Mechanisms of alternative telomere elongation in human cancer cells

Dissertation

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By

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Abstract

Telomeres are DNA-protein structures that cap the ends of chromosomes and are important modulators of genomic stability. Telomeres shorten with each round of cell division and must be actively maintained in cells with high rates of proliferation, such as cancer cells. Cancer cells can elongate telomeres with the telomerase enzyme or in a telomerase-independent manner termed alternative lengthening of telomeres (ALT). ALT uses recombination to copy telomeric templates to lengthen shortened chromosome ends, although the exact functions and mechanisms of ALT are still loosely defined. While some proteins are essential for ALT, the role of other proteins implicated at the telomeres is still unknown. Due to structural and functional similarities to the BLM helicase, mutated in Bloom syndrome and required for ALT, this work asked whether the WRN helicase, mutated in Werner syndrome, is also required for ALT. Short interfering RNA knockdown of WRN in immortalized human cell lines demonstrates that WRN is necessary for ALT in some, but not all, ALT cell lines. In cells that require WRN for ALT, WRN knockdown results in telomere shortening and a loss of ALT characteristics. The requirement for WRN in ALT correlates with an interaction between WRN and the BRCA1 tumor suppressor. These results imply that ALT cells can use different mechanisms of alternative telomere maintenance and that these mechanisms make use of unique protein complexes. In a more clinical scope, this work also asked whether
telomere maintenance mechanisms within human sarcoma tumors, which are known to have a high incidence of ALT, are variable. Both immunohistochemical and biochemical methods were used to evaluate human sarcomas for the presence of telomerase-expressing cells and cells with characteristics of the ALT pathway. This work has shown that ALT and telomerase are not mutually exclusive in some human sarcomas and that some tumors have a high degree of tumor heterogeneity in regards to telomere maintenance, with cells using either mechanism within the tumor. ALT is used by an estimated 15% of human tumors. Therefore, understanding ALT mechanisms will be important for the development of effective therapeutic strategies to inhibit ALT tumors. The clinical use of telomerase inhibitors in recent clinical trials warrants a close inspection of telomere maintenance in human tumors to get true measures of accurate therapeutic responses.
Dedication

It has been over a decade since she passed, but my grandmother’s spirit and memory have never left. Her battle with cancer was the catalyst for my decision to study cancer research; it is in her name and memory that I could not and would not give up.

In loving memory of Mary Joan Witham Sandy and Jesse Sandy
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Chapter 1: Literature Review

DNA is the blueprint for the structure of a cell to a person’s susceptibility for disease. Because of this immensely important role, fidelity must be preserved throughout the various processes of DNA metabolism. DNA replication and repair requires helical double-stranded DNA to be unwound to allow access to each stand by enzymes known as helicases. Furthermore, DNA fidelity is protected at the chromosome ends by DNA-protein structures called telomeres. Telomeres cap the ends of each chromosome and differentiate the end from a DNA double strand break, which would otherwise elicit a DNA damage response. Telomeres also prevent the loss of DNA at chromosome ends and are required for proper chromosome segregation and nuclear localization, DNA repair, cell viability, and genetic stability. Proper genomic maintenance is thus heavily intertwined with the functions of both helicases and telomeres.

I. Telomeres and their maintenance

I.1 Telomere structure

Telomeres are comprised of repetitive noncoding DNA sequences, although the specific nucleotide sequence can vary with the type of organism. Vertebrate telomeres consist of (TTAGGG)$_n$ repeats (Moyzis et al., 1988) that span the last 10-15 kb of each
Due to the complementary TTAGGG sequence, the telomere is composed of one G-rich (TTAGGG) and one C-rich (CCCTAA) DNA strand. The 3’ G-rich strand ends as a single strand overhang, which loops back and invades the upstream telomeric double stranded region (Griffith et al., 1999) to form a T-loop (Figure 1). The size of the T-loop can vary, but the conformation protects the chromosome end by hiding the end and preventing its recognition as a DNA break.

Several proteins help the telomere achieve this conformation and protect the chromosome end (Figure 1). The single-stranded end of each telomere is bound by a protein known as protection of telomeres 1 (POT1). In addition, the telomere is bound directly by telomere repeat binding factors 1 (TRF1) and 2 (TRF2), which help the DNA end form and stabilize the T-loop structure. POT1, TRF1, and TRF2 are three components of the shelterin complex, a 6-protein complex that binds to telomeres and forms a protective cap. Also included are the RAS-proximate protein 1 (RAP1), TRF1-interacting nuclear factor 2 (TIN2), and tripeptidyl peptidase 1 (TPP1), which do not bind directly with telomeric DNA but instead are linked to the complex through their interactions with POT1, TRF1, and TRF2 (de Lange 2005).
I.2 Telomere maintenance

Telomeres shorten with each round of cell division as cells replicate due to the inability of the DNA replication machinery to proceed to the very end of each DNA strand, a process called the end-replication problem (Harley et al., 1990). Loss of telomeric DNA eventually induces cellular senescence (Bodnar et al., 1998), suggested to be one process by which normal aging occurs as telomere lengths are shorter in older individuals (Allsopp et al., 1992). Immortalized cells activate expression of a telomere maintenance mechanism, such as the protein telomerase, to counteract the persistent loss of telomeric DNA. Telomerase enzymatically adds telomere repeats to shortened...
chromosome ends. In humans, telomerase is constitutively active in germline cells and stem cells, while expression in somatic tissues is repressed (Kim et al., 1994).

The catalytically active telomerase enzyme is composed of two subunits each of two different proteins: a reverse transcriptase (TERT) and dyskerin (Cohen et al., 2007). Active telomerase includes two subunits of an RNA component (TERC) that serves as a telomere sequence template. Telomerase enzymatically catalyzes the addition of telomere repeats to the 3’ end of a shortened chromosome using TERC as a template for nucleotide addition to the leading strand. DNA polymerases then complete lagging strand synthesis from the newly elongated template, completing telomere repeat addition. Dyskerin is a pseudouridine synthase that binds to TERC to help process and stabilize the RNA component of telomerase (Chen et al., 2004; Mitchell et al., 1999).

Telomeres can also be maintained in the absence of telomerase through the alternative lengthening of telomeres (ALT) pathway. Telomerase-independent telomere maintenance was first discovered in the yeast Saccharomyces cerevisiae. Most yeast with mutations in est1, required for yeast telomere maintenance by recruiting telomerase, exhibit telomere shortening and subsequent cell death. However, some are able to escape senescence and elongate telomeres in a telomerase-independent manner, seemingly analogous to ALT mechanisms (Lundblad and Blackburn, 1993). Yeast elongate telomeres in the absence of telomerase by utilizing two different Rad52-dependent pathways: Rad51-dependent type I and Rad50-dependent type II (Teng and Zakian, 1999; Le et al., 1999). Type I telomere elongation generates cells with short telomeric repeats
and amplified subtelomeric Y’-elements; type II telomere elongation generates cells with long heterogeneous telomeric repeats.

Mammalian cells also utilize ALT (reviewed in Henson et al., 2002) as its prevalence in human tumors and immortalized cell lines has been observed (Bryan et al., 1997). Some evidence suggests ALT-like characteristics in non-neoplastic human cells (Slatter et al., 2012) and in early stages of development (Liu et al., 2007). Recent studies have also shown that roundworms can survive using ALT alone (Lackner et al., 2012); however, further studies are needed to determine the prevalence of ALT in normal mammalian cells.

I.3 ALT characteristics

The current cellular requirement for ALT classification is the ability to maintain telomeres throughout several rounds of cell division without activity of the telomerase enzyme; however, certain characteristics consistent in most ALT cells allow other means for their classification. Cells that use ALT processes typically have highly heterogeneous telomere lengths in contrast to the more uniform lengths of cells expressing telomerase. On average, telomerase-positive cells have homogenous telomere lengths of 5-10 kb (Bryan et al., 1995; Park et al., 1998), while ALT telomeres average 20 kb and range about 3-50 kb in length (Bryan et al., 1995, 1997; Grobelny et al., 2000). ALT cells contain both linear and circular extrachromosomal telomeric DNA repeats (ECTR) (Cesare and Griffith, 2004) that may function as recombination templates to extend telomere repeats. Circular ECTRs may form through deletion of telomeric T-loops by aberrant homologous recombination (Wang et al., 2004), destabilizing telomeres and
explaining the diverse lengths of ALT telomeres. In addition, aberrant recombination itself is characteristic of ALT cells, as telomeric sister chromatid exchange (T-SCE), a form of telomere recombination, is highly elevated in cells that use ALT (Londono-Vallejo et al., 2004). Furthermore, ALT cells display rapid changes in telomere length that may be consistent with the effects of recombination (Murnane et al., 1994; Perrem et al., 2001).

ALT cells are also characterized by ALT-associated promyelocytic (PML) nuclear bodies (APBs) (Yeager et al., 1999). These dynamic protein foci are distinguished from normal PML nuclear bodies by the inclusion of telomeric DNA and telomeric proteins, such as TRF1 and TRF2 (Yeager et al., 1999). TRF1 and TRF2 bind telomeric repeats to prevent chromosomal end fusions and to form the T-loop structure by securing the single-stranded DNA end (Griffith et al., 1999). These protein foci may be directly involved in alternative telomere elongation, as they contain telomeric DNA, telomeric proteins, homologous recombination proteins, and are sites of DNA synthesis (G. Wu et al., 2000). APBs are observed in ALT cells mostly in late G2- and M-phases of the cell cycle (Grobelny et al., 2000; G. Wu et al., 2000) when ALT is proposed to occur. Furthermore, treating ALT cells with DNA damaging agents increases APB numbers and ECTR DNA, suggesting overlapping roles between both telomere maintenance and DNA repair (Fasching et al., 2007). To date, the mechanisms of ALT are not fully elucidated, but evidence suggests recombination (Azam et al., 2006; Dunham et al., 2000) in which the 3’ single-stranded end of a telomere invades another
telomere and lengthens via recombination. Both inter-telomere (Dunham et al., 2000) and intra-telomere (Muntoni et al., 2009) recombination have been observed in vitro.

1.4 ALT proteins

Depletion of the MRE11-RAD50-NBS1 (MRN) complex, structural maintenance of chromosomes 5/6 (SMC5/6) complex, or the BLM helicase—proteins known to be involved in homologous recombination—results in telomere shortening and a loss of ALT characteristics in ALT cells lines, suggesting these proteins are integral in telomerase-independent telomere maintenance. Suppression of the MRN complex in IIICF/c and Saos-2 immortalized human ALT cell lines by over-expression of SP100 results in shortened telomeres and loss of APBs (Jiang et al., 2005). Confirming the importance of this complex in ALT, direct inhibition of NBS1 by RNA-interference produces similar results (Zhong et al., 2007) and also impairs the formation of ECTR in GM847, Saos-2, and WI-38 VA-13/2RA ALT cells (Compton et al., 2007). The SMC5/6 complex is required for localization of telomeres to APBs in U-2 OS cells, with RNA interference-mediated depletion of the complex causing subsequent telomere shortening and cellular senescence in another ALT cell line, SUSM1 (Potts and Yu, 2007). Helicase proteins are also integral to recombination, as access to each strand occurs through DNA unwinding. RNA interference-mediated knockdown of BLM helicase induces telomere shortening and growth arrest in Saos-2 ALT cells, but is dispensable for telomere maintenance in cells using telomerase (Bhattacharyya et al., 2009). BLM also interacts with topoisomerase IIIα (Wu et al., 2003); depletion of topoisomerase IIIα by RNA
interference inhibits BLM and TRF2 expression, subsequently inducing telomere
dysfunction in MRC5-V1 and U-2 OS ALT cells (Temime-Smaali et al., 2008).

Other proteins are involved in ALT although their loss does not induce telomere
shortening. These proteins may not be involved directly in telomere elongation by
recombination, but instead may function in other roles. This category includes flap
endonuclease 1 (FEN1), MUS81, replication protein A (RPA), Fanconi anemia
complementation group D protein 2 (FANCD2) and FANCA, Ku70/80, x-ray repair
cross-complementing protein 3 (XRCC3), and RAD51D. RNA interference-mediated
loss of FEN1, a protein normally involved in DNA processing during replication, from
U-2 OS ALT cells increases telomeric DNA damage response (Saharia and Stewart,
2009) and implicates this protein in telomere protection. Similarly, MUS81 loss induces
growth arrest, increases signal-free telomere ends, and inhibits telomere recombination in
GM847, Saos-2, and U-2 OS ALT cells (Zeng et al., 2009). Although overall telomere
shortening is not observed after MUS81 loss, these results suggest that MUS81 functions
in telomere maintenance. Growth arrest and accumulation of single-stranded telomeric
DNA in U-2 OS and GM847 ALT cells are induced upon RPA knockdown, suggesting
that this protein helps to process telomere ends (Grudic et al., 2007). Knockdown of
FANCD2 or FANCA induces telomere loss and inhibits telomere recombination in
GM847 and U-2 OS ALT cells (Fan et al., 2009). Knockdown of the Ku70/80 complex
impairs the growth of CCL75.1 ALT cells and reduces ECTR, although its loss does not
induce telomere shortening (Li et al., 2011). XRCC3 normally functions to resolve
Holliday junctions and is also required for the maintenance of ECTR in GM847 and WI-
38 VA-13/2RA ALT cells (Compton et al., 2007). Although Ku70/80 and XRCC3 do not seem to be required for telomere length maintenance, both proteins are implicated in telomere dynamics and may be required to preserve the fidelity or structure of ALT telomeres. Knockdown of RAD51D, another protein involved in Holliday junction resolution, in WI-38 VA-13/2RA ALT cells induces telomere dysfunction and apoptosis (Tarsounas et al., 2004).

Furthermore, studies combining methionine restriction and RNA interference technologies have identified PML, TRF1, TRF2, TIN2, RAP1, and the MRN complex as requirements for APB formation in IIICF/c ALT cells (Jiang et al., 2007). TRF2 is required for U-2 OS ALT cells, as loss induced telomere shortening and senescence activation (Stagno D’Alcontres et al., 2007). However, as a component of the telomere-protective shelterin complex (along with TRF1, TIN2, and RAP1), TRF2 is required for telomere maintenance in all cells rather than specifically for those using ALT. Other proteins are implicated in ALT through telomeric localization in ALT cells; these are summarized in Nabetani and Ishikawa (2011). Further studies will determine the functions of these proteins and their requirement for ALT.

In addition to telomeric proteins and DNA, APBs contain a variety of proteins important for cellular replication and recombination, including BLM (Yankiowski et al., 2000), WRN (Johnson et al., 2001), RAD51, RAD52, replication protein A (RPA) (Yeager et al., 1999), RAD50, MRE11 (Zhu et al., 2000), and NBS1 (G. Wu et al., 2000; Zhu et al., 2000). RAD50, RAD51, and RAD52 are pertinent for telomerase-independent
telomere maintenance in yeast, suggesting there may be similarities in yeast and mammalian ALT systems (Yeager et al., 1999; Zhu et al., 2000).

II. A focus on recQ-like helicases in ALT: BLM and WRN

BLM and WRN are members of the recQ-like family of helicase proteins, a group of ATP- and Mg$^{2+}$-dependent enzymes that catalyze the unwinding of DNA in the 3’→5’ direction and are required for genome maintenance. recQ-like helicases (from one to several) are present in all species examined to date, first discovered in Escherichia coli, and including Saccharomyces cerevisiae, mouse, and man—this conservation underscores the importance of the recQ-like helicases in genomic fidelity. Five such helicases exist in humans: RECQL1, WRN (RECQL2), BLM (RECQL3), RECQL4, and RECQL5 (Figure 2). Three of these (WRN, BLM, and RECQL4) are implicated in diseases characterized by genomic instability: BLM is mutated in Bloom syndrome (BS), WRN in Werner syndrome (WS), and RECQL4 in Rothmund-Thomson syndrome. All are autosomal recessive diseases characterized by genomic abnormalities and increased cancer incidence. Strikingly, BLM and WRN share similar homology, DNA substrate preference, and protein binding partners. These similarities suggest overlapping and/or cooperative functions in DNA metabolism.
Figure 2: Human recQ-like helicases.
The human genome encodes 5 recQ-like helicases: RECQL1, WRN, BLM, RECQL4, and RECQL5. All proteins share the central helicase domain; BLM and WRN have the greatest similarity in amino acid composition, structure, and size.

II.1 Clinical relevance: Bloom syndrome

Dr. David Bloom first characterized BS in 1954, after recognizing similar clinical features in Ashkenazi Jewish patients in his dermatology practice (Bloom, 1954). All BS individuals display proportionally small stature, a high-pitched voice, and narrow face with small lower jaw. Sun sensitivity often causes pigmentation changes and vessel
dilation on the face, resulting in a characteristic butterfly pattern of redness on the cheeks and nose. Immunodeficiency and reproductive complications (including sterility in males and late menarch and early menopause in females) are also observed in those with BS. Associated with the syndrome is diabetes, lung disease from repeated infections, and, most notably, cancer. Affected individuals have an increased incidence of many neoplasms, which occur much earlier than in the general population (German and Ellis, 2002). BS is relatively rare, with less than 300 cases reported worldwide to date; however, the disease incidence is more common in the Ashkenazi Jewish population due to a founder effect, with an incidence reaching close to 1 in 50,000 individuals (German et al., 1977) and a carrier frequency of about 1 in 100.

II.2 Clinical relevance: Werner syndrome

WS is named after the German scientist who first described the disease symptoms in 1904, Dr. Otto Werner (Werner, 1904). WS is an autosomal recessive disorder with adult onset of untimely aging during the second or third decade of life. Affected individuals generally display cataracts, short stature, skin pathologies, and graying or thinning hair. Along with premature aging symptoms, WS patients are affected by diseases more prevalent in an older population: cardiovascular disease, diabetes, and cancer. While most cancers in the general population are epithelial in origin, WS individuals are more likely to develop mesenchymal cancers, particularly of the muscle and connective tissues (Goto et al., 1996; Schellenberg et al., 2002). While not as rare as BS, WS is a relatively uncommon genetic disease, with less than 1500 cases reported
worldwide. The incidence of WS is elevated in the Japanese population and accounts for
the majority of known cases (Yamamoto et al., 2003).

**II.3 BLM and WRN**

The *BLM* gene was mapped to chromosome 15q26.1 via somatic crossover point
mapping and encodes a protein of 1,417 amino acids (Ellis et al., 1995a). *BLM* has three
conserved sequence elements (Figure 2): a helicase domain, a RECQ C-terminal domain
(RQC), and a helicase and RNaseD-like C-terminal domain (HRDC) (Morozov et al.,
1997). Central to the protein, the helicase domain is conserved throughout the recQ-like
helicases. The RQC domain is situated adjacent to the helicase domain and is believed to
have a pivotal role in mediating protein-protein interactions (Brosh et al., 2001a; von
Kobbe et al., 2002; Bachrati and Hickson, 2003). The HRDC domain functions as a
DNA binding domain and structure recognition domain (Morozov et al., 1997; Liu et al.,
1999). A nuclear localization sequence (NLS) is present near the C-terminus of BLM,
directing the protein to the nucleus (Kaneko et al., 1997).

The helicase domain of recQ-like helicases confers DNA unwinding ability, a
necessary component of DNA metabolism machinery. BLM prefers substrates that
resemble recombination intermediates, including Holliday junctions (Karow et al., 2000),
triplex DNA (Brosh et al., 2001b), and D-loops (van Brabant et al., 2000). Many other
DNA substrates are unwound by BLM, including quadruplex DNA (Sun et al., 1998),
DNA/RNA heteroduplexes (Popuri et al., 2008), 3’ tailed duplexes, forked duplexes, and
double strand DNA containing a single strand bubble (Mohaghegh et al., 2001).
Mutation within *BLM* results in loss of protein and loss of these helicase functions,
promoting the disease state. A variety of different mutations in BLM have been identified, all resulting in absent BLM (Ellis et al., 1995a; German and Ellis, 2002). A specific BLM mutation is found within the Ashkenazic and Sephardic Jewish populations and is hence termed BLM\textsuperscript{Ash} (Ellis et al., 1995a). Other mutations within BLM segregate specifically with given populations, indicating consanguinity or founder effects of each mutation (German et al., 2007).

Positional cloning strategies identified WRN (Yu et al., 1996) after it was mapped to chromosome 8p12 (Goto et al., 1992). WRN encodes the 1,432 amino-acid-long WRN helicase protein (Gray et al., 1997; Suzuki et al., 1997). WRN unwinding activity, similar to that of BLM, acts on a variety of substrates that include Holliday junctions (Constantinou et al., 2000), triplex DNA (Brosh et al., 2001b), D-loops (Orren et al., 2002), quadruplex DNA (Fry and Loeb, 1999), DNA/RNA heteroduplexes (Suzuki et al., 1997), 3’ tailed duplexes (Shen et al., 1998a; Mohaghegh et al., 2001), forked DNA (Suzuki et al., 1997), and bubbled DNA (Mohaghegh et al., 2001). In addition to its helicase, RQC, and HRDC domains, WRN is unique in the recQ family for its exonuclease domain (Shen et al., 1998b; Huang et al., 1998) (Figure 2). This domain has unusual substrate specificity that includes bubble DNA, single strand loops, stem-loop DNA (Shen et al., 2000), 3’ recessed strands of DNA/DNA or DNA/RNA duplexes, nicks and gaps (Huang et al., 2000), and a single mismatched nucleotide on the 3’ recessed end of DNA (Kamath-Loeb et al., 1998). Numerous unique mutations of WRN have been identified in patient samples, including nonsense, frameshift, and insertion-deletion mutations (Huang et al., 2006). All mutations result in a C-terminal truncated
protein. The C-terminus of WRN also contains an NLS (Matsumoto et al., 1997); some mutations prohibit C-terminal truncated WRN from localizing to the nucleus for proper function.

II.4 Cellular manifestations of BLM or WRN absence

Mutation of BLM causes increased somatic cell recombination and the accumulation of mutations throughout the genome. Cytogenetic abnormalities of BS cells include chromosomal breaks and an increased frequency (5-10 fold over normal cells) of sister chromatid exchanges (SCE) (Ellis et al., 1995b). SCE are formed by aberrant homologous recombination between sister chromatids, implicating BLM in their suppression (Wang et al., 2000b). Stalled replication forks can also be resolved by homologous recombination, implicating BLM in their resolution. In the absence of BLM, recombination is used to bypass an unresolved stalled fork (Yankiowski et al., 2000). No other disease displays increased SCE, making BS unique in this feature. Quadriradial formations between homologous chromosomes are frequent in BS cells and are cytogenetically pathognomonic for BS (German 1964). These structures also indicate increased homologous recombination events. BS cells are further characterized by increased micronuclei formation (Rosin and German, 1985), reflecting chromosomal breakage. BS cells also display increased telomeric associations of homologous chromosome arms (Lillard-Wetherell et al., 2004).

BLM has numerous functions within the cell in DNA replication, repair, and recombination. BLM is required for precise, error-free repair of DNA double strand breaks, through both non-homologous end-joining (Rünger and Kraemer, 1989; Langland
et al., 2002; Gaymes et al., 2002) and homologous recombination (Sengupta et al., 2003). BLM localizes to nucleoli, to telomeres, and to promyelocytic leukemia (PML) bodies (Yankiwicki et al., 2000; Sanz et al., 2000). It localizes to sites of double strand DNA breaks at replication forks (Davalos and Campisi, 2003), supporting its role in DNA repair and replication. BLM varies in expression during the cell cycle, with high nuclear expression during late S-phase and persisting throughout G2/M phases. There is little to no detectable expression during G1-phase (Dutertre et al., 2000).

Cells from WS individuals, similar to BS, are characterized by genomic instability. WS cells display elevated rates of chromosomal translocations and deletions (Fukuchi et al., 1989) and an accelerated loss of replicative capacity (and thus initiation of cellular senescence) (Faragher et al., 1993), which can be prevented by telomerase expression (Wyllie et al., 2000). Increased cellular senescence and telomere shortening may be a direct cause of age-related pathologies in both WS patients and the rest of the population, with age of onset differing between these two groups. WS cells exhibit variegated translocation mosaicism, suggesting an increase of non-homologous recombination (Salk et al., 1981). Cultured WS cells also display an extended S-phase (Poot et al., 1992), suggesting a function for WRN in S-phase of the cell cycle. WRN expression peaks at G2/M-phases, although the protein is expressed steadily throughout the cell cycle (Kitao et al., 1998). Cellular localization is predominantly nucleolar (Marciniak et al., 1998), with nucleoplasmic translocation and focus formation following DNA damage (Sakamoto et al., 2001). Analogous to BLM, WRN functions in DNA repair. WRN physically and functionally interacts with the apurinic/apyrimidinic
exonuclease 1 (APE1) (Ahn et al., 2004), required for base excision repair (BER), supporting its function in oxidative DNA damage repair. Non-homologous end-joining (NHEJ), one of two mechanisms for DNA double strand break repair (DSB), requires the DNA-PK complex of Ku70/80 proteins and DNA-PKcs and the X4L4 complex of XRCC4 and DNA ligase IV. WRN interacts with the X4L4 complex (Kusumoto et al., 2008) and the Ku heterocomplex of Ku70/80 (Cooper et al., 2000; Li and Comai, 2000); each complex stimulates WRN exonuclease activity. WRN is also implicated in DSB repair in pathways of homologous recombination (Saintigny et al., 2002).

II.5 Interacting protein partners of BLM and WRN

BLM interacts with numerous other proteins (Figure 3). It co-localizes with PML nuclear bodies (Ishov et al., 1999; Zhong et al., 1999; Yankiwski et al., 2000) and interacts directly with topoisomerase IIIα (Johnson et al., 2000; L. Wu et al., 2000; Hu et al., 2001), RAD51, RPA (Bischof et al., 2001), NBS1 (Davalos and Campisi, 2003), the tumor suppressor protein 53 (p53) (Garkavtsev et al., 2001), small ubiquitin-like modifier (SUMO) (Eladad et al., 2005), DNA polymerase δ (Selak et al., 2008), MutS homolog 6 (MSH6) (Pedrazzi et al., 2003), MutL homolog 1 (MLH1) (Langland et al., 2001), the ataxia-telangiectasia and Rad3-related kinase (ATR) (Davies et al., 2004), flap endonuclease 1 (FEN1) (Sharma et al., 2004), WRN (von Kobbe et al., 2002), and RNA polymerase I (Grierson et al., 2012). Many of these proteins function in homologous recombination and DNA repair. BLM is a component of the BRCA1-associated genome surveillance complex (BASC), a complex that includes the ataxia-telangiectasia mutated
kinase (ATM), MRE11, NBS1, MutL homolog 1 (MLH1), MutS homolog 2 (MSH2), MSH6, RAD50, and the DNA replication factor C (Wang et al., 2000a).

**Figure 3: BLM and WRN interacting protein partners.**
Proteins that interact with only BLM are shown in the black box on the left; proteins that interact with only WRN are shown in the white box on the right. Central proteins in the gray box interact with both BLM and WRN.

Numerous protein partners of WRN have also been identified (Figure 3). These include DNA polymerase δ, topoisomerase I (Szekely et al., 2000), proliferating cell nuclear antigen (PCNA) (Lebel et al., 1999), RPA (Brosh et al., 1999), FEN1 (Brosh et
al., 2001a), RAD51, RAD54B (Otterlei et al., 2006), ATR (Pichierri et al., 2003), p53 (Spillare et al. 1999; Blander et al., 2000), SUMO1 (Kawabe et al., 2000), BRCA1 (Cheng et al., 2006), MSH2/MSH6, MSH2/MSH3, MLH1/PMS2 (Saydam et al., 2007) and Werner helicase interacting protein (WHIP) (Kawabe et al., 2001). Many of these proteins also have roles in DNA replication, repair, and recombination. Interactions of WRN with the Ku heterocomplex and RPA that stimulate exonuclease activity and helicase activity, respectively, suggest functions in DNA damage responses (Orren et al., 2001).

II.6 BLM and WRN at the telomere

The sole Saccharomyces cerevisiae recQ-like DNA helicase, Sgs1p, is implicated in telomere maintenance. Similar to BLM and WRN, Sgs1p suppresses DNA hyper-recombination (Heo et al., 1999) and unwinds Holliday junctions and G-quadruplexes (Bennett et al., 1999; Sun et al., 1999). Similar to loss of BLM, sgs1-Δ increases SCE frequency (Onoda et al., 2000). sgs1-Δ yeast lacking telomerase display accelerated aging indicators, including increased telomere shortening rates, cell cycle arrest, and increased cell death (Sinclair et al., 1997; Johnson et al., 2001), but can be corrected by over-expression of human BLM (Lillard-Wetherell et al., 2005). sgs1-Δ yeast display high rates of recombination, especially affecting repeated sequences such as telomeres (Watt et al., 1996). BLM and WRN can each complement the increased recombination observed in yeast lacking Sgs1p (Yamagata et al., 1998), again suggesting conserved functions of the recQ-like family members.
Sgs1p is important for telomere elongation by the type II pathway, as yeast deficient for both telomerase and sgs1 escape senescence via the type I pathway (Johnson et al., 2001). Indeed, BLM can complement Sgs1p deficiency in yeast mutant for both telomerase and sgs1, and restore telomere elongation via the type II pathway (Lillard-Wetherell et al., 2005).

BLM and WRN helicases associate with known DNA repair proteins at telomeres. For example, the non-homologous end-joining (NHEJ) proteins Ku70, Ku80, and DNA-PK Cs are necessary for proper telomeric function. Yeast strains with disrupted genes encoding Ku proteins lose telomeric repeat sequences, have disrupted telomere organization, and develop telomeric dysfunction (Boulton and Jackson, 1996; Laroche et al., 1998; Porter et al., 1996). Mammalian cells without these NHEJ proteins display telomere end-to-end fusions and genomic instability (Bailey et al., 1999), also suggesting a role of NHEJ DNA damage/repair proteins in normal telomere maintenance. TRF2, implicated in DSB repair, is necessary to protect telomere ends (van Steensel et al., 1998). Homologous recombination proteins, pertinent for the resumption of stalled replication forks, may be required to resolve stalled forks that occur in the G-rich telomere DNA; recQ-like helicases may enable recombination-based resolution of such stalled forks (Azam et al., 2006).

Both WRN and BLM are implicated in the ALT pathway, as both localize to APBs and associate with telomeric proteins TRF1 and TRF2 in ALT cells (Stavropoulos et al., 2002; Lillard-Wetherell et al., 2004; Opresko et al., 2002). TRF2 stimulates the unwinding helicase activity of BLM and WRN, and allows prolonged unwinding of
telomeric substrates (Opresko et al., 2002; Lillard-Wetherell et al., 2004). Colocalization of BLM and TRF2 occurs within APBs and increases with bromodeoxyuridine (BrdU) incorporation during late S- and G2-phases of the cell cycle when ALT is thought to occur (Lillard-Wetherell et al., 2004). This overlap with BrdU incorporation indicates synthesis of telomere DNA within APBs. BLM also directly binds telomeric DNA within nuclear bodies; deletion of the DNA interaction domain of BLM interrupts proper cell cycle progression (Schawalder et al., 2003). WRN also specifically binds telomeric DNA sequences (Opresko et al., 2004) and co-localizes exclusively in ALT cells with APB-associated proteins, including PML, RAD52 (Baynton et al., 2003), and NBS1 (Johnson et al., 2001). POT1, which binds single-stranded telomeric DNA and regulates telomere length, also facilitates BLM and WRN unwinding activity of telomeric D-loop and forked duplexes structures, which are otherwise poor substrates. POT1 stimulates both BLM and WRN to unwind and release the single-stranded chromosome end from a telomeric D-loop structure (Opresko et al., 2005). In addition, the MRN complex of MRE11, RAD50, and NBS1 localizes to telomeres with Ku 70/80 and DNA-PKcs. WRN also interacts with FEN1, a protein required for ALT telomere stability (Saharia and Stewart, 2009).

WRN and BLM are involved in telomeric maintenance. WS cells have normal telomere lengths, although passage in culture reveals elevated rates of telomere shortening and senescence induction at inconsistent telomere lengths (Schulz et al., 1996; Tahara et al., 1997). Cells lacking functional WRN helicase activity increase sister telomere loss (Crabbe et al., 2004). These cytogenetic abnormalities are reversed by
expression of telomerase (Wyllie et al., 2000; Crabbe et al., 2004), suggesting the necessity of WRN for proper telomeric maintenance in a telomerase-independent context. Mouse embryonic fibroblasts mutated for both WRN and TERT (telomerase) accumulate telomere sister chromatid exchanges (T-SCE) and double minute chromosomes in vitro, suggesting that WRN normally prevents abnormal telomeric recombination. Overexpression of BLM within mammalian ALT cells increases detectable telomeric signals (Stavropoulos et al., 2002), suggesting a role for BLM in ALT. Short interfering RNA (siRNA)-mediated knockdown of BLM in ALT cells results in loss of APBs and telomere shortening and suggests BLM is directly involved in recombination-mediated telomere elongation (Bhattacharyya et al., 2009). Consistent with a model in which BLM and WRN are necessary for telomere maintenance, both helicases catalyze not only DNA unwinding but DNA strand annealing. These data signify a role for the recQ-like helicases in telomere repair and maintenance, as the helicases could render telomeric DNA accessible for repair machinery and anneal the strands back together once the damage has been repaired (Machwe et al., 2005).

In vivo data also support a role for BLM and WRN in telomeric functions. Mouse models that ablate Wrn or Blm in combination with telomerase function result in similar pathologies to human diseases. These mouse models display telomere dysfunction such as end-to-end fusions and large deletions, pointing to telomere abnormalities for a subset of the pathologies observed in WS and BS. The absence of premature aging in the context of telomere dysfunction in BS may be explained by BLM expression in proliferative tissues, such as the thymus and testis (Kitao et al., 1998), where telomerase
is also expressed to mask the effects of BLM deficiency (Du et al., 2004). In contrast, WRN is more widely expressed at low levels throughout the body, although high expression is also observed in the pancreas and testes (Kitao et al., 1998). Mouse models deleted for both Wrn and a functional telomerase develop WS pathologies and DNA damage, suggesting that some of the WS pathologies may be due to telomere depletion. These mice are strongly affected by changes in mesenchymal tissues, which are often sites of cancer development in WS individuals (Chang et al., 2004).

Experimental evidence suggests a direct interaction of BLM and WRN, and similar mechanistic roles in DNA metabolism. Both proteins colocalize in ALT cell lines (von Kobbe et al., 2002), although some co-localization occurs in telomerase-positive cell lines (Yankiwski et al., 2000). Immunofluorescence demonstrates co-localization in punctuate nuclear foci and some staining of BLM or WRN in cellular nucleoli. These interactions only occur in a subset of cells, suggesting a transient interaction between BLM and WRN.

**III. Telomere maintenance in human tumors**

Continuous telomere erosion throughout life has important implications for aging and the diseases associated with aging. As such, cancer cells must actively maintain their telomere length to support high rates of proliferation and permit cellular immortalization. Current hypotheses predict that following the primary genomic alterations that confer enhanced proliferative capacity, the increased rate of cell cycle completion pressures neoplastic cells to activate a telomere maintenance mechanism
(Figure 4). Although tumorigenesis can be achieved through oncogenic transformation of mammalian cells, telomere maintenance is required to prevent telomere crisis and cellular senescence induced by telomere shortening (Sun et al., 2004). Telomere dysfunction itself can favor cancer initiation (Farazi et al., 2003; Artandi et al., 2000; O’Hagan et al., 2002) but limits progression (Farazi et al., 2003), suggesting that additional genetic changes and cellular factors are required. Furthermore, human skin, breast and colon cancer precursor legions demonstrate evidence of telomere dysfunction, absent in corresponding malignant legions, through colocalization of DNA damage markers with the telomere (Suram et al., 2012). These results suggest telomere shortening in neoplastic cells precipitates telomere dysfunction and pressures activation of a telomere maintenance mechanism for tumor cell immortalization. While telomere maintenance is not necessarily an activator of tumorigenesis, it represents a bottleneck that limits the lifespan of rogue cells. The activation of a telomere maintenance mechanism thus permits cellular immortalization and tumor progression.
Figure 4: Activation of a telomere maintenance mechanism.
Neoplastic mutations allow relaxation of cell cycle controls with increased proliferation and telomere shortening (red circles represent telomeres on green chromosomes). Once critically short, telomeres signal the activation of a telomere maintenance mechanism to permit tumor cell immortalization (top) or the cell will exit the cell cycle through senescence and/or apoptosis (bottom, shaded cell).
Numerous studies have evaluated the telomere maintenance mechanisms in place in a variety of human tumors (Henson and Reddel, 2010; Heaphy et al., 2011). Although 85% of human tumors use telomerase (Shay and Bacchetti, 1997), TMM in human tumors varies greatly according to tumor type (Table 1). ALT is over-represented in mesenchymal cancers; osteosarcomas have the highest incidence of ALT examined thus far, with 59% of osteosarcoma cases exhibiting ALT characteristics (Bryan et al., 1997; Henson et al., 2005; Ulaner et al., 2003; Sanders et al., 2004). Many, but not all, soft tissue sarcomas display a preponderance (27% overall) of ALT characteristics, along with low-grade (grade 1-3) astrocytoma (37%), diffuse malignant pleural mesothelioma (17%) and gastric carcinoma (38%). The variability of ALT frequency within tumor types suggests there are inherent differences between cell origins or mutational mechanisms that allow preference of one telomere maintenance mechanism over the other. Accordingly, mesenchymal stem cells express little to no detectable telomerase expression (Zimmermann et al., 2003) and may predispose cells from this lineage to utilize ALT. Although cell-specific differences may exist, the question of how cells activate one mechanism over the other remains unresolved.
Table 1. ALT incidence by tumor type

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*From Henson and Reddel, 2010; Heaphy et al., 2011*

Previous studies show that both varieties of telomere maintenance can occur within the same cell upon experimental manipulation *in vitro*. Exogenous expression of functional telomerase within ALT cells results in the elongation of short telomeres by telomerase, although the cells still retain ALT characteristics (Perrem et al., 2001; Ford et al., 2001; Cerone et al., 2001; Grobelny et al., 2001). Fusion experiments of both telomerase-positive and ALT cells demonstrate that telomerase-positive cells contain ALT inhibitors other than telomerase itself, as these hybrid cells suppress ALT and maintain telomeres exclusively with telomerase (Perrem et al., 2001). Conversely, other studies with hybrid cells show a suppression of telomerase activity and telomere maintenance by ALT, suggesting ALT cells contain telomerase inhibitors (Katoh et al., 1998). Similar results have not been observed *in vivo*.

The correlation between TMM and patient prognosis clinically varies for each tumor type. Patients with glioblastoma multiforme have a better prognosis when the tumor uses ALT (Hakin-Smith et al., 2003; Henson et al., 2005); however, patients with
neuroblastoma (Onitake et al., 2009), liposarcoma (Costa et al., 2006), or malignant fibrous histiocytoma (Matsuo et al., 2009) show a worse prognosis when the tumor is ALT-positive. Several studies have identified no significant difference in patient survival in regards to TMM in osteosarcoma, soft tissue sarcoma, malignant fibrous histiocytoma (Henson et al., 2005), or diffuse malignant peritoneal mesothelioma (Villa et al., 2008). The prognostic value of TMM may vary with tumor type according to other patient or tumor variables.

Telomere maintenance is crucial for the survival of tumor cells and so has attracted the interest of pharmaceutical companies. A telomerase inhibitor therapy, Imetelstat (GRN163L), is marketed by Geron and is currently in Phase 2 clinical trials. Imetelstat is an oligonucleotide that functions by binding and competing with the telomerase RNA component hTERC for the active site within the telomerase enzyme (Röth et al., 2010). Imetelstat has been proven effective at inhibiting the growth of tumor cells in vitro (Brennan et al., 2010; Goldblatt et al., 2009; Hochreiter et al., 2006; Joseph et al., 2010; Marian et al., 2010) and completed Phase 1 clinical trials, demonstrating encouraging drug tolerance and effectiveness. Phase 2 clinical trials include breast cancer, non-small cell lung cancer, essential thrombocythemia, and multiple myeloma. Recent studies have demonstrated that loss of telomerase expression in established tumors promotes survival by the ALT pathway (Hu et al., 2012), implicating telomere maintenance as a critical component of tumor immortalization and a plasticity in TMM. This work suggests that complex therapeutic interventions may be necessary to inhibit both telomerase and ALT to prevent tumor cell survival.
Chapter 2: Thesis Rationale and Research Objectives

Telomere maintenance is a critical step in the immortalization of tumor cells and can occur through expression of the enzyme telomerase or by recombination-mediated ALT pathways. Although telomerase has been well characterized over the past two decades, the mechanisms of ALT are still largely unknown. Understanding how recombination maintains telomere lengths in tumor cells is very important for the development of therapeutics to limit tumor cell immortalization and thus inhibit cancer progression.

The goals of this work are two-fold: microscopically, to examine the proteins involved in ALT telomere maintenance; and macroscopically, to identify how tumor cells use telomere maintenance to promote immortalization through telomerase and/or ALT. The BLM helicase is absent in Bloom syndrome, an inherited disorder characterized by proportional dwarfism and cancer predisposition. The closely related WRN helicase is absent in Werner syndrome (WS), an inherited disorder characterized by accelerated aging and cancer predisposition. BLM and WRN interact physically and functionally with telomeres and telomeric proteins; BLM is required for ALT (Bhattacharyya et al., 2009), while data from one cell line, however, intimated that WRN may not be. The telomeres of WS patients undergo early senescence and do so at longer telomere lengths
than unaffected individuals (Schulz et al., 1996; Tahara et al., 1997). Mutation of Wrn in mouse models does not mimic Werner syndrome unless combined with telomere shortening through Terc mutation (Du et al., 2004), suggesting telomere dysfunction is a contributing factor for disease manifestation. WRN is required for the DNA damage response at exposed telomere ends of immortalized cell lines, likely through exonucleolytic degradation of the telomeric overhang (Eller et al., 2006). Furthermore, similarities in structure and function of BLM and WRN suggested the hypothesis that WRN is also required for ALT. Short-interfering RNA (siRNA) was used to inhibit WRN expression in human immortalized ALT cell lines. Telomere-specific assays were used to measure telomere length and ALT characteristics of these cells following WRN loss. These experiments demonstrated that some, but not all, required WRN for ALT to occur and that its loss can lead to the induction of alternative ALT pathways to maintain telomeres. Protein localization and preliminary protein-protein interactions suggest that the BRCA1 protein may facilitate these pathways.

More than 85% of human tumors use telomerase to maintain telomeres; the remaining 15% use ALT mechanisms (Shay and Bacchetti, 1997). Previous studies evaluating TMM in human tumors have identified a subset of tumors that do not classify readily as only telomerase-positive or ALT-positive (reviewed in Henson and Reddel, 2010). These tumors display either mixed characteristics of both mechanisms or neither. In addition, recent work suggests that inhibition of telomerase in mouse tumors can pressure the activation of ALT mechanisms (Hu et al., 2012), suggesting that TMM may not be a static characteristic of tumors. The notion that tumors may be composed of cells
with either TMM has important implications for human tumor therapies, as telomerase inhibitors are being tested in clinical trials. The second goal of this work was to test the hypothesis that human tumors can contain some cells expressing telomerase and some cells with ALT characteristics. Close examination of human sarcoma tumors was performed using immunohistochemical staining for both telomerase and ALT markers followed by their analysis with an enhanced imaging system. Immunohistochemical results were validated with a small set of frozen osteosarcomas by both immunohistochemistry and biochemical assays to confirm the ability of ALT and telomerase to co-exist within the same tumor.
Chapter 3: A variable requirement for the WRN helicase in human cell lines using ALT suggest multiple mechanisms of telomere maintenance in the absence of telomerase

I. Introduction

Erosion of chromosome ends persists with each round of cell division due to the inability of the replication machinery to proceed completely to the chromosome end. Telomeres protect the terminal chromosome ends by buffering the loss of coding DNA from this erosion and by concealing the chromosome end from recognition as a DNA double strand break. Although most human somatic cells do not maintain their telomeres, cancer cells activate a telomere maintenance mechanism to support growth and immortalization. A majority of cancers activate telomerase, while a subset maintains telomeres in the absence of telomerase. This telomere maintenance mechanism is termed alternative lengthening of telomeres, or ALT.

Yeast cells can survive without telomerase in two RAD52-dependent forms (Lundblad and Blackburn, 1993): type I survivors are RAD51-dependent and have short telomere repeats and amplified Y’ telomere elements, while type II yeast are RAD50-dependent and have long heterogeneous telomere repeat tracts. Immortalized mammalian cells without detectable telomerase expression are classified as ALT. ALT characteristics include heterogeneous telomere lengths, extrachromosomal telomeric repeats and ALT-associated PML bodies (APBs), although these characteristics are variable (Fasching et
Clear evidence suggests that ALT uses recombination to add telomeric repeats to the chromosome terminus (Dunham et al., 2000). Homologous or non-homologous chromosome ends can supply a (TTAGGG)_n template for telomeric recombination. Alternatively, sister chromatids or extrachromosomal telomeric sequences, abundant in both linear and circular forms in ALT cells, may provide templates for telomere elongation by de novo addition (linear) or rolling circle amplification (circular). Both inter-telomeric copying between chromosomes (Dunham et al., 2000) and intra-telomeric copying within the same chromosome (Muntoni et al., 2009) have been shown. Given these options, multiple telomerase-independent maintenance mechanisms may be used by mammalian cells, reminiscent of the yeast type I and II pathways.

Numerous DNA repair proteins localize to the telomere in ALT cells and are assimilated in APBs. APBs are distinguished from normal PML bodies by the inclusion of telomeric proteins and telomere DNA (Yeager et al., 1999) and are sites of bromodeoxyuridine (BrdU) incorporation (G. Wu et al., 2000). APBs are considered sites of telomere elongation and/or dynamics in ALT cells. DNA repair proteins, such as the MRN complex (G. Wu et al., 2000), localize to APBs and may function to recognize telomeric damage inherent in ALT cells or may actively participate in telomeric recombination. Two other DNA repair proteins, the RecQ-like BLM (Yankiwski et al., 2000) and WRN (Johnson et al., 2001) helicases localize to APBs.

Bloom syndrome (BS) and Werner syndrome (WS) are inherited chromosome instability disorders marked by predisposition to cancer. The genes mutated in BS and
WS, *BLM* and *WRN*, respectively, encode proteins of similar structure that carry out essential roles in DNA replication and repair. Both helicases unwind telomeric DNA sequences and structures *in vitro* (Lillard-Wetherell *et al*., 2004; Opresko *et al*., 2004), suggesting that they unwind the t-loop to allow telomere elongation. BLM (Yankiwski *et al*., 2000) and WRN (Opresko *et al*., 2004) interact with telomeric proteins and telomere DNA in immortalized human cell lines. The telomeric proteins TRF2 (Stavropoulos *et al*., 2002; Opresko *et al*., 2002; Lillard-Wetherell *et al*., 2004) and POT1 (Opresko *et al*., 2005) interact with BLM and WRN to stimulate helicase activity using telomeric substrates *in vitro*.

Evidence suggests that telomere dysfunction contributes to disease pathology in both BS and WS: BS cells exhibit telomeric associations (Lillard-Wetherell *et al*., 2004), while cultured WS cells undergo early senescence (Schulz *et al*., 1996; Tahara *et al*., 1997) and exhibit increased sister telomere loss (Crabbe *et al*., 2004). Exogenous telomerase expression prevents these phenotypes of WS cells (Wyllie *et al*., 2000; Crabbe *et al*., 2004), indicating shortened telomeres may contribute to disease progression. *Wrn*-deficient mice lack the WS disease phenotype (Lombard *et al*., 2000), although late generation mice develop phenotypes that closely mimic WS when *Wrn* is mutated in combination with *Terc*, the RNA component of telomerase (Chang *et al*., 2004). Mice only display these phenotypes in the context of shortened telomeres, suggesting that these manifestations are associated with shortened telomeres. Mutation of *Blm*, *Wrn* and *Terc* accentuates telomere dysfunction, suggesting that all proteins function at the telomere (Du *et al*., 2004).
Our previous work showed that BLM is required to maintain telomeres in ALT cells, but not in telomerase-positive cells (Bhattacharyya et al., 2009). The similarities in structure and in vitro function of BLM and WRN suggested that WRN may also be required for ALT. A previous study concluded that WRN is dispensable for ALT function based upon a single WS cell line, AG11395, that displays ALT characteristics (Fasching et al., 2005). AG11395 is SV40-immortalized, incorporates SV40 sequences into its telomeric repeats and fails to form APBs. Transfection of WRN into AG11395 cells results in APB formation, gain of telomeric sequences and the conversion of type I-like telomeres (with SV40 sequences interspersed) to type II-like telomeres (Siddiqa et al., 2012). We asked whether WRN is required for ALT by investigating its role in three ALT cell lines. Our results show that two of these three ALT cell lines require WRN to maintain telomeres. This variability suggests that different ALT cells use different pathways of telomerase-independent telomere maintenance, and that these pathways employ unique subsets of proteins.

II. Materials and Methods

Cell lines.

Immortalized human cell lines WI-38 VA-13/2RA, Saos-2, U-2 OS, HeLa and MCF7 were obtained from ATCC. Saos-2 and U-2 OS are ALT cell lines derived from osteosarcomas; WI-38 VA-13/2RA is an ALT cell line derived from lung fibroblasts; HeLa is a telomerase-positive cell line derived from a cervical adenocarcinoma; and
MCF7 is a telomerase-positive cell line derived from a breast adenocarcinoma. Saos-2, HeLa, and MCF7 cells were grown in Dulbecco’s Modified Eagle Medium (Invitrogen); WI-38 VA-13/2RA cells were grown in Minimal Essential Medium (Invitrogen); and U-2 OS cells were grown in McCoy’s 5A Medium (Invitrogen). All cells were grown in media containing 10% fetal bovine serum (HyClone) and maintained at 37°C with 5% CO₂.

**siRNA knockdown.**

ON-Target Plus pooled siRNAs targeting WRN (Dharmacon) were reverse transfected into cultured cells using Dharmafect 1 transfection reagent (Dharmacon) per manufacturer’s directions at a final concentration of 50nM. Cells were passaged into fresh media containing siRNAs every 5 days. Pooled scrambled sequence control siRNAs (Dharmacon) are non-targeting and were transfected at 50nM as above. TRF2 siRNA (Santa Cruz) was transfected at 30nM as above. BLM siRNA (Ambion) was transfected at 40nM using Lipofectamine 2000 transfection reagent (Invitrogen) per manufacturer’s directions.

**Western blot.**

Whole cell extracts were collected by lysing cells directly in 95°C 1X SDS loading buffer, sonicating 10 seconds and then boiling for 5 minutes at 95°C. Extracts were separated by standard procedures using SDS-PAGE (Bio-Rad) and transferred to a PVDF
membrane (Millipore). Membranes were blocked with a 5% solution of nonfat dry milk in 1X TBST and subsequent incubations were carried out in 2% milk. For western blotting, primary antibodies used were: anti-WRN (1:2000, Abcam ab66606), anti-BLM (1:1000, Bethyl Laboratories A300-110A), anti-lamin B (1:1000, Santa Cruz sc-6217), anti-TRF2 (1:1000, Imgenex IMG-124A), anti-p53 (1:1000, Santa Cruz sc-126), anti-BRCA1 (1:100, Santa Cruz sc-642). Secondary antibodies used were: horseradish peroxidase-conjugated goat-anti-mouse, goat-anti-rabbit, and rabbit-anti-goat antibodies (Jackson ImmunoResearch Laboratories) at a concentration of 1:10,000.

**TRAP assay.**

Telomerase activity was measured using the TRAPeze kit (Millipore) per manufacturer’s directions. Extracts were collected from cells in cold 1X CHAPS lysis buffer. To prevent interference by PCR inhibitors in the cell extracts, samples were phenol-chloroform extracted after template elongation and before PCR amplification. Products were separated on 10% non-denaturing PAGE gels and dried on a BioRad Model 583 gel dryer. Gels were exposed to phosphor screens (GE) overnight and imaged on a Typhoon 9410 variable mode imager (GE).

**qRT-PCR assay.**

DNA extracts were collected from cells using the Genomic DNA Isolation kit (Trevigen). Telomeric DNA was amplified from 30 ng total genomic DNA using Sybr green PCR
master mix (Applied Biosystems) as previously described (Cawthon, 2009). ALB was amplified as a single copy gene control. Both PCR reactions were performed in triplicate wells on an Applied Biosystems 7900HT Fast-Real Time PCR machine. Amplified products were visualized on a 4% agarose gel to verify expected products.

*C-circle assay.*

Total DNA was extracted from cells using the DNeasy kit (Qiagen) and analyzed for C-circles as previously described (Henson et al., 2009). Because C-circle levels vary in different ALT cell lines, 100 ng of DNA was used for Saos-2 cells, and 1000 ng for VA-13 cells, HeLa cells and MCF7 cells. To control for DNA input, one sample was undigested and not subjected to the C-circle assay (total telomere DNA), while another sample was digested with Hinfl/Rsal restriction enzymes and ExoI/ExoV/Exol exonucleases, and subjected to the C-circle assay. C96 is a synthetic C-circle that serves as a positive control (Henson et al., 2009). Products were separated on a 0.8% agarose gel and transferred to a nylon charged membrane (Hybond). Telomeric products were visualized by hybridization with a 32P-end labeled (CCCTAA)3 probe at 42°C in UltraHyb-Oligo hybridization buffer (Ambion). Membranes were washed and exposed to a phosphor screen (GE) overnight and imaged on a Typhoon 9410 variable mode imager (GE).
**Immunofluorescence.**

Cells were grown on glass coverslips (Fisher), washed in PBS, and fixed in 4% paraformaldehyde for 15 minutes at room temperature. For immunocytochemistry, cells were permeabilized in 0.25% Triton-x-100 in PBS and washed before blocking in 10% normal goat serum and subsequent staining with the indicated antibodies in 1% BSA/0.1% Tween/PBS: anti-TRF2 (Imgenex), anti-PML (Abcam), or anti-γH2AX (Millipore). Cells were washed in 0.1% Tween/PBS and primary antibodies were visualized with fluorescent AlexaFluor (Invitrogen) secondary antibodies. For FISH, cells were dehydrated after fixation in an ethanol series, rehydrated in 2X SSC washes, and hybridized with a Cy-3 conjugated PNA telomeric probe (Panagene) at 67μg/mL in 70% formamide/2X SSC. Cells were washed in 0.1% Tween in PBS at 55°C, followed by a rinse in 0.1% Tween/2X SSC at room temperature. Coverslips were then rinsed in water, air-dried, and mounted with VectaShield DAPI mounting medium (Vector Labs) onto glass slides (Fisher). Cells were imaged using a Zeiss AxioVert 200M with an attached AxioCam MRm camera. Fifty cells each from three independent experiments were analyzed blindly for colocalization.

**Statistical analysis.**

The two-sample t-test was used to analyze significance for qRT-PCR results and immunofluorescent assay by comparing each treatment group to the control for each cell line with an assumption of unequal variances.
III. Results

Continuous WRN knockdown shortens telomeres in VA-13 and U-2 OS, but not Saos-2, ALT cell lines.

Telomeres shorten an average of 50-100 bp per cell division without an active TMM (Harley et al., 1990; Allsopp et al., 1992; Vaziri et al., 1993, 1994). To investigate whether WRN is necessary to maintain telomeres in the absence of telomerase, we continuously knocked down WRN in two telomerase-positive human cell lines, HeLa and MCF7, and two ALT human cell lines, WI-38 VA-13/2RA (hereafter referred to as VA-13) and Saos-2. Stable clones were first generated using two pSilencerWRN shRNA plasmids generously provided by Dr. Patricia Opresko; although multiple cell clones were generated that exhibited antibiotic resistance, WRN continued to be expressed in all clones as assessed by western blot (Appendix A). As an alternative strategy, pooled WRN siRNAs were reverse-transfected into cells every 5 days to maintain a continuous transient knockdown (Appendix B). Cells were maintained in culture for at least 30 population doublings (PD) with continuous transient WRN knockdown to allow changes in telomere length to occur. WRN expression was consistently reduced by over 90% in all cell lines (Figure 5, top panels). BLM and WRN are highly homologous; therefore western blotting verified that WRN siRNAs were specific to WRN and did not affect BLM expression levels (Figure 5, middle panels). Cells were transfected with pooled scrambled control (SC) siRNAs as a negative control. No growth defects were observed
in cells transfected with *WRN siRNAs* in comparison to SC *siRNAs* or untransfected cells. Cells were analyzed for characteristics of telomere maintenance mechanisms.

![Western Blot Image](image)

**Figure 5: WRN siRNA knockdown is effective and specific.**

Pooled WRN siRNAs or scrambled control (SC) siRNAs were transfected into immortalized human cell lines and whole cell extracts were collected 48 hours after transfection. Each lysate was separated by SDS-PAGE and western blotted with antibodies to WRN (top), BLM (to ensure specificity of the *siRNAs*, middle), and lamin B (as a loading control, bottom).

To examine the requirement for WRN in telomere maintenance, relative telomere lengths of cells maintained with continuous *WRN* knockdown were measured. Genomic DNA was extracted from cells at 4, 20 and 54 populations doublings (PD) after initiation of *WRN* knockdown and subjected to telomere repeat analysis via quantitative real-time PCR (qRT-PCR) (*Appendix C*) (Cawthon, 2009). qRT-PCR allows a quantitative relative telomere length measurement from a small amount of DNA, in comparison to telomere restriction fragment Southern blotting (TRF Southern). qRT-PCR confirmed previously reported changes in telomere length after stable *BLM* knockdown in Saos-2 cells (*Figure 6*) (Bhattacharyya *et al.*, 2009). A significant reduction in relative mean
Relative telomere length in two telomerase-positive cell lines was unchanged in response to continuous transfection with SC (HeLa \( p = 0.506 \); MCF7 \( p = 0.415 \)) or \( WRN \) \( siRNAs \) (HeLa \( p = 0.195 \); MCF7 \( p = 0.987 \)) at all PDs sampled (Figure 6). HeLa cells were maintained in culture for 58 PD and MCF7 cells were maintained for 32 PD. In contrast, VA-13 ALT cells were unable to maintain telomere lengths without WRN. Telomere lengths remained stable for 20 PD, but then steadily shortened to almost 50% of their original length by 54 PD \( (p = 0.0004) \). Remaining cells in culture declined in health until no cells survived at 66 PD, presumably due to progressively shortening telomeres. At this time, there were no longer enough cells to collect a DNA sample. No significant change in telomere length in untransfected controls or SC \( siRNA \) transfected VA-13 cells was measured \( (p = 0.670) \), indicating the telomere length fluctuations were a specific response to \( WRN \) knockdown (Figure 6). In contrast, Saos-2 ALT cells steadily maintained long telomere lengths despite similar \( WRN \) knockdown efficiency (Figure 6 and Figure 5). Saos-2 cells were maintained in culture for 124 PD without change in telomere length \( (p = 0.732) \) or in response to SC \( siRNAs \) \( (p = 0.146) \). These results suggest that VA-13 cells require WRN for alternative telomere maintenance and that Saos-2 cells do not.
Relative telomere length is reduced in VA-13 cells following WRN knockdown as measured by quantitative real time PCR (qRT-PCR). The difference in the cycle threshold (Ct) between a telomere-specific PCR reaction and a single copy gene (ALB) PCR reaction is calculated for each sample as the ΔCt, which represents the average relative telomere repeat length. Telomere length in Saos-2 ALT cells is decreased after stable BLM knockdown as measured by TRF Southern blot (Bhattacharyya et al., 2009). Here, we confirm these length changes by qRT-PCR. Relative telomere lengths of VA-13, but not Saos-2, ALT cells is decreased after continuous WRN knockdown at 54 PD. Relative lengths depicted represent the final measurement for each cell type.

We asked whether our results were unique to VA-13 cells and transfected the U-2 OS ALT cell line with WRN siRNAs. U-2 OS ALT cells, although established from an osteosarcoma, maintain an intact p53 pathway while most other ALT cell lines are mutated or null for p53. U-2 OS cells died in culture in response to WRN knockdown
after 17 PD. We speculate that increased genomic DNA instability due to loss of WRN induced apoptosis, as an increase in cleaved poly-ADP ribose polymerase (PARP) was detected following transfection with WRN siRNAs (Figure 7A). U-2 OS cells underwent apoptosis before a change in telomere length could be detected in the absence of WRN. These data suggested that WRN may be required for ALT in U-2 OS cells, but that p53-mediated apoptosis prevents the survival of these cells in culture in the absence of WRN. U-2 OS cells were simultaneously transfected with siRNAs to reduce both WRN and p53; western blots demonstrated that both proteins were effectively knocked down (Figure 7B). Cells were maintained in culture with continuous transient knockdown as described previously. A significant reduction in telomere length was detected at 77 PD in cells transfected with WRN and p53 siRNAs (Figure 7C).
Figure 7: WRN is required for ALT in U-2 OS cells.
(A) WRN siRNA-transfection induces apoptosis in U-2 OS cells as shown by the cleaved PARP 48 hours after transfection. Whole cell extracts were separated by SDS-PAGE and western blotted with antibodies to WRN to confirm the knockdown, cleaved PARP to examine apoptosis induction and lamin B (loading control). Treatment with camptothecin is a positive control for the induction of apoptosis. (B) Knockdown of WRN in combination with p53 allows cells to survive in culture. Whole cell extracts were separated by SDS-PAGE and western blotted with antibodies specific to WRN, p53 and lamin B (loading control). (C) qRT-PCR shows that telomeres shorten after 77 PD in culture in the presence of WRN and p53 siRNAs. Average relative telomere repeat lengths are graphically represented as the difference between a telomere-specific PCR reaction in comparison to the single copy gene ALB PCR reaction as delta C_t.

The ability of a cell or tumor to switch telomere maintenance mechanism is not well understood, although recent publications suggest that this may occur (Hu et al., 2012). Ballal et al. (2009) reported that telomerase expression increased in cell cultures
following *BRCA1* knockdown. To rule out telomerase reactivation as a mechanism by which Saos-2 ALT cells maintain their telomeres without WRN, the telomere repeat amplification protocol (TRAP) assay was used to assess telomerase activity in cell extracts. Non-transfected Saos-2 cells do not express telomerase and exhibit no activity in the TRAP assay. Transfections with SC (Figure 8, lanes 9-10) or *WRN* siRNAs (Figure 8, lanes 11-12) do not alter these results. Positive controls for the TRAP assay included extracts from telomerase-positive MCF7 (Figure 8, lanes 1-4) and HeLa (Figure 8, lanes 5-8) cells. Activity is absent in VA-13 (Figure 8, lanes 13-16) and U-2 OS ALT cell lines (Figure 8, lanes 19-30). These results support the conclusion that WRN is not required for ALT in Saos-2 cells.
Figure 8: Telomerase activity of siRNA-transfected cell lines does not change following WRN knockdown.
Figure 8: Telomerase activity of siRNA-transfected cell lines does not change following WRN knockdown.
Protein extracts from indicated cells were processed and evaluated using the telomere repeat amplification protocol (TRAP) assay. IC represents the internal control band for the assay, with the laddered bands representing telomerase products. HeLa (lanes 1-4) and MCF7 cells (lanes 5-8) are telomerase-positive cell lines and depict clear laddering, while Saos-2 (lanes 9-12), VA-13 (lanes 13-16) and U-2 OS (lanes 19-30) are ALT cells and lack telomerase activity. A heat denatured negative control is included for each cell type. In addition, negative (lane 17, lane 31) and positive (lane 18, lane 32) TRAP kit controls are included on the far right of the gel.

Loss of WRN prevents APB formation in VA-13 and U-2 OS cells.
ALT characteristics of VA-13 and U-2 OS cells were analyzed following WRN knockdown. Extrachromosomal telomere repeat (ECTR) DNA is abundant in ALT cells and is most commonly present in a partially single-stranded, C-rich form termed C-circles (Henson et al., 2009). C-circles are an accurate predictor of ALT activity. C-circles can be measured in vitro with an assay that promotes rolling circle amplification of C-circles to produce a large and abundant telomeric product (Henson et al., 2009). We used the C-circle assay to examine ECTR in VA-13 and U-2 OS cells after transfection with SC or WRN siRNAs and found no differences in the prevalence of C-circles (Figure 9). This result held true for multiple time points after continuous WRN siRNA knockdown, including 4, 20 and 54 PD. Positive C-circle results were obtained from Saos-2 cells and negative results from both HeLa and MCF7 cells. The C-circle status of all cell lines tested was unaffected by siRNA transfection, suggesting that loss of WRN does not grossly influence the presence of c-circles in the cells tested. WRN knockdown
in CCL75.1 ALT cells has previously been shown not to alter the levels of T-circles in these cells (Li et al., 2011).

**Figure 9:** C-circle levels following WRN knockdown in ALT cell lines do not change. Total DNA extracted from HeLa, Saos-2, VA-13 and U-2 OS cell lines at 20 PD was examined for ECTR by the C-circle assay. Total telomeric DNA is shown in the bottom panel as an input control. Only C-circle products are visualized in the top panel after linear DNA is digested and subjected to the C-circle assay. Telomeric products are visualized with a $^{32}$P-end labeled (CCCTAA)$_3$ probe. C96 is a synthetic C-circle and serves as a positive control for the assay, while DNA from telomerase-positive HeLa cells represents a negative control.

APBs are also characteristic of ALT cells and are quickly responsive to changes in ALT activity (Jiang et al., 2005; Potts and Yu, 2007; Zhong et al., 2007), suggesting that these nuclear bodies indicate the telomere maintenance mechanism used by cells. PML co-localizes with the telomeric protein TRF2 within APBs, so APBs were visualized in fixed VA-13, Saos-2 and U-2 OS cells by immunofluorescence with antibodies to TRF2 and PML following WRN or SC siRNA transfection (**Figure 10**).
APBs sharply decline in ALT cells upon *BLM* knockdown (Bhattacharyya *et al.*, 2009), so these cell lines were also transfected with *BLM siRNAs* to provide a positive control. Control VA-13 (48.7% of cells), Saos-2 (29.8% of cells) and U-2 OS (61.9% of cells) cells displayed significant colocalization of TRF2 with PML, while both telomerase-positive cell lines lacked appreciable APBs. Forty-eight hours following *WRN* knockdown, a 50.1% reduction in the formation of APBs in VA-13 cells (*p*=0.027) and a 20.0% reduction in the formation of APBs in U-2 OS cells was observed (*p*=0.023); Saos-2 cells displayed no reduction in APBs (*p*=0.698); telomerase-positive cells displayed no change (HeLa *p*=0.613; MCF7 *p*=0.211). *BLM* knockdown significantly reduced APB levels in all ALT cell lines (59.1% reduction in VA-13, *p*=0.020; 19.1% reduction in U-2 OS, *p*=0.002; and 61.1% reduction in Saos-2, *p*=0.007) and had no effect on the telomerase-positive cell lines (HeLa, *p*=0.502; MCF7, *p*=0.937).
Figure 10: ALT-associated PML body (APB) formation is impaired following *WRN* knockdown in VA-13 and U-2 OS cells.

**A**

![Graph showing the percentage of cells with APBs under different conditions.]

**B**

![Images showing immunofluorescence staining of PML, TRF2, and DAPI in different cell lines under different treatments.]

continued
Figure 11: ALT-associated PML body (APB) formation is impaired following \textit{WRN} knockdown in VA-13 and U-2 OS cells.

(A) A graphical representation of at least 3 independent experiments depicts the percentage of cells displaying APBs in two telomerase-positive cell lines, HeLa and MCF7, and three ALT cell lines, VA-13, Saos-2 and U-2 OS. All cells were transfected with scrambled control (SC) \textit{siRNAs}, \textit{WRN siRNAs}, or \textit{BLM siRNAs}. We have previously shown a reduction in APBs following \textit{BLM} knockdown (Bhattacharyya \textit{et al}., 2009), so transfection with \textit{BLM siRNAs} served as a positive control. Cells were fixed 48 hours after \textit{siRNA} transfection and were immunofluorescently labeled with antibodies to PML and TRF2. Colocalization of PML/TRF2 foci was scored as a positive indication of APBs. Western blots confirm the ability of \textit{BLM siRNAs} to reduce BLM expression. (B) Representative confocal micrograph images of cells from each treatment group as quantified in A. PML is labeled in red, TRF2 in green, and the nucleus is stained with DAPI in blue.

\textit{WRN} is required for ALT telomere maintenance in VA-13 cells.

Telomere lengths drastically shortened after prolonged \textit{WRN} knockdown in VA-13 cells. We asked if re-expression of endogenous WRN could reverse this telomere loss. After a 50\% reduction in telomere length following 54 PD of continuous \textit{WRN siRNA} knockdown, a subset of VA-13 cells was separated and grown in culture for an additional 54 PD without further \textit{siRNA} transfection. Samples of HeLa cells transfected with SC and \textit{WRN siRNAs} were also allowed to recover without further \textit{siRNA} transfections as a negative control. Telomere length in VA-13 cells stabilized at the shortened 50\% length and remained steady for an additional 54 PD in the absence of \textit{WRN} repression. Telomere lengths did not change in the control VA-13 cells after recovery from SC \textit{siRNA} \((p=0.622)\) and no significant change was observed in HeLa cells. Although WRN was re-expressed in cells at levels comparable to untransfected cells, the VA-13 telomeres were unable to elongate to control lengths \((p=0.002)\) in these recovered cells.
(Figure 11). These results suggest that WRN is necessary to maintain telomeres in the absence of telomerase in VA-13 ALT cells.

![Graph showing telomere length changes](image)

**Figure 12:** WRN expression prevents telomere loss in VA-13 cells.

After 54 PD of continuous WRN knockdown (after telomeres had shortened by almost 50% of the original length), WRN siRNA transfections of a subset of HeLa and VA-13 cells ceased and cells were allowed to recover in culture for an additional 54 PD. Average relative telomere repeat lengths were then measured. qRT-PCR results are graphically represented as the difference between a telomere-specific PCR reaction in comparison to the single copy gene albumin PCR reaction as delta C_t. Although WRN expression was restored in the absence of siRNAs, WRN was unable to elongate the shortened VA-13 telomeres but was able to prevent further telomere loss, as measured by qRT-PCR. HeLa telomere lengths remained unchanged.

**WRN does not cap telomere ends.**

The shelterin protein complex—composed of TRF1, TRF2, TIN2, TPP1, RAP1, and POT1—caps the telomere and hides the chromosome end from recognition as DNA
damage. Loss of shelterin components results in a robust DNA damage response at the telomere and telomere dysfunction (d'Adda di Fagagna et al., 2003; Takai et al., 2003; Kim et al., 2004; Hockemeyer et al., 2005). Telomere dysfunction-induced foci (TIFs) form at telomeres in response to DNA damage and are visualized by co-localization of DNA damage response proteins, including γH2AX and 53BP1, with the telomere. ALT cells maintain basal levels of telomere dysfunction (Nabetani et al., 2004) and display TIFs in the absence of DNA damaging treatment. As WRN knockdown results in telomere loss in VA-13 and U-2 OS ALT cells and because restoration of WRN expression following telomere shortening in VA-13 cells failed to return telomeres to original lengths, we tested whether WRN may act as a telomere capping protein. Colocalization of γH2AX with the telomere was analyzed in control untreated cells and those transfected with SC, WRN or TRF2 siRNAs. Telomere hybridization was validated by co-staining with antibodies to the telomeric protein TRF2 and γH2AX staining was validated by treating a subset of cells with 2 μM camptothecin, which induces DNA double strand breaks (Appendix D). TRF2 knockdown served as a positive control for the induction of TIFs and resulted in a significant uncapping of telomeres in all cell lines (HeLa $p=0.004$; MCF7 $p=0.018$; VA-13 $p=0.047$; Saos-2 $p=0.042$; U-2 OS $p=0.001$). WRN knockdown did not alter TIFs in any cell line tested (HeLa $p=0.124$; MCF7 $p=0.597$; VA-13 $p=0.742$; Saos-2 $p=0.345$; U-2 OS $p=0.819$) (Figure 12).
Figure 13: WRN does not cap the telomere end.

A

% of cells with TIFs

-10 0 10 20 30 40 50 60 70 80 90

HeLa MCF7 VA-13 Saos-2 U-2 OS

+SC siRNA +WRN siRNA +TRF2 siRNA

p=0.004 p=0.018 p=0.042

p=0.047 p=0.001

66 kD 67 kD

SC siRNA: + - TRF2 siRNA: - +

α-TRF2 α-lamin B

B

Telomere γH2AX DAPI

Telomere γH2AX DAPI

Telomere γH2AX DAPI

HeLa MCF7 VA-13 Saos-2 U-2 OS

+SC siRNA +TRF2 siRNA +WRN siRNA

continued
**Figure 14: WRN does not cap the telomere end.**

(A) Two telomerase positive cell lines, HeLa and MCF7, and three ALT cell lines, VA-13, Saos-2 and U-2 OS, were transfected with scrambled control (SC) siRNAs, WRN siRNAs, or TRF2 siRNA. As a member of the shelterin complex, TRF2 knockdown was a positive control. Cells were fixed 48 hours after siRNA transfection and were subjected to fluorescent *in situ* hybridization (FISH) with a Cy3-labeled telomeric PNA probe and then immunofluorescently labeled with an antibody to phosphorylated histone 2A (γH2AX). At least 3 independent experiments were averaged to yield the percentage of each cell type depicting telomere dysfunction-induced foci (TIFs). Western blots confirm the ability of TRF2 siRNAs to reduce TRF2 expression. (B) Representative confocal micrograph images of cells from each treatment group as quantified in A. The telomere is labeled with a Cy3-PNA probe in red, γH2AX in green, and the nucleus is stained with DAPI in blue.

*Differential requirement for WRN in ALT reflects differences in WRN protein interactions.*

Finally, we tested whether protein partners of WRN in ALT cells correlated with the different WRN requirements in ALT cell lines. BLM colocalizes with BRCA1 in APBs in ALT cell lines, while WRN interacts with BRCA1 in response to interstrand crosslinks (Cheng *et al.*, 2006). ALT cells were examined for an interaction between WRN and BRCA1. Immunoprecipitation-western experiments using synchronized cell extracts with anti-BRCA1 and anti-WRN antibodies revealed a robust interaction between WRN and BRCA1 in VA-13 ALT cells, while no interaction was detected in Saos-2 ALT cells (Fig 13). Reversing the antibodies for immunoprecipitations generated similar results. This interaction was most prominent in cells synchronized in G2/M-phases of the cell cycle when ALT is thought to occur. Immunofluorescent staining confirmed the interaction between WRN and BRCA1 in VA-13 ($p=0.004$) and U-2 OS ($p=0.0007$) cells.
(Fig 14). BRCA1 localizes to APBs in ALT cells (Wu et al., 2003). Transfection of WRN siRNAs results in decreased localization of BRCA1 to APBs 48 hours after knockdown in VA-13 cells ($p=0.004$) but not in Saos-2 cells ($p=0.376$) (Fig 15), suggesting that WRN plays a role in APB assembly in VA-13 and U-2 OS cells.

**Figure 15: WRN interacts with BRCA1 in VA-13 ALT cells by immunoprecipitation.**

Nuclear extracts were immunoprecipitated with antibodies to BRCA1 and the immunoprecipitated proteins were subjected to western blotting with an antibody to WRN (bottom panels). Immunoprecipitation with anti-anti-BLM serves as a positive control for the interaction with WRN, while an anti-BRCA1 immunoprecipitation from WS cells and an anti-IgG immunoprecipitation serve as negative controls. Immunoprecipitation input lanes (10% of the total extract) of WRN and BRCA1 are shown in the top panels.
Figure 16: WRN interacts with BRCA1 in VA-13 and U-2 OS ALT cells by immunofluorescence.

A

B

continued
Figure 17: WRN interacts with BRCA1 in VA-13 and U-2 OS ALT cells by immunofluorescence.

A. Telomerase-positive cell line HeLa and three ALT cell lines, VA-13, Saos-2 and U-2 OS, were fixed and immunofluorescently labeled with antibodies to BRCA1 and WRN. Percent of cells with colocalized foci were averaged from three independent experiments. 

B. Representative micrograph images of labeled cells as quantified in A. Antibodies label WRN green and BRCA1 red, and the nucleus is stained with DAPI in blue.

Figure 18: BRCA1 localization to APBs is decreased upon WRN knockdown in VA-13 and U-2 OS ALT cells.

WRN knockdown inhibits BRCA1 localization to APBs. Telomerase-positive HeLa cells and three ALT cell lines, VA-13, Saos-2 and U-2 OS, were transfected with SC or WRN siRNAs and fixed 48 hours after transfection. Fixed cells were immunofluorescently labeled with antibodies to BRCA1 and PML and scored for colocalization of these signals. Percent of cells with colocalized foci were averaged from three independent experiments.

IV. Discussion

The ALT pathway maintains telomeres in the absence of telomerase, but its precise mechanism or mechanisms are largely unknown. A small number of proteins
have proven to be essential for ALT. Loss of MRN complex components (Jiang et al., 2005; Zhong et al., 2007), the SMC5/6 complex (Potts and Yu, 2007), or the BLM helicase (Bhattacharyya et al., 2009) inhibits ALT and result in telomere shortening. Loss of other ALT-related proteins does not shorten telomeres but leads to other types of telomere dysfunction (reviewed in Cesare and Reddel, 2010). Here, we show the WRN helicase, mutations in which lead to Werner syndrome (WS), is required for alternative telomere maintenance in some, but not all, ALT cell lines. Our results suggest there are at least two different telomerase-independent telomere maintenance mechanisms in ALT cells.

Previous studies have suggested a function for WRN at the telomere through its interactions with telomeric proteins and DNA structures. Our results reveal that WRN is necessary for telomere maintenance in VA-13 and U-2 OS cells by promoting the formation of APBs, as a defect in APB formation was observed subsequent to telomere shortening following WRN knockdown. In support of this, the AG11395A immortalized cell line, a WS cell line that maintains telomeres by ALT, lacks APBs (Fasching et al., 2005). Expression of WRN in AG11395 cells results in the formation of APBs, a gain of telomere sequences and the conversion of type 1-like telomeres to type II-like telomeres (Siddiq et al., 2012). These data suggest that WRN may be required for APB formation and the maintenance of specific types of ALT telomeres. APBs are a site of BrdU incorporation (G. Wu et al., 2000), suggesting that some ALT cells may use APB-localized proteins for telomere elongation. Known functions of WRN in DNA recombination (Saintigny et al., 2002; Baynton et al., 2003) would plausibly support a
role for its function in recombination-mediated telomere elongation within APBs. Previous studies have also demonstrated that suppression of APBs correlates with telomere shortening (Jiang et al., 2005). Other cell lines, such as Saos-2, may not require WRN either for the formation or function of APBs. C-circle experiments suggest that WRN is not involved in the formation/degradation of ECTR and that ECTR levels do not directly account for telomere length changes.

The ALT-specific requirement for WRN in VA-13 and U-2 OS cells correlates with an interaction between WRN and BRCA1. WRN interacts with BRCA1 in response to interstrand crosslinks, while BRCA1 can stimulate both WRN exonuclease and helicase activity in vitro (Cheng et al., 2006). WRN interacts with BRCA1 specifically at the telomeres of U-2 OS ALT cells in response to resveratrol treatment (Rusin et al., 2009), indicating that a variety of cellular situations can facilitate their interaction. BRCA1 has been implicated in telomere maintenance as BRCA1 deficiency results in telomere loss and telomere dysfunction in T-cells (McPherson et al., 2006). Here we confirm an ALT-specific interaction between WRN and BRCA1 in VA-13 and U-2 OS ALT cells and suggest this interaction facilitates the formation of APBs and telomere maintenance in the absence of telomerase. Taken together, our data suggest that WRN is involved in some forms of ALT by promoting the formation of APBs, possibly though an interaction with BRCA1.

The ability of WRN to prevent telomere loss in VA-13 and U-2 OS ALT cells may be accomplished by suppressing aberrant recombination within telomeric tracts. Telomere sister chromatid exchanges (T-SCE) are frequent in ALT cells (Bechter et al.,
In addition, T-SCE levels are increased after BLM or WRN knockdown in telomerase-negative human fibroblasts (Hagelstrom et al., 2010), implicating both RecQ-like helicases in suppressing aberrant recombination in ALT. Recombination within telomere tracts can result in genomic instability and telomere dysfunction, as following the loss of POT1 (Wu et al., 2006). Although we did not detect a change in ECTR following WRN knockdown, the C-circle assay may be limited in its ability to detect small changes in the levels of C-circles. Previous studies have demonstrated an increase in ECTR in the absence of WRN supporting its function in preventing telomere recombination (Li et al., 2008). Additionally, WRN functions to resolve telomeric D-loops in vitro (Opresko et al., 2004) and resolve G4 structures (Mohaghegh et al., 2001), assumed to arise within the G-rich telomeric DNA and interrupt DNA metabolism. Loss of WRN may promote the stalling of DNA replication machinery within the G-rich telomere strand, increasing recombination within the telomere to bypass the stalled region.

We have shown that the WRN helicase is required to maintain telomeres in the absence of telomerase in some, but not all, immortalized human ALT cell lines. Our data suggest that different ALT cell lines maintain telomeres using different pathways and that the ALT designation represents a variety of telomerase-independent mechanisms. These mechanisms may reflect differential use of templates for telomere recombination, including preferences for ECTR, homologous chromosomes or sister chromatids. Finally, our work argues for a variable requirement for DNA repair proteins in ALT mechanisms.
Chapter 4: Human sarcomas are mosaic for telomerase-dependent and – independent telomere maintenance mechanisms

I. Introduction

Telomere maintenance within cell populations is thought to proceed either with or without telomerase, although some evidence suggests each mechanism may not be mutually exclusive in human tumors. While most tumors exhibit clear evidence for a particular TMM, almost every published study to classify the TMM of human tumors has also identified a small subset of tumors that are not definitively ALT or telomerase-positive (Bryan et al., 1997; Ulaner et al., 2003; Sanders et al., 2004; Henson et al., 2005; Yan et al., 2002; Johnson et al., 2005; Costa et al., 2006; Matsuo et al., 2009; Villa et al., 2008; Else et al., 2008; Gupta et al., 1996; Hakin-Smith et al., 2003; Omori et al., 2008). These tumors display mixed results with regard to telomere length, telomerase activity, or the presence of APBs. Some tumors display long, heterogeneous telomere lengths on a TRF Southern blot, suggestive of ALT, but test telomerase-positive with the TRAP assay; various combinations of results with the three assays have been observed. This heterogeneity could suggest that tumors contain mixed populations of cells; indeed, extensive heterogeneity has been observed within the same tumor (Henson et al., 2005). In this sense, these seemingly inconsistent results actually reflect the limitations of the assays employed to examine telomere maintenance: the extracts created from these
tumors reflect gross mixtures of the tumors themselves. If separate cell populations exist within these tumors, this heterogeneity would be lost. *In situ* methods would need to be employed to individually distinguish these populations within a single tumor sample. Therefore, we asked whether human sarcomas could demonstrate mosaicism for TMM by evaluating tumors at a cellular level.

II. Materials and Methods

*Tumor samples.*

De-identified frozen human osteosarcoma samples were obtained from the Human Tissue Resource Network at The Ohio State University under IRB protocol 2009 E0409. Human cancer tissue microarrays (T262, T263, BO241, BO481, and CO483) were purchased from US Biomax.

*TRAP assay.*

Frozen tumor samples were mechanically homogenized in cold 1X CHAPS lysis buffer (Millipore) on ice, