DEFINING MULTIPLE STEPS IN HUMAN

TELOMERE END PROCESSING

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DEDICATION

Dedicated to my grandmother Faiting, and my parents Tanbulan and Waiche Chow for their continuous support and encouragement.

DEFINING MULTIPLE STEPS IN HUMAN TELOMERE END PROCESSING

By

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DISSERTATION

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DEFINING MUTIPLE STEPS IN HUMAN

TELOMERE END PROCESSING

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ABSTRACT

Telomere overhangs are essential for chromosome end protection and telomerase extension, but how telomere overhangs are generated is unknown. Due to the classic end replication problem, leading DNA daughter strands are initially blunt while lagging daughters are shorter by at least the size of the final RNA primer, which historically is believed to be located at extreme chromosome ends. We developed a variety of new approaches to define the steps in the processing of these overhangs. Understanding the number and nature of the overhang processing events is crucial in establishing the roles of candidate proteins involved. We here define these steps in normal human cells. We show the final lagging RNA primer is positioned ~7 nt from chromosome ends (not at

the extreme ends), and is not removed for ~1hr following replication. Therefore, the location of the RNA primer, rather than its size, is a primary driving force for telomere shortening. Moreover, we demonstrate that telomere end-processing occurs in two distinct phases following telomere duplex replication. During the early phase, which occupies 1-2 hours following telomere replication, several steps occur on both leading and lagging daughters. Leading telomere processing remains incomplete until late S/G2 when the C-terminal nucleotide is specified referred to as the late phase. Furthermore, in human cancer cells under maintenance condition, telomerase extension is uncoupled from C-strand fill-in.

These results uncover crucial mechanistic details of the DNA end-replication problem as well as several specific steps in telomere overhang processing. These results also indicate the presence of previously unsuspected complexes and signaling events required for the replication of the ends of human chromosomes. The findings and the methods developed will now provide the basis for examining candidate factors that may function to regulate particular steps in telomere length homeostasis with implications in both cellular aging and cancer.

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LIST OF ABBREVIATIONS

bp	Base pair(s)
BrdU	5-Bromodeoxyuridine
DKC	Dyskeratosis Congenita
DNA	Deoxyribonucleic Acid
hTERT	Human Telomerase Reverse Transcriptase (Protein Component)
hTR	Human Telomerase RNA (template RNA Component)
IdU	Iododeoxyuridine
IPF	Idiopathic Pulmonary Fibrosis
kb	Kilobase Pair(s)
M1	Mortality Stage 1
M2	Mortality Stage 2
MEF	Mouse Embryonic Fibroblast
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RNP	Ribonucleoprotein
SMARD	Single-molecule Analysis of Replicated DNA
STELA	Single Telomere Length Analysis
TIF	Telomere-induced Foci
TRAP	Telomeric Repeat Amplification Protocol
TRF	Telomere Restriction Fragment

CHAPTER ONE

General Introduction and Literature Review

The History of Telomere Biology

Telomere and Telomerase

The extremities of all eukaryotes linear chromosomes ends are structures called “telomeres”, derived from the Greek telos (end) meres (part) (Fig. 1-1). Over the past few decades, the intricate involvements of telomeres as a “cap” at chromosomal ends in the maintenance of genome integrity, as well as its impact on the aging and cancer development have been widely recognized (Bianchi and Shore, 2008; de Lange, 2005; Levy et al., 1992). The consequences of dysfunctional telomeres lead to various diseases such as dyskeratosis congenital, idiopathic pulmonary fibrosis, bone marrow failure, and cancers (Calado and Young, 2009). Telomeres are regulated by the catalytic activity of a ribonucleoprotein enzyme named “telomerase” through a reverse transcription mechanism. A growing number of telomere-specific binding proteins (called the shelterin complex) with significant influences in telomere function and telomerase action have been discovered (Bianchi and Shore, 2008; de Lange, 2005; Linger and Price, 2009). Studies regarding the roles of telomerase in cancer have rapidly diverged the field into multiple directions to explore the implications of telomeres

and telomerase in stem cells, aging, developmental processes and disease states (Buseman et al., 2012; Calado and Young, 2009; Flores and Blasco, 2010; Joseph et al., 2010). The potential of telomerase inhibition as a cancer therapy has been expeditiously recognized and is currently under evaluation in phase II clinical trials (Buseman et al., 2012). As telomere biology is emerging to an exciting frontier in both basic and translational research, the 2009 Nobel Prize in Medicine was awarded to Elizabeth Blackburn, Carol Greider, and Jack Szostak for their early contributions in elucidating how chromosome ends are protected and the identification of telomerase (Varela and Blasco, 2010).

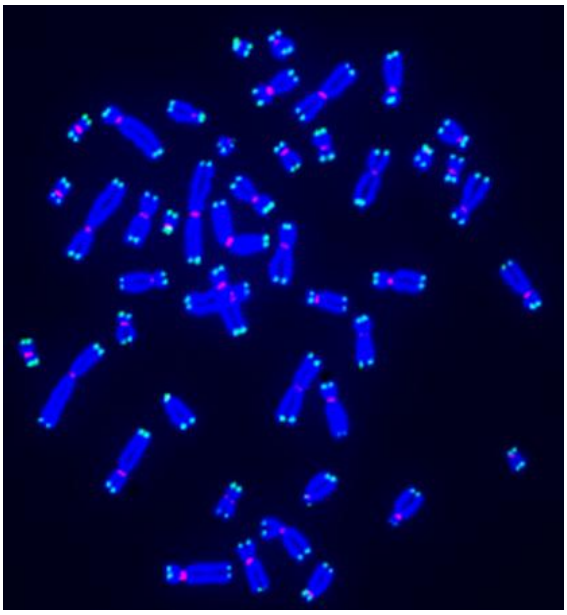

Figure 1-1. A metaphase spread of human chromosomes. Chromosomes DNA in blue (DAPI staining). Telomeres are in green (fluorescent PNA probe). Centromeres stained in Red (centromere probe). Chromosomes at metaphase have replicated but have not yet been separated into daughter cells; therefore, four dots were observed per chromosomes. Courtesy of Ying Zou.

The telomere story started as early as 1881 when the great German biologist August Weismann insightfully speculated, though without evidence, that somatic cells in vertebrates have a limited doubling potential (Weismann, 1891). However, this paradigm was nearly dismissed when a different concept was championed by the French Nobel laureate Alexis Carrel in 1921 (Carrel and Ebeling, 1921). Dr. Carrel claimed his group was able to culture chicken cells in vitro for decades by feeding the cells a chicken embryo tissue extract which unknowingly contained fresh living cells everyday (Carrel and Ebeling, 1921). This experimental design error led to the general concept of indefinite division of vertebrate cells in culture for over three decades.

In 1938, Herman Muller (Muller, 1938) first demonstrated that natural chromosome ends are distinct from deoxyribonucleic acid (DNA) double stranded breaks produced by ionizing radiation in fruit flies using cytogenetic analysis. In 1941, Barbara McClintock (McClintock, 1941) reported the same phenomena in maize cells. Breakage induced free ends are often healed by fusing together, thus resulting in genome rearrangements and instability. However, the chromosome ends, referred to as “telomeres” by Muller and “natural ends” by McClintock, remain intact with no fusions. Telomeres received little attention until the 50s-60s when a series of observations unfolded that telomeres are not simply unique structures but also had crucial functions in cell biology. In 1953, Watson and Crick discovered the duplex helix structure of DNA (Watson and Crick, 1953). In

1961, Leonard Hayflick and Paul Moorhead performed a series of experiments that challenged Carrel's concept of infinite division capacity of human cells in culture. Equal numbers of old male versus young female fibroblasts were mixed with unmixed population as controls. As the old male cell population remembered their age, they entered a non-diving stage when their inherit limit was reached, called replicative senescence. Only female cells were left in the mixed populations, and thus took over the culture. This finite proliferative lifespan of normal cells was named the "Hayflick limit" (Hayflick and Moorhead, 1961). They proposed the concept of a "replicometer" as the molecular clock which counts the limited number of cellular division.

As the mechanisms of DNA replication continued to unravel, it became apparent that eukaryotes chromosomes consisted of linear DNA molecules (Lark, 1969; Richardson, 1969). In 1971, Alexey Olovnikov, a Russian theoretical biologist proposed that a cell might undergo senescence because chromosomal ends cannot be fully replicated, and therefore small amount of DNA is lost during every cell division (Olovnikov, 1971, 1973). One year later, Watson reported DNA polymerase could not completely replicate the 3'end of a liner duplex DNA. The incomplete replication, if not compensated, would eventually lead to loss of genetic material at chromosome ends (Watson, 1972). This phenomenon, referred to as the "end replication problem", presented a unique challenge to the DNA

replication machinery. Interests in solving the end replication problem languished with the bloom of technological advances in the 80s.

In 1978, Elizabeth Blackburn, who was previously a graduate student in Fred Sanger's lab at Cambridge where DNA sequencing was invented, moved to the US and became a postdoctoral fellow in the lab of Joseph Gall. Blackburn applied the new sequencing technology and determined the first telomere sequence from extra-chromosomal ribosomal DNA (rDNA) in single-cell ciliated protozoa, *Tetrahymena thermophila* (Blackburn and Gall, 1978). *Tetrahymena* telomere consisted of 20-70 tandem hexameric repeat sequence of TTGGGG/AACCCC (Blackburn and Gall, 1978). As *Tetrahymena* can divide infinitely, Blackburn reasoned that a mechanism must exist to prevent loss of DNA due to the end replication problem. It was then discovered that telomere repeats were added to chromosome ends in actively dividing yeast (Shampay et al., 1984; Szostak and Blackburn, 1982). Seven years later, Blackburn and her graduate student Carol Greider discovered the existence of an enzyme activity in the cell extract of *Tetrahymena* that was capable to catalyze *de novo* addition of new repeats onto telomeric ends as a solution to the end replication problem. It was animally called "telomere terminal transferase" (Greider and Blackburn, 1985). The observation that this transferase was inhibited by RNase, and a low-abundance RNA was co-purified with the partially purified active enzyme led to the identification of telomerase being a ribonucleoprotein complex (Greider and

Blackburn, 1987). The *Tetrahymena* RNA template was soon sequenced, and was closely associated with the protein component of the terminal transferase, later named "telomerase" (TERT) (Greider and Blackburn, 1989).

In the subsequent years, telomere structures similar to that of the *Tetrahymena* were identified in various eukaryotic organisms. *Saccharomyces cerevisiae* consisted of telomeres with irregular sequences of TG1-3 (Blackburn, 1990; Shampay et al., 1984; Wellinger and Sen, 1997). In 1988, human telomere sequences 5'-TTAGGG-3' was identified (Moyzis et al., 1988). The T_2AG_3 sequence was conserved among 91 eukaryotic species ranging from reptiles, fish, amphibians to mammals (Meyne et al., 1989). Common features of all telomeres include tandem repeat of short sequences of G-rich and its complementary C-rich strands, while the number of repeats and the actual sequence may vary from species to species (Blackburn, 2006). The fact that duplex telomeres contain unique repetitive sequences, yet then the ends are protected from nuclease degradation (Blackburn and Chiou, 1981), suggested that non-histone, telomeric-specific proteins may be involved in proper capping of chromosomal ends.

The first protein identified to bind to duplex region of telomeres was yeast Rap1p (Buchman et al., 1988). Rap1p was originally identified to be a transcription factor (Santoro et al., 1988) while also associated with telomeres *in vivo*, suggesting proteins function in other cellular mechanisms also participate in telomere structure and function (Conrad et al., 1990). Rap1p was also found to be

a negative regulator of telomere length (Conrad et al., 1990; Lustig et al., 1990). The amount of Rap1p acts as a counting mechanism to regulate telomere lengths (Krauskopf and Blackburn, 1996; Marcand et al., 1997). The first telomere binding protein discovered in the ciliate *Oxytricha nova* was TEBP, which was a two subunit protein bound to the ss 3' G-overhang (Gottschling and Zakian, 1986). TEBP homologs have been found in budding yeast (Cdc 13) (Lin and Zakian, 1995; Nugent et al., 1996), human and fission yeast (POT1) (Baumann and Cech, 2001). Another mammalian telomere duplex binding protein is TRF1 (Chong et al., 1995), which also serves as a negative regulator of telomere length (van Steensel and de Lange, 1997). TRF2, similar to TRF1, was also found to bind duplex telomere sequence (Bilaud et al., 1997; Broccoli et al., 1997). TRF2 not only regulates telomere lengths but is also important in the formation of secondary structures at telomeric ends (de Lange, 2004; Smogorzewska et al., 2000). Together with Rap1, POT1, TRF1, TRF2, two additional proteins work together to protect mammalian telomeres, called the shelterin complex, which will be discussed in details in a later section.

The first protein component of telomerase was identified in yeast mutant with an ever-shortening telomere (*Est*) phenoptype (Lundblad and Szostak, 1989). *Est* mutants exhibit telomere loss, and eventually result in senescence and cell death over many population doubling. It was not until the mid-90s that the RNA and the protein component of human telomeres were cloned. The human RNA

template for telomerase (hTR) was cloned in 1995 (Feng et al., 1995) while the human catalytic subunit of telomerase (hTERT) was cloned in 1997 (Harrington et al., 1997; Nakamura et al., 1997). hTR is expressed in all human cells, but only cells with telomerase activity expresses hTERT (Ducrest et al., 2002; Feng et al., 1995; Harrington et al., 1997; Nakamura et al., 1997; Takakura et al., 1998).

With respect to telomere and telomerase in humans, it has been known that cancer cells, similar to *Tetrahymena*, can divide indefinitely; therefore, mechanisms must exist to maintain immortality in cancer cells. In the late 80s Cook and Smith observed different human tissues vary in telomere lengths. Telomeres of X/Y chromosomes from adult somatic cells were shorter than those in germ cells, suggesting telomerase activity was present in germ cells but not somatic cells (Cooke and Smith, 1986). In 1989, Greg Morrin detected telomerase activity in human cancer Hela cells. One year later, Calvin Harley showed that telomeres in normal cultured human cells shorten progressively until cells approach the Hayflick limit (Harley, 1991). These observations though correlative linked telomeres and replicative senescence. Though this correlation was strong, direct evidence supporting the hypothesis that telomere shortening was the cause of replicative senescence was still lacking as human telomerase had not yet been cloned at the time. In the early 90s, Jerry Shay and Wooding Wright proposed a theory relating cellular replicative senescence to aging and cancer,

which they later confirmed with experimental evidence in 1998 (to be discussed in details in the section *Replication Senescence* of this chapter).

In 1994, Jerry Shay and Woodring Wright developed a highly sensitive PCR-base assay to measure telomerase activity, named Telomere Repeat Amplification Protocol (TRAP). This technical innovation led to the detection of telomerase in over 90% of human tumors (Kim et al., 1994), which had propelled the telomere field from replication and aging into the enormous field of cancer studies. In 1995, the Shay and colleagues suggested that telomerase activities in cancers as a hallmark for cancer development, and could be a biomarker for cancer prognosis (Hiyama et al., 1995). Examinations of a hundred advanced neuroblastoma patients identified tumors with high telomerase activity showed genetic alteration and unfavorable prognosis. However, patients with low telomerase activity showed favorable prognosis without genetic alternation. Surprisingly, tumors with no detectable telomerase spontaneously regressed (Hiyama et al., 1995); thus, drawing immediate attention for telomerase as an attractive potential target for the development of anti-cancer therapies.

With the mounting evidence for the establishment of telomere shortening and telomerase as regulators for cellular senescence and cancer, new efforts in understanding the medical implications of telomerase began to emerge. Dysfunctional telomeres had been implicated in various diseases such as dyskeratosis congenita, pulmonary fibrosis and aplastic anemia (Calado and

Young, 2009). Dyskeratosis congenita (DKC) was the first human disease found to be linked to dysfunctional telomeres, in which mutations were identified in dyskerin, a protein that associates with the functional RNA (hTR) of telomerase (Dez et al., 2001; Pogacic et al., 2000). Additional mutations in telomerase components or telomere associated proteins were identified to be associated with DKC (Calado and Young, 2009). DKC inherited from ectodermal dysplasia, is characterized by nail dystrophy, abnormal skin pigmentation, and leukoplakia of the tongue (Calado and Young, 2009), along with very short telomeres (Vulliamy et al., 2001). In addition, a subset of sporadic patients with aplastic anemia also has short telomeres (Yamaguchi et al., 2003; Yamaguchi et al., 2005). Mutations in hTR and hTERT have been identified in 10% of these patients (Yamaguchi et al., 2003; Yamaguchi et al., 2005). Furthermore, mutations in hTERT have been found in 15% of patients with idiopathic pulmonary fibrosis (IPF) (Armanios et al., 2007; Mushiroda et al., 2008; Tsakiri et al., 2007). Likewise IPF patients also exhibit short telomere phenotype. Dysfunctional telomeres seem to interplay with various physiological process and the disease states. Continued efforts in the field focus on several exciting avenues: the basic molecular mechanisms of telomere end protection, how telomerase action serves to protect chromosome ends, impact of dysfunction telomeres in human disease states, regulations of telomerase in cancer, and the implications of anti-telomerase cancer therapies.

Replicative Senescence

When the Hayflick limit was first proposed in 1961 suggesting there was a counting mechanism in cells that defined a finite lifespan of cultured normal human cells, named "replicative senescence", it was strongly challenged by many scientists because this contradicted the accepted concept of "cells are immortal in culture" as originally proposed by the Nobel Laureate Alexis Carrel in 1921. In 1975, Woodring Wright, a graduate student of Hayflick, identified the replicometer being located in the nucleus (Wright and Hayflick, 1975). However, the molecular basis of this replicometer was unknown. Fifteen years later, Harley and colleagues initiate a correlation between telomeres and replicative senescence when they reported that telomeres in normal cells shorten until cells reach the Hayflick limit (Harley et al., 1990), providing a correlation but not cause-and-effect relationship. In 1989, Woodring Wright and Jerry Shay proposed a mortality stage 1 (M1) and mortality stage 2 (M2) model (Wright et al., 1989). In 1992, they summarized this two-stage mechanism (Fig. 1-2), which determines the senescence or immortalization states of a cell population, and its relationship between telomere, aging and cancer (Wright and Shay, 1992). This theory proposed that as normal diploid cells divide in culture, their telomeres shorten gradually until a critical length is reached. The cells then growth arrest at cell cycle checkpoints, and enter M1 stage in which they undergo replicative senescence. This is probably due to a DNA damage signal from one or a few

uncapped shortened telomeres. However, some cells are able to block cell cycle checkpoints by inactivating p53 or p16/Rb pathway. These cells continue to divide and lose more telomeres (Shay et al., 1991). Eventually, these cells will lose telomeres on most of the ends and reach a crisis stage, named M2. Cells in M2 are characterized by genomic instability due to chromosome end fusions, and most cells undergo apoptotic cell death. Only rare cells manage to escape this crisis stage by activating telomerase to maintain telomere lengths and these cells become immortal. This model was also correlative and did NOT prove that short telomeres caused replicative senescence. In addition, Shay and colleagues showed that 90% of human cancer has upregulated telomerase activity (Kim et al., 1994). The fact that cancer cells could escape replicative senescence and became immortal by activating telomerase immediate sparked interest in the potential of an anti-telomerase cancer therapy. While replicative senescence is driven by telomere lengths, cultured cells can stop dividing with long telomeres due to inadequate culture conditions (independent of cell division counts), referred to stress-induced senescence or stasis (Parrinello et al., 2003). These quiescent cells stay in G0 stage, and are prompted by differentiation and/or growth arrest signals. For example, cultured fibroblasts in low serum condition will prompt the cells to exit the cell cycle until nutrients are restored.

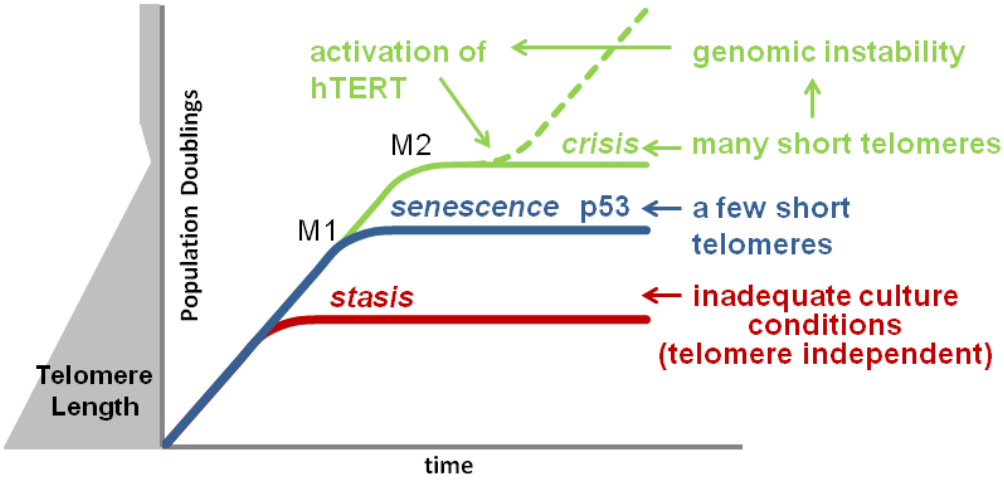

Figure 1-2. Replicative senescence model: Mortality Stage 1 (M1) or Mortality Stage 2 (M2). Telomeres of normal diploid somatic cells shorten during each cell division. Inadequate cell culture conditions results in telomere-independent cellular senescence, called stasis. When a few telomeres reach a critically short length, DNA damage repair mechanisms trigger a growth arrest called replicative senescence, or Mortality Stage 1 (M1). However, some cells will acquire mutations to inactivate DNA damage checkpoints in the p53 and p16/Rb pathways. These cells can bypass M1 and continue cell division as their telomere shorten even more until many telomeres become critically short with increased chromosomal instability resulting in a state of crisis or Mortality Stage 2 (M2), and many of these cells undergo apoptotic cell death. Occasionally, some rare cells can activate telomerase to stabilize telomere length; thus, allowing cells to bypass M2/crisis.

The RNA component of telomerase, hTR template, was cloned in mid-90s and encompassed 11 nucleotides (5'-CUAACCCUAAC-3') complementary to the human telomere sequence (TTAGGG)n for telomere elongation. hTR was detected in both normal and immortal human cells, while there was a subtle increase of hTR expression in immortal cell (Feng et al., 1995). hTR is crucial for proper telomerase function because introduction of anti-sense oligonucleotide in

Hela cells resulted in cell death after 20-30 population doubling (Feng et al., 1995). The catalytic component of telomerase hTERT was cloned in 1997 (Harrington et al., 1997; Nakamura et al., 1997), and hTERT was only detected in telomerase positive cells (Kim et al., 1994). This led to the hypothesis that telomerase activity can be restored in telomerase negative normal human cells simply by expressing telomerase.

Meanwhile, the Shay/Wright lab has been working firmly to test the hypothesis that telomere shortening is the cause of replicative senescence. In 1996, they reported that when different cell types were fused together, the cell type with shorter telomeres limit the replicative lifespan of the hybrid (Wright et al., 1996). However, the highly unnatural setting of a fused hybrid cell line limited a firm conclusion. Weinrich, Shay, Wright and colleagues reported that the expression of hTR and hTERT were the minimal components to reconstitute telomerase activity *in vitro* (Weinrich et al., 1997). The following year, a landmark publication by Shay/Wright provided a final, definitive confirmation for the telomere shortening and cellular senescence hypothesis (Bodnar et al., 1998) proposed by Olovnikov more than twenty years ago (Olovnikov, 1971, 1973), at least in the level of cultured cells.

Shay/Wright and colleagues over-expressed exogenous hTERT into telomerase negative normal fibroblasts as well as retinal pigmented epithelial cells, and extended the cellular lifespan of these cells well passed the Hayflick

limit compared to the negative controls. This was the first report that cells could be immortalized by expression hTERT alone (Bodnar et al., 1998). Telomerase was not detected in most normal human cells, with a few exceptions (Shay and Bacchetti, 1997), but most cancer cells have activated telomerase to escape replicative senescence. They also demonstrated that expression of hTERT into pre-M1 cells as well as in pre-M2 cells was sufficient to permit cells to grow indefinitely. These results provided for the first time direct evidence that telomerase were the cause of the M1 and M2 senescence and crisis arrest, since expression of hTERT alone was sufficient to bypass the growth arrest.

What in evolution terms favors replicative senescence versus letting our cells have everlasting lifespan? One popular explanation is that the lack of detectable telomerase in normal human cells serves as a defense to protect cells from developing cancers. Normal cells need sufficient division for normal growth and repair of damaged tissues. However, each time a cell divides there is an increasing possibility of accumulating mutations and if sufficient alterations occur, cancer would ensure. Thus, if a cell reach the Hayflcik limit before enough mutations have been accumulated to process to cancer, this would in essence be a very powerful anti-cancer protection mechanism, especially in large and long-lived species (Shay and Wright, 2000, 2001). In contrast, mice have much longer telomeres than humans, but live only two-three years in captivity. It has been proposed that mice do not use replicative aging to count cell divisions. This leads

to the question concerning the role of replicative aging among organisms, and whether mice or human represent a common mammalian paradigm. One hypothesis is that in mice, very long telomere is used as a buffer or protect free-radical induced telomere breakage, while replicative aging is abandoned because mice to do not live long enough to develop cancer. Moreover, invertebrates such as amphibians, fish and reptiles etc. have telomerase activity in some somatic tissues, and it has been suggested that telomerase functions to maintain the extensive regenerative potentials (Gomes et al., 2010). Though there is rather strong evidence supporting the relevance of replicative senescence of *cultured cells* and telomere length, the direct evidence linking replicative senescence and organismal aging remains controversial, and is of continuous interest and research efforts.

Approximately 10% of human tumors escape replicative senescence but lack detectable telomerase expression, and have been found to maintain telomeres through a recombination pathway named alternative lengthening of telomere (ALT) (Bryan et al., 1997; Hanahan and Weinberg, 2000; Murnane et al., 1994). There have also been debates over the therapeutic value of potential anti-telomerase treatments for cancer. It has been claimed that telomerase expression was detected in normal human cells (Masutomi et al., 2003). There has been arguments that telomerase expression protects cells from cancer because mutations occurred during genome rearrangement resulting in cancer can happen

when telomeres are short (Harley, 2002). Thus in some instances short telomeres may protect against cancer, and in other cases the greatly shortened telomeres may lead to increased genomic instability and an increase risk of cancer.

End Replication Problem

The end replication problem proposed that the extreme ends of chromosomes cannot be fully replicated during DNA replication (Olovnikov, 1971, 1973; Watson and Crick, 1953). Replication machinery synthesizes eukaryotic chromosomal DNA by a semi-conservative mechanism, in which the newly replicated DNA contains one old strand (parental strand) and one new strand (daughter strand) (Fig 1-3). Linear parental chromosomes consist of two strands of DNA in opposite orientations, and there are two distinct strand synthesis mechanisms because the daughter strand can only be synthesized in a 5'to 3' direction. The leading strand is synthesized continuously in the direction of the replication fork progression all the way to the end of a chromosome. In contrast, the complementary lagging strand synthesis is discontinuously replicated by utilizing Okazaki fragments in the opposition direction of the fork movement. Each Okazaki fragments start with an RNA primer and are extended into short DNA fragments. Following synthesis, RNA primers of the Okazaki fragments are removed and ligated together to generate an interrupted daughter strand. The removal of the terminal RNA primer will generate a 10-13 nt gap (if the primer is being positioned at the extreme chromosome ends) or up to 200 nt gap (if the

primer is positioned randomly with respect to extreme chromosomal ends). Thus, lagging strand synthesis fails to fully replicate the 3' ends of chromosomes; therefore, everytime DNA replicates, there is a net loss of DNA, known as the end replication problem.

Following replication, processing of telomeric ends is crucial to generate unique structures for proper capping of the chromosomal ends; therefore, chromosomal ends can be distinguished from DNA double strand (ds) breaks to prevent catastrophic genome instability. However, the mechanisms involved in human telomere end processing are unknown. In subsequent chapters of this dissertation, the steps involved in human end processing will be detailed.

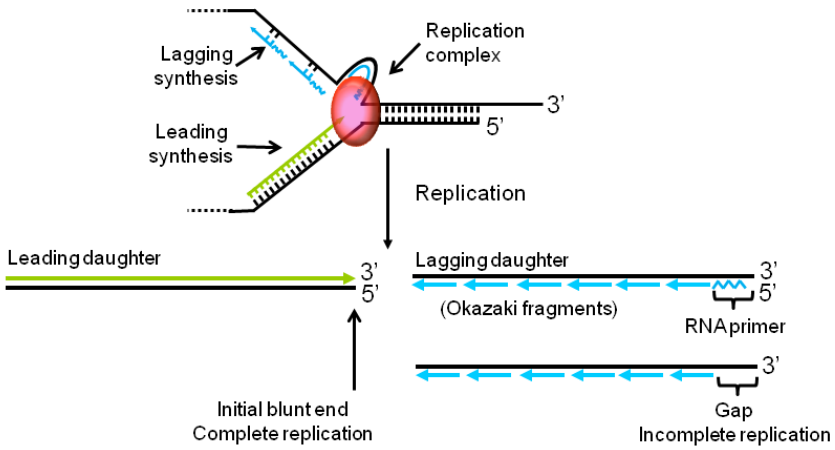

Figure 1-3. The end replication problem. Semi-conservative replication consists of leading and lagging daughter strands syntheses. Upon duplex DNA replication, the leading strand is fully replicated to the very end of the chromosome and is initially blunt. In contrast, lagging strand daughters are replicated by Okazaki fragments. The end of a chromosome cannot be fully replicated. Removal of the terminal RNA primer of the final Okazaki fragment generates a gap. Therefore a small amount of DNA is lost every cell division, and his has been named the "end replication problem".

Structures of Telomere

Telomere End Structure

Telomeres of eukaryotes consist of short G-C pairs tandem repeats at their chromosome extremities, with the first being reported in *Tetrahymena* (Blackburn and Gall, 1978). While all telomeres of vertebrates contain duplex 5'-TTAGGG-3' repeat, they vary in length from species to species. In some lower invertebrates, the telomeres average about 20 kb while in human the range is 5-15 kb in human kb (Cooke and Smith, 1986; de Lange et al., 1990; Dionne and Wellinger, 1996; Greider, 1999a, b; Stewart et al., 2003). However in rodents such as mice, they can be up to 100kb (Kipling and Cooke, 1990). Very little is known about what factors are important in setting telomere lengths but some generalizations have been made (Gomes et al., 2010). Telomeres are capped by telomere specific binding protein to prevent chromosomal end-to-end fusions and to maintain genome integrity (Bielinsky and Gerbi, 1999; de Lange, 2005; Levy et al., 1992).

Adjacent to the telomeres is a subtelomeric region, comprising a class of low copy, mosaic, and generally longer repeats. Subtelomeric sequences are highly polymorphic among different chromosome ends. Human subtelomere regions have variable repeats ranging from 1 kb to 200 kb. The large copy number of polymorphisms (CNPs) makes them challenging to sequence and assemble. It has been reported that human subtelomeric regions also contain an X

region, a restriction-endonuclease-resistant segment (2 to 4 or more kb) (Steinert et al., 2004). An important unanswered question is whether/how the large subtelomeric structural variations that are prone to deletions, translocations and duplications, contribute to gene regulation, genome instability and disease phenotypes.

It turns out that extreme chromosomal ends are not blunt. Duplex telomeres terminate with a 3' single-stranded G-rich protruding tail (G-overhang) extended beyond the duplex telomere region. G-overhangs have been identified in various organisms such as ciliates, yeast, worms, plants, mouse and human, while recent studies have identified C-overhangs in *C.elegans*, mouse and human cells engaging in the ALT pathway (Oganesian and Karlseder, 2011). Mammalian G-overhangs range in size from 12-300 nts (Chai et al., 2006a; Chai et al., 2005; Cimino-Reale et al., 2001; Keys et al., 2004; Wright et al., 1997).

The G-overhang is thought to function to hide chromosome ends from the DNA repair machinery by inserting (strand-invading) into the duplex telomere DNA to form a lariat-like T-loop (Telomere loop) structure (detected by electron microscopy) and a small D-loop (displacement loop), which are then bound by telomeric proteins (Fig. 1-4, 1-5) (de Lange, 2005; Griffith et al., 1999; Stansel et al., 2001). However, it has not been excluded that some region of the C-strand can also invade, forming a holiday junction. T-loops resemble a DNA recombination intermediate similar to those formed during homologous

recombination or the site during re-initiation of a stalled replication fork. T-loops have also been found in various other organisms: the end of micronuclear chromosomes of *Oxytricha nova* (Laporte and Thomas, 1998; Murti and Prescott, 1999), at the ends of mitochondria DNA of yeast *Candid1a parapsilosis* (Tomaska et al., 2002), at the telomeric ends of human cells (Griffith et al., 1999), *Trypanosome brucei* (Munoz-Jordan et al., 2001), *Pisum sativum* (Cesare et al., 2003), *Caenorhabditis elegans* (Raices et al., 2008), chicken and mouse (Nikitina and Woodcock, 2004). Their sizes range from 1 kb in *Trypanosomes* (Munoz-Jordan, 2001) up to 50 kb in *Pisum sativa* (Cesare et al., 2003; de Lange, 2004; Griffith et al., 1999; Stansel et al., 2001), suggesting they are evolutionarily conserved for telomere maintenance. *In vitro* modeling suggests that at least 6 nt are needed to form a T-Loop. Telomere binding protein, TRF2, have been suggested to stabilize T-Loop junctions (Griffith et al., 1999; Stansel et al., 2001) by facilitating G-overhang invasion (Amiard et al., 2007), and therefore, stabilizing the junction structure to be resolved by helicases (Nora et al., 2010; Poulet et al., 2009). In addition, a number of *in vitro* studies have shown that the guanine rich (G-rich) repetitive sequences are capable of forming stable four-stranded structures known as G-quadruplexes, held together by "G quartets" (Tang et al., 2008). They are formed by four sequences of triple G's stacked in a square arrangement stabilized by Hoogsteen hydrogen bonding, though their existence and functions *in vivo* remain uncertain. The detailed structure of T-loop

formation, unfolding and folding dynamics during DNA replication and how T-loop affects telomerase action are mostly unknown. Current T-loop detection has mostly relied on electron microscopy, which requires massive input materials. The development of a robust, sensitive assay for T-loop detection would certainly be a big leap in advancing our understanding in telomere end structures and their impact on telomere action.

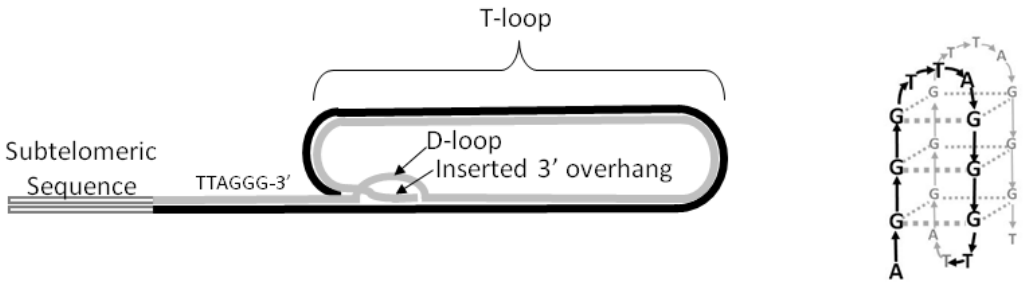

Figure 1-4. Telomere overhang structure. (Left) In native mammalian telomeres, the 3'-G-rich overhang is inserted into the double-stranded telomeric region, forming a large T-loop and a smaller D-loop. (Right) Free single stranded G-rich DNA containing triplet Gs can form structures called G-quadruplexes, one form of which is shown. Sets of four G's can form stacked planar structures stabilized by Hoogsteen hydrogen bonding. It is believed that these G4 structures are involved in some aspect of telomere biology *in vivo*, but their role is not yet clear. The inserted overhang present in T-loops is not single stranded and would not form quadruplexes (except, perhaps, at the very end), while the single-stranded G-rich sequences in the D-loop could potentially form quadruplexes.

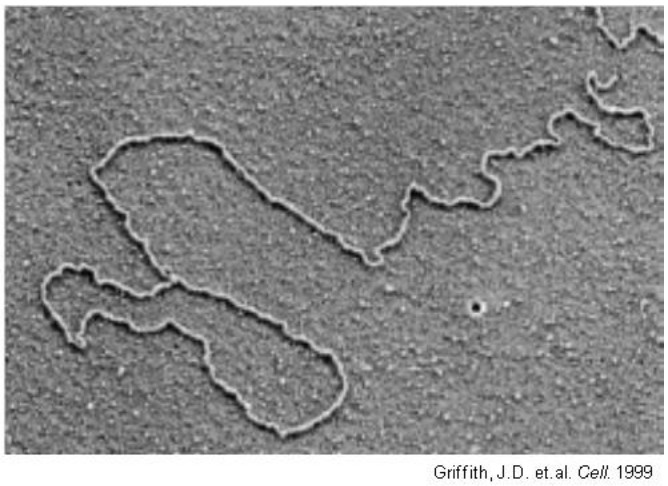

Figure 1-5. Telomere loop (T-loop) shown by electron microscopy. Courtesy of Griffith, J.D (Griffith et al., 1999).

Besides T-Loop formation, G-overhangs also serve as crucial priming sites for telomere extension by telomerase. Processing of G-overhangs has been well documented in simple model systems, such as yeast and ciliates, with genetic and biochemical approaches (Fan and Price, 1997; Jacob et al., 2003; Wellinger et al., 1993a, b).

Saccharomyces cerevisiae overhang processing regulation is cell cycle dependent (Dionne and Wellinger, 1996). Yeast telomeres replicate late in S phase. Yeast have transient long overhangs (>30 nt) independent of telomerase activity (Wellinger et al., 1993a, b), yet the overhangs are processed to a final length of 12-14 nt (Larrivee et al., 2004). This implies the presence of a nuclease resection processing mechanism. The yeast extreme G-terminus shows no terminal nucleotide specificity (Forstemann et al., 2000), and the C-terminal specificity (if it exists in yeast) has yet to be determined. Proteins and nucleases involved in the molecular steps in overhang processing still remain largely unknown. However, a few factors have been shown to affect overhang lengths. Ku 70/80 heterodimer (main function to repair double stranded breaks by NHEJ) and Cdc 13 (bind to ss G-overhang) have both been reported to influence overhang length by inhibiting C-strand resection (Booth et al., 2001; Gravel et al., 1998). Mre11 deletion in budding yeast resulted in smaller overhangs and telomere shortening (Larrivee et al., 2004). It has been proposed that the blunt ended leading strands in yeast could be converted to ss 3' G-overhangs which

Mre11 (involved in double stranded break repair) functions in cooperation with Dna2, Sgs1, Sae2 and Exo1 by mechanisms similar to the resection that occurred in double stranded breaks (DSB) (Dewar and Lydall, 2010; Larrivee et al., 2004; Mimitou and Symington, 2008, 2009; Takata et al., 2005; Tomita et al., 2004). However, it is unknown if similar mechanisms are involved in human cells.

Though G overhangs have been observed in various organisms, how human overhangs are generated is still largely unknown. In mammals, upon replication, telomere shortening results by two major causes: incomplete lagging strand synthesis and further telomere end processing on daughter strands. In lagging daughter synthesis, a gap is left between the final Okazaki fragment RNA priming event and the end of a chromosome (the end-replication problem) (Cech and Lingner, 1997; Levy et al., 1992; Lingner et al., 1995). Leading daughter strands should initially be blunt ended following DNA replication. Both leading and lagging ends (if further processing mechanism on the lagging daughters exists) are hypothesized to be processed by hypothetical helicases or nucleases to generate longer overhangs (Price, 1997). Virtually nothing is known about the difference in the biogenesis of leading versus lagging overhangs in human cells. It is generally assumed that further telomere processing by nuclease resection involves the replication machinery on both daughter strands.

Telomere overhangs are single-stranded regions at the extreme chromosome ends, and a telomere overhang is a required substrate for telomerase

extension. We know remarkably little about how telomere overhang maturation is coupled with replication and when overhangs are generated in human. Telomere overhangs are hypothesized to be generated first by a lagging-strand-specific end replication problem resulting in incomplete DNA synthesis upon removal of the final lagging RNA primer, which led to the model presented in all current textbooks: the final RNA primer is positioned at the extreme chromosome terminus. However, the presence and location of the final lagging RNA primer have not been directly shown in any organism. Many of these above questions will be addressed in later chapters.

In mammals, leading overhangs are shorter than lagging overhangs (Chai et al., 2006a). Moreover, leading overhangs are longer in telomerase-expressing cells compared to those in normal cells, suggesting the leading overhangs are processed differently from lagging overhangs following telomerase extension (Chai et al., 2006a). For decades, inadequate techniques have limited the signal detection in studying human telomere overhang structure due to the considerably larger number of chromosomes in humans, and relatively much longer telomeres relative to the overhang in human cells. Therefore, much of the work in overhang processing was pioneered in lower eukaryotes. A few methods have been developed to measure overhang length to study the nature of leading and lagging overhang processing as described below.

Both the non-denaturing hybridization assay (McElligott and Wellinger, 1997) and the HPA method (Tahara et al., 2005) only determine the relative strength of overhang signals with respect to total DNA. Techniques such as T-OLA (Telomeric Oligonucleotide Ligation Assay), is best for the determination of the longest overhang length (Cimino-Reale et al., 2001). Electron microscopy requires inputs of huge amounts of telomeric DNA and its detection limit is only down to ~75 nt (Wright et al., 1997), which is longer than most leading overhangs. The primer extension-nick translation method (PENT) can detect the presence of overhangs but it is deficient in providing information for short overhangs (Makarov et al., 1997). Overhangs can also be measured by the protein gp32 overhang protection assay, which was previously developed in our lab (Chai et al., 2005). However, the overhang quantification is limited to 45nt or above due to the presence of low molecular weight sizes, leading to the overestimation of the overhang lengths. In 2007, a new promising overhang assay was published from our lab using the commercially available Kamchatka crab double duplex-specific nuclease (DSN) (Zhao et al., 2007), which can accurately detect overhangs as short as 12 nt (with a residual 6bp ds DNA remained intact).

Determining which nucleases function in G-overhang processing, and what factors regulate their activity have remained a substantial challenge. Mutations and deficiencies of certain proteins have implicated differential defects in telomere integrity. Members of the shelterin complex as well as DNA repair /

processing proteins could be potential candidates. POT1 (a single stranded telomere binding protein) associated with the telomere throughout S-phase (Verdun et al., 2005) and showed a decrease in overhang sizes with randomized ends in bulk DNA while no telomere fusion was observed (Hockemeyer et al., 2005). The precise role of POT1 remains unknown.

Furthermore, a few helicases and nucleases have also been found to influence either leading or lagging overhangs processing. Cells lacking RecQ helicase WRN (3'-5' exonuclease) exhibited lagging specific deletions of telomeres from sister chromatids (Crabbe et al., 2004). Suppression of DNA-dependent protein kinase (DNA-PKcs) resulted in leading specific end fusions (Bailey et al., 2001; Zhang et al., 2005), but the exact roles of these proteins in normal telomere replication and overhang processing are unknown. The mammalian MRN complex (Mre11-Rad50-Nbs1) has been implicated in protecting leading strand telomeres in mice; deficiency in Mre11 resulted in leading telomere end fusions (Attwooll et al., 2009; Deng et al., 2009; Dimitrova and de Lange, 2009). Interestingly, Mre11 contains 3'->5' nuclease activities but it is not known how this nuclease activity functions in overhang processing (Cotta-Ramusino et al., 2005; Dewar and Lydall, 2010; Paull and Gellert, 1998). Mre11 interacts with TRF2 mostly in G2 phase of the cell cycle (Zhu et al., 2000). Deficient Mre11 induced dysfunction telomeres required the absence of TRF2 at the telomeres (Attwooll et al., 2009; Deng et al., 2009; Dimitrova and de Lange,

2009). Likewise in humans, as it is in mice, dominant negative mutant TRF2 resulted in leading end fusions (Bailey et al., 2001; van Steensel et al., 1998), and resulted in decreased overhang lengths (van Steensel et al., 1998). Apollo, an Artemis paralog, a 5'->3' exonuclease activity in vitro, interacted with TRF2 and exhibited a role in telomere protection and prevention of senescence (Lenain et al., 2006; van Overbeek and de Lange, 2006; Ye et al., 2010). Interestingly, Apollo has been shown to interact with Mre11 and Rad50 (Bae et al., 2008). The involvement of Apollo in C-strand resection following leading strand synthesis in mice has been reported (Lam et al., 2010; Wu et al., 2010), but its roles in human cells have yet to be elucidated. Nevertheless, the precise roles of factors function in ss 3' G-overhang processing remain to be identified.

Structures of Telomere Binding Protein

To maintain genome integrity, telomeres must be capped by telomere binding proteins and be protected from cellular DNA damage response pathways. Some telomere binding protein complexes binds to the G-overhang, while others bind to the duplex telomere DNA. These proteins have been well characterized in both yeast and humans. While the composition and structure of the complex subunits vary among species, their functions remained conserved (Bianchi and Shore, 2008). An overview of telomere binding protein members in various

organisms are compared below, with in depth description focused on the mammalian system.

Budding yeast *S. cerevisiae*

Telomeres in budding yeast are bound by two distinct protein complexes (Fig. 1-6): The CST complex that binds the G-overhang and the Rap1 complex that binds the DNA duplex (Linger and Price, 2009; Lundblad, 2006). Cdc13 binds to G-overhangs and recruits two binding partners (Stn1 and Ten1), forming the Cdc13/Stn1/Ten1 (CST) complex (Gao et al., 2007). CST deficiency results in C-strand degradation and accumulation of G-overhangs (Garvik et al., 1995; Grandin et al., 2001; Grandin et al., 1997). The CST complex also plays a role in telomere replication (Bianchi et al., 2004; Pennock et al., 2001). In addition, Rap1, together with Rif1 and Rif2, binds the double-stranded telomeres and functions to regulate telomere length through a negative feedback loop (Hirano et al., 2009). Increase binding of Rif1 and Rif2 at telomeres inhibits telomerase recruitment and prevents further elongation (Hirano et al., 2009). Disruption of Rap1 binding results in lengthening of the telomeric sequence, and a faster turnover rate of the terminal repeats. Moreover, Rap1 can also recruit a second group of proteins – Sir2, Sir3 and Sir 4, which enhances the formation of heterochromatin, telomere silencing and influence positioning of the telomeres at the nuclear periphery (Lundblad, 2006).

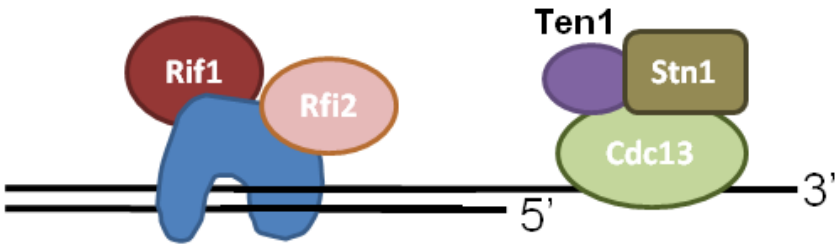

Figure 1-6. Telomere protein complexes in *S. cerevisiae*. See text for details.

Fission yeast *S. pombe*

Telomeric protein compositions in fission yeast are quite different from those in budding yeast (Fig. 1-6 & 1-7) (Linger and Price, 2009). The G-overhang of fission yeast is bound by the Pot1 complex (Pot1/Tpz1/Poz1/Ccq1) (Baumann and Cech, 2001; Miyoshi et al., 2008) (Fig. 1-7). This Pot1 complex interacts with the double-stranded binding protein complex Taz1/Rap1 to form a higher order complex structure that is essential for telomere capping and telomerase recruitment (Kanoh and Ishikawa, 2001). Interestingly, many telomere binding proteins in fission yeast are similar to those in mammals (Baumann and Cech, 2001), making them an important model system. Recent studies have also identified the CST components Stn and Ten1 in fission yeast to be important for telomere capping (Martin et al., 2007).

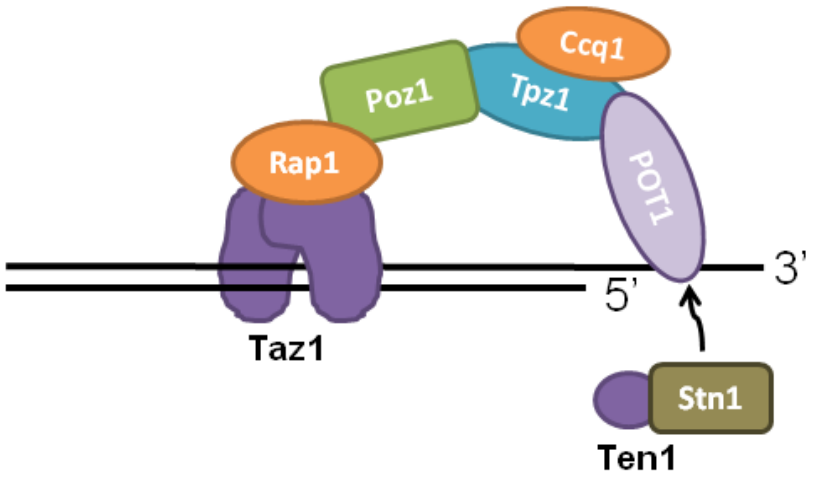

Figure 1-7. Telomere protein complexes in *S. pombe*. See text for details.

Mammalian

Human telomeres and perhaps all mammalian telomeres are bound by a six-subunit protein complex known as shelterin, which is composed of TRF1, TRF2, TIN2, RAP1, TPP1, and POT1 (Fig. 1-8) (Liu et al., 2004a; Palm and de Lange, 2008). Besides limited homology in Rap1, most of the shelterin components do not share sequence similarities with telomere proteins from budding yeast (Li et al., 2000). Human POT1 binds the single-stranded G-tail (Baumann and Cech, 2001), while TRF1 and TRF2 bind duplex telomeric DNA (Bianchi and de Lange, 1999; Bianchi et al., 1997; Chong et al., 1995). TIN2 and TPP1 serve as a bridge between the double-stranded DNA binding TRF1/TRF2 and the single-stranded DNA binding POT1 (Houghtaling et al., 2004; Liu et al., 2004b), similar to the higher order complex observed in fission yeast. The

shelterin complex protects telomeres from being recognized as DNA damage. POT1 inhibits ATR-mediated damage responses (Churikov and Price, 2008; Denchi and de Lange, 2007; Guo et al., 2007) while TRF2 inhibits ATM damage responses (Denchi and de Lange, 2007). Rap1 is known to interact with TRF2 (Liu et al., 2004b; Ye et al., 2004b). TRF2 is also required in the assembly of t-loops by assisting in the strand invasion of the single-stranded G-overhang into the double-stranded region of the telomere (de Lange, 2005; Smogorzewska and de Lange, 2004).

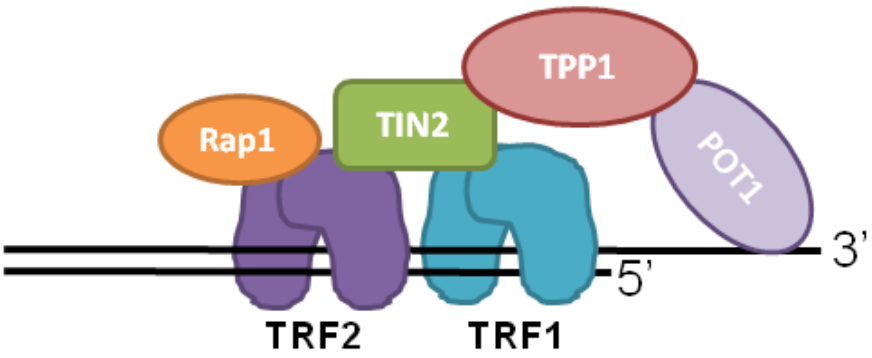

Figure 1-8. Shelterin telomere protein complexes in mammals. See text for details.

TRF1 (TTAGGG Repeat-binding factor 1) was the first human telomere binding protein to be identified (Zhong et al., 1992). TRF1 consists of three domains: a dimerization domain (TRFH or TRF homology domain) (Fairall et al., 2001), an N-terminal acidic domain and a C-terminal Myb motif in the DNA

binding domains with high affinity for binding to duplex telomere region (Bianchi and de Lange, 1999; Bianchi et al., 1997; Chong et al., 1995). Myb motifs have been found in mammalian TRF2 (Broccoli et al., 1997), and also in telomere duplex binding protein from a few other organisms such as *S.pombe* Taz1, Arabidopsis TRFLs and Trypanosome TRF (Bilaud et al., 1997; Broccoli et al., 1997; Cooper et al., 1997; Karamysheva et al., 2004; Li et al., 2005). Human TRF1 can be alternatively spliced into a smaller protein, Pin2, which 20 amino acids are missing from the linker region (Shen et al., 1997). TRF1 is a negative regulator of telomere length through a telomerase-dependent pathway (Smogorzewska et al., 2000; van Steensel and de Lange, 1997), and binds bipartite telomeric site composed of two 5'-YTAGGGTTR-3' half-sites (Bianchi et al., 1999). Mouse TRF1 knockout models are embryonic lethal, suggesting it is essential for telomere structure or that it may also have other functions (Karlseder et al., 2003). Conditional TRF1 deletion leads to fragile telomere site and hinder telomere replication (Sfeir et al., 2009). PinX1 has been reported to both interact and stabilize TRF1 at mitosis (Kishi et al., 2001; Yonekawa et al., 2012).

TRF2, TRF1 paralog, contains a TRFH dimerization, a C-terminal Myb domain, and an N-terminal basic domain as suppose to an acidic N-terminal in TRF1 (Broccoli et al., 1997). Despite the similarities in TRFHs, TRF1 and TRF2 do not form heterodimers. TRF2 does form a homodimer (Broccoli et al., 1997; Fairall et al., 2001). TRF2 also interacts with TIN2 and Rap1 (Li et al., 2000).

TRF2 was reported to facilitate strand invasion of G-overhangs (Amiard et al., 2007; Fouche et al., 2006) in T-loop formation *in vitro* (Khan et al., 2007; Nora et al., 2010; Poulet et al., 2009; Stansel et al., 2001), and stabilize the holiday loop junction (Nora et al., 2010; Poulet et al., 2009), while TRF1dimer is thought to generate bending of the telomere to allow T-loop formation (Bianchi et al., 1997). TRF2 may also be involved in chromatin remodeling at the telomeres because TRF2 over-expression in mouse keratinocytes resulted in aberrant nucleosomal organization at the telomeres (Benetti et al., 2008). Over-expression of a dominant negative TRF2 led to at least 30% loss of telomere G-overhang signal, increased nonhomologous end joining (NHEJ)-dependent chromosome end fusions, ATM and p53 mediated apoptosis or senescence (Smogorzewska et al., 2002; van Steensel et al., 1998). In addition, dominant negative TRF2 leads to a DNA damage response that accumulates telomere-induced foci (TIFs) containing 53BP1, Nbs1 and phospo-ATM (d'Adda di Fagagna et al., 2003; Takai et al., 2003). TRF2 knockout, similar to TRF1, is embryonic lethal (Celli and de Lange, 2005; Karlseder et al., 2003). Conditional TRF2 deletion in MEFs resulted in chromosome end fusions and DNA damage responses, suggesting an essential role of TRF2 in telomere protection (Celli and de Lange, 2005). Recently, TRF2 has been shown to be involved in non-telomeric DNA damage response as accumulation of TRF2 has been shown to occur at non-telomeric double stranded breaks (Bradshaw et al., 2005). TRF2 has also been implicated in facilitating the

early stages of homologous recombination (HR) to repair non-telomeric DNA damage (Mao et al., 2007), and maybe regulated by ATM (Huda et al., 2009). However double stranded breaks alone was not sufficient for TRF2 recruitment (Williams et al., 2007). Nevertheless, it is apparent that TRF2 mainly functions to protect telomeres and inhibit DNA damage response.

TIN2 (TRF1-interacting nuclear protein) was identified in a yeast two-hybrid screen (Kim et al., 1999). TIN2 serves as a bridge between TRF1 and TRF2 to form a central hub of the shelterin complex (Houghtaling et al., 2004; Ye et al., 2004a). TPP1/POT1 is required to tether to TIN2 for telomere protection (Takai et al., 2011). TIN2 stabilizes TRF1 on the telomeres, and protects it from degradation driven by Tankyrase. TIN2 over-expression results in telomere shortening in telomerase positive cells, probably due to the accumulating of TRF1 and TRF2 (both are negative regulators of telomere length) (Ye and de Lange, 2004).

hRap1 (TRF2-interacting protein), is a highly diverged ortholog of the yeast Rap1. Budding yeast Rap1contains two Myb domains, and binds DNA as a homodimer (Konig and Rhodes, 1997; Rhodes et al., 2002). hRap1 has four domains: a BRCT domain, a Myb HTH motif (a coiled region), and a C-terminal protein interaction domain (RCT) (Li et al., 2000). hRap1, resembles TRF1 and TRF1, but only consists of one Myb domain (Hanaoka et al., 2001). TRF1 and TRF2 form homodimer to bind to DNA duplex (Court et al., 2005). However,

hRap1 does not dimerize, and does not bind DNA through its own Myb domain. Instead, hRap1 interacts with telomere localization by interacting with TRF2 through the RCT domain (Hanaoka et al., 2001; Li et al., 2000). The interaction partners of hRap1 BRCT domain and Myb domain and their function are largely unknown (Hanaoka et al., 2001; Li and de Lange, 2003). Over-expressing deletion mutants missing the BRCT or the Myb domain decreases the telomere length heterogeneity (Li and de Lange, 2003). Furthermore, Rap1 has also been reported to function in NHEJ (Sarthy et al., 2009) and telomeric homology-directed recombination (Sfeir et al., 2010).

POT1 (Protection of Telomeres 1) was identified by sequence homology to the ciliate telomere binding protein (TEBPα) (Baumann and Cech, 2001). The two OB (oligonucleotide/oligosaccharide-binding) folds of POT1, together with various heterogeneous nuclear ribonucleoproteins (hnRNPs) bind the single-stranded G-overhang (Baumann and Cech, 2001; Ishikawa et al., 1993). *In vitro* assays show POT1 has high affinity for single-stranded telomeric sequence 5'-TAGGGTTAG-3' (Loayza et al., 2004). POT1 crystal structure reveals the first OB fold binds firmly to the first 6 nt, and the second fold protects the terminal end at TAG-3' (Lei et al., 2002; Lei et al., 2003; Lei et al., 2004; Loayza et al., 2004). POT1 is localized to the telomere throughout the cell cycle (Wei and Price, 2004). Furthermore, the OB fold of POT1 is not the only way by which POT1 interacts with the telomeres. POT1 is recruited to the telomere by its

interaction with TPP1 (linking POT1 and TIN2) (Houghtaling et al., 2004; Liu et al., 2004b; Ye et al., 2004b). Over-expression of a POT1 deletion mutant lacking the OB fold results in telomere elongation (Loayza and De Lange, 2003). This suggests that POT1 interact with TRF2 and TPP1 (Baumann and Price, 2010; Kendellen et al., 2009) to confer negative telomere length regulation. This also suggests the shelterin-T-loop confirmation functions to limit telomerase extension (Kendellen et al., 2009). In addition, POT1 can also act as a positive regulator to facilitate telomerase action (Colgin et al., 2003; Lei et al., 2005) such that the POT1-TPP1 heterodimer directly interacts to regulate telomere length by recruiting telomerase to the telomere and increasing telomerase processivity and the efficiency of telomerase translocation (Baumann and Price, 2010; Kendellen et al., 2009; Latrick and Cech, 2010; Wang et al., 2007). POT1-TPP1 may prevent the recruitment of the DNA damage machinery by outcompeting other DNA damage regulators such as RPA (Barrientos et al., 2008; Denchi and de Lange, 2007). Deficiency in POT1 resulted in a telomere damage response leading to telomere fusions, shortened 3' overhangs and altered C-strand processing (Hockemeyer et al., 2005). Finally, POT1 may function to couple leading and lagging strand synthesis during telomere replication (Arnoult et al., 2009).

TPP1 (PTOP/PIP1/TINT1) is the most recent of the six-member shelterin complex to be identified (Houghtaling et al., 2004; Liu et al., 2004b; Ye et al.,

2004b). TPP1 contains two domains: a central domain that binds to POT1 and a C-terminal TIN2 binding domain, making TPP1 a bridge between POT1 and TIN2. TPP1 stabilizes the TRF1-TIN2-TRF2 complex (O'Connor et al., 2006). The TPP1-POT1 interaction is important for telomerase recruitment and telomere length regulation (Baumann and Price, 2010; Hockemeyer et al., 2007; Xin et al., 2007). TPP1 has a nuclear export signal, which may control the accumulation of the shelterin proteins in the nuclease. It has been reported that the TIN2-TPP1-POT1 complex is present in both the nucleus and the cytoplasm (Chen et al., 2007).

The shelterin complex has direct roles in chromosomal end protection and telomere length regulation (de Lange, 2002; Linger and Price, 2009). Stoichiometry of the shelterin subunits may affect the assembly and function of the shelterin complex (Takai et al., 2010). In addition, various DNA repair factors have be localized to the telomeres such as DNA-dependent protein kinase (DNA-PK), which is thought to be recruited by interaction of TRF proteins with its Ku 70 subunits (Hsu et al., 1999; Hsu et al., 2000; Song et al., 2000). MRN (Mre11/Rad50/Nbs1) associates with TRF2 (Zhu et al., 2000), and has also been shown to co-localize with telomeres and TRF1 (Lombard and Guarente, 2000; Wu et al., 2000). Other than telomere binding proteins, replication, damage repair proteins, and many other proteins can also be present at the telomeres (Dejardin and Kingston, 2009).

In 2009, about two hundred such proteins were identified when segments of telomere chromatin were purified from human cells followed by mass spectrometry. Many of these proteins have known non-telomeric functions, but in most cases the telomeric roles of these proteins have yet to be characterized. One thought is that some may be involved in arranging telomeric chromatin structure at higher complexity, while others may possibly be responsible in transcribing telomere repeat containing RNA (TERRA) (Linger and Price, 2009).

The Telomerase RNP Complex and Telomere Elongation

The discovery of telomerase activity, which extends telomere ends, provides a solution to the end replication problem (Greider and Blackburn, 1985, 1989). The human telomerase holoenzyme contains two core components, hTERT (catalytic component) (Harrington et al., 1997; Nakamura et al., 1997; Weinrich et al., 1997) and its associated hTR or hTERC (functional telomerase RNA) (Feng et al., 1995), which together are sufficient to reconstitute an active enzyme in rabbit reticulocyte lysates (Weinrich et al., 1997). hTR is ubiquitously expressed in most telomerase-negative human cells; therefore, introducing exogenous hTERT alone would normally be able to render active telomerase (Weinrich et al., 1997). The assembly of telomerase RNP, however, is much more complex as it requires many other regulatory and structural proteins present in the reticulocyte lysates (Cong et al., 2002).

hTR has been identified in a variety of species including ciliates, yeast and vertebrates. Although its primary sequence is greatly diverse, its secondary structure is conversed (Chen et al., 2000; Chen and Greider, 2004). The templates of all TRs are conserved, which consists of a single-stranded region that pairs with the telomeric end to allow repeat synthesis. For example hTR has 11bp that pairs with the end of the telomeres (Greider and Blackburn, 1985, 1989). The template is flanked by a 5' boundary domain, which serves to limit reverse transcription past the end of the template (Chen and Greider, 2003), and a 3' pseudoknot domain, which roles that are still largely unknown (Ly et al., 2003).

TERT is upregulated in most human cancer cells (Kim et al., 1994), and is conserved among various species. It contains different domains involved in catalysis, dimerization, RNA binding, telomere recruitment and nuclear localization (Cech et al., 1997; Harrington et al., 1997; Lingner et al., 1994; Morin, 1989; Nakamura and Cech, 1998; Nakamura et al., 1997; Shippen-Lentz and Blackburn, 1989; Zahler and Prescott, 1988). hTERT contains a phylogenetically conserved reverse transcriptase (RT) motif (Lingner et al., 1997). This RT motid is flanked by N-terminal and C-terminal extension domains that are specific to telomerase. N-terminal extension (NTE) is subdivided into two domains separated by a nonconserved linker (Drosopoulos and Prasad, 2010). These two domains are the N-terminal-most TEN (TERT essential N-terminal)/GQ/RID1 (RNA interaction domain-1) domain and the

TRBD (TERT RNA binding domain)/RID2 (RNA interaction domain-2) domain. The nucleotide addition (polymerization) is catalyzed by the RT domain (Bryan et al., 2000; Drosopoulos and Prasad, 2010). The C-terminal extension (CTE) plays a role in the processivity of nucleotide addition (Hossain et al., 2002; Huard et al., 2003) and dimerization of TERT (in human) (Autexier and Lue, 2006).

Furthermore, the TERT family also has domains that function in telomerase recruitment and nuclear localization (Arai et al., 2002; Cech et al., 1997; Harrington et al., 1997; Nakamura et al., 1997). The expected mass of hTR and hTERT is about 280kD, but the size of telomerase enzyme is measured at about 1.5 mega dalton (Schnapp et al., 1998), suggesting the association of other putative proteins with telomerase. Assembly of functional telomerase requires the chaperones Hsp23 and Hsp90, which are thought to function in translocation after synthesis of each telomere repeat (Holt et al., 1999). Dyskerin (a TR associated protein) has been shown to be required for telomerase activity in *in vitro* (Cohen et al., 2007). Telomerase also interacts with 14-3-3 proteins, which are thought to assist in telomerase recruitment to the nucleus (Seimiya et al., 2000). Other telomerase holoenzyme associated proteins include heterogeneous nuclear snoRNA binding proteins (hnRNPs) A1, C1, C2 and D (Eversole and Maizels, 2000; Ford et al., 2000; LaBranche et al., 1998), ribosomal protein L22 (Aigner et al., 2000), vault protein TEP1, and the double-stranded RNA binding protein hStau (Le et al., 2000). Telomerase also associates with Cajal bodies in order to

be delivered to hTR foci at telomeres and to extend telomeres (Cristofari et al., 2007; Venteicher et al., 2009). TIN2 and TPP1, have been reported to be required to recruit hTR foci to the telomeres (Abreu et al., 2010).

The telomerase RNP binds to the G-overhang and is involved in catalyzing telomere elongation by adding GGTTAG repeats, and translocating to the terminal of the newly added repeats. The cycle continues until TERT dissociates from the telomere (Fig. 1-9) (Cong et al., 2002; Kelleher et al., 2002). Telomerase recruitment may be part of a negative feedback loop with the shelterin complex as the negative regulator for telomerase activity (De Boeck et al., 2009; Smogorzewska and de Lange, 2004). The negative feedback loop suggests that longer telomere recruits more shelterin complex, and therefore limit further telomere extension. This model is thought to partially maintain telomere length homeostasis in stem and germ cells as they have active telomerase (Smogorzewska and de Lange, 2004). For example, TRF1 knockdown resulted in telomere extension with no increase in telomerase expression suggesting that TRF1 is a negative telomere length regulator (van Steensel and de Lange, 1997). TRF1 has been proposed to act through a protein counting model, such that longer telomeres contain more TRF1 bound, thereby reducing the access of telomerase to elongate telomeres. TRF1 binding proteins, Tankyrase and TIN2, also function in telomere length regulation. Dominant-negative TIN2 results in telomere elongation while TIN2 over-expression leads to telomere shortening (Kim et al.,

1999). Tankyrase over-expression results in TRF1 degradation and telomere elongation, while cells deficient in Tankyrase stabilize TRF1 and telomeres shorten (Smith and de Lange, 2000). Rap1, similar to TRF1, TIN2, also function as a negative regulator of telomere length (Li and de Lange, 2003; Loayza and De Lange, 2003; Smogorzewska and de Lange, 2004; van Steensel and de Lange, 1997). POT1 can regulate telomere length both positively and negatively, but this depends on the situation (see POT1 description under telomere binding protein). Other telomerase-associated proteins that influence telomere length include Nbs (Bai and Murnane, 2003; Ranganathan et al., 2001), Rad54 (Jaco et al., 2003), DNA-PKcs (Espejel et al., 2002; Espejel et al., 2004), Suv 39 (Garcia-Cao et al., 2004), hEST1 (Reichenbach et al., 2003) and retinoblastoma (RB) proteins (Gonzalo and Blasco, 2005). Though many factors have been found to regulate telomere length; their precise roles and mechanisms remain poorly understood.

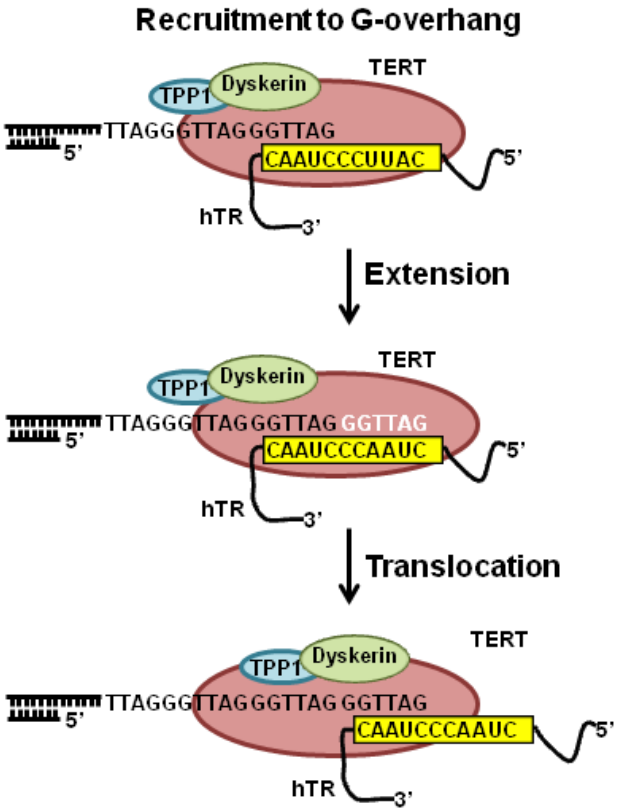

Figure 1-9. Schematic representation of telomerase extension. Telomerase ribonucleoprotein is recruited to the telomere. The template region of hTR 5'CUAAC-3' aligns to 5'-GTTAG-3' of the telomere. Then, hTERT extends the telomere by adding complementary telomere repeats to the hTR template region. Finally, the hTERT holoenzyme translocates. This extension process is repeated in each cell cycle.

Telomeres in cancer cells are at steady state almost always have shorter lengths compared to that of normal cells, suggesting telomerase within cancer cells is just enough to maintain telomeres to a stage where they would not be recognized as broken DNA. Telomere length homeostasis in yeast indicates that telomerase preferentially binds to the shortest telomeres (Teixeira et al., 2004).

This suggests that telomeres have two states, extendible state permissive for telomerase extension while long telomeres are in a non-extendible state inhibiting telomerase action. A protein counting mechanism was proposed to set the threshold between short and long states (Marcand et al., 2000; Teixeira et al., 2004). However in humans, under steady state telomere length maintenance conditions, human telomerase extends most chromosomal ends, and is not preferentially recruited to the shortest telomeres (Zhao et al., 2009). Each telomere is elongated by a one telomerase molecule that extends ~60 nt in a processive manner (Zhao et al., 2011a). POT/TPP1 interact with telomerase to function processively to extend an average of 48 nt of repeats to telomeric *ends in vitro* (Latrick and Cech, 2010). However, when telomeres are lengthening by either over-expression of exogenous telomerase or during recovery of artificially shortened telomeres, multiple telomerase molecules function on each chromosomal end to extend telomere length through a distributive mechanism (Zhao et al., 2011a). Processing of telomerase by Cajal bodies may also influence processivity (Zhao et al., 2011a).

Telomeres are unique dynamic structures and their lengths are tightly regulated. Regulation of telomerase recruitment/action on telomeres varies with its context and depends on various different factors for its proper maintenance. The detailed mechanisms of telomere length homeostasis, and the interplay among the factors involved in telomere length maintenance are largely unknown.

Developing tools to visualize the dynamics of single telomeres will enhance our understanding of the mechanisms of telomerase extension.

Telomere, Telomerase and Cancer Therapeutics

The fact that cancer cells can be immortalized by activating telomerase to escape replicative senescence makes telomerase an attractive target for cancer therapy. Potential telomerase-based cancer therapies include telomerase inhibitors, telomerase immunotherapy, and targeting telomerase-expressing cells with oncolytic viruses.

Telomerase inhibitors

In the 90s, a biotech company Geron, was established to focus on researching telomeres and telomerase to develop cancer therapy. Geron developed a compound, GRN163L (or Imetelstat), which essentially is a telomerase template antagonist that blocks the active site, thereby inhibiting telomerase activity. Since then, GRN163L has also been reported to inhibit tumor growth in cell lines of diverse origins, such as glioblastoma tumor-initiating cells, multiple myeloma, breast and pancreatic stem cells, as well as liver and lung cancer cells. This compound has recently entered phase II clinical trial (Buseman et al., 2012; Dikmen et al., 2005; Jackson et al., 2007; Ouellette et al., 2011; Shay and Wright, 2005). In addition, there have been some concerns that targeting

telomerase to limit cancer cell proliferation may also target normal proliferating stem cells. It is because other than cancer cells, telomerase activity has also been identified in a subset of normal human cells such as normal bone marrow stem cells, adult pluripotent stem cells (Engelhardt et al., 1997), fetal tissue, germ cells, testes, activated peripheral blood lymphocytes, transient amplifying skin epidermis, proliferating intestinal crypt cells (Wright et al., 1996), and some immune cells (Broccoli et al., 1995). However the levels of telomerase activity in these cells are significantly less than that in cancer cells (Wright et al., 1996). In addition, most telomerase positive cancer cells have much shorter telomeres and potentially a relatively faster proliferation rate compared to most stem cells, which are slower growing with longer telomeres, and therefore making it possible for differentiate normal cells from being targeted (Ouellette et al., 2011).

Telomerase immunotherapy

As telomerase is a marker for cancer, we may be able to teach our immune system to attack these cells, leaving healthy cells unharmed (Greten et al., 2010). To date, a few hTERT-based vaccines have been developed, with GV1001 and GRNVAC1 being the most advanced vaccines. GV1001 has been applied for pancreatic cancer, hepatocellular carcinoma and non small cell lung cancer (NSCLC) in phase I/II clinical trials (Greten et al., 2010; Harley, 2008; Kyte, 2009). Meanwhile, GRNVAC1 has completed its phase II clinical trials in

metastatic prostate cancer and acute myelogenous leukemia (information provided by clinicaltrials.gov). Both have shown to be well tolerated and stimulate hTERT-specific T-cell responses in numerous patients. So far, results are encouraging in these limited-tested cancers. Continuous effort of similar studies in other cancers shall review the potential of telomerase immunotherapy in a broader spectrum of cancers.

Targeting telomerase positive cells with oncolytic viruses

This approach applies the utilization of hTERT promoter as a switch to control the replication of a lytic virus to target telomerase positive cells. The viruses are modified such that genes required for replication are controlled by the hTERT promoter (Irving et al., 2004; Kawashima et al., 2004; Lanson et al., 2003). Therefore, these modified viruses can only be replicated in cells that express telomerase to induce cytolysis. One example being virus OBP-31, which has been reported to inhibit tumor growth in mice xenografts with human lung, prostate and liver cancer cells, and is currently under phase I clinical trials in various types of cancers (Nemunaitis et al., 2010). A second strategy is to use hTERT promoter to drive the expression of suicidal genes e.g. pro-apoptotic proteins (Liu et al., 2002; Majumdar et al., 2001; Schepelmann et al., 2007)

CHAPTER TWO

A New Method to Measure Telomere Replication

Introduction

Telomeres are 5'-TTAGGG-3' repeat arrays at the ends of linear chromosomes that serve as binding sites for the shelterin complex to prevent chromosomal end-to-end fusions and degradation. Failure to properly replicate telomeres in cell division critically inhibits the regeneration of this nucleoprotein, which eventually results in catastrophic genome instability associated with uncapped telomeres. Average lengths of adult human telomeres are 5-15 kb (Dionne and Wellinger, 1996; Greider, 1999b; Stewart et al., 2003).

The unique ability of telomeric chromatin to form various unusual protection-based architectures may propose challenges for the progression of replication fork during telomere replication. Repetitive telomeric and subtelomeric regions are one of the most vulnerable foci prone to replication stress (Nakamura et al., 2005). Moreover, single-stranded G-tract human telomere DNA is capable to adopt secondary intermolecular (Miura and Thomas, 1994) and intramolecular G-quadruplex structures (Zhang et al., 2009), potentially hindering replication fork progression. Other hypothetical structures inferred from in vitro studies include four way junctions, D-loops and triple helical

structures. It has been reported that replication forks can naturally pause or stall at telomeres in yeasts and human (Gilson and Geli, 2007). Studies have suggested that telomere binding proteins impede replication fork progression (Ohki and Ishikawa, 2004), while others suggested that telomere-binding proteins functions to coordinate replication fork progression in preventing stalled forks (Miller et al., 2006; Sfeir et al., 2009). Roles of many telomere binding proteins in replication fork progression have yet to be elucidated.

Methods have been developed to monitor the replication fork progression during S phase. Fluorescently labeled peptide nucleic acid (PNA) $(CCCTAA)_3$ probes followed by flow cytometry (Flow-FISH) monitor telomere lengths at various S phases (Hultdin et al., 1998). 2D gels have been applied to analyze the structures of replication fork progression (Brewer and Fangman, 1987; Nabetani and Ishikawa, 2009). SMARD (single-molecule analysis of replicated DNA), a technique extended from Dynamic Molecular Combing (DMC) (Herrick et al., 2000), was used to analyze individual DNA molecule fork progression rate of specific loci with known lengths. SMARD has recently been adapted to examine the progression of telomere replication (Sfeir et al., 2009), where replicating DNA was monitored with fluorescent from incorporation of halogenated nucleotides in S phase such as IdU and CIdU, together with a C-rich FISH-PNA probe to identify telomeric DNA fibers (Sfeir et al., 2009). However, most of the present techniques often involve cumbersome experimental procedures. In

particular, SMARD requires extensive alignments of hundreds of individual telomeres to be analyzed under the microscope at a given time. We developed a simple approach to efficiently progression of telomere replication fork, and found that the timing it took to replicate telomeres examined by this new assay correlated with the telomere length analysis by TRF. Application of this assay should enhance the identification of factors needed to facilitate or inhibit fork progression difficulties during telomere replication.

Results

To examine the telomere replication fork progression rate, replicating telomeres of synchronized cells were labeled with pulses of 5-bromodeoxyuridine (BrdU – a thymidine analog) upon releasing into S phase. BrdU was differentially incorporated into daughter telomeres such that two BrdU molecules were incorporated per leading repeat (TTAGGG) versus only one BrdU incorporated per each complimentary lagging repeat (CCCTAA). In some cases, iododeoxyuridine (IdU), which has a higher density than BrdU, was used to increase resolution (data not shown). Leading and lagging strands were then separated on a cesium chloride gradient. Aliquots of the gradient fractions were hybridized with a telomere specific probe on a slot blot to differentiate leading versus lagging from unreplicated telomeres. Leading yielded heavier density as compared to that of lagging daughters followed by unreplicated DNA (Fig. 2-1

top panel). The minimal time it took to resolve distinct leading versus lagging peaks indicated the time needed to fully replicate a telomere. Partially replicated DNA would not appear as distinct peaks. For examples, ~25% replicated telomeres would correspond to densities between unreplicated and lagging peaks; ~75% replicated telomeres would fall into densities between fully replicated lagging and leading daughter strands; meanwhile, ~50% replicated telomeres would have the same density as replicated lagging daughter strands (Fig. 2-1 bottom panel).

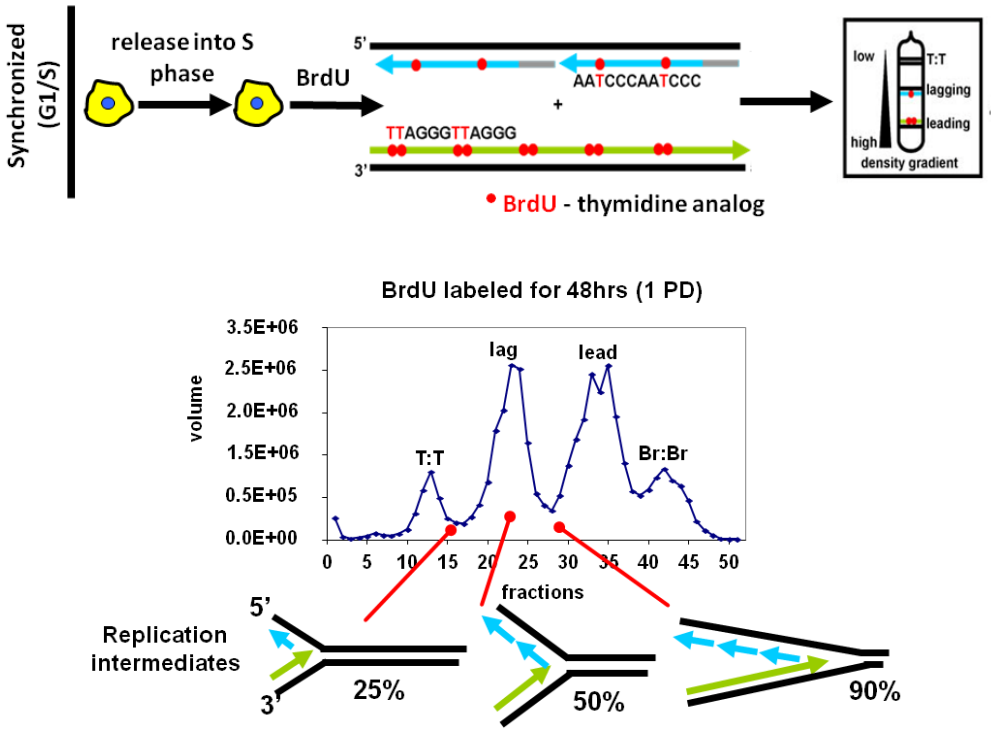

Figure 2-1. Schematic representation of strategy to isolate leading and lagging daughters using cesium chloride gradients in BJ cells. Top panel) Cells were synchronized at G1/S, released into S phase for 3 hours and labeled with BrdU for various time periods thorough S phase. Differential BrdU incorporation allowed for separation of leading and lagging strands on a cesium

chloride gradient. Leading strand replication produces the highest density, followed by lagging, while unreplicated DNA has the lowest density. Bottom panel) Only fully replicated telomeres were resolved into distinct leading and lagging peaks while partially replicated telomeres yielded various intermediated densities.

Cell lines with various telomere lenghts (measured by TRF) were used to study telomere replication using the CsCl centrifugation strategy. These cell lines include Hela cells with short telomeres (~4.2kb), BJ cells with medium telomere lengths (~7.2 kb) and H1299 cells with long telomeres (~10.3kb) (Fig. 2-2 top panel). Synchronized cells were released into S-phase with fresh medium for 3 hrs followed by a 15 min labeling with IdU. IdU is a denser thymidine analog compared to BrdU, and was used in replacing BrdU for achieving maximal peak resolution. First round of CsCl gradient centrifugation with 15 min IdU labeling in all three cell lines did not resolve distinct leading and lagging peaks as expected because only a very small amount of DNA would have been replicated in just 15-min (Fig. 2-2 bottom panel). Fractions with densities corresponding to leading and lagging peaks were combined and rebanded on a second CsCl gradient centrifugation to eliminate most of the unreplicated background DNA peak. To be considered as fully replicated telomeres, both leading and lagging peaks should appear simultaneously; the large lagging only peaks indicated mostly replicated intermediates (Fig. 2-1 bottom panel).

Distinct leading and lagging replicated telomeres appeared in Hela with a 15 min labeled, “maybe” a tiny BJ leading peak, and no leading peak in H1299

(Fig. 2-2 bottom panel). For BJ, a minimal leading peak was observed between 15 and 30 min. H1299 resolved clear leading peaks between 30 to 60 min. The timing it took to resolve distinct leading and lagging peaks correspond to the measured telomere lengths in Hela (Fig. 2-1 top panel, Fig. 2-3), BJ (Fig. 2-1 top panel, Fig. 2-4) and H1299 (Fig. 2-top panel, Fig. 2-5).

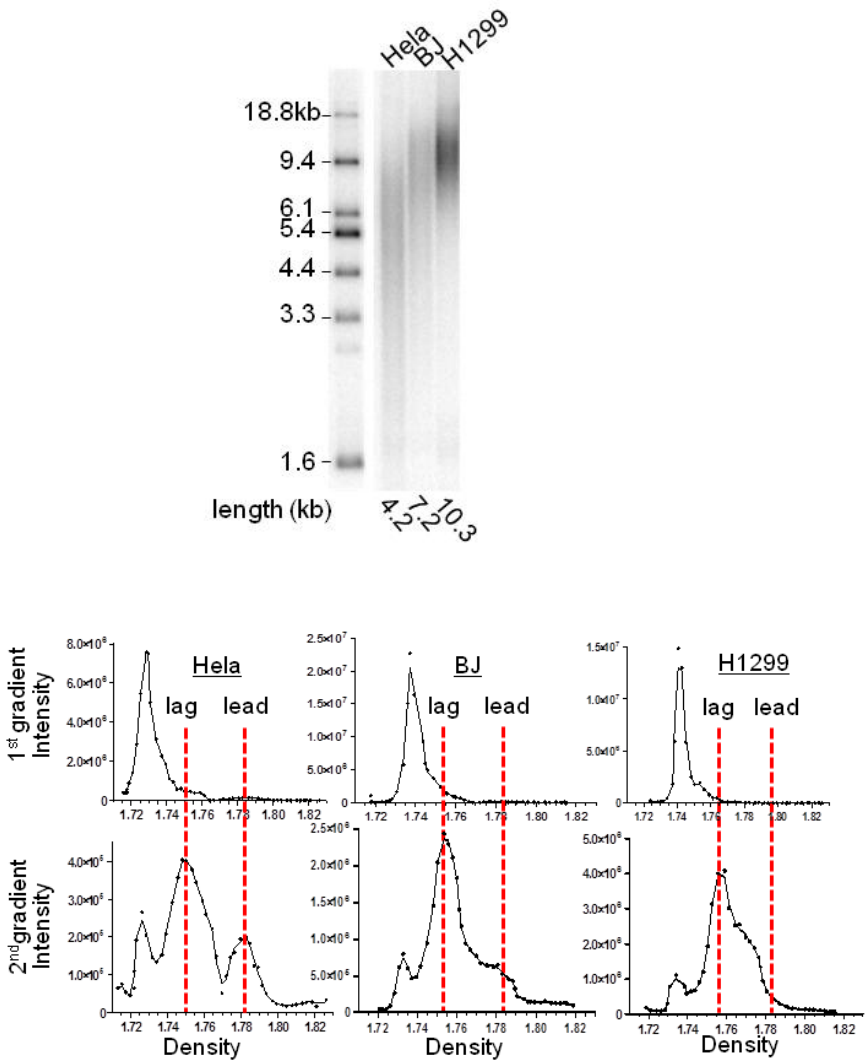

Figure 2-2. Telomere length and telomere replication in Hela, BJ and H1299. Top) Telomere lengths measured by TRF. Hela is ~4.2kb, BJ is ~ 7.2kb, and H1299 is ~10.3kb. Bottom) Timing to resolve distinct leading and lagging peaks is proportional to the measured telomere lengths during a15 min IdU labeling of Hela, BJ and H1299. DNA peaks were identified upon hybridizing aliquots of the fractions collected from the gradient with a telomere specific C-rich probe on a slot blot.

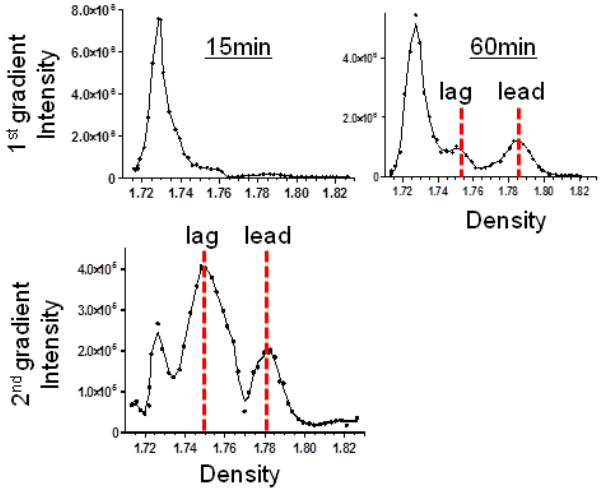

Figure 2-3. Hela telomere DNA completes replication by 15 min. Hela cells were labeled with IdU for 15 and 60 min respectively. At 15 min, little DNA had replicated with majority being unlabeled as shown by the high unreplicated peaks. Therefore, leading and lagging fractions from a first gradient were combined and rebanded on a second gradient to better resolve the peaks. Distinct leading versus lagging peaks are detected by 15 min IdU labeling on a second CsCl gradient, suggesting some Hela telomeres have completed duplex telomeric replication.

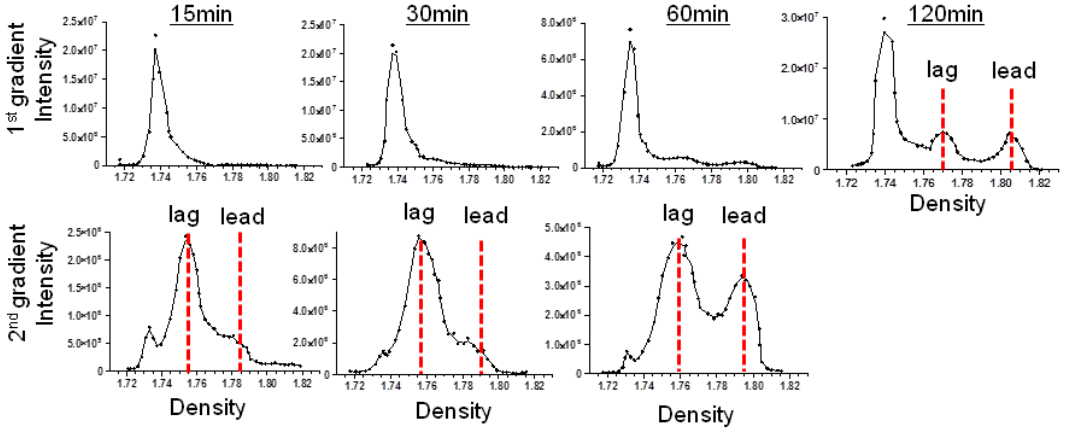

Figure 2-4. BJ telomere DNA completes replication between 15 to 30 min. BJ cells were labeled with IdU for 15, 30, 60 and 120 min respectively. For 15, 30 and 60 min, little DNA had replicated with majority being unlabeled as shown by the unreplicated peaks. 30 min label contained a minimal peak. Therefore, leading and lagging fractions from a first gradient were combined and rebanded on a second gradient to better resolve the peaks. Some BJ telomeres have completed duplex telomere replication between 15 to 30 min.

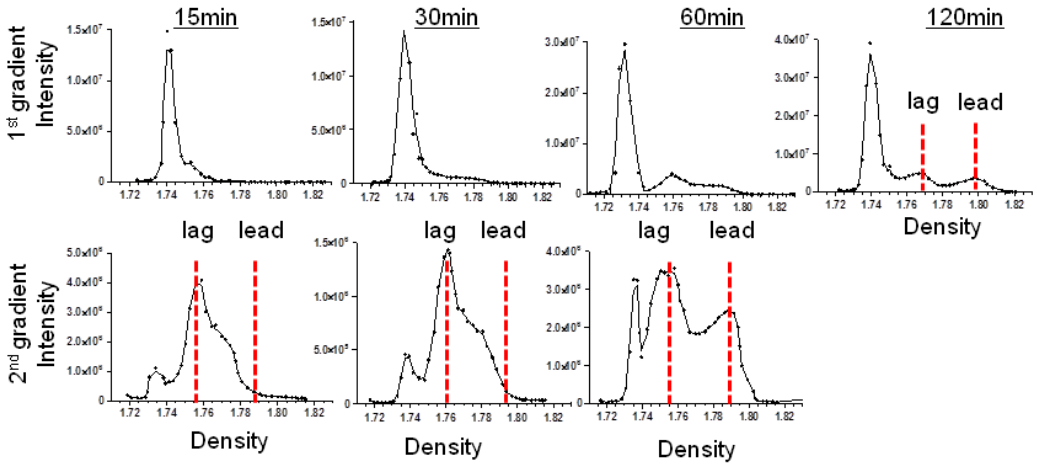

Figure 2-5. H1299 telomere DNA completes replication between 30 to 60 min. H1299 cells were labeled with IdU for 15, 30, 60 and 120 min respectively. For 15, 30 and 60 min, little DNA had replicated with majority being unlabeled as shown by the unreplicated peaks. Therefore, leading and lagging fractions from a first gradient were combined and rebanded on a second gradient to better resolve the peaks. Some H1299 telomeres have completed semi-conservative duplex replication between 30 to 60 min.

Conclusions

We have developed a new, simple assay to study the effect of candidate proteins in telomere replication fork progression in human cell culture by CsCl centrifugation. Complicated telomere chromatic structures appear to pose challenges for replication machinery (Gilson and Geli, 2007). Proper telomere replication is critical because stalled forks at dysfunctional human telomeres are likely to accelerate the genomic instability leading to tumorigenesis.

A few helicases and nucleases have been reported to play a role in telomere replication. RecQ helicases BLM and WRN, FANCJ family helicase RTEL, as well as nuclease FEN1 are known to recover stalled replication forks.

Depletion of these proteins results in telomere loss by FISH or replication re-initiation assay (Crabbe et al., 2004; Ding et al., 2004; Saharia et al., 2010). 5'-3' helicase is required for telomere replication fork progression in yeast (Ivessa et al., 2002). *S. cerevisiae* 5'-3' helicase Pif1 has been shown to resolve G-quadruplex structure to prevent replication fork stalling (Paeschke et al., 2011). Subunits of CST complex is needed for proper telomere replication (Price et al., 2010). *Pombe* Taz1 (ortholog of mammalian TRF1 and TRF2) is required for telomere replication by 2D gels (Miller et al., 2006). TRF1 depletion in mice also results in stalled telomere replication forks using SMARD (Sfeir et al., 2009). Meanwhile, other studies have suggested telomere binding proteins inhibit, rather than enhance, telomere replication fork progression. DNA binding N-terminus domain of Rap1 has been suggested to hinder replication fork progression in yeast (Makovets et al., 2004). Expressions of human TRF1 and TRF2 in an *in vitro* SV40 replication system block replication a linear telomere plasmid (Ohki and Ishikawa, 2004). Nevertheless, the role of TRF1 in telomere replication in human has yet to be elucidated. The involvements of other telomere binding proteins in regulating telomere replication are still largely unknown. Identification of proteins required to ensure efficient telomere replication can now be efficiently examined with this newly developed technique.

It has been estimated that rates of mammalian replication forks progression is about 2kb/min (Anglana et al., 2003; Jackson and Pombo, 1998).

However, it is unknown whether the unique protection-based architecture of telomeres inherently prolonged replication progression rates at mammalian telomere as compared to bulk genomic DNA. The presented technique can also possibly be applied to address this question upon by designing a way to measure bulk DNA replication using the same approach as a control. This technique can measure "average" telomere replication rates by knowing the average telomere lengths and the minimal time it takes for distinct peaks to appear. Individual telomeres replication at different time throughout S phase in human (Wright et al., 1999). Efforts to develop single molecule high throughput approaches to specifically follow replication of single telomeres would be of enormous value in advancing the studying of the roles of candidate proteins in telomere replication.

Materials and Methods

Cell Culture and Cell Cycle Synchronization. BJ foreskin fibroblasts were cultured at 37˚C in 5% CO_2 in high glucose DMEM medium (Hyclone, Logan, UT) containing 15% Comic Calf Serum (Hyclone, Logan, UT). For synchronization, logarithmically growing cells were washed twice with 1xPBS, pre-synchronized with low serum (high glucose DMEM with 0.1% fetal bovine serum and 20mM HEPES) for 48 hr, and then fed fresh medium containing 15% fetal bovine serum and 2 μg/ml aphidicolin (A.G. Scientific, San Diego, CA) for 24 hr. Cells were then washed with 1xPBS (3x), released into fresh medium for 2

hr, and then labeled with 100μM 5-bromo-2'-deoxyuridine (BrdU). For pulse-chase experiments, cells were washed twice with 1xPBS and chased with 100μM thymidine.

Hela cervical carcinoma and H1299 lung cancer cells were cultured at 37˚C in 5% CO2 in high glucose DMEM medium with 10% cosmic calf serum (CS) (Hyclone, Logan, UT). Exponentially growing Hela cells were synchronized with 2mM thymidine (Sigma, St. Louis, MO) for 19 hr (16 hr for H1299), washed with 1xPBS (3x), and incubated with fresh medium for 9 hr (8 hr for H1299). 2mM thymidine was added again for 16 hr (14 hr for H1299). Cells were washed with 1xPBS (3x) and released into fresh medium with 100μM BrdU for 0-10 hr.

Genomic DNA Isolation. Genomic DNA was purified by Qiagen Blood & Cell Culture Midi Kit (Valencia, CA). Precipitated DNA was wash with 70% ethanol (2x), and suspended in 10mM Tris-HCl pH-8. DNA was dissolved at 37˚C overnight.

Telomere Probes Generation. 24 nt probes containing six ^{32}P-dC or six ^{32}P-dG were synthesized as described (Herbert et al., 2003).

CsCl Separation of Leading and Lagging Telomeric Daughters. CsCl gradient separation was performed as described (Zhao et al., 2011c) with modifications. In brief, 500 µg purified genomic DNA was digested with HinfI and RsaI overnight in 250 µl and terminated by the addition of EDTA to 10mM. DNA was mixed with CsCl solution (density of 1.79 g/ml with 5mM Tris-HCl pH8, 2mM EDTA) to obtain a final density of ~1.760-1.770 g/ml, and added to a polyallomer quick-seal centrifuge tube (Beckman, Brea, CA). Samples were centrifuged at 25˚C and 55,000 rpm for 20 hr using a VTi-80 vertical rotor (Beckman, Brea, CA). Fractions of the sample were collected and aliquots were denatured and hybridized with a telomere specific probe on a slot blot to identify the fractions that contained telomere DNA. The corresponding densities were obtained by measuring the refractive index. Leading DNA was located at a density of 1.790 - 1.800 g/ml, lagging at 1.760 - 1.770 g/ml, and unreplicated at 1.740 - 1.750 g/ml.

CHAPTER THREE

Telomere Overhang Processing in Normal Cells

Introduction

Adult human telomeres contain approximately 5-15 kb of hexameric DNA repeats (5'-TTAGGG-3') ending with 3' single-stranded G-rich overhangs ranging in sizes from ~12-300 nucleotides (nt) (Chai et al., 2006a; Chai et al., 2005; Cimino-Reale et al., 2001; Dai et al., 2010; Keys et al., 2004; Makarov et al., 1997; Stewart et al., 2003; Wright et al., 1997; Zhao et al., 2008). Invasion of the 3'G-overhangs into duplex telomere DNA form a lariat-like t-loop structure that protects chromosome ends from being recognized as DNA double-stranded breaks (Griffith et al., 1999). Telomeres can be lost during each cell division due to the inability to replicate the 3' ends of chromosomes, known as the end-replication problem. Telomerase, a reverse transcriptase, adds telomeric repeats to the 3' chromosome ends to compensate for the telomere loss from the end-replication problem. Nevertheless most of the telomeres repeats are maintained by semi-conservative DNA replication. In cancer and stem cells, this overhang provides the ss substrate required for telomerase extension. This G-overhang can be inserted into homologous double-stranded (ds) regions, resulting in a lasso-like telomere loop (t-loop) structure thought to prevent chromosome ends from being recognized as ds breaks (Griffith et al., 1999).

The mechanism generating these overhangs has been sought for many years. Many DNA damage signaling factors such as ATM, ATR, the MRN complex and their homologues in other species (Chai et al., 2006b; Denchi and de Lange, 2007; Deng et al., 2009; Dimitrova and de Lange, 2009; Goudsouzian et al., 2006), helicases (Bonetti et al., 2009; Li et al., 2009; Tomita et al., 2004). telomere associated proteins (Ballal et al., 2009; Churikov et al., 2006; Garvik et al., 1995; Grandin et al., 2001; Grandin et al., 1997; Nugent et al., 1996; Price et al., 2010; Song et al., 2008; van Steensel et al., 1998; Wu et al., 2006), and nucleases (Tom and Greider, 2010; Tomita et al., 2003; Wu et al., 2010) have been found to affect overhang length. However, it is very difficult to interpret the mechanism of action of these factors without knowing the specific steps involved in overhang generation. Some of these factors may affect overhang length by influencing the action of telomerase (Chai et al., 2006b; Zhao et al., 2009), while others may affect additional overhang processing steps. The number, nature and timing of the steps involved in producing an appropriately sized G-rich 3'-overhang following telomere replication in the absence of telomerase remain unknown. How overhang maturation is coupled with telomere replication also remains undefined. In particular, the differences in the biogenesis of leading versus lagging overhangs have not been characterized. It is generally assumed that the nuclease activity of C-strand resection is tightly associated with the

replication machinery, and is dependent on the alteration of telomere chromatin during replication fork progression.

Short G-overhangs have been observed in several organisms, e.g. ~14 nt in ciliates (Jacob et al., 2001; Klobutcher et al., 1981) and ~12-14 nt in *S. cerevisiae* (Larrivee et al., 2004). This is approximately the size of the RNA primer laid down by polymerase alpha during lagging strand synthesis (Kunkel and Burgers, 2008; Nick McElhinny et al., 2008). Together with the original hypothesis of the end-replication problem by Watson (Watson, 1972) and Olovnikov (Olovnikov, 1973) about 40 years ago, this has led to the model presented in most modern textbooks: the final primer of Okazaki fragment synthesis is positioned at the very end of the chromosome. However, the presence and location of the final RNA primer has not yet been directly demonstrated in any organism

In the absence of telomerase, the size of the overhangs represents a reduction in the size of the C-strands. In the absence of damage to telomeres, this determines the rate of telomere shortening, and the amount that telomerase would have to extend to compensate and maintain telomere length (Zhao et al., 2009). In yeast, the rate of telomere shortening in the absence of telomerase (estimated at 3-5 bp/division) (Marcand et al., 1999) is close to what would be expected based upon the observed 12-14 nt overhangs (Larrivee et al., 2004), and telomerase only adds ~44 nt to ~7% of the ends (Teixeira et al., 2004), for an average addition that matches the shortening rate. The rate of telomere shortening in cultured

telomerase-negative human cells is 50-100 bp/division, which is also close to what would be expected when the average overhang in telomerase-negative cells is ~60-70 nt (~30 nt for the daughters of leading strand synthesis and ~100 nt for the daughters of lagging strand synthesis) (Zhao et al., 2008). In human cancer cells, telomerase adds ~60 nt to every chromosome (Zhao et al., 2009), again closely matching the shortening rate. There are two alternate mechanisms thought to explain why the length of telomere loss per cell division in human cells is considerably longer than a simple result of the end-replication problem upon removal of terminal RNA primers. The first assumes that the RNA primer is positioned at the very terminal end, and both leading and lagging daughters are processed by nuclease resection on the C-strands. An alternative model would be that the initial lagging overhang reflects the position of the final Okazaki RNA priming event as the replication complex runs off its linear template. The removal of the RNA primer could then generate an initial overhang that is almost the size of the fully processed overhang. Meanwhile, the initially blunt leading daughters would be processed by nucleases or helicases to generate mature overhangs (Sfeir et al., 2005). It is unknown when leading overhangs are processed from the blunt ends, or whether overhang processing events happen simultaneously or involve multiple steps.

ChIP analysis has shown that the association of a variety of proteins at telomeres change at S/G2 in normal diploid cells (Verdun et al., 2005; Verdun

and Karlseder, 2006), suggesting that some processing events might occur at that time. Uncovering the steps that regulate the processing of human telomeric overhangs is thus fundamental in understanding the roles of candidate proteins involved, and potentially manipulating rates of replicative ageing and telomerase action.

In the present study, combinations of modified and newly developed biochemical assays were designed to monitor the dynamics of overhangs sizes, RNA primers on lagging daughters, and the C-terminal nucleotides during a single cell cycle. We found that lagging daughters had an almost mature overhang size very soon after replication, and were capped by a terminal RNA primer for approximately one hour following replication prior to its removal, leaving a specified C-terminal nucleotide. In contrast, leading daughters only developed a mature size 1-2 hours after replication and did not acquire a specific C-terminal sequence until S/G2. We present a model characterizing overhang processing in two distinct phases to summarize the complex series of steps by which human linear chromosomal ends are replicated.

Results

Mature overhang sizes are present by 2 hours after replication

In order to establish the window within which mature overhang sizes were established, we examined the end-structure in telomeres labeled for increasing times with 5-bromodeoxyuridine (BrdU). Normal human foreskin fibroblast BJ cells were synchronized at the G1/S interface with an efficiency of 65%-75%, with an S phase of about 6-8 hr (Fig. 3-1).

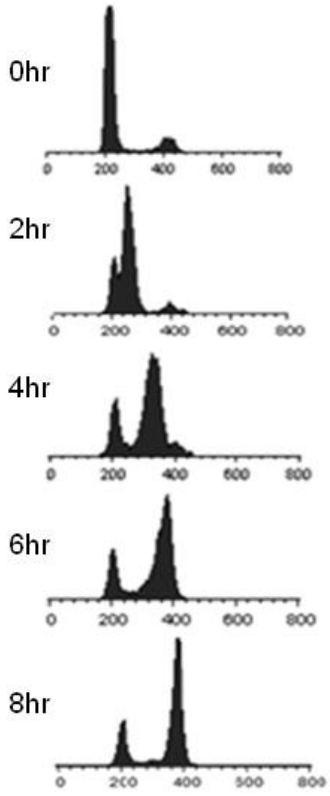

Figure 3-1. FACS analysis of BJ fibroblasts synchronized at G1/S. Cells are synchronized at the G1/S interface with an efficiency of 65%-75%.

Telomeres in BJ cells replicate throughout S-phase, but there is a delay of approximately 2 hr before significant numbers of telomeres begin to replicate (Wright et al., 1999). Cells were first released into S-phase in fresh medium for 2 hr, before treating with BrdU (Fig. 3-1). This procedure maximized the amount of BrdU-incorporated into the newly synthesized telomeres during short periods. Cesium centrifugation of samples labeled for 2, 4, 6 and 8 hr showed distinct leading versus lagging peaks as expected (Fig. 3-2). Cells labeled for one population doubling ([PD], ~48 hr) served as a positive control for fully processed overhang sizes. The fractions corresponding to leading versus lagging telomeres were then pooled and analyzed.

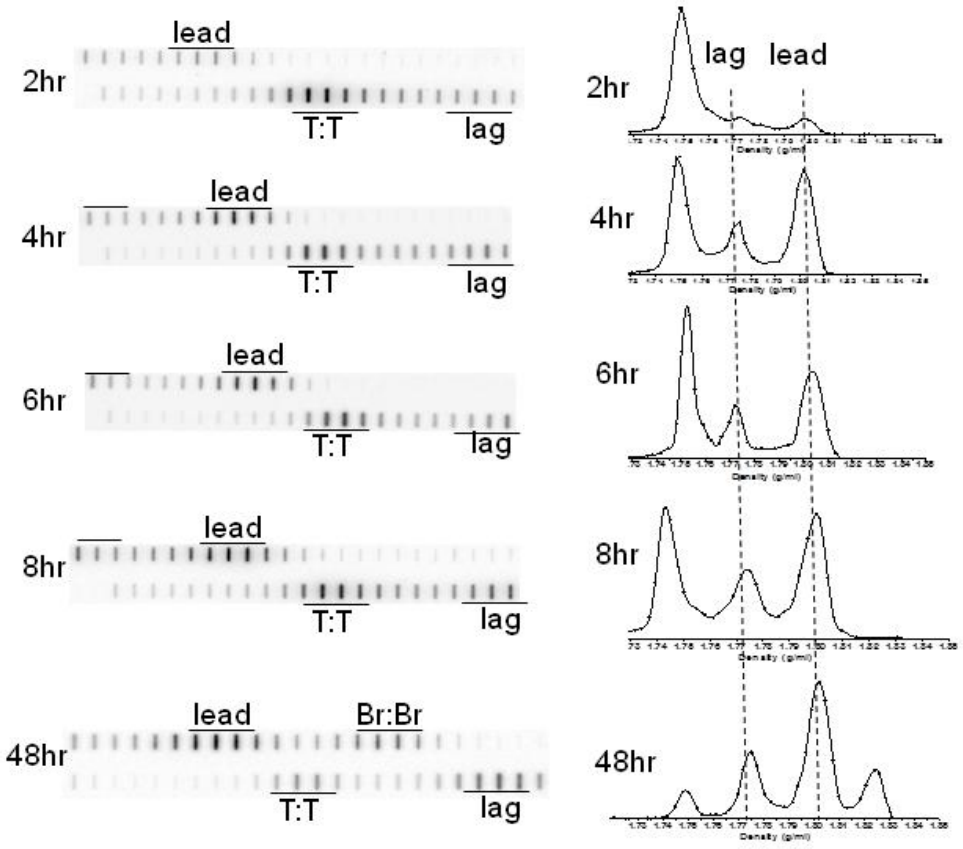

Figure 3-2. Separation of leading versus lagging daughter strands using CsCl centrifugation. Left panel) Aliquots of the fractions are collected and hybridized with telomere specific probe on a slot blot. Right panel) Quantification of the slot blot. 48 hr represents the 1 population doubling (PD) control.

Two independent approaches were used to quantify overhang lengths. Signals from ss overhangs hybridized to a telomere probe under native conditions were normalized to the total telomere signal obtained after the ds DNA was denatured. This native in-gel hybridization approach measures changes in overhang abundance (Fig. 3-3). Alternatively, duplex specific nuclease (DSN)

digests ds telomere DNA to less than 10 bp in length with no effect on ss telomere G-overhangs (Fig. 3-3), allowing the quantitative determination of overhang sizes as short as 12 nt (Zhao et al., 2008). Due to the heterogeneity of overhangs, a wide range of sizes were distributed on a denaturing gel.

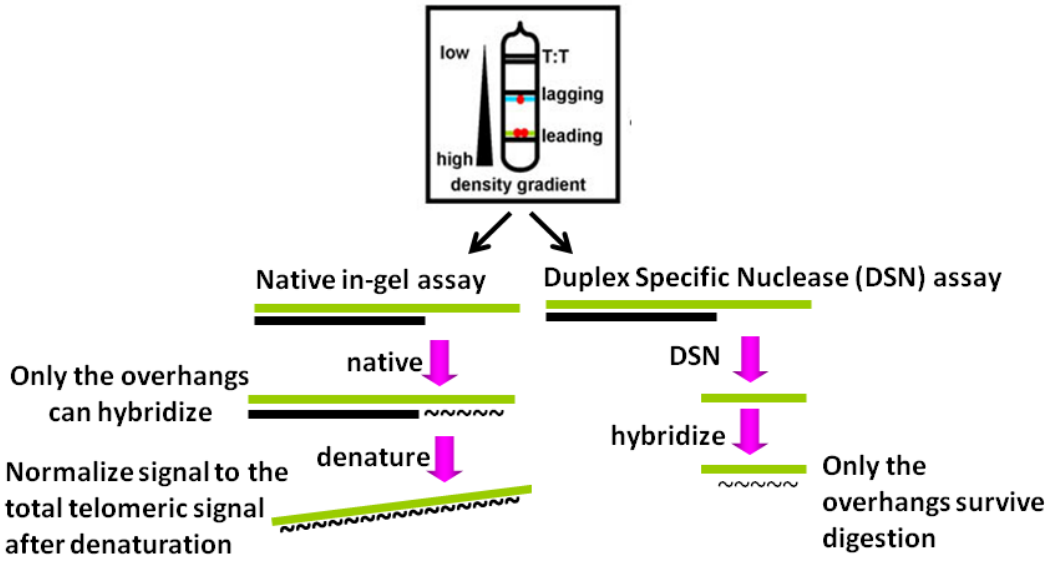

Figure 3-3. Approaches to analyze overhang lengths. Upon purifying leading and lagging daughters by cesium chloride gradients, overhangs were analyzed by two independent methods. For the native-in-gel assay, telomeres were hybridized under native conditions to detect overhangs. Gels were then denatured and probed for total telomeric signals as internal loading controls for normalization. In the DSN assay, purified leading and lagging were digested with DSN, leaving only the overhangs for detection.

In control cells labeled with BrdU for one population doubling (1 PD), lagging overhangs were ~3 fold more intense than leading overhangs after signal normalization on in-gel hybridization (Fig. 3-4 top panel). Average leading overhangs were ~30 nt and lagging were ~100 nt by the DSN assay (Fig. 3-4

middle panel). In both assays, a 3'→5' Exonuclease (ExoI) was used as a negative control to digest 3' overhangs. These controls were consistent with our previous observations (Chai et al., 2006a; Zhao et al., 2008). Using the same analyses, we showed leading and lagging overhangs were processed to nearly mature sizes within 2 hr (0 – 2 hr released, 2 – 4 hr BrdU incorporation) (Fig. 3-4 bottom panel).

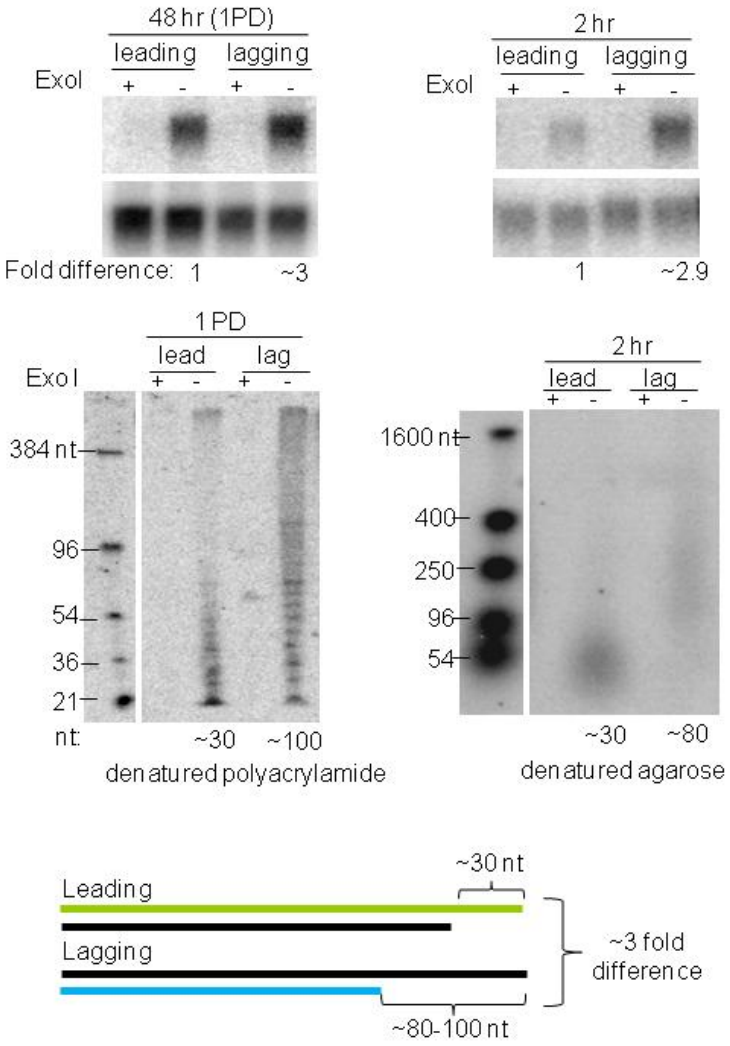

Figure 3-4. In-gel hybridization of telomeres for 1 PD (left column) versus 2 hr (right column). Top panel) The native gel was first hybridized to show the ss overhang, then denatured and re-hybridized to show the total telomeric signal. Overhang signals are normalized to the total genomic input (native / denatured). Both 2 hr and 1 PD lagging overhangs have 3 times overhang signals as compared to that of the leading overhangs. Middle panel) DSN of 1 PD on denatured polyacrylamide gel (left) versus 2 hr on alkaline agarose gel (right). Both 2 hr and 1 PD lagging overhangs have 3 times overhang signals as compared to that of the leading overhangs. Bottom panel) Summary of the expected fold difference between leading vs lagging overhangs signals of both 1 PD & 2 hr, and overhang length (nt) obtained, which indicates the mature overhang sizes.

Lagging, but not leading overhangs have almost mature overhang size immediately following replication

We then determined whether overhangs were processed to mature sizes immediately following replication at the earliest time point we could analyze. Only fully replicated telomeres can be resolved into distinct peaks on a CsCl gradient, since partially replicated telomeres have densities ranging from that of unreplicated DNA to midway between the leading and lagging peaks. 500 μg genomic DNA from a 1 hr BrdU label (0 – 2 hr released, 2 – 3 hr BrdU incorporation) generated only small leading and lagging peaks (Fig. 3-5). Fractions containing leading and lagging peaks from two independent CsCl gradient centrifugations (~1 mg total genomic DNA) were combined and rebanded on a second CsCl gradient to better resolve the different fractions. A hypersensitive telomere probe was developed to maximize the sensitivity of overhang signal detection (Fig, 3-6, Materials & Methods). Distinct leading and lagging peaks were present, indicating that some telomeres had been completely replicated within 1 hr (Fig. 3-5).

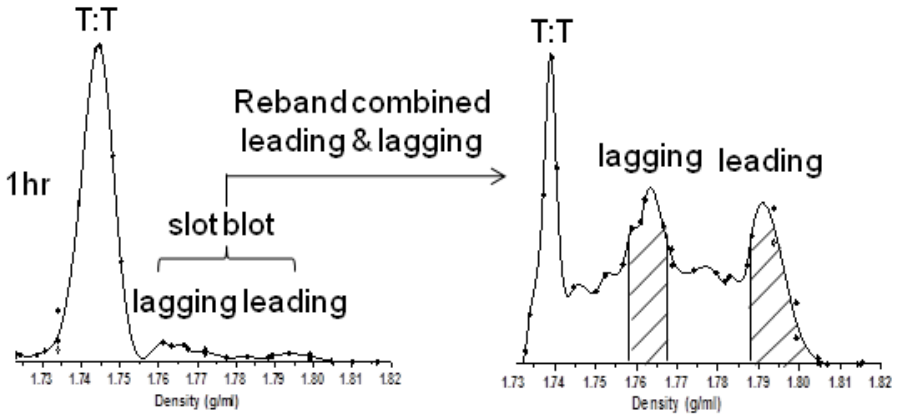

Figure 3-5. Strategy to obtain 1 hr labeled BJ with minimal BrdU labeled telomeres. The small amount of 1 hr labeled BJ telomeres on a 1st CsCl gradient are combined and rebanded on a 2nd CsCl gradient for higher resolution of fully replicated leading and lagging peaks (shaded area).

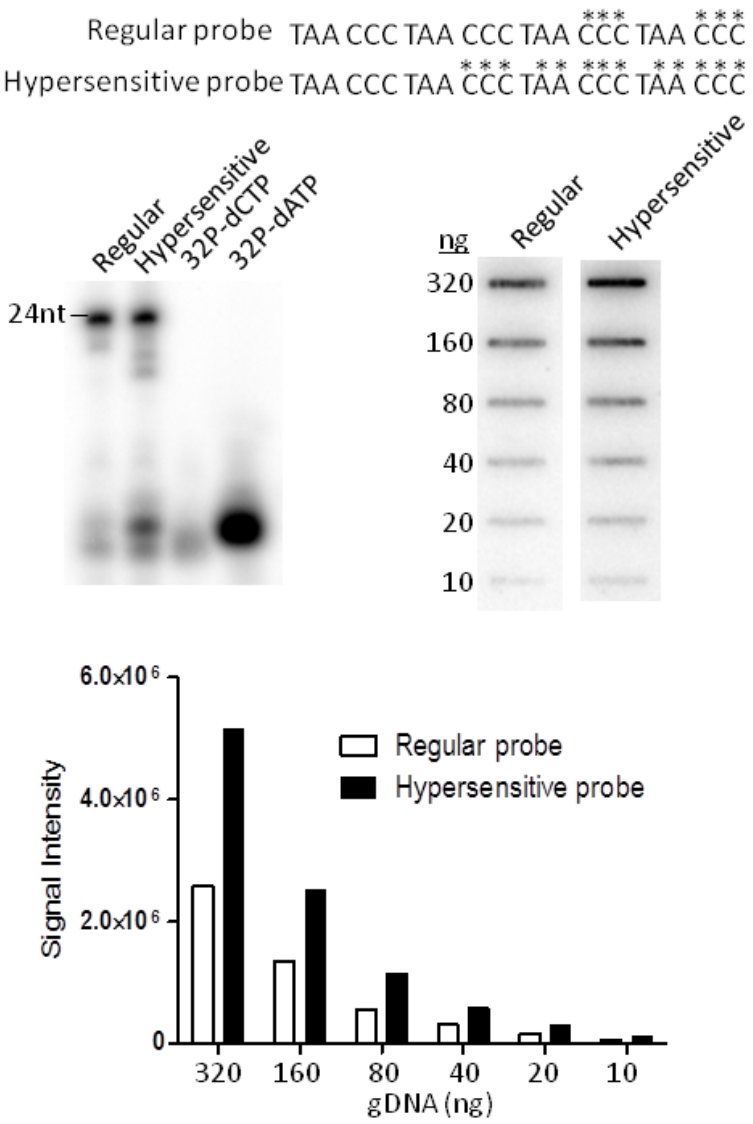

Figure 3-6. Validation of a hypersensitive telomeric probe. Top panel) Regular probe contains 6 radioactive ^{32}P per 24 nt telomeric sequence. Hypersensitive probe contains 13 radioactive ^{32}P per 24 nt telomeric sequence. Middle Panel) 24 nt telomere probe is resolved on a denatured polyacrylamide gel (left). Genome DNA with two fold dilutions from 320 ng to 10 ng were denatured and hybridized with either regular or hypersensitive probe on a slot blot. Quantification is shown in bottom panel.

By in-gel hybridization, 1 hr and 2 hr labeled lagging telomeres had similar overhang signals (0.8 versus 1.0, normalized signals), indicating that lagging telomeres had almost mature overhang sizes at the earliest time point we could analyze following replication (Fig. 3-7 top panel, left). However, the overhang signal from 1 hr leading daughters was an average of ~15 fold less intense than the almost mature 1 hr lagging overhangs (Fig. 3-7 top panel, right), far less than the roughly 3-fold difference found in the mature overhangs control (Fig. 3-4 & 3-7). Leading overhangs were thus not processed to almost mature lengths until an additional hour had passed.

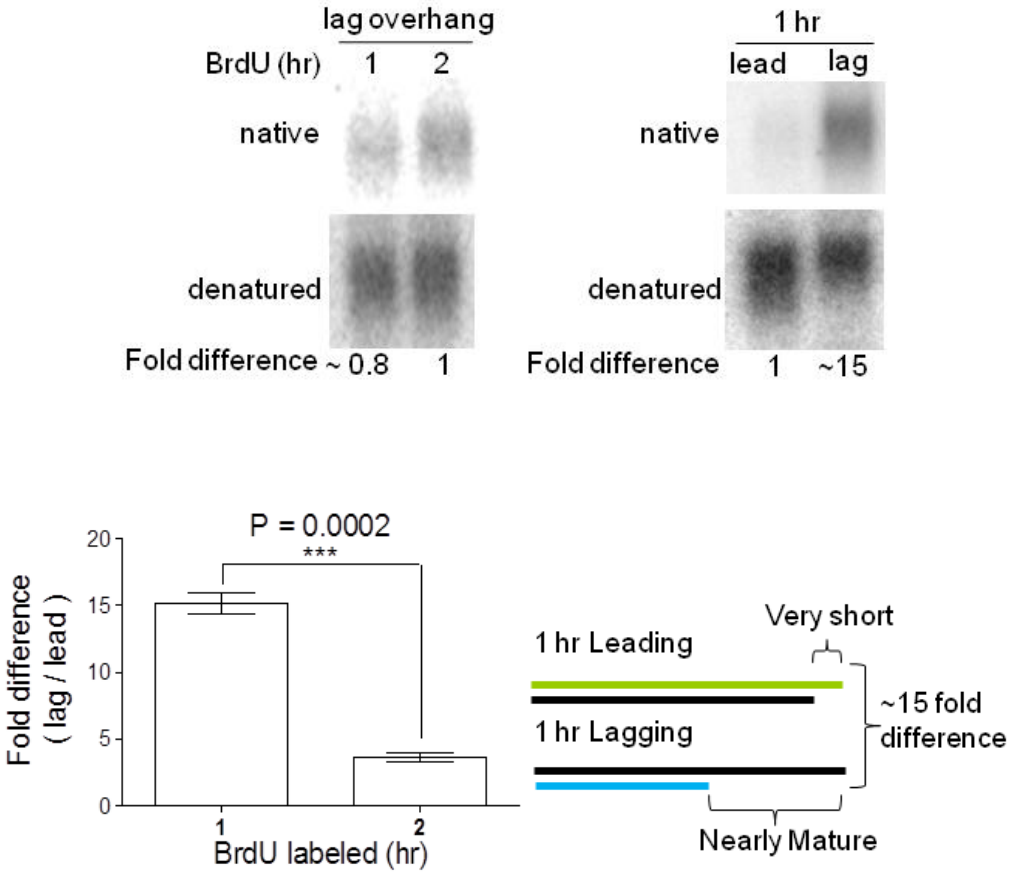

Figure 3-7. Lagging overhang size is almost mature following replication but leading overhang size maturation is delayed. Top panel) In-gel hybridization comparing overhangs of lagging daughters at 1 hr versus 2 hr (left), and overhangs of leading versus lagging daughters at 1 hr (right). Bottom panel) Quantification of (lagging/leading) fold difference comparing 1 hr versus 2 hr controls from 3 independent experiments using two-tailed Student's *t*-test with *** P<0.001 (P = 0.0002), 99% confidence level. Error bars represent SEM. Summary of the results of 1 hr leading versus lagging daughter strands.

The terminal lagging Okazaki RNA primers remain for about 1 hr after replication on lagging daughters

In order to determine whether the final primer of Okazaki fragment synthesis was still present immediately following replication or whether nuclease processing (that would remove this primer and rapidly generate the long overhang on lagging daughters) had occurred, we developed two independent assays.

i) Lambda exonuclease assay to monitor lagging terminal RNA primers

Lambda exonuclease (Lambda exo) is a highly processive 5'→3' exonuclease that removes 5' mononucleotides from ds DNA. We first determined that lambda exo was unable to digest RNA in RNA/DNA heteroduplexes. 18 nt DNA or RNA oligos 5' labeled with ^{32}P-γ ATP were annealed to an excess of complementary 88 nt oligos to generate dsDNA or heteroduplex substrates with a 3' ss overhang (Fig. 3-8). The radioactive DNA oligos in ds DNA were digested; however, lambda exo was unable to remove even the single 5'-ribonucleotide in the heteroduplex (Fig. 3-8).

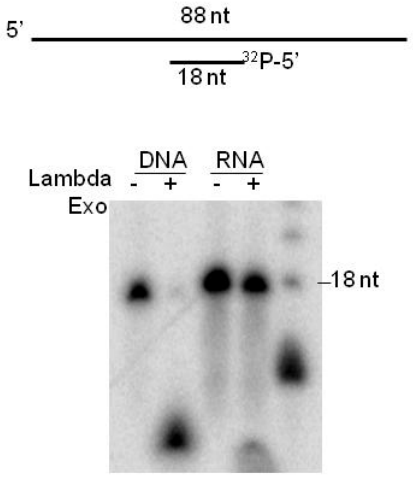

Figure 3-8. In vitro validation that lambda exo cannot digest RNA. 18 nt either DNA or RNA oligos, which has been end-labeled with ^{32}P, was preannealed to its complementary strand (88 nt) to generate duplex substrate for lambda exo digestion. DNAoligo is sensitive but RNA oligo is resistant to lambda exo digestion.

The C-strand of leading daughter terminates in DNA, and was used as a positive control for the action of lambda exo on telomeres. Leading daughters showed the expected decrease in size and 50% decreased intensity of the C-strand following lambda exo digestion (Fig. 3-9). However, the C-strand of lagging daughters from Hela cells labeled for 2 hr immediately upon release into S were resistant to digestion, but were digested after 4 hr and 10 hr labeling periods (Fig. 3-9). Quantification demonstrated that the 2 hr lagging daughters retained most of the C-strand signal, while the 4 hr lagging daughters had approximately 50% C-strand signal reduction (Fig. 3-9).

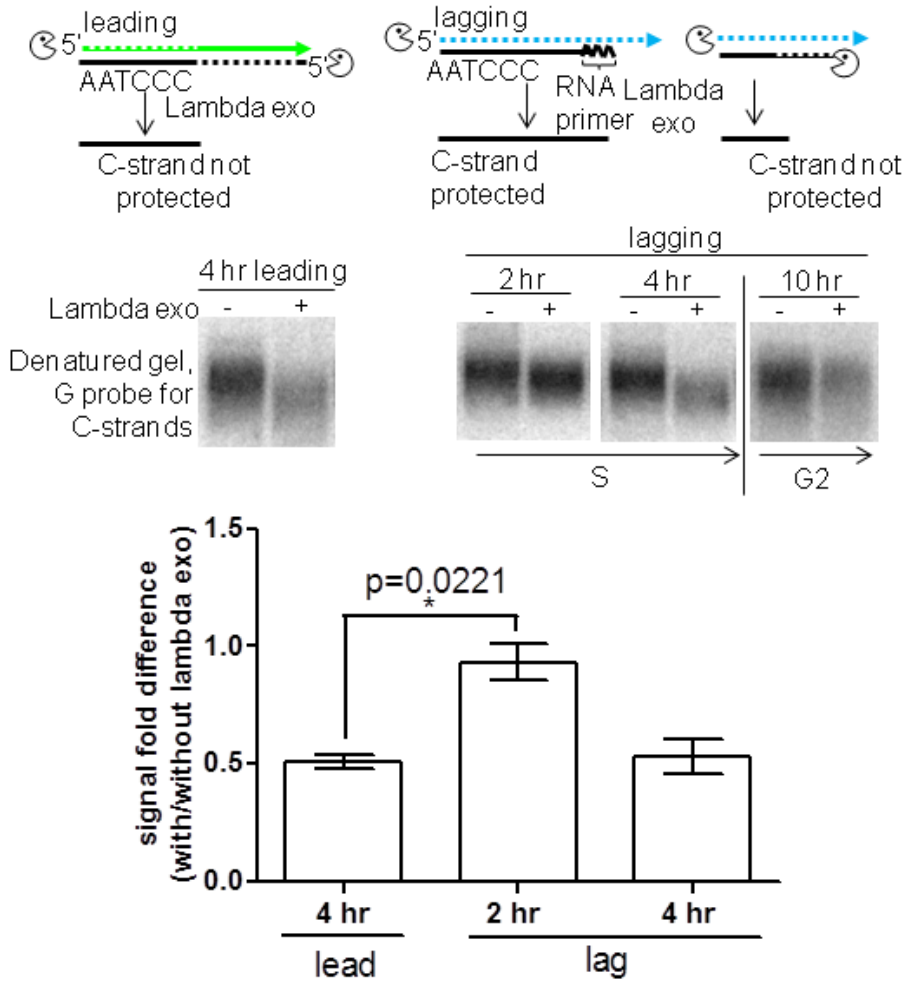

Figure 3-9. RNA primers remain on lagging C-strands following replication – lambda exonuclease assay. Top panel) Positive control: leading 4 hr Hela telomeres are lambda exo sensitive (left). Dynamics of Hela lagging RNA primers across S-phase (right). Lagging telomeres are resistant to lambda exo at 2 hr. Bottom panel) 3-5 independent experiments are quantified by comparing lambda exo signal fold differences of lagging 2 hr versus 4 hr to that of the control leading 4 hr using two-tailed Student's *t*-test with * $P<0.05$ ($P = 0.0221$), 99% confidence level. Error bars represent SEM.

As a control, lagging telomeres with intact RNA primers should to be sensitive to T7 exo digestion. T7 exo digestion produced the expected digestion of both C-rich and G-rich strands. T7 exonuclease (T7 exo) is a 5'→ 3' exonuclease with low processivity which degrades both RNA and DNA from RNA-DNA heteroduplexes (Fig. 3-10). These results indicated that something, presumably an RNA primer, was inhibiting lambda exo but not T7 exo for at least an hour following the completion of lagging strand replication.

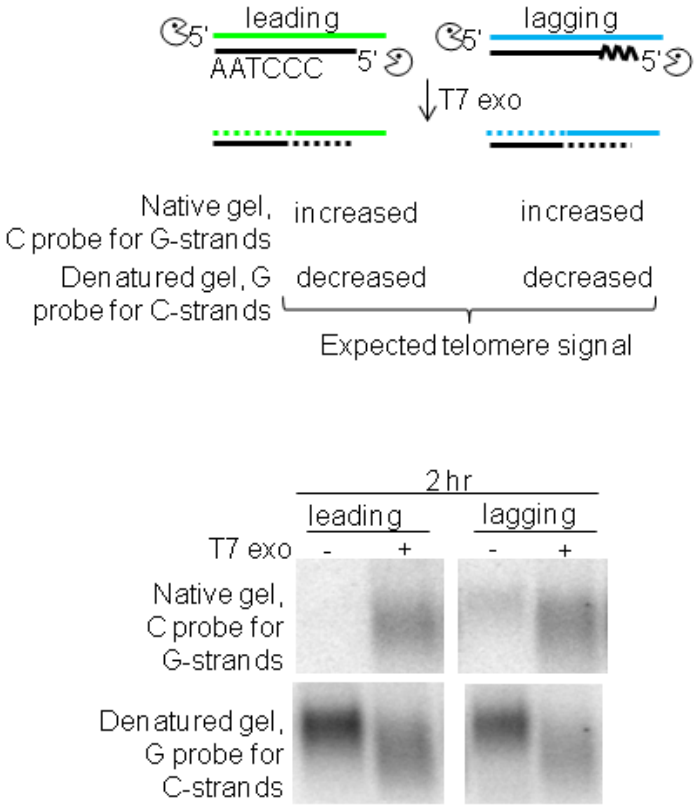

Figure 3-10. Sensitivity of 2 hr leading and lagging daughter strands to T7 exo. Top panel) Predicted results of T7 exo, which digests 5' mononucleotides of both DNA and RNA. Bottom panel) 2 hr leading and lagging both are sensitive to T7 exo.

ii) Pull-down assay to monitor lagging terminal RNA primers

The identification of RNA as the block to lambda exonuclease digestion was established by the ability of RNase HII (an endonuclease that nicks heteroduplexes at multiple sites within the RNA) to release purified lagging C-strands following ligation of biotinylated oligonucleotides to their 5' end (Fig. 3-11 top panel). A mixture of six biotinylated telorette oligos (as used for STELA in Fig. 3-12 to 3-14) was ligated to either purified leading (control) or lagging Hela DNA, and retrieved using streptavidin-coated magnetic beads. RNase HII should release telomeres anchored to the beads via ligation to RNA but not those in which the telorette oligo is ligated directly to DNA. We first verified the assay was telomere-specific and ligase-dependent by ligating purified leading 4 hr labeled telomeres to either a mixture of the six telorette oligos or biotinylated random oligos (Fig. 3-11 middle panel). Retrieved telomeres were then released using formamide. Only the telorette oligos yielded a C-strand signal, and the signal was dependent on the inclusion of ligase (Fig. 3-11 top panel). The experiment was then repeated using 2 hr labeled DNA (0 – 2 hr BrdU label upon release into S-phase) and RNase HII release rather than formamide (Fig. 3-11 bottom panel). Leading control telomeres showed no detectable release while lagging telomeres were liberated (Fig. 3-11 bottom panel). This confirmed that

RNA was still present on telomeric lagging daughters for a significant time following replication.

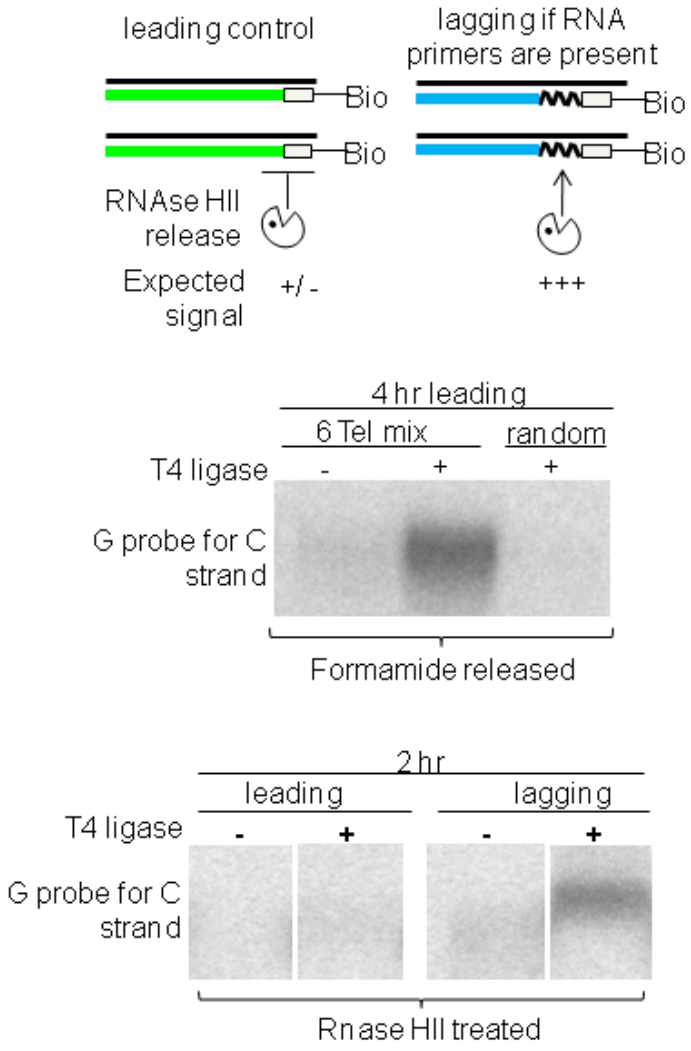

Figure 3-11. RNA primers remain on lagging C-strands following replication – pull-down assay. Top panel) A mixture biotinylated C-telorette oligos are ligated to leading or lagging Hela telomeres, and pulled down with streptavidin beads followed by RNase HII treatment to released ligated products containing RNA. Middie panel) Ligation is specific to the six C-telorette oligos but not the random oligos. Bottom panel) 2 hr lagging but not leading strands are released by RNase HII.

Leading terminal nucleotide is specified at S/G2

Fully processed overhangs exhibit a specific C-terminal nucleotide (Sfeir et al., 2005). We then asked whether the processing of leading daughters C-strands to produce this CCAATC-5' terminal sequence occurred simultaneously as overhang sizes mature. This timing was monitored using a modified STELA (Baird et al., 2003; Sfeir et al., 2005) (Fig. 3-12) with two independent experimental designs.

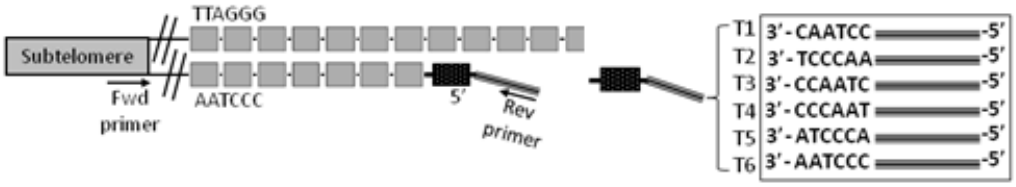

Figure 3-12. Approaches to perform modified STELA to determine terminal nucleotide. Individual C telorettes representing the six permutations of the hexameric C-rich repeat are ligated in separate reactions to the same amount of DNA, and amplified using a forward Xp/Yp subtelomere specific primer and a reverse Teltail primer. Only the C-telorette that anneals adjacent to the last base of the complimentary strand will be ligated to the end of the telomere, and thus produces a PCR product.

Cells were either BrdU labeled for increasing periods of time starting at 2 hr after release into S phase (Fig. 3-13 left column) or BrdU labeled for 3 hr at the time of release into S phase and chased with thymidine for increasing periods of time (Fig. 3-13 right column). Replicated leading daughters were then isolated on CsCl gradients and analyzed. Both protocols indicated that C-strand 5' ends of leading daughters were initially random though mature overhang sizes have already been generated, and were only processed so that most ended in CCAATC-5' late in S phase or S/G2 (Fig. 3-13).

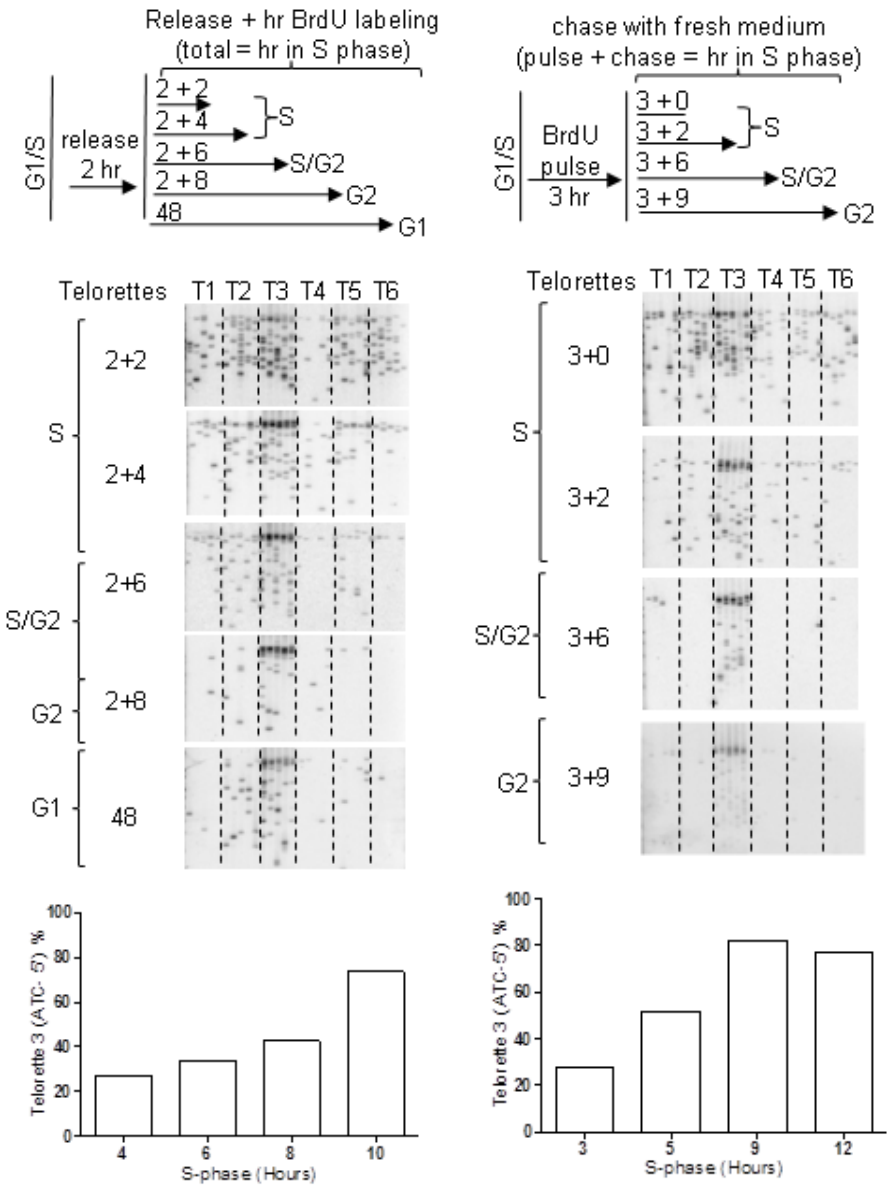

Figure 3-13. Leading daughters C-strand terminal nucleotides are random until S/G2. Top left panel) Approach: Synchronized BJ fibroblasts released into S phase for 2 hr are labeled for 2, 4, 6, 8 or 48 hr. Middle left panel) 2 hr leading strands (2 + 2 hr in S-phase) show randomized ends but become specified for ATC-5' (T3) at S/G2. Bottom left panel) Quantification. Top right panel) Approach: Synchronized BJ are pulsed for 3 hr with BrdU, and chased for 0, 2, 6, 9 hr in fresh media. Middle right panel) 3 hr BrdU pulse results in randomized ends (28% T3 preference) that become specified at S/G2 (3 + 6 hr) with 82% ATC-5' (bottom right panel).

Lagging terminal nucleotide is specified rapidly

In contrast to leading ends, the terminal nucleotides of lagging daughters were specified within 2 hr after replication (0 – 2 hr released, 2 – 4 hr BrdU incorporation). Cells were labeled for increasing periods of time starting at 2 hr after release into S phase (Fig. 3-14 top panel). The sequence CCAATC-5' was predominant even at the earliest times examined (Fig. 3-14 middle and bottom panels).

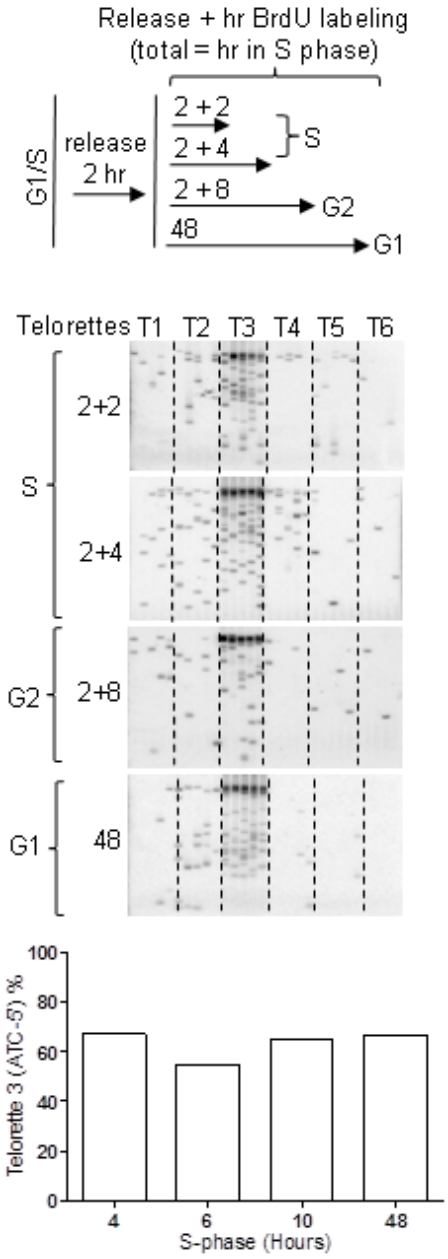

Figure 3-14. Lagging C-strands terminal nucleotides are rapidly specified. Top panel) Approach: Synchronized BJ are released into S phase for 2 hr in fresh media followed by BrdU labeling for 2, 4, 8 hr with 48 hr as a positive control. Middle panel) ATC-5' preference is present at all time points ranging from 55%-67% (Bottom panel).

Conclusions

Telomeres require 3' overhangs for chromosome end protection. The details of how these overhangs are generated in normal human cells are unknown. The present results provide important insights implicating several distinguishable steps in telomere end-processing occur in two distinct phases of any given telomere summarized in Fig. 3-15. Since human telomeres replicate throughout S-phase (Ten Hagen et al., 1990; Wright et al., 1999), there can be many hours intervening between the first and second phases of end-processing. The telomeric G-strand is always generated by leading strand synthesis, and should end in GGTTAG since 80%-85% of fully processed telomeres have C-strands ending in the complementary sequence CCAATC-5' (Sfeir et al., 2005). However, approximately equal amounts of G-ends of the sequence GGTTAG, GGTTA and GGTT were found in normal diploid cells (Sfeir et al., 2005). This is consistent with the concept that the replication apparatus simply runs off the ends and often fails to incorporate the final one or two nucleotides at the extreme termini (Sfeir et al., 2005). Although this does not rigorously exclude the possibility of G-strand processing, we believe the initial result of leading strand synthesis usually has a 0, 1 or 2 nt 5' overhang due to the challenges of making fully blunt-ended products. The early phase lasts approximately one hour following the completion of basic replication (the ability to physically separate the daughters of leading and lagging

strand synthesis). We show that the final RNA primer of lagging daughter telomere is not positioned at the extreme end of chromosome as it is usually illustrated in modern textbooks. Rather, it is positioned ~70-100 nt from the end, and is not removed for about one hour following replication. The end-replication problem in human cells is thus not primarily produced by the inability to synthesize the 10-14 nt gap caused by the removal of the RNA primer, but rather by the ~70-100 nt gap resulting from the inability to position the final RNA priming event at the extreme end of a telomere. Furthermore, the resection of the initially blunt daughter of leading strand synthesis is not tightly coupled to replication, but it is a prolonged process across the early phase. The specification of the terminal nucleotide of the C-strand (late processing phase) occurs at different times for leading and lagging daughter telomeres.

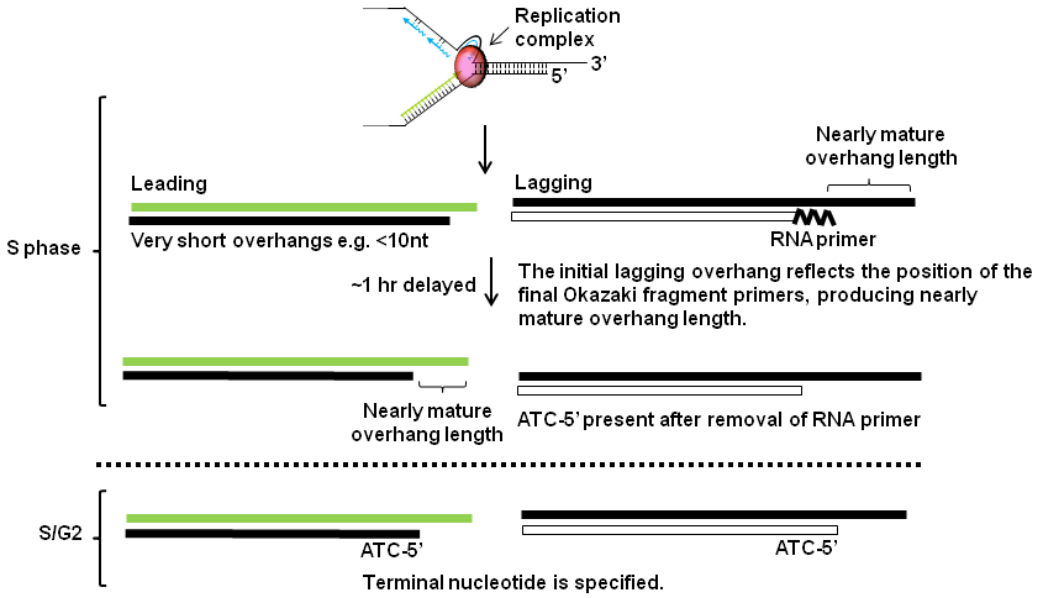

Figure 3-15. Early and late steps in telomere overhang processing in normal human cells: The position of the final RNA primer drives telomere shortening. See text for details.

Materials and Methods

Cell Culture and Cell Cycle Synchronization - See chapter 2.

Genomic DNA Isolation - See chapter 2.

CsCl Separation of Leading and Lagging Telomeric Daughters - See chapter 2.

Generation of Hypersensitive Telomere Probes. A hypersensitive C-probe was designed to incorporate thirteen total radioactive nucleotides (nine ^{32}P-dC + four ^{32}P-dA). T3C3+3 (5'-TTTCCCTAA) was used instead of T3C3+9 (5'-TTTCCCTAACCCTAA) and annealed to GTU4 (5'-GGGUUAGGGUUAGGGUUAGGGAAA) at 90˚C for 1 min; 20˚C for 15 min;

17˚C for 15 min. The synthesis reaction (Herbert et al., 2003) was modified as follows: 3.1 µl (8x Roche buffer M), 1 µl annealed template oligo (1.7 pmol/µl), 1 µl of dTTP (1.25mM stock, final 50µM), 7 µl ^{32}P-dCTP (3000 Ci/mmol), 4 µl ^{32}P-dATP (3000 Ci/mmol), 7.9µl Millipore H_2O, and 1µl Klenow (5 U/µl) were combined in a final volume of 25µl. After 20˚C extension for 30 min, and 95˚C for 5 min (to inactivate Klenow to prevent probe degradation upon UDG treatment) the reaction was cooled to room temperature. 0.5µl uracil deglycosylase (UDG) (1 U/µl) was added to degrade the GTU template, incubated at 37˚C for 15 min, and then UDG was inactivated at 95˚C for 10 min. Free isotopes were then removed using an Illustra Microspin G-25 column (GE Healthcare, Piscataway, NJ).

Telomere Overhang Analysis by In-gel Hybridization. 5 µg purified leading or lagging DNA was briefly run into an 0.8% agarose gel in 1xTAE so that telomeres were not significantly separated by size and remained as a relatively tight band. DNA that had been digested with 10U Exonuclease I (20 U/µl) (Epicentre Biotechnologies, Madison, WI) in a 20 µl reaction for 37 ºC for 1 hr, terminated by adding 0.5 µl of EDTA (0.5M stock), served as a negative control. The gel was dried, prehybridized (6x SSC, 5x Denhardt's solution, 0.5% (w/v) SDS), and hybridized with C-probe for G-overhangs under native condition. Upon obtaining the signal from the native hybridization, the gel was denatured

(0.5M NaOH and 1.5M NaCl for 1 hr), rinsed with distilled water (3x), neutralized (0.5M Tris-HCl pH8 and 1.5M NaCl for 30 min) and then hybridized again with the C-probe to obtain the denatured signal for total telomere input.

Overhang Analysis by Duplex Specific Nuclease (DSN) Assay. DSN assay was performed as described (Zhao et al., 2008; Zhao et al., 2011b). In brief, 2-5 μg of purified leading versus lagging was digested with 2U of DSN (Duplex specific nuclease, Evrogen, Russia) at 37˚C for 2 hr. For negative controls, 10U ExoI (Epicentre Biotechnologies, Madison, WI) was added to the genomic DNA at 37˚C for 1 hr prior to DSN digestion. DSN digestion was stopped by adding EDTA to a final concentration of 25mM. For denaturing polyacrylamide gels a 1:1 ratio of deionized formamide was added. DNA was then heated at 65˚C for 5 min and loaded on a 6% polyacrylamide gel with 8M urea. The gel was run at 15 V/cm in 0.5x TBE until the bromophenyl blue dye front migrated two thirds into the length of the gel. DNA was electro-transferred onto a Amersham Hybond™-N+ membrane (GE Healthcare, Piscataway, NJ) with 0.5x TBE. The membrane was air-dried, UV crosslinked, hybridized to a telomere specific probe as described (Herbert et al., 2003) and the membrane exposed on a Phosphorimager (GE Healthcare, Piscataway, NJ). Alkaline agarose gels (1% agarose made with 50mM NaOH and 1mM EDTA) were run in 50mM NaOH and 1mM EDTA at 4˚C with a low voltage (1–2 V/cm) until the dye has migrated ~6–8 cm. DNA was

capillary-transferred to Amersham hybond™-XL (GE Healthcare, Piscataway, NJ) and processed as above. Average overhang sizes were calculated using the formula mean average length = $\sum(Int_i) / \sum(Int_i / MW_i)$, where Int_i = signal intensity and MW_i =molecular weight of the DNA at position *i* (Chai et al., 2006a; Zhao et al., 2008).

Modified Single Telomere Length Analysis (STELA). STELA was performed as described (Sfeir et al., 2005) with modifications. Ligations were performed using each of the six C-telorette oligos corresponding to six permutations of the hexameric telomere repeat. 500 ng purified leading or lagging daughters were incubated in a 10 µl reaction (1x T4 ligase buffer, 0.001µM individual C-telorettes, 50 U NEB T4 ligase) at 35˚C for 20 hr. Multiple PCR amplifications were performed with 0.5 ng ligated DNA and 0.5µM primers (XpYpE2 forward and C-Teltail reverse primers) using Abgene Hi-Fidelity PCR Master 2x Mix AB-0792 (Thermo Scientific) in a final volume of 25 µl for 28 cycles of 94˚C for 15s, 65˚C for 30s, and 68˚C for 15 min. Amplified products were resolved on a 0.7% agarose with 1X TAE, and capillary-transferred onto Amersham Hybond™-N+ membrane overnight. Membranes were fixed at 80˚C for 2 hr and hybridized with a subtelomeric probe generated by PCR amplification with Xp/YpE2 and Xp/YpB2 primers and ^{32}P labeled by random priming. The membrane was then exposed to a Phosphorimager screen.

Oligonucleotides and primers:

XpYpE2 (forward primer subtelomeric): 5'-TTGTCTCAGGGTCCTAGTG-3'

XpYpB2 (reverse primer subtelomeric): 5'-TCTGAAAGTGGACC(A/T)ATCAG-3'

C-telorette 1: 5'-TGCTCCGTGCATCTGGCATCCCCTAAC-3'

C-telorette 2: 5'-TGCTCCGTGCATCTGGCATCTAACCCT-3'

C-telorette 3: 5'-TGCTCCGTGCATCTGGCATCCCTAACC-3'

C-telorette 4: 5'-TGCTCCGTGCATCTGGCATCCTAACCC-3'

C-telorette 5: 5'-TGCTCCGTGCATCTGGCATCAACCCTA-3'

C-telorette 6: 5'-TGCTCCGTGCATCTGGCATCACCCTAA-3'

C-teltail (reverse primer): 5'-TGCTCCGTGCATCTGGCATC-3'

Lambda Exonuclease Assay. 2 µg purified leading or lagging DNA was digested with 1 µl lambda exonuclease (10 U/µl) (Epicenter Biotechnologies, Madison, WI) for 1.5 hr at 37˚C. DNA was run on a 0.8% agarose gel in 1xTAE at 10 V/cm for 40 min, denatured with an alkaline solution containing 0.5M NaOH and 1.5M NaCl for 30 min, rinsed 3x with distilled water and dried at room temperature. The gel was then neutralized with 0.5M Tris-HCl pH8 and 1.5M NaCl for 30 min and hybridized at 42˚C overnight with G-telomere probes to detect the C-strands.

RNA Primer Pull-down Assay. Prior to CsCl gradient centrifugation, genomic DNA was digested with RsaI and HaeIII generating only blunt ends to minimize background. 2 μg purified leading or lagging DNA was ligated at 16˚C overnight to 0.5μM (final concentration) of a mixture of the 6 telorette oligos (Sfeir et al., 2005) that were biotinylated. DNA was pulled down using Dynabeads kilobaseBINDER kit (Invitrogen, Carlsbad, California) at room temperature overnight. DNA was released by 7.5 U RNase HII (NEB, Ipswich, MA) for 2 hr. Control DNA was released using 95% formamide and 10mM EDTA pH8.2 at 90˚C for 10 min.

CHAPTER FOUR

Overhang Processing and Telomerase Action in Human Cancer Cells

Introduction

Telomere end processing to generate G-overhangs is independent of telomerase (Greider, 1999a). In telomerase positive cancer cells, overhang is elongated by telomerase action. Maintenance of telomere length homeostasis is an intricate balance between telomere shortening (due to end replication problem and nuclease resection) and extension. Human telomerase loses 50-200 bp per cell division due to end replication problem and C-strand resection, eventually telomeres become critically short results in cellular senescence (Smogorzewska and de Lange, 2004). Cancer cells overcome senescence by the action of telomerase. How telomerase extends telomeres is unknown. To extend telomeres, both strands of the replicated duplex DNA need to be elongated. The G-strand is elongated by telomerase through reverse transcription, but when and how telomerase is recruited to the telomeres following duplex DNA replication is unknown. Telomerase action results in transient long G-overhangs. The complementary C-strand extension is thought to be carried out by mechanisms similar to lagging strand synthesis to reduce the overhangs, and complete the replication of duplex telomere DNA. Extension of the C-strand, a process called

"fill-in" is crucial to prevent accumulation of the excessively long ss G-overhang catastrophic to genome integrity (Price et al., 1994).

Inhibition of polymerase α and β during telomere replication led to increased length of the G-strand and altering C-strand length in Euplotes, (Price, 1997). Deficient lagging strand replication machinery in *S. cerevisiae* resulted in telomerase-dependent increase in telomere length (Adams and Holm, 1996; Adams Martin et al., 2000). The absence of Pol α, δ and DNA primase prevented the extension of G-strand telomere. Marcand *et al.* showed that the extension of artificially shortened telomere couples with the duplex telomere replication at late S phase (Marcand et al., 1999).

Increasing evidence has suggested that the C-strand fill-in mechanism is under tight regulation. In Euplotes, the C-strand are exactly 84 nucleotide in length (Price et al., 1994). In human, up to ~80% C-strand terminates in CCAATC-5', while the G-terminal nucleotide is less precise (Sfeir et al., 2005). In *S. cerevisiae*, telomeres replicate late in S phase. The process of C-strand fill-in is tightly coupled with telomerase extension of the G-strand to regulate telomere length (McCarroll and Fangman, 1988). However, it is unknown if the model in yeast holds up in human. The fact that human telomeres replicate throughout S-phase (Ten Hagen et al., 1990; Wright et al., 1999) provides a window of opportunity to study whether telomerase extension occurs in a late S/G2 process versus tightly coupled to the duplex telomere replication. The

kinetics dynamics of the telomere extension by telomerase in human is unknown. Understanding the mechanisms of telomerase action and C-strand fill-in are keys questions that impact the human telomere length homeostasis.

Human telomeres are much longer than many model organisms, and cannot be easily sequence; thus more challenging to study. Key questions in understanding the mechanisms of telomerase action and C-strand fill-in have yet to be answered. Several independent assays were developed to study the timing of G-strand extension following duplex telomere replication and C-strand fill-in. Telomerase extends telomeres within 30 mins of duplex telomere replication. Unexpectedly, C-strand fill-in is delayed until late S-phase; thus, resulting in this unexpected two-step model for telomere extension. Candidates involved in telomerase extension may be potential targets for the development of telomerase inhibitor cancer therapies.

Results

To provide direct telomerase action during S phase, we monitored the BrdU incorporation of lagging overhangs (Fig. 4-1). The parental G-strand is the template for lagging daughter synthesis. Before telomerase action, lagging C-strands initially only contain thymidine when synchronized at G1/S. Only parental single stranded overhangs would survive DSN digestion, conferring a low density (thymidine only) on a CsCl gradient. If C-strand fill-in is delayed following telomerase action, the newly synthesized telomere will be a thymidine-BrdU hybrid represented by intermediate density. Only the newly synthesized GGTTAG sequences will contain BrdU. As C-strand is being fill-in, depending on the amount of C-strand extension, the originally thymidine containing parental G-overhang may be converted to duplex DNA, leaving only the fully BrdU substituted telomeres.

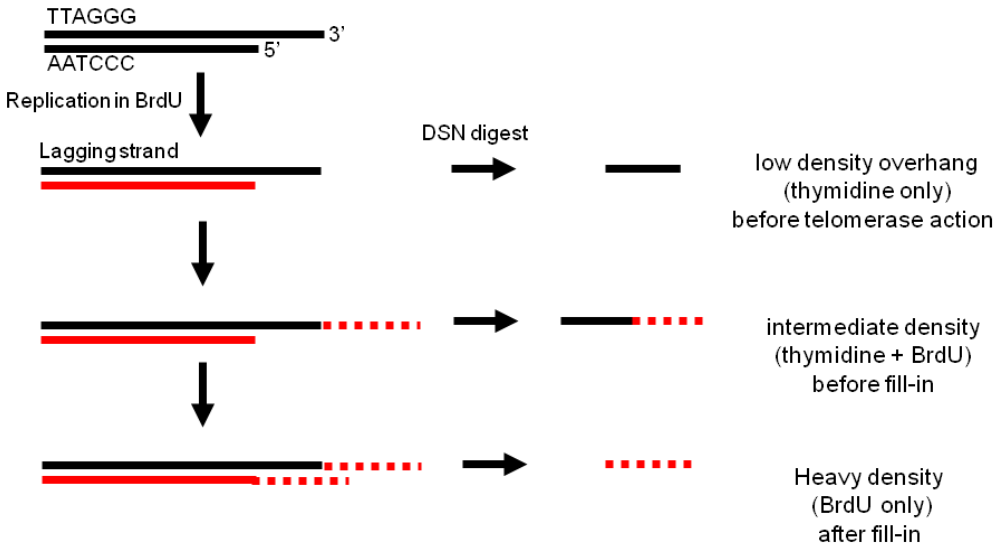

Figure 4-1. Approach to study telomerase action on newly synthesized lagging daughter telomeres. Replicated telomere (black – parental strand; red – BrdU incorporated daughter strand) was digested with DSN with remaining overhangs for analysis. Unextended lagging strands prior to telomerase action contain only thymine, and therefore have low densities. Upon telomerase extension, parental lagging G-strands extended by telomerase result in a hybrid of thymidine and BrdU, which have intermediate densities. After C-strand filled-in, only BrdU incorporated overhangs remain, resulting in heavy densities.

On the other hand, since the G-strand for leading is the newly synthesized daughter strand. Leading daughter G-strand will always be fully substituted with BrdU independent of whether telomerase extension has been accomplished or not (Fig. 4-2).

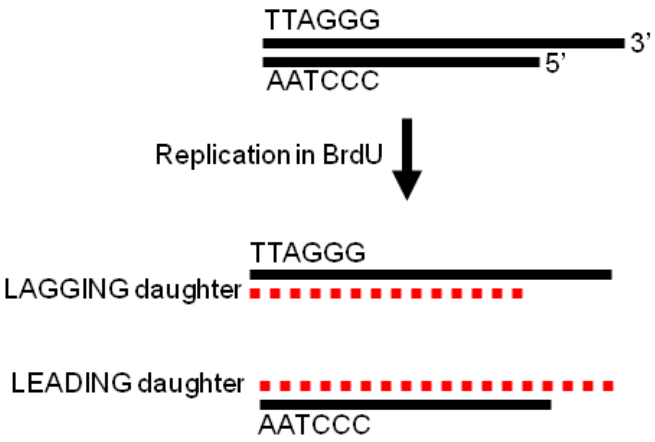

Figure 4-2. Differences between leading versus lagging BrdU incorporated daughter strands. Lagging parental G-strand contains only thymidine, while the newly synthesized C-rich daughter strand has BrdU incorporation. In contrast, leading parental strand is the C-strand; the newly synthesized G-strand has BrdU incorporation. Therefore, digestion with DSN always yields a heavy density peak whether or not the G-overhang has been extended by telomerase.

Uncoupled of C-strand Fill-in from Telomere Replication in Hela

The timing of telomerase extension and C-strand fill-in was monitored by releasing synchronized Hela cells at G1/S. Increasing intermediate accumulated till 4 to 6 hr after release. The intermediate peak had shifted to high density by 8 hr. The appearance of the intermediate peak identifies the early timing of telomerase action while the late appearance of heavy peak at S/G2 suggested the C-strand fill-in is uncoupled from telomerase action (Fig4-3).

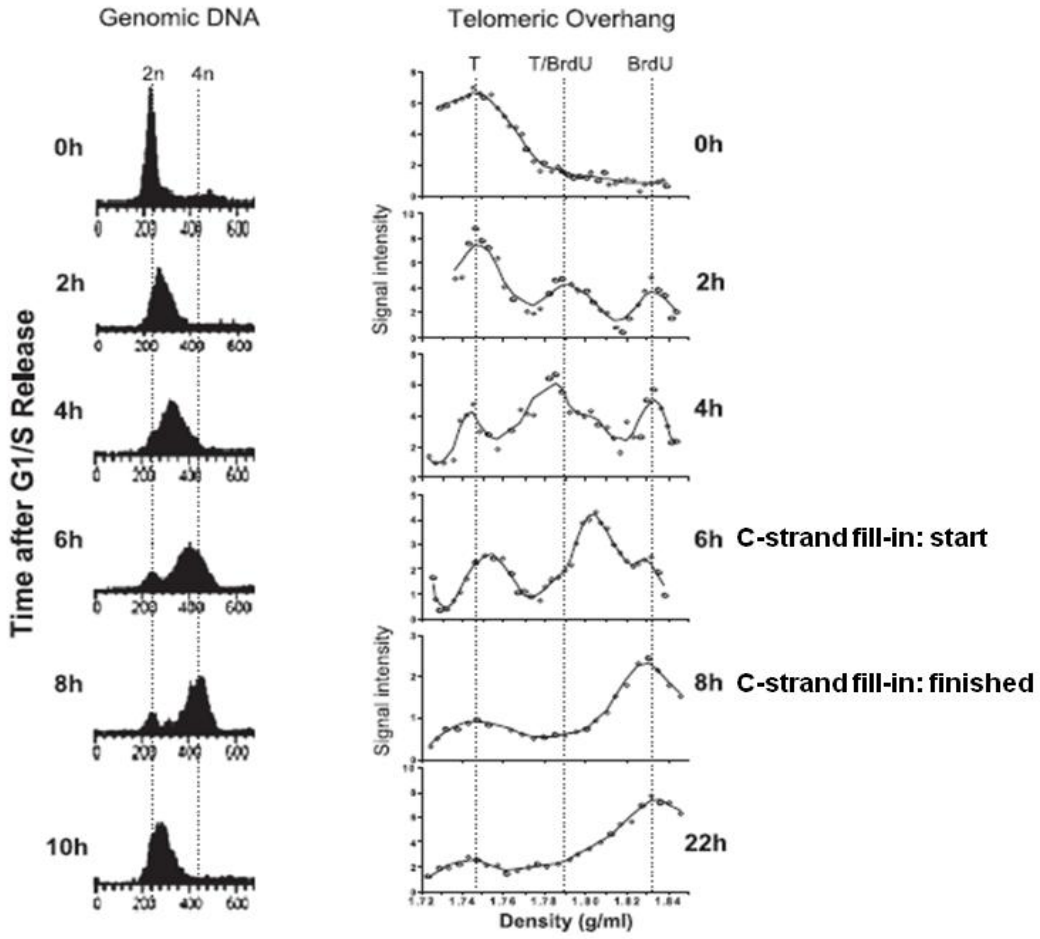

Figure 4-3. Telomerase extension versus C-strand fill-in in Hela cells. Left panel) FACS analysis showing S-phase progression of synchronized Hela cells upon release into S-phase for various time periods. Right panel) Overhang densities throughout S-phase. Intermediate density shifted to heavy density indicating start of C-strand fill-in (6 hr) until it is completed (8 hr).

Transient Long Overhangs in Mid S-phase after Telomerase Extension

Given the results obtained in Fig. 4-3, we would predict to observe transient long G-overhang after telomerase action prior to C- strand fill-in during S. Next, we examined the G-overhang length in synchronized Hela cells during S phase quantitatively using DSN. Average overhang sizes were compared at various times upon release into S/G2. Heterogeneous overhang sizes within cells showed a wide distribution of sizes on a polyacrylamide gel (Fig.4-4). Overhang sizes were calculated as mentioned in chapter 3. Average overhang sizes increased from ~65 nt to ~105 nt by 6 hr into S phase, and decreased back to ~65 nt by 10 hr into S phase (Fig. 4-4).

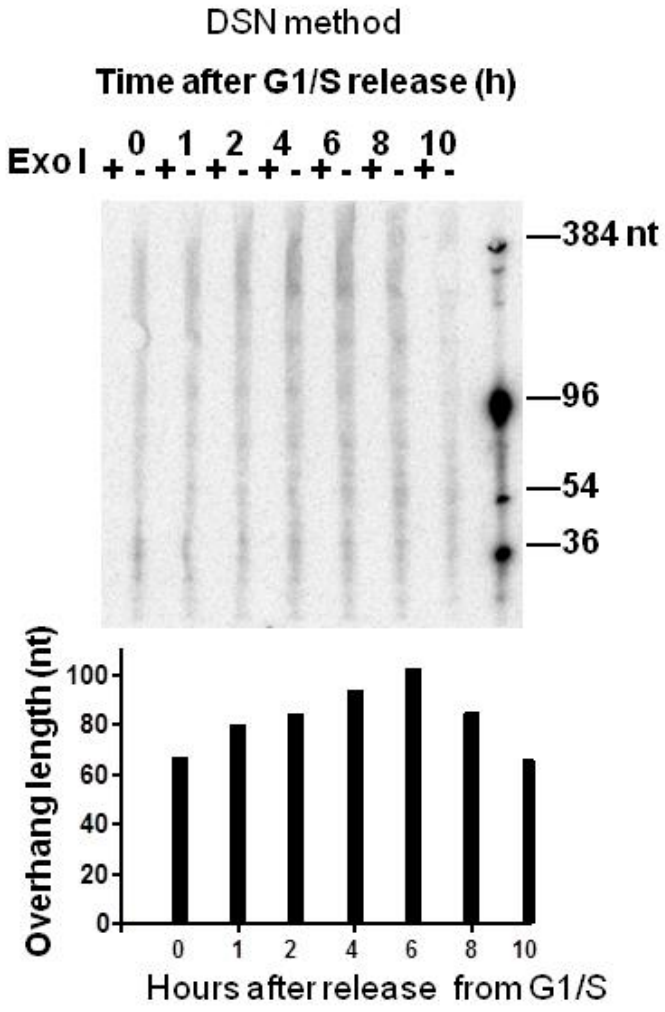

Figure 4-4. Elongation of overhang during S-phase in Hela cells by DSN. Top panel) Total genomic DNA was collected from synchronized Hela cells that have been released at various times into S-phase. DNA was first digested with DSN and negative control was further digested with ExoI to remove 3' overhangs and analyzed by a denaturing polyacrylamide gel. Bottom panel) Overhang dynamics across S-phase was quantified.

Alternatively, the results from DSN were confirmed qualitatively using native in-gel hybridization.

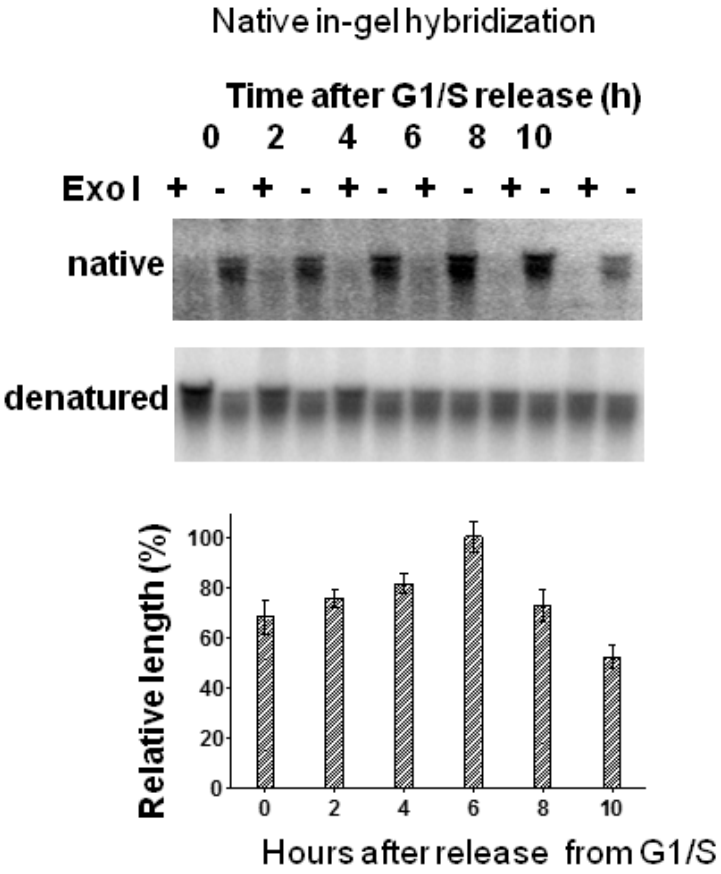

Figure 4-5. Overhang elongation in S-phase of Hela by in-gen hybridization. Top panel) Total genomic DNA was collected from synchronized Hela cells that have been released at various times into S-phase. Negative controls were digested with ExoI. Overhang signals were normalized to the total genomic signals obtained upon denaturation of the gel. Bottom panel) Quantification of overhang length dynamics across S-phase. Results are plotted as % of maximum value.

Telomerase extends telomere within 30 min of replication

Next, we asked how quickly telomerase acted following replication. We examined overhang lengths in telomeres that were labeled for a very short BrdU pulse. Hela was synchronized and released into S phase either for a 30 min BrdU pulse in mid S phase or labeled continuously for 8 hours with BrdU. Average size of the elongated leading and lagging G-overhangs within the 30 min of replication was longer compared to that of the 8 hr on both leading and lagging telomeres (Fig. 4-6). The increased overhang length of both leading and lagging strands were consistent with the hypothesis that telomerase extended both leading and lagging daughter strands.

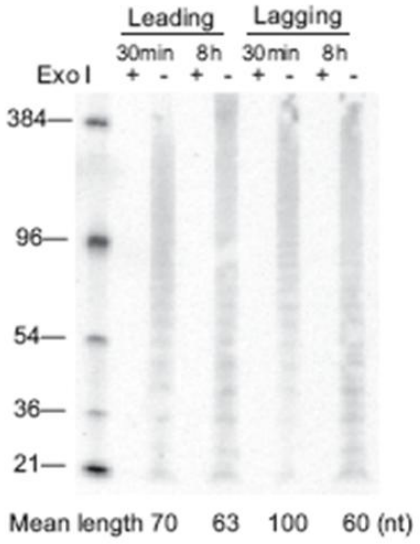

Figure 4-6. Telomerase act within 30 min of BrdU incorporation. DSN analysis of leading versus lagging with BrdU labeled for 30 min versus 8 hr. Mean overhang sizes are indicated below the gel.

Conclusions

Telomerase is important in maintaining telomere length in most cancer cells. We found telomerase acted rapidly following replication of the telomere duplex. Furthermore, after telomerase action, there was a transient long overhang, and finally the C-strand fill-in occurred at late S/G2. The unanticipated delay of C-strand fill-in draws attention for being a possible therapeutic window in cancer cells that express telomerase; thereby factors important for C-strand fill-in will be an attractive target for potential cancer therapies.

Telomerase is likely to act upon completion of telomere replication

There are two possible models in regards to the timing of telomerase action with respect to telomere replication. The first model predicts that telomerase extend telomeres after duplex telomere replication has been completed. The second is that telomerase may act prior to the completion the duplex telomere replication (Chakhparonian and Wellinger, 2003). Moreover, it has been previously speculated that telomere replication might be stalled during S phase, and it is not until late S/G2 that both telomere replication and overhang generation are completed (Verdun and Karlseder, 2006). If so, this would mean that the transient longer overhangs observed during S-phase could be due to telomerase action on a stalled replication fork prior to completion of the duplex telomeres. However, detailed data analysis concludes that telomerase acts after duplex telomere has completed its replication. First, we showed that a 15 min IdU

labeled Hela showed distinct leading versus lagging separated peaks on a CsCl gradient (Fig. 2-3), indicating completion of telomere replication (at least for some telomeres) within 15 mins. Second, if telomerase acts prior to completion of duplex telomere replication, normal lagging strand synthesis would replicate the C-rich strand (complementary to the G-rich strand which contains the protruding overhang) much into the extended overhangs. Therefore, the intermediate density peak would not be present in Fig. 4-3 (right panel) due to early elongation of the C-strand. We also did not observe early C-strand elongation by STELA (data not shown).

Telomerase acts within 30 min of duplex telomere replication.

In yeast, it has been proposed that telomerase action is coupled with telomere duplex replication (Teixeira et al., 2004), and that progression of the replication fork at telomere is required for proper telomerase extension (Dionne and Wellinger, 1998; Marcand et al., 2000). Furthermore, *de novo* telomere extension required factors from the replication machinery such as polα, polβ and primase (Diede and Gottschling, 1999). In human, chromosomes replicate asynchronously throughout S-phase; *in situ* cytological studies have also demonstrated that hTERT and hTR only associate with a few telomerase molecules at any particular time during S-phase (Jady et al., 2006; Tomlinson et al., 2006), which leads to the prediction that human telomerase is recruited

quickly after telomere replication. Our data in Hela cells confirm the predicted model that telomerase extended telomeres within 30 min of duplex telomere replication.

Possible C-strand fill-in mechanism

A possible mechanism for C-strand fill in would be by the convention lagging strand synthesis machinery, where RNA primer is laid down and polα extended to fill-in the C-strand. As the average size of Okazaki fragment is ~100 nt, this would predict a rapid transition from intermediate to heavy density from mid S to S/G2 during the fill-in step. Rather, we observed a peak between the intermediate and heavy densities at 6hr (Fig. 4-3 right panel), suggesting an incremental fill-in model, which mechanism has yet to be elucidated.

Materials and Methods

Cell culture and synchronization – see Materials and Methods in chapters 2 & 3

Genomic DNA purification – see Materials and Methods in chapters 2 & 3

CsCl chloride centrifugation – see Materials and Methods in chapters 2 & 3

CHAPTER FIVE

Discussions and Future Directions

Telomeres generate many problems for DNA replication. In addition to the classic end-replication problem, the ability of the G-rich strands to form quadruplex structures makes telomeres hypersensitive to fork stalling (Hao and Tan, 2002; Lipps and Rhodes, 2009; Paeschke et al., 2011). An additional facet of telomere replication is the processing of the ends to produce appropriately sized 3' G-rich overhangs. These overhangs are essential for telomerase action. Proper packaging of the ends into structures (t-loops) that mask the chromosome ends from the DNA repair machinery, and their sizes are the primary driving force behind the rate of telomere shortening in normal somatic cells. However, because of the sequence repetitive nature and the lack of appropriate restriction sites in telomeres, we know remarkably little known about the number and nature of the steps involved in end-processing in human. Although many candidate factors have been shown to influence overhang dynamics over the past twenty years, there has been little progress in resolving specific mechanisms of action of these factors without an understanding of the different processing steps involved. Just as understanding homologous recombination requires a definition of its intermediate DNA structures, understanding telomere end protection requires a

definition of the different steps of overhang processing. Due to the different modes of leading versus lagging daughter strands synthesis as presented by the end replication problem, we hypothesized that leading and lagging overhang processing involve independent mechanisms. In this study, we have established a variety of novel approaches to identify the timing and steps of these overhang processing steps in both normal and cancer cells. We have now established a platform of expected "intermediate steps/structures and when they appear "during telomere overhang processing. Future directions include identifying the players required to generate each intermediates. The ultimate goal is to identify specific step(s) and/or factor(s) unique to overhang processing in cancer cells as potential cancer therapy targets. The following discussion will summarize our current telomere replication and overhang processing models in normal versus cancer cells, and highlights some further considerations and investigation concerning several aspects in the understanding of telomere overhang processing.

The Position of the Final RNA Primer Drives Telomere Shortening

Eukaryotic Okazaki fragments are approximately 100-200 nt in length (Balakrishnan and Bambara, 2011; Bielinsky and Gerbi, 1999; Blumenthal and Clark, 1977; Burgers, 2009; Okazaki et al., 1968; Sakabe and Okazaki, 1966). If the replication apparatus simply ran off the end of the telomere while the position of the final RNA primer is random, then lagging daughters should have the final

RNA priming event occur at approximately half of the average fragment size (or 50-100 nt from the end). We observed that the final lagging RNA primer remained intact immediately following replication, with an overhang size of about 80 nt at that time, consistent with the above prediction. This resolves a long-standing question about the mechanism of overhang generation at lagging daughter telomeres that is fundamental to our understanding of telomere shortening. The end-replication problem has traditionally been formulated as the inability to copy the 10-14 nt at the end of the chromosome after the final lagging Okazaki fragment RNA primer has been removed. Our results demonstrate this is not the case in human cells. Although the final overhang size in *S. cerevisiae* is 12-14 nt (Larrivee et al., 2004), this actually does not represent the site of the final Okazaki priming event because both leading and lagging strands have a transient >30 nt overhang that is later filled-in by an unknown mechanism to generate the final overhang (Wellinger et al., 1996; Wellinger et al., 1992, 1993b). Although in some organisms it may be possible for the conventional replication machinery to position the final lagging RNA primer at the very terminus, the data do not support this interpretation in either yeast or humans. Since the gap produced by removal of the RNA primer only contributes ~10% to the size of the human overhang, the end-replication problem in human should now be revised to state that the conventional replication complex cannot copy the gap between the final RNA priming event and the end of the chromosome.

Under unstressed conditions, the position of the final priming event of replication (rather than processing that degrades the C-strand) is thus the most important intrinsic step in and a primary driver in the rate of telomere shortening in human cells.

The RNA primer on lagging strands is removed during the early phase of processing following replication. The first nucleotide incorporated during RNA primer synthesis contains a 5' triphosphate. Neither T7 exonuclease nor T4 ligase should be able to function on an RNA containing a 5' triphosphate (Engler, 1982; Kerr and Sadowski, 1972), yet both of these enzymes are able to function on the RNA-capped DNA in our assays. We infer that there is a rapid initial processing of the RNA primer after replication that removes at least the triphosphate, followed by a delay of approximately 1 hr before the rest of the RNA is removed in the early phase.

Factors Involved in C-strand Fill-in as a Potential Target for Cancer Therapy

Human telomeres replicate throughout S-phase. In normal human cells, telomere overhangs are processed in two distinct phases following replication. The early phase occurs during S phase and it is accomplished as mature overhang lengths are established. The late phase happens in S/G2, when the C-strand terminal nucleotide is specified, resulting in the completion of overhang

processing. In telomerase positive human cancer cells, we have previously shown that telomerase extends leading strands at the shortest BrdU incorporation time (30 min pulse) we could measure (Zhao et al., 2009). Telomerase requires at least 6 nt of ss G-rich overhangs to function (Lai et al., 2001); so we infer that there is a very rapid processing event that generates an initial overhang. However, we were unable to detect meaningful overhang signals from leading strands in normal diploid cells after a 1hr label. Unless the timing is different in normal vs tumor cells or there is an additional rapid processing event that is specifically present in telomerase positive tumor cells, this initial overhang must be very short. The nearly mature size of the leading overhang was only detected later, after an additional hour. Moreover, telomerase extension is uncoupled from C-strand fill-in, and this fill-in step currently seems to be a unique process identified in cancer overhang processing. Factors potentially involved in the C-strand fill-in such as the CST complex may be potential targets to accelerate telomere shortening in cancer cells.

One drawback to the use of anti-telomerase drugs (e.g. GRN163L) in the treatment of cancer is the length of time needed for such drugs to inhibit cell growth or induce apoptosis. It would be optimal to minimize the delay from the time of GRN163L treatment to the time when cell killing occurs. Cultured cells showed different rates of telomere shortening (Counter et al., 1992; Harley et al., 1990). Studies have shown that after the initial treatment of GRN163L, many

population doublings are required before sufficient shortening occurs to cause apoptosis in breast adenocarcinoma cells (Gellert et al., 2006). If targeting CST can accelerate the rate of telomere shortening in cancer cells while having minimal effect on normal cells, this may greatly enhance the efficiency for GRN163L treatments. The development of anti-telomerase drugs is likely to be needed to use in conjunction with faster-acting anti-cancer drugs.

Do Normal Cells Undergo Transient Resection and C-strand Fill-in Similar to Cancer Cells?

Fill-in of the telomerase-extended G-strands is delayed until S/G2 in tumor cells (Zhao et al., 2009). Normal diploid cells do not have extended G-strands requiring fill-in, but they also exhibit processing events during this second phase of telomere replication. The C-rich strand terminal nucleotides of leading daughter telomeres remain random until S/G2, when CCAATC-5' specification is acquired. In contrast, lagging C-strands exhibit specific ends once the RNA primer is removed during the early phase following replication.

In normal cells, mature overhang sizes of lagging (~100 nt) are roughly 3 times longer than that of leading (~30 nt) (Fig. 3-4). However, fully processed leading and lagging overhangs in telomerase positive cells have approximately the same sizes (~60-70 nt) (Fig. 4-6) (Chai 2006). However, initially extended lagging overhang sizes (~100 nt) are longer than that of leading (70 nt) (Fig. 4-6).

This would make sense because presumably lagging daughters would have a longer overhang than the initially blunt ended leading daughters, which is processed to yield shorter overhangs. Our current data in telomerase positive cells observed the net, average increase of overhang length sizes, and was unable to dissect the contribution of C-strand resection (if any) versus telomerase elongation. It is important to understand to what extent the C-strand is processed during S-phase (i.e. whether the C-strand also contains a transient long G-overhang that is being fill-in similar to that in yeast).

We cannot exclude the possibility that C-strand fill-in may also occur in normal cells. As leading daughters have DNA in contrast to lagging daughters which have RNA at their C-terminus, it is possible that entirely different mechanisms are used to generate C-strand end-specificity on leading versus lagging daughters reflecting the activation of separate enzymatic complexes. However, if the phenomenon of "the presence of a transient overhang independent of telomerase in yeast" (Dionne and Wellinger, 1996; Wellinger et al., 1996; Wellinger et al., 1992, 1993b) also exists in human, this mechanism presumably requires an RNA primer, possibly produced by polymerase alpha recruited by the CST complex (Price et al., 2010). In this scenario, the following model is proposed: First a helicase unwinds the duplex telomere DNA in conjunction with exonuclease action; meanwhile, single-stranded binding proteins such as POT1, RAP or hnRNP are loaded on the G-overhangs. When a threshold is reached,

there will be a transient resection, and leading strands would acquire an RNA primer at their C-strand termini. Since, leading daughters acquire an almost mature overhang size within ~1 hr of replication, any transient resection and fill-in at S/G2 would thus need to produce little net change in overhang size. It is possible that both leading and lagging daughters undergo a transient resection, so that the final end-specification of both strands could occur at S/G2 by the same mechanism.

To test this hypothesis, a preliminary experiment can be performed by harvesting synchronized normal cells at 0, 2, 4, 6, 8, and 10 hr into S-phase, followed by lambda exo digestion on the genomic DNA at these time points. If there is a transient resection followed by a C-strand fill-in, it will predict the genomic DNA collected from mid S phase to S/G2 may have signal retention upon lambda exo digestion, indicating the presence of a RNA primer during the fill-in.

If the C-strand fill-in mechanism does in fact occur in normal cells, the next important question is whether normal and cancer cells use the same factor(s)/mechanism for C-strand fill-in and whether there is a need to re-evaluate the potential of targeting the C-strand fill-in as a cancer-specific therapy.

An alternative model predicts that once the threshold of single-stranded binding protein is reached, another endonculease is recruited for C-terminal specification, and there is no transient resection on the C-strand. This will also

predict that leading and lagging daughters are likely to used different mechanism for C-terminal specification.

T-loop Dynamics during S-phase

Properly generated telomere overhangs are required to form T-loop structures to cap the telomere for chromosome end protection. It is unknown whether t-loops refold following replication or remain unfolded following replication until the end of S-phase. In the context of normal cells, one model is that both leading and lagging telomeric daughters contain unfolded T-loops for at least 1-2 hours following replication until mature overhang sizes are generated. The mechanism prevents the single-stranded overhang at telomeres from initiating an ATR-mediated DNA damage response during this time is likely to be POT1 mediated, but that remains to be determined. With respect to cancer cells, where uncoupling mechanism of telomerase action and C-strand fill-in is observed, it is uncertain whether T-loops remain unfolded until after the C-strand fill-in or that T-loops can be refolded immediately after replication, and unfold again for the C-strand fill-in during late S/G2. The later is more consistent with the observations that telomeres are associated with factors (ChIP analysis) involved in DNA damage at late S/G2 (Verdun et al., 2005). The fraction of T-loop during S phase is also unknown. To date the only available T-loop assay is based on electron microscopy, which requires massive DNA input in the milligram range. The

development of a robust biochemical T-loop assay would there for be ideal in examining T-loop dynamics during S-phase.

In Search of Potential Candidates Involved in Overhang Processing

Apollo has been implicated in leading strand C-strand resection in mice (Lam et al., 2010; Wu et al., 2010). The Mre11-Rad50-Nbs1 (MRN) complex has been suggested to protect leading strand telomeres following telomere replication in mice; Mre11 deficiency results in leading end-to-end telomere fusion (Attwooll et al., 2009; Deng et al., 2009; Dimitrova and de Lange, 2009). However, the roles of the MRN complex in human overhang processing have yet to be confirmed. The telomere binding shelterin complex such as TRF1, TRF2, TIN1, POT1, TPP1, RAP1, as well as factors involved in DNA damage sensing/repair, telomerase action/telomere length regulation such as MRN (3'-5' exonuclease, and endonuclease), ATM, ATR, XRCC4, Dna2, Ku 70, FEN1 (Flap-endonuclease I), DNA-PKcs, Exonuclease I (5'-3' exonuclease); as well as Apollo (5'-3' exonuclease), Werner (3'-5' exonuclease), Artemis (5'-3' exonuclease), and TCAP1 are all possible regulators for telomere replication and overhang processing steps. In normal cells, these steps include generation of mature overhang length, terminal nucleotide specification telomerase etc. In cancer cells, these steps include telomerase recruitment, processivity of telomerase and translocation of telomerase holoenzyme.

In addition, about two hundred such proteins were identified to associate with telomere chromatin in human cells using mass spectrometry. Though many seem to have known non-telomeric functions, the telomeric roles of these proteins have yet to be characterized. Most of the biochemical assays described to monitor overhang processing in this study only allow for average dynamics of all telomeres to be analyzed. Telomeric ends are highly dynamic during telomerase action, and telomeres exist in very low abundance (1/6000) of total genomic DNA. Moreover, human telomeres replicate throughout S phase and different telomeres replicate at different times. It would be most ideal to develop high throughput single-molecule-based assays with more robust signal to noise ratios for analysis. Nevertheless, these newly developed biochemical tools described in the above chapters can certainly be powerful tools to allow us to analyze candidate factors to further dissect the mechanistic details of telomere overhang processing.

Summary:

Understanding the mechanistic steps in telomere overhang processing has been a fundamentally important, yet neglected question in the telomere field for years due to the lack of appropriate sensitive biochemical tools. The series of separate specific steps presented in this study introduce crucial mechanistic details of the DNA end-replication problem and telomere overhang processing required

to successfully reproduce the proper telomere end structures for chromosome end-capping and telomerase action. This will now provide the basis for examining the roles of many candidate factors that influence telomere biology with implications in both cellular aging and cancer.

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