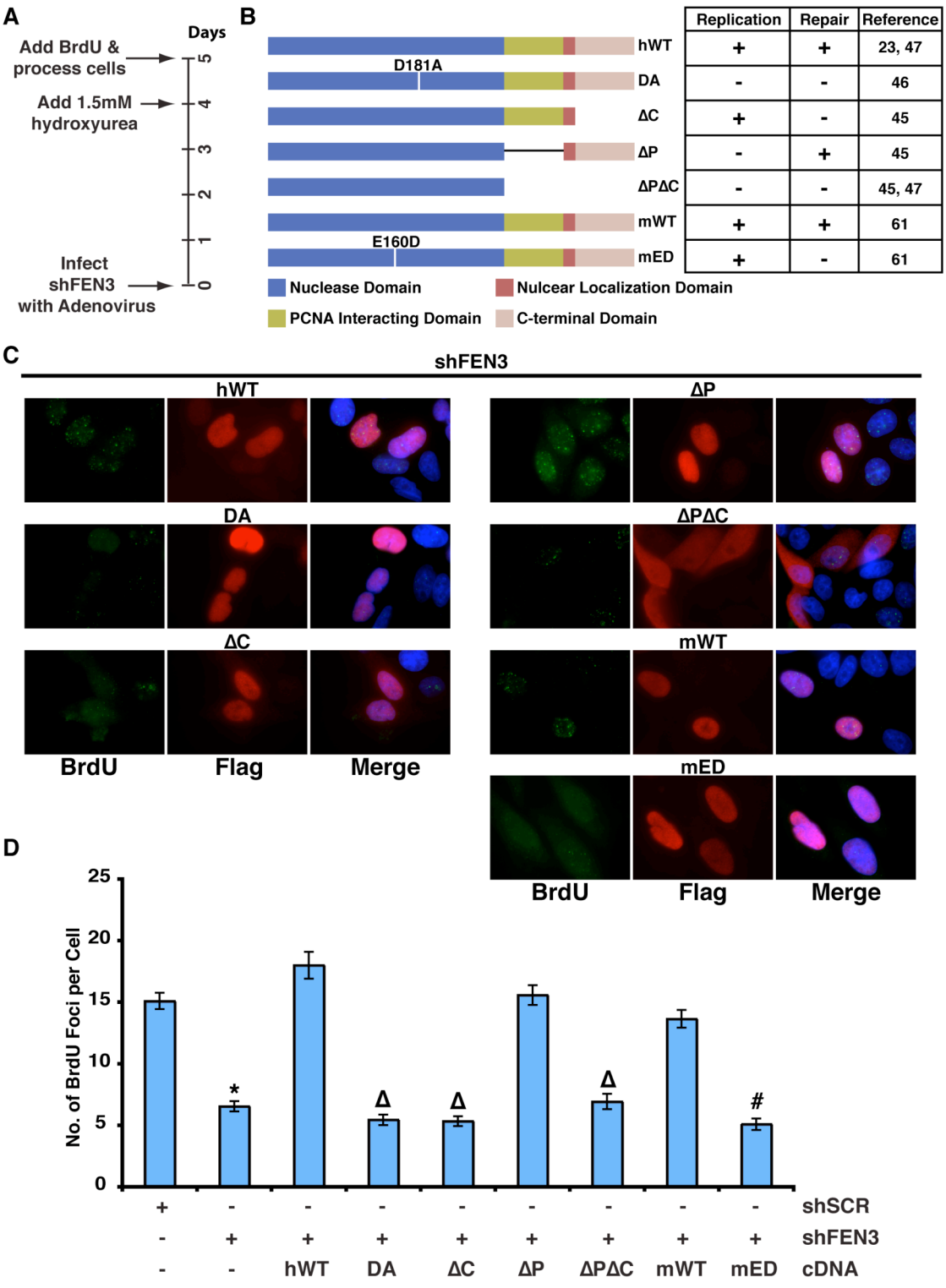

Figure 3.2. The gap endonuclease activity and C-terminal of FEN1 are essential to re-initiate stalled replication forks.

(A) Timeline of experimental procedure given in days. (B) Schematic showing the different mutants of FEN1 used in the subsequent experiments with the different domains of FEN1 and the deletions/mutations. Inferences on whether the different mutants of FEN1 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. (C) Representative images showing BrdU incorporation, after exposure to HU, in FEN1 depleted cells with the indicated adenovirus treatment. Immunofluorescence was conducted using anti-BrdU (green), anti-FLAG (red) and DAPI (blue). (D) Quantification of the number of BrdU foci per cell in FEN1-depleted HeLa cells with the indicated adenovirus. Only cells exogenously expressing FLAG-tagged FEN1 (marked by the red cells in C) were quantified. A minimum of a 75 cells was counted for each condition and the experiment was conducted twice (a representative experiment is presented). The error bars show SEM (*$P<0.0001$ compared to shSCR; $^{\Delta}P<0.0001$ compared to hWT; $^{\#}P<0.0001$ compared to mWT).

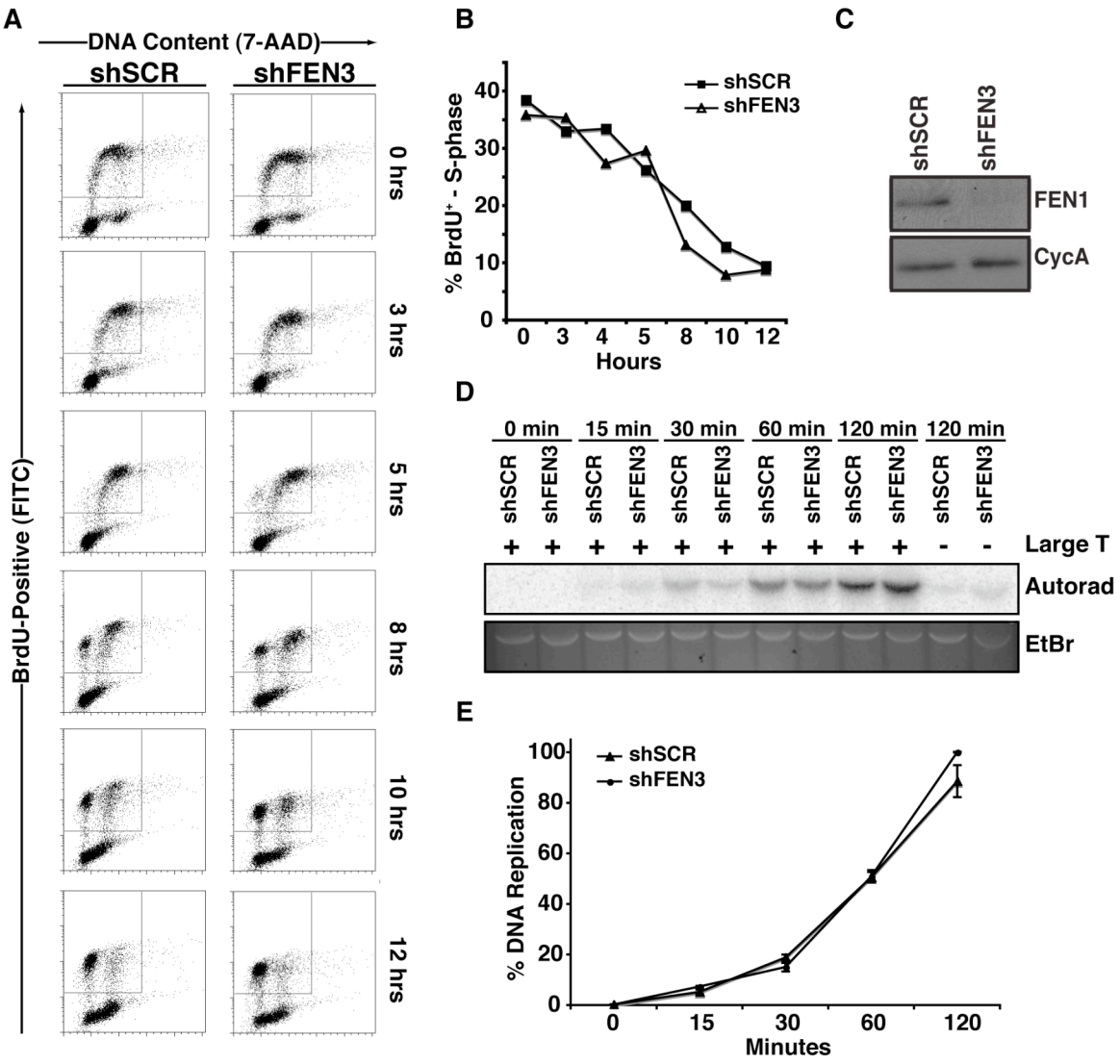

Figure 3.3. FEN1 depletion does not affect S-phase progression and *in vitro* DNA replication.

(A) Progression of cells through the different phases of the cell cycle. HeLa cells expressing shSCR or shFEN3 were labeled with BrdU for one hour and analyzed at the indicated times using the anti-BrdU antibody (FITC-conjugated) and 7-amino-actinomysin D (7-AAD) to mark DNA content. (B) Graph showing the quantification of the number of BrdU-positive cells in S-phase. This experiment was conducted twice (a representative experiment is shown). (C) Western blot of S100 lysates from control and FEN1-depleted HeLa cells. CycA (Cyclophilin A) (lower panel) is shown as a loading control. (D) SV40 Large T dependent *in vitro* DNA replication assay was conducted using lysates from control (shSCR) and FEN1-depleted (shFEN3) HeLa cells as described in the methods. The assay was stopped at 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. A representative experiment is shown. (E) Quantification of the replication products at the indicated times. Two independent experiments were conducted in duplicate and the average of the four experiments is shown. The error bars represent SEM.

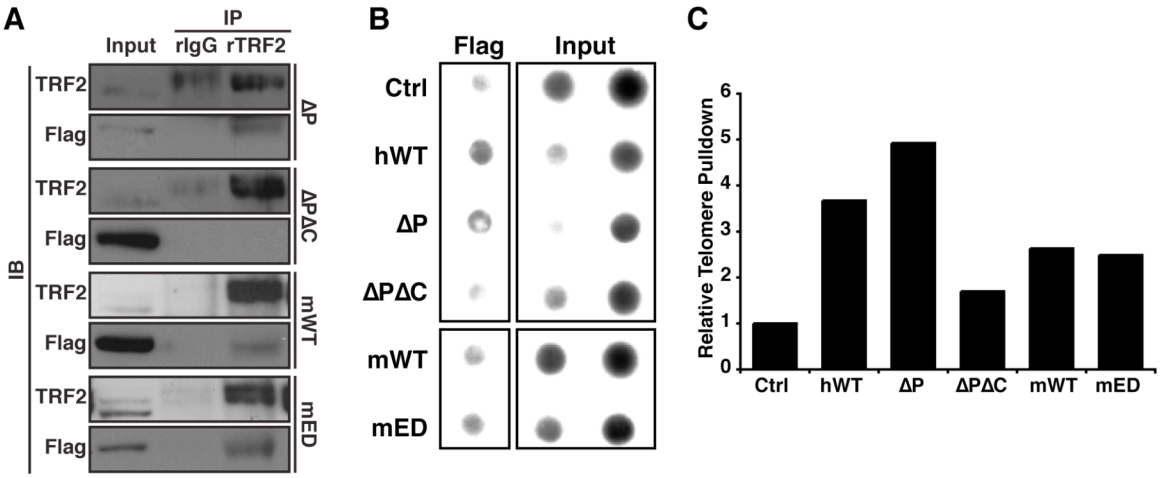

Figure 3.4. FEN1 mutants interact with TRF2 and localize to the telomere.
(A) FEN1 mutants interact with TRF2. 293T cells transfected with flag-tagged mouse wildtype FEN1 (mWT) or FEN1 mutants, ΔP, ΔPΔC and mED and subjected to immunoprecipitation (IP) with an anti-TRF2 antibody as described in the methods. The presence of TRF2 and the FEN1 mutants were detected by immunoblot (IB) using anti-TRF2 and anti-Flag antibodies, respectively. The input lane indicates 10% of total protein used per immunoprecipitation. (B) FEN1 mutants localize to the telomere. Representative ChIP analysis of 293T cells (Ctrl) or 293T cells transfected with wildtype FEN1 (hWT or mWT) or FEN1 mutants (ΔP, ΔPΔC and mED), subjected to immunoprecipitation with the M2 flag antibody. Precipitated DNA was probed for the presence of telomeric sequences as described in the methods. The inputs indicate 0.1% and 0.2% of the total protein extract. (C) Quantification of the representative ChIP assay. Percent of telomere pulldown was calculated using input DNA and the control pulldown percentage was set to 1.

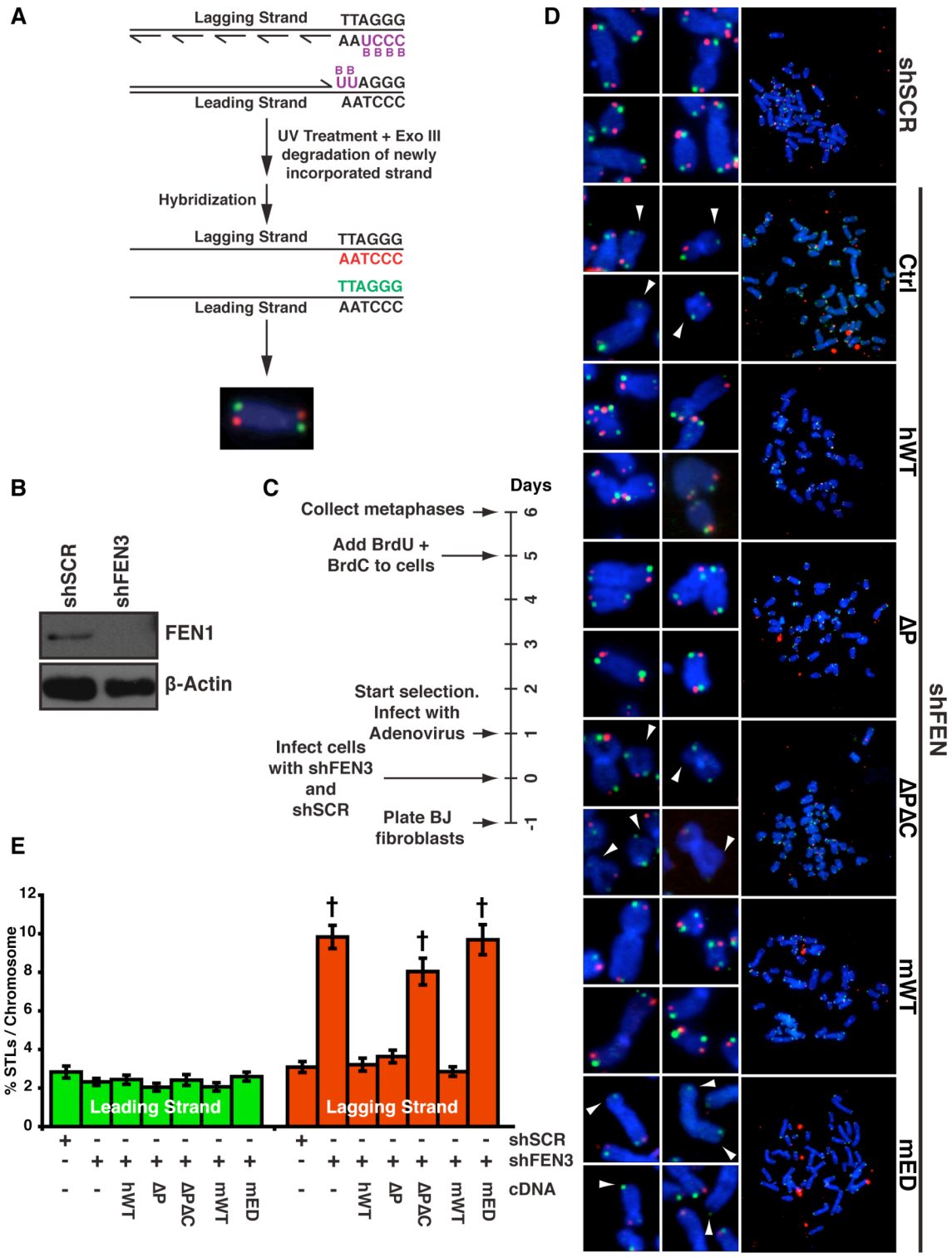

Figure 3.5. The gap endonuclease activity of FEN1 is essential for its function at the telomere.

(A) Chromosome Orientation – Fluorescent *in situ* hybridization (CO-FISH) schematic. Newly synthesized DNA strands incorporate BrdU and BrdC. UV and ExoIII treatment results in degradation of newly synthesized DNA containing BrdU and BrdC, and the template strands are hybridized with Cy3-$[CCCTAA]_3$ (red, lagging strand) and FLU-$[TTAGGG]_3$ (green, leading strand) probes. (B) Western blot of FEN1 (upper panel) from BJ fibroblasts infected with shSCR and shFEN3. β-Actin (lower panel) is shown as a loading control. (C) Timeline of experimental procedure given in days. (D) Representative CO-FISH of metaphases from BJ fibroblasts expressing shSCR or shFEN3 and the indicated FEN1 proteins. Ctrl refers to cells that do not express exogenous FEN1 protein. Color schemes are as described in (A). DNA was stained with 4',6-diamidino-2-phenylindole (DAPI; blue). The arrowheads indicate missing telomeres. (E) Quantification of sister telomere losses on metaphase chromosomes following depletion of endogenous FEN1 and expression of the indicated FEN1 protein, depicted as percentage of chromosomes with missing leading and lagging 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 SEM.

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CHAPTER 4: FEN1 CONTRIBUTES TO TELOMERE STABILITY IN ALT-POSITIVE TUMOR CELLS

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Abstract

Abrogation of telomere stability through loss of function mutations in telomere binding proteins contributes to genomic instability and cancer progression. Recently, Flap endonuclease 1 (FEN1) was shown to contribute to telomere stability in human cells that had not yet activated a telomere maintenance mechanism, suggesting that abrogation of FEN1 function influences the transformation process by compromising telomere stability and driving genomic instability. Here, we analyze the telomeres in human cancer cells following FEN1 depletion. We show that FEN1 is required for telomere stability in cells that rely on the alternative lengthening of telomere (ALT) mechanism. Indeed, FEN1 depletion resulted in telomere dysfunction, characterized by formation of telomere dysfunction-induced foci (TIFs) and end-to-end fusions in ALT-positive cells. In contrast, no telomere phenotype was observed in telomerase-positive cells upon FEN1 depletion, suggesting that ongoing telomerase activity protected telomeres. In consonance with this, we found that expression of the catalytic component of telomerase (hTERT) but not an inactive allele rescued telomere dysfunction upon FEN1 depletion in ALT cells. Our data suggests that mutations that arise in FEN1 impact telomere stability and genome fidelity by promoting telomere fusions and anaphase-bridge-breakage cycles that further drive genome instability and thereby contribute to the transformation process.

Results and Discussion

Loss of function mutations in genes involved in detection, signaling, and repair of DNA damage correlate with increased genomic instability and cancer incidence. Proper

maintenance of telomere function is critical to genomic stability. As a functional DNA-protein complex, the telomere distinguishes the end of a chromosome from a *bona fide* double strand break. Destabilization of telomere structure compromises its function and renders it susceptible to the actions of the DNA repair machinery, often leading to chromosome end-to-end fusions (de Lange, 2005). Telomeric fusions result in anaphase bridge-breakage cycles, which contribute to genomic instability and drives the transformation process (Artandi & DePinho, 2000).

The telomere consists of repetitive double and single stranded DNA (TTAGGG) and six core proteins referred to as Shelterin (or Telosome) (de Lange, 2005; Liu et al., 2004) that together shield the telomere from the DNA repair machinery. In addition to the Shelterin components, a growing list of accessory proteins localize to the telomere and play essential roles in telomere maintenance (Blasco, 2005). For example, ATM, WRN and Ku influence telomere stability where mutation and/or depletion of these proteins result in cancer syndromes (Blasco, 2005). Together these data underscore the importance of these DNA replication and repair proteins in telomere maintenance and high fidelity maintenance of the genome.

Recently, we demonstrated that Flap endonuclease 1 (FEN1) is a telomere binding protein that plays an important role in maintaining telomere stability in human cells (Saharia et al., 2008). RNAi-directed depletion of FEN1 led to sister telomere loss (STL) that was restricted to telomeres replicated by lagging strand DNA synthesis (Saharia et al., 2008). FEN1 is a multifunctional nuclease that participates in replication (Li et al.,

1995), long-patch base excision repair (Prasad et al., 2000), homologous recombination (Kikuchi et al., 2005), re-initiation of stalled replication forks and DNA degradation in apoptotic cells (Zheng et al., 2007; Zheng et al., 2005). Work in yeast revealed that disruption of the FEN1 homolog, *Rad27*, results in a DNA mutator phenotype and telomere dysfunction (Parenteau & Wellinger, 1999; Parenteau & Wellinger, 2002; Tishkoff et al., 1997). Similarly, mice heterozygotic for FEN1 display a mutator phenotype and are predisposed to develop neoplasias (Kucherlapati et al., 2007). Given that the initiation and development of cancer results in part from accumulation of genetic instability and that telomere dysfunction can contribute to this instability, abrogation and/or mutation of genes such as FEN1 may contribute to this process. Indeed, such a role for FEN1 was suggested by a recent report demonstrating that knock-in of a FEN1 mutant gene identified in human cancers resulted in cancer predisposition in a murine model (Zheng et al., 2007). Specifically, when a FEN1 mutant that abrogates a repair function known as the gap endonuclease (GEN) activity was knocked into the analogous murine locus, animals developed several pathologies including lung tumors (Zheng et al., 2007). This observation together with our previous findings, raise the possibility that FEN1 depletion (and/or mutation) impacts genomic stability by abrogating telomere stability and in this way contributes to the transformation process.

FEN1 is required for telomere stability in ALT cells. In previous work we found that FEN1 depletion in somatic cells that have not activated a telomere maintenance mechanism led to telomere dysfunction that was compensated for by ectopic expression of the catalytic component of telomerase (hTERT) (Saharia et al., 2008). This

observation raised the possibility that FEN1 depletion or mutation might impact telomere stability in transformed cells that utilized the ALT mechanism of telomere maintenance. Thus, we investigated whether FEN1 depletion affected telomere stability in a human osteosarcoma cell line (U2OS) that is telomerase negative and maintains its telomeres via the recombination-dependent ALT mechanism (Bryan et al., 1997). To control for possible off-target effects associated with RNAi, we utilized two independent lentiviral constructs expressing short hairpin RNAs (shRNA) targeted to FEN1's coding region and 3' untranslated region (shFEN and shFEN3, respectively). In addition, a short hairpin consisting of a scrambled sequence (shSCR) was also introduced into these cells and functioned as a negative control.

Following transduction, FEN1 protein expression was determined by Western blot analysis. Expression of the two hairpins (shFEN and shFEN3) led to a significant reduction in FEN1 protein levels (**Figure 4.1A**). To determine the effect of FEN1 depletion on telomere stability, metaphase spreads were prepared and analyzed for telomere dysfunction. Metaphases were labeled using fluorescent *in situ* hybridization (FISH) with telomere (red) and centromere (green) probes and analyzed (**Figure 4.1B**). Analysis of metaphase spreads revealed that FEN1 depletion led to telomere dysfunction characterized by chromosomal end-to-end fusions that retained telomeric sequences at the fusion points (**Figure 4.1B**). U2OS cells expressing shSCR displayed 0.1 telomere fusion events per cell. In contrast, FEN1 depletion resulted in a significant increase in the number of telomere fusions observed in U2OS cells to 0.33 ($P<0.001$) and 0.38 ($P<0.01$) events in the shFEN and shFEN3 expressing cells, respectively (**Figure 4.1C**).

Moreover, the percentage of cells having one or more fusion events increased from 6.7% in the shSCR expressing cells to 27% and 33%, respectively in the shFEN and shFEN3 expressing cells.

The telomeric impact of FEN1 depletion was not unique to U2OS cells. Indeed, depletion of FEN1 in a second ALT cell line GM847 (**Figure 4.1A**) also resulted in a significant increase in telomere dysfunction (**Figure 4.1B-E**). GM847 cells infected with a control virus displayed 0.07 telomeric fusions. In contrast, expression of shFEN and shFEN3 led to 0.8 and 0.57 telomeric fusions, respectively with 50-60% of the metaphases analyzed displaying one or more telomeric fusion ($P<0.0001$; **Figure 4.1C**).

Several groups have demonstrated that DNA damage foci referred to as telomere dysfunction-induced foci (TIFs) are readily detectable when telomere stability is compromised (d'Adda di Fagagna et al., 2003; Takai et al., 2003). Therefore, to confirm the presence of telomere dysfunction upon FEN1 depletion, we examined cells for the presence of γH2AX foci at telomeres. As expected, we found that FEN1 depletion led to an increase in TIFs (Figures 1D and 1E). In GM847 cells infected with a control hairpin, we noted that 27.9% of the cells had greater than 5 TIFs per cell, whereas upon infection with shFEN, the number of cells with greater than 5 TIFs increased to 78.2% (**Figure 4.1E**). Together these data demonstrate that FEN1 contributes to telomere stability in immortal cells and that its depletion leads to telomere dysfunction in cells that maintain their telomeres via the ALT mechanism.

FEN1 depletion in telomerase positive cells does not impact telomere stability. Human cancer cells maintain stable telomere lengths through activation of either ALT or the telomerase enzyme. Above we demonstrate that FEN1 depletion leads to telomeric fusions in ALT cells. In addition, in a previous report we demonstrated that FEN1 depletion in mortal human fibroblasts led to sister telomere losses (STL) that were rescued by expression of catalytically active telomerase. Together, these data argue that tumor cells that have activated telomerase would be insensitive to FEN1 depletion. To test this hypothesis directly, we examined how FEN1 depletion impacted telomere stability in telomerase-positive cells. HeLa cells, a cervical cancer cell line that utilizes telomerase for telomere maintenance, were transduced with viral vectors expressing shSCR, shFEN, or shFEN3. Upon FEN1 depletion (**Figure 4.2A**), cells were analyzed for telomeric fusions as described above. As expected, depletion of FEN1 did not result in telomeric fusions (**Figure 4.2B** and **4.2C**). Similar results were observed in a second telomerase positive ovarian cancer cell line, 36M (**Figure 4.2**). These results indicate that cells that utilize endogenous telomerase for telomere maintenance are insensitive to FEN1 depletion at the telomere.

Catalytically active telomerase rescues FEN1 depletion at the telomeres. Depletion of FEN1 in cells that maintain stable telomeres via the ALT mechanism resulted in telomere dysfunction. In contrast, telomere stability was unperturbed in telomerase-positive cells following FEN1 depletion. These results were reminiscent of our earlier findings that telomerase rescued sister telomere loss in cell lines that had not yet activated a telomere maintenance program (Saharia et al., 2008). To determine whether the catalytic activity

of telomerase was required to protect telomeres in cells that utilized the ALT mechanism, we expressed the catalytic component of telomerase (hTERT) in GM847 cells (GM847-hTERT) (**Figure 4.3A**) (Hahn et al., 1999). Expression of hTERT reconstitutes telomerase activity in these cells, leading to lengthening of the shortest telomeres (Grobelny et al., 2001; Hemann et al., 2001; Perrem et al., 2001; Teixeira et al., 2004).

Introduction of shRNA constructs targeting FEN1 into GM847-hTERT cells resulted in a significant reduction in protein expression (**Figure 4.3B**). Analysis of metaphase spreads from cells expressing the FEN1 hairpins compared to those expressing a control hairpin did not reveal an increase in telomere dysfunction (**Figure 4.3C**). To determine whether it was the telomere extension activity of telomerase that compensated for FEN1 depletion at the telomeres as suggested by our earlier work, we utilized a catalytically inactive, dominant negative allele of hTERT (DN-hTERT) (**Figure 4.3A**). This allele was chosen because previous work demonstrated that it had no impact on telomere stability in GM847 cells (Stewart et al., 2002). In contrast to that observed in GM847-hTERT cells, FEN1 depletion in GM847-DN-hTERT cells resulted in increased telomeric fusions (**Figure 4.3B** and **4.3C**). FEN1 depletion increased the number of telomere fusion events per cell from 0.15 events in control cells, to 0.55 and 0.53 in cells expressing the two hairpins against FEN1 ($P<0.01$; **Figure 4.3C**). There was also a large increase in the percentage of metaphases possessing one or more fusions (46% versus 13% in the control cells). The inability of DN-hTERT to rescue FEN1 depletion at the telomere indicates that the catalytic activity of telomerase is important for this rescue and suggests that telomeric extension by telomerase is important in the absence of FEN1.

FEN1 is a structure specific endonuclease that acts in DNA replication and repair. Here, we assessed the role of FEN1 in the context of telomere stability. We found that depletion of FEN1 in cancer cells that maintain their telomeres via the ALT mechanism results in telomere dysfunction characterized by increases in the number of TIFs and telomeric fusions. In contrast, FEN1 depletion did not lead to telomere dysfunction in telomerase positive cells. Telomere fusions observed in ALT cells were rescued by the expression of catalytically active telomerase but not a catalytically dead enzyme. Given our earlier work demonstrating that single telomeres were lost in pre-crisis human cells upon FEN1 depletion (Saharia et al., 2008) this result suggests that the ability of telomerase to elongate the shortest telomeres was protective. Together these results suggest that abrogation of FEN1 function in telomerase-negative tumor cells results in increased genomic instability by compromising telomere stability that may contribute to tumor progression.

DNA replication is a challenging cellular event that is prone to errors that can result in loss of genomic fidelity. Sequences that offer significant challenges to the DNA replication machinery are repetitive DNA sequences, particularly those containing triplicate repeats (Fouche et al., 2006), which hinder replication fork progression. Therefore, it is not surprising that the repetitive G-rich nature of the telomere presents a challenging template for the replication machinery (Gilson & Geli, 2007). Indeed, this has been underscored by *in vitro* DNA replication systems that have shown that replication of telomeric sequences is less efficient than randomized sequences due to a

significant increase in stalled DNA replication forks within telomeric sequences (Ohki & Ishikawa, 2004). For these reasons, the impact of loss of function mutations in genes that facilitate replication fork progression and restart would be expected to have a profound impact at the telomere. Indeed this has been observed with the Werner protein (Crabbe et al., 2004) as well as FEN1 (this study). FEN1 functions with the Werner protein to process branch migrating structures that resemble stalled replication forks (Sharma et al., 2004). Therefore, given that unresolved stalled replication forks lead to DNA double strand breaks (Branzei & Foiani, 2005), loss of Werner or FEN1 activity would be expected to result in telomere loss and subsequent end-to-end fusions.

Our previous work demonstrated that FEN1 depletion led to sister telomere losses but no significant telomeric fusions were observed (Saharia et al., 2008). Why then do we observe telomeric fusions in cells that utilize the ALT mechanism? Telomeres within ALT cells are in a constant state of flux, where they undergo rapid elongation and shortening (Londono-Vallejo et al., 2004). This dynamic fluctuation results in chromosome ends with extremely short telomeres that are unlikely to adequately protect telomere ends from recognition by DNA damage surveillance mechanisms. As a result, telomeres within ALT cells are recognized as DNA damage, as evidenced by the presence of TIFs or γH2AX foci at many telomeres (**Figure 4.1D** and **4.1E**, shSCR) (Nabetani et al., 2004). Depletion of FEN1 appears to exacerbate telomere dysfunction by producing signal free ends in fibroblasts (Saharia et al., 2008) and increasing the number of TIFs in ALT cells (**Figure 4.1D** and **4.1E**), thus leading to additional substrates capable of participating in end-to-end fusions. Because telomerase acts on the

shortest telomeres (Forstemann et al., 2000; Marcand et al., 1999; Ouellette et al., 2000) it would be recruited to those chromosome ends that experienced a catastrophic loss due to a stalled and unresolved replication fork or failure to cap the telomere. Telomerase could then extend the short telomeres, stabilizing them and rescuing telomere dysfunction. These studies suggest that FEN1 mutation contributes to the transformation process by increasing genomic instability through telomere loss and subsequent end-to-end fusions. Further, abrogation of FEN1 function in tumor cells that do not utilize telomerase may result in additional genomic instability leading to progression of the neoplastic state.

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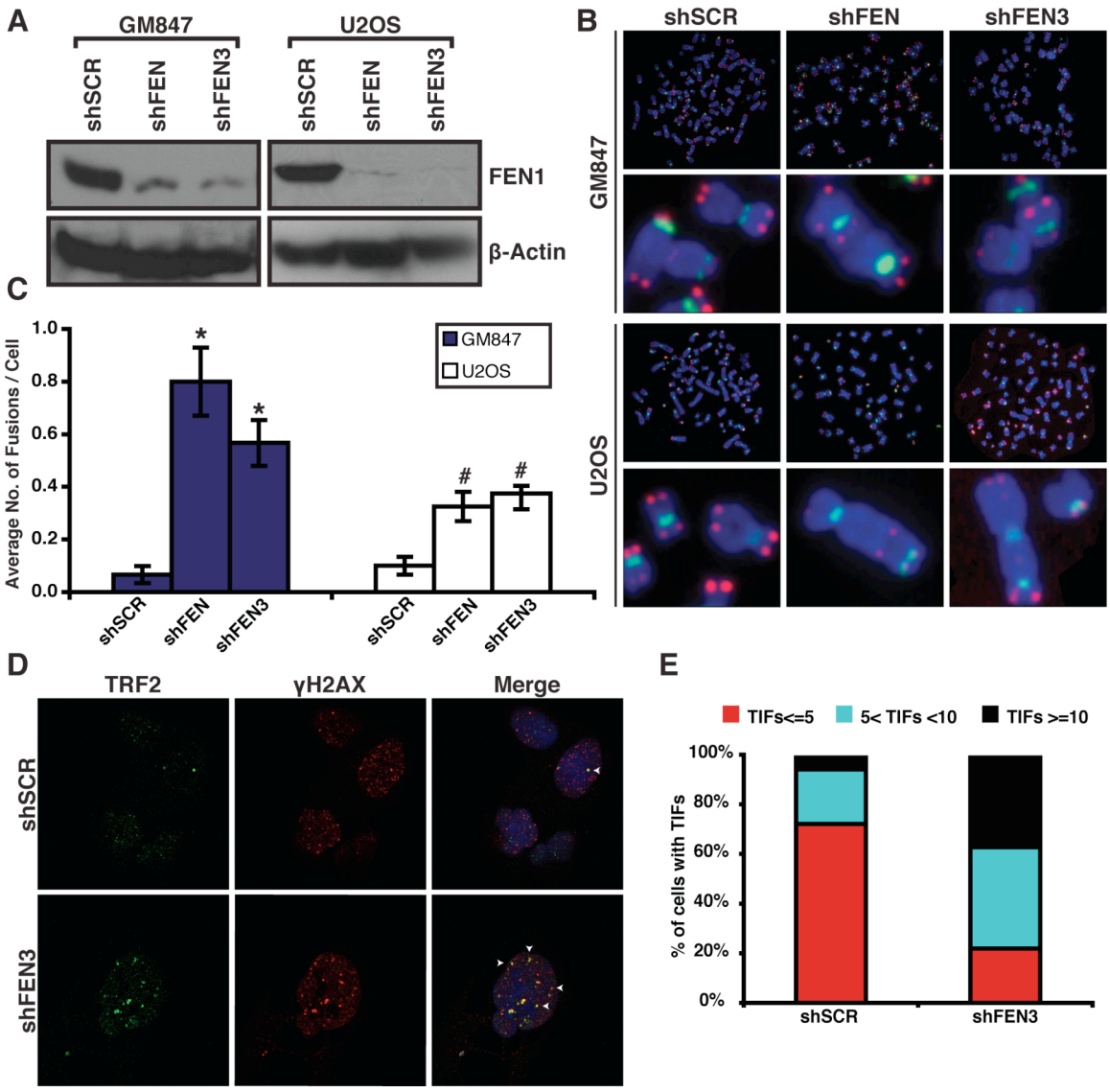

FIGURE 4.1. FEN1 depletion leads to telomere dysfunction in ALT cells.
(A) Two independent shRNAs targeting FEN1 (shFEN and shFEN3) and one consisting of a scrambled sequence (shSCR) were introduced by lentiviral infection into GM847 and U2OS cells and FEN1 expression was determined by western blot analysis. **(B)** Representative metaphases from GM847 and U2OS cells following indicated shRNA expression. FISH analysis was conducted using Cy3-labeled TTAGGG probes (in red) and FITC-labeled centromere probes (in green). DNA was stained using 4',6-diamidino-2-phenylindole (DAPI; in blue). The lower panel shows a higher magnification image of the metaphase chromosomes. **(C)** Quantification of telomere fusion events observed after indicated treatments of GM847 (blue bars) and U2OS (white bars) cells. A minimum of 60 metaphases was analyzed per treatment in a blinded fashion. Statistical analysis was conducted using the Wilcoxon Two-Sample Test. (*P<0.001; #P<0.01). **(D)** FEN1 depletion increases TIF formation in GM847 cells. Immunofluorescence was conducted using anti-TRF2 (green: Santa Cruz, CA, USA; H-300), anti-γH2AX (red: Upstate, NY,

USA; 05-636) and DAPI (blue). Confocal images were acquired on a Zeiss Axiovert 200 microscope. **(E)** TIF quantification in GM847 cells. A minimum of a 100 cells was counted for each condition and the average for two experiments is presented. Cell culture, western blot analysis, viral constructs and production as well as metaphase preparation and statistical analyses were as previously described (Saharia et al., 2008; Stewart et al., 2003; Stewart et al., 2002).

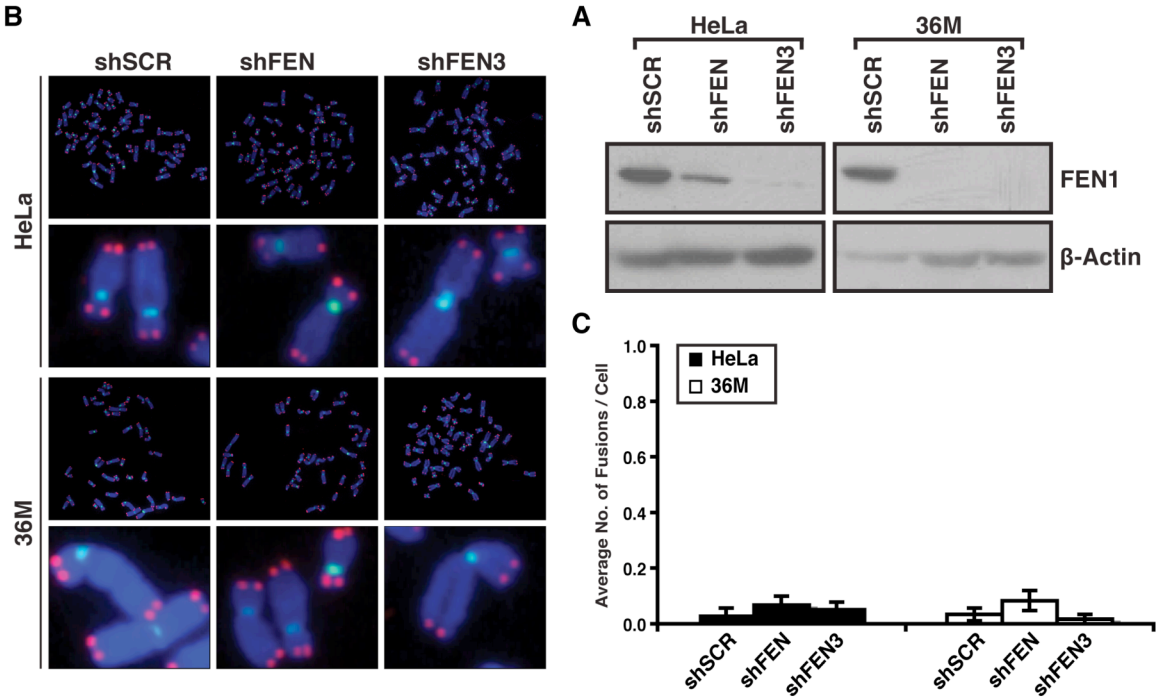

FIGURE 4.2. FEN1 is not essential for telomere stability in telomerase positive cells. **(A)** Western blot analysis reveals that introduction of two different shRNAs targeted to FEN1 leads to reduction in FEN1 protein levels in HeLa and 36M cells. **(B)** Representative metaphases from HeLa and 36M cells following shRNA expression. **(C)** Quantification of telomere fusion events observed following shRNA expression in HeLa (white bars) and 36M (black bars) cells. A minimum of 60 metaphases was analyzed per treatment in a blinded fashion. Statistical analysis was conducted using the Wilcoxon Two-Sample Test.

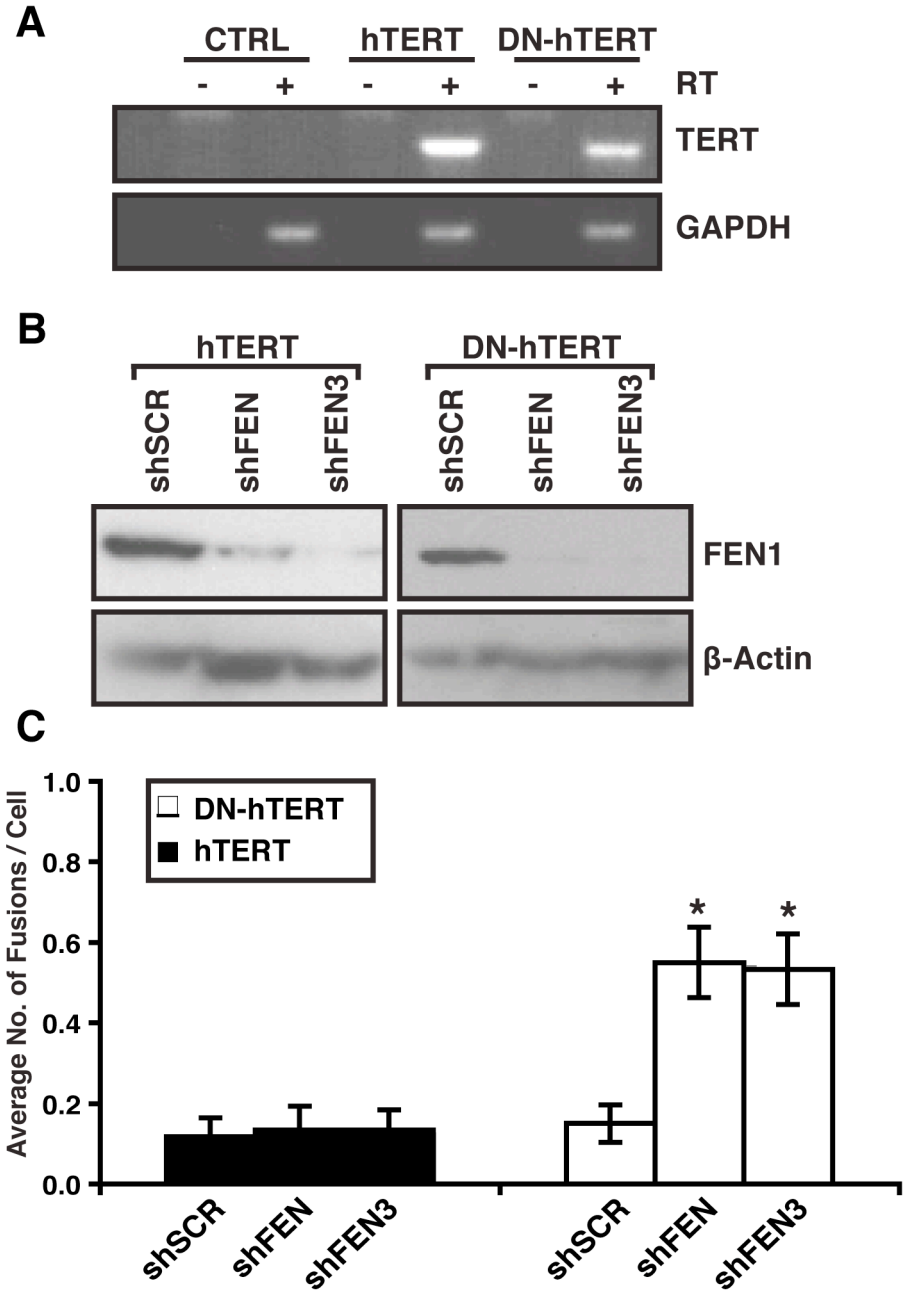

FIGURE 4.3. Catalytically active telomerase rescues telomere instability upon FEN1 depletion.

(A) Reverse transcriptase (RT) PCR demonstrating exogenous expression of the catalytic component of telomerase (hTERT), dominant negative hTERT (DN- hTERT), or uninfected (CTRL). RNA isolation, PCR and primers were as previously described (Hahn et al., 1999). **(B)** Western blot analysis reveals that introduction of two different shRNAs targeted to FEN1 leads to reduction in FEN1 protein levels in hTERT and DN-hTERT cells. **(C)** Quantification of telomere fusion events following shRNA expression in hTERT (white bars) and DN-hTERT (black bars) cells. A minimum of 60 metaphases

was analyzed per treatment in a blinded fashion. Statistical analysis was conducted using the Wilcoxon Two-Sample Test (*P<0.01).

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CHAPTER 5:

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Summary

Telomeres need to be replicated with high fidelity to ensure genomic stability. Lack of fidelity will lead to loss of telomere integrity, its recognition as a DNA double strand break, end-to-end fusions, fusion-breakage-bridge cycles and ultimately to genomic instability. Considering that genomic instability is a driving force for cancer, high fidelity telomere replication is critical for tumor suppression. Flap Endonuclease 1 (FEN1) is a structure specific metallonuclease that performs several roles in DNA metabolism. In Chapters 2, 3 and 4 I demonstrate that FEN1 is critical for telomere stability. FEN1 depletion led to telomere dysfunction characterized by recognition of the telomeres as DNA double strand breaks (DSBs), activation of a DNA damage response (DDR) at telomeres, loss of single sister telomeres (STLs) in primary fibroblasts and telomere fusions in ALT-positive cancer cells. Despite the impact on telomeres, no significant defects were observed on DNA replication and cell cycle progression, indicating a telomere specific role for FEN1 that is independent of its role in Okazaki fragment maturation. The work presented in the preceding chapters thus allows us to postulate that FEN1 contributes to telomere stability by ensuring the efficient resolution of stalled replication forks at mammalian telomeres. The following sections underscore the significance of this work and how it contributes our understanding of telomere biology, genome stability and tumorigenesis.

5.2 FEN1 in DNA Replication and Repair

FEN1 is an important DNA replication and repair protein involved in genome maintenance. In an *in vitro assay* utilizing purified proteins, FEN1 was identified as one of the ten essential components required for DNA replication [1]. However, deletion of the FEN1 gene in several different organisms does not inhibit DNA replication, which continues with relatively minor defects. Indeed, *rad27Δ* in *S. cereviseae*, *rad2Δ* in *S. pombe*, and FEN1 deletion in chicken DT-40 cells does not inhibit bulk DNA replication [2-4]. However, deletion of mammalian FEN1 leads to early embryonic lethality in mice [5], suggesting a critical function for FEN1 in mammalian cells. My work demonstrates that FEN1 depletion in human cells does not affect DNA replication *in vitro* or progression through S-phase (Chapter 3). Furthermore, FEN1 depletion, in the absence of telomere dysfunction, does not lead to genomic instability in primary fibroblasts (Chapter 2) indicating that another nuclease, such as Dna2 or Exo1, is able to compensate for FEN1 depletion during Okazaki fragment maturation [6-10]. The aforementioned studies utilized complete gene knockouts to investigate FEN1 function whereas my work in human cells has utilized virus-based shRNA technology to deplete FEN1 from the cells, which does not completely eliminate the endogenous protein. Therefore, it is possible that the small amount of FEN1 remaining is sufficient for uninhibited DNA replication. However, FEN1 depletion does affect telomere stability, indicating that there is functional loss of this protein. Only construction of human cells with FEN1 deletion from the genomic locus will conclusively address whether it is essential for human DNA replication.

Cytogenetic analysis upon FEN1 depletion in human telomerase-positive cells, lacking telomere dysfunction, revealed few aberrations indicating an absence of genomic instability. However, this is a macro view and therefore only one facet of genomic instability. Work in yeast and in mice show that FEN1 also plays a critical role in maintaining stable microsatellite and triplet repeats in the genome [11, 12]. This observation was species-specific, as the same was not seen in drosophila upon FEN1 deletion [13, 14]. Additionally, a recent study in human cells observed no expansion of the CAG Huntington's locus upon FEN1 depletion [15], indicating that human cells do not replicate the genomic instability phenotypes observed in other organisms. Together, these studies support our observations on the lack of genomic instability in telomerase-positive cells upon FEN1 depletion. However, a more cautious analysis of the different micro-satellite, mini-satellite and rDNA repeat regions via Southern analysis of genomic DNA from FEN1-depleted human cells [15] needs to be conducted to investigate genome stability.

Although our data indicates that FEN1 is not essential for Okazaki fragment processing in human cells, work from Chapter 3 demonstrates a significant novel role for FEN1 in DNA repair through the re-initiation of stalled replication forks. This work supports recent studies suggesting that FEN1 plays a role in the re-initiation of stalled replication forks [16, 17]. It was previously shown that treatment of cells with cisplatin or mitomycin C (MMC), DNA cross-linkers that stall replication forks,

increased FEN1 association with WRN, a RecQ helicase, and led to the co-localization of this complex to the stalled forks [16]. Stalled replication forks can be converted to Holliday junctions (HJs) through fork regression, branch migration and the annealing of nascent DNA strands [18]. Together the WRN-FEN1 complex is able to process these branch migrating structures known as "chickenfoot" structures [16, 18]. FEN1 also possesses a gap endonuclease (GEN) activity, which can be stimulated by WRN and enhances the cleavage and resolution of chickenfoot and bubble-shaped DNA structures [17]. One can speculate that the telomeric lagging strand forms a putative G-quadruplex (G4) lesion, stalls the moving replication fork that regresses and forms chickenfoot structures, which can now be processed by the WRN-FEN1 complex to re-initiate the stalled replication fork. Furthermore, evidence from *E.coli* DNA replication and repair demonstrates that upon stalling of a replication fork, the RecQ DNA helicase and RecJ, a 5' to 3' exonuclease, can process the regressed replication fork intermediate to reinitiate replication [19-21]. The mammalian homologs of the RecQ-RecJ proteins that participate in the processing of stalled replication forks have not yet been identified. However, we could reason that such function could be assumed by a RecQ helicase such as WRN or BLM and a RecJ nuclease, FEN1. This hypothesis could be tested by gene replacement experiments of the *E. coli* genes with their mammalian counterparts.

Though the above studies suggest a role for FEN1 in the re-initiation of stalled replication forks, this work is the first to conclusively demonstrate it. FEN1's role in fork re-initiation suggests that its depletion should sensitize cells to replication stress-causing

reagents such as HU, mitomycin C (MMC) and cisplatin. Indeed, recent work has demonstrated that FEN1-depleted cancer cells are sensitive to cisplatin [22]. Our knowledge of FEN1 function in the re-initiation of stalled replication forks is still in its nascent stages and the experiments suggested herein will give us a greater understanding of its role in the re-initiation of stalled replication forks and at telomeres.

5.3 Telomere Replication: FEN1 at the ends

Replication of telomeres inherently possesses several "high risk" elements (**Figure 1.4**). Telomeric DNA consists of a highly repetitive sequence and has a greater probability of forming secondary structures. In addition, telomeres are replicated from a single unidirectional fork and remain unreplicated if the fork collapses. The intricacies in telomere replication are compounded by the presence of the T-loop, which needs to be resolved before replication and reformed post-replication. To address these unique challenges the DNA replication machinery undertakes specialized action for efficient telomere replication and stability [23, 24].

Indeed, several studies have indicated that telomere replication and stability is influenced by an increasing number of DDR proteins. Recent work demonstrates that during replication of human telomeres the ATR-dependent DDR machinery is recruited together with the DNA replication machinery [25]. The ATR-dependent repair machinery is postulated to be present at the telomere in response to stalled replication

forks, leading to their resolution and efficient telomere replication. FEN1 localizes to the telomere during telomere replication [25]. It is possible that FEN1 impacts telomere stability in two ways that are not mutually exclusive. It could be recruited to the telomere in S-phase for DNA replication with PCNA and the replication machinery for the processing of Okazaki fragments [26]. Alternatively, it could engage in the efficient resolution of stalled replication forks with the ATR-dependent repair machinery [16, 17]. Our data do not negate the possibility of either scenario of FEN1 function at the telomere. However, they do demonstrate that the FEN1 function in the re-initiation of stalled replication forks is essential to telomere stability whereas FEN1's ability to interact with PCNA and process Okazaki fragments is dispensable for telomere stability.

Although the results presented suggest the presence of stalled replication forks at human telomeres after FEN1 depletion, this has not been directly shown. Direct evidence demonstrating the presence of stalled replication forks upon FEN1 depletion in human cells has been difficult to obtain due to technical hurdles. Below, I have outlined some of the techniques that may be utilized to investigate the presence of stalled replication forks at telomeres upon FEN1 depletion. First, 2D gel electrophoresis of the replication intermediates of human telomeres would identify the presence of stalled forks, if present, upon FEN1 depletion. However, this approach is technically challenging and given the stochastic nature of the observed phenotype, would make the analysis extremely difficult. Alternatively, taking advantage of the model organism, *S. pombe*, where 2D gel electrophoresis of telomere replication intermediates is simpler due to fewer

chromosomes [27], we can investigate whether *rad2Δ* (the FEN1 homolog) cells have stalled replication forks at their telomeres. Given that human FEN1 can compensate for its yeast homolog [28], this technique will also provide a clear way to investigate the role of certain mutations in telomere replication.

Thirdly, the most direct method to investigate the presence of stalled replication forks at human telomeres upon FEN1 depletion is through single molecule analysis of replicating DNA (SMARD) [29]. This technique double labels replicating telomeres with two BrdU analogs that are temporally separated, allowing the direct measurement of the number of stalled replication forks per number of replicating telomeres. SMARD analysis affords us the opportunity to investigate the role of FEN1 in the re-initiation of stalled replication forks both at the telomere and away from it [29]. Finally, a simpler but indirect method for investigating the presence of stalled replication forks is to identify ATR, ATRIP or phosphorylated-RPA foci at telomeric DNA [29, 30]. If FEN1 depletion leads to increased numbers of stalled replication forks at telomeres, I would predict increased localization of ATR-dependent DDR machinery there. The experiments outlined above will provide further evidence of stalled replication forks at telomeres upon FEN1 depletion.

Recently, telomeres have also been identified as fragile sites [29]. This result suggests that fragile site expression (fragile sites are said to be 'expressed' when they exhibit cytogenetic abnormalities such as gaps and breaks on metaphase chromosomes) at

telomeres might contribute to the inefficient replication of telomeres and lead to loss of single sister telomeres (STLs). Fragile sites are usually expressed upon the induction of replication stress and in the absence of certain proteins that help maintain those sites, such as BLM [31]. Considering the complexity of telomere replication and the stalling of replication forks traversing the telomere, I propose that the telomere provides a natural form of replication stress. My results indicate that FEN1 depletion leads to an increase in replication stress at telomeres, suggesting a role for it suppressing fragile site expression at telomeres. To investigate FEN1's role in the maintenance of telomere fragile sites, we could monitor fragile site expression at telomeres (presence of abnormal telomere signals) in FEN-1 depleted cells. Additionally, it will be interesting to subject FEN1-depleted cells to further replication stress such as exposure to low levels of aphidicolin, an inhibitor of DNA polymerases α and δ, and analyze fragile site expression on metaphase chromosomes, both at and away from the telomere. If FEN1 were involved in the fragile site maintenance at the telomere and across the genome, we would expect an increase in fragile site expression. Alternatively, the increase in fragile telomeres may be a secondary effect of FEN1 depletion and we would then expect to see no modulation of fragile site expression on a genomic scale upon inducing replication stress.

5.4 FEN1, TRF2 and the RecQ helicases, BLM and WRN

Interestingly, expression of a C-terminal deletion mutant of FEN1, which abolishes interaction with WRN [32], cannot rescue FEN1 depletion at the telomeres in

non-immortal cells. The C-terminus is also essential for its interaction with the BLM protein [32] and TRF2 (Chapter 2). Given the importance of FEN1's C-terminus for telomere function and its ability to interact with BLM, WRN and TRF2, it is critical to determine which of these proteins recruits FEN1 to the telomere. One hypothesis is that all these proteins, in conjunction with other unidentified players, mediate telomere replication and the following experiments will allow us to investigate this.

Similar to WRN, BLM is able to unwind G4 DNA, is critical for the re-initiation of stalled replication forks and has recently been shown to be important for the efficient replication of telomeres [29, 33-35]. The BLM-FEN1 complex may play a role similar to the WRN-FEN1 complex in alleviating replication stress during telomere replication. Therefore, BLM may be able to partially compensate for WRN loss at the telomeres. Deletion of *Blm*, similar to *Wrn* deletion, accentuates the pathology of later generation telomerase RNA component (mTERC) knockout mice indicating a functional role for BLM at mammalian telomeres [36]. It is of interest to investigate whether BLM deficiency has a similar phenotype to the FEN1 and WRN deficiency in human cells, i.e., loss of single sister telomeres specifically from the lagging strand. Any compensation by BLM for WRN can be investigated through epistasis analysis by the simultaneous depletion of the two proteins. If BLM partially compensates for WRN depletion at the telomere, simultaneous loss will lead to an additive effect on telomere dysfunction. Given that FEN1 may also be recruited to the telomeres by TRF2 [37, 38], RecQ helicase-independent function of FEN1 at the telomere may be investigated through

similar epistasis analyses with the simultaneous depletion of FEN1 or BLM or both in WRN-deficient cells. An increase in telomere dysfunction upon FEN1 depletion in cells without both BLM and WRN would suggest a role for FEN1 in telomere maintenance independent of the two RecQ helicases.

TRF2's interaction with FEN1 [37, 38] may directly engage the latter to actively repair and/or replicate telomeres. Interestingly, TRF2 also interacts with and stimulates the activities of WRN and BLM RecQ helicases [39-41]. This raises the possibility that these proteins act concordantly to enhance replication and repair of telomeres. Interestingly, a recent study demonstrated that TRF2 increases branch migration of HJ intermediates, suggesting that this promotes formation of chickenfoot structures in the context of a stalled replication fork at telomeres [42]. TRF2, which binds these chickenfoot structures, may engage the WRN-FEN1 complex to act upon and resolve them [16]. In effect, TRF2 could enable this complex to efficiently restart stalled replication forks at telomeres.

5.5 FEN1: A Tumor Suppressor

Several reports have demonstrated a significant role for FEN1 in oncogenesis. FEN1 overexpression has been associated with human lung, prostate, brain and breast cancer [22, 43-46]. Strikingly, FEN1 haploinsufficiency increases cancer incidence in mice with a heterozygous Adenomatous polyposis coli (APC) background [47, 48].

Interestingly, mutations have also been detected in a variety of human tumors that abolish the GEN and EXO, but not the FEN, activities of FEN1 [49]. Furthermore, when Shen and colleagues knocked-in one of the detected E160D (mED) human cancer mutations into mice, the transgenic animals spontaneously developed tumors indicating that FEN1 is a tumor suppressor gene [49]. A similar study conducted on a different strain of mice with the same mED mutation in FEN1 revealed a different spectrum of cancers [50]. Although in the first study [49], tumorgenesis was attributed to incomplete DNA fragmentation during apoptosis, an alternate hypothesis for this phenotype may also been proposed [50]. Our results with the FEN1 mED mutation (Chapter 4) suggest that the increased genomic instability and cancer incidence in mice with this mutation is caused by telomere dysfunction. Indeed, murine cells expressing the mED mutant have telomere dysfunction evidenced by telomere end-to-end fusions [51]. Additionally, comparative genomic hybridization of the resulting tumors in the E160D mice showed genomic instability with changes in chromosome copy number, chromosomal rearrangements, gains and losses [50]. Telomere-dysfunction induced genomic instability is a known driving force for tumorigenesis [52-54], suggesting a similar mode of transformation in the E160D mutant mice. However, evidence for telomere dysfunction in the FEN1 mED murine cells is not conclusive and further work is required to demonstrate the nuclease deficiency associated with the FEN1 E160D mutation and its effect on telomere stability in mice. These studies will allow us to further investigate the effect of FEN1 E160D mutation on genomic stability and cancer incidence in both the mouse and human.

As described above, FEN1 is a tumor suppressor. Conversely, FEN1 depletion and/or inhibition kills human colorectal cancer cells that have a Rad54B deletion [55], raising the possible therapeutic utility of FEN1 inhibition. The study demonstrates that cancer cells with a Rad54B deletion have increased chromosomal instability and can be effectively and selectively killed by depleting synthetic lethal genes such as FEN1 [55]. To this end, specific small molecule inhibitors of FEN1 have been identified and characterized in mammalian cells, making FEN1 inhibition a distinct possibility for cancer therapeutics [56, 57]. However, my data suggest that this approach be taken with caution. Indeed, telomerase-positive tumor cells, which represent the vast majority of all tumor cells do not appear to be affected by FEN1 depletion. Nonetheless, this approach may have utility in telomerase-negative, ALT-positive tumor cells, which display telomere dysfunction and cell death upon FEN1 depletion (Chapter 3).

5.6 Conclusions

High fidelity replication and maintenance of the genome is fundamental to the preservation of life. Genome stability is intricately linked to the faithful maintenance of telomere stability. Telomere replication, an essential but relatively unexplored component of telomere maintenance, is a complicated task. Telomere chromatin poses several challenges to the moving replication fork causing natural stalling/pausing. For these reasons, loss of function mutations or depletion of genes that facilitate replication fork progression and restart would be expected to have a profound impact at the telomere.

This thesis identifies complications associated with telomere replication and characterizes one protein, FEN1, as a necessary component of the telomeric replication machinery. The results presented herein demonstrate that FEN1 works in a complex with a RecQ helicase (WRN, BLM or both) to efficiently resolve stalled replication forks at sites of stalling. The inability to efficiently resolve stalled forks at the telomere leads to the drastic loss of the distal end of the telomere causing telomere dysfunction. Therefore, FEN1 mutation/depletion increases telomere dysfunction, leading to an increase in genomic instability, a known driving force for cancer. My model provides an explanation for the increased cancer incidence observed in mice and humans with mutations in the FEN1 gene. Conversely, telomere dysfunction and subsequent genomic instability associated with FEN1 depletion/inhibition can be exploited to selectively target telomerase-negative tumor cells harboring other critical mutations.

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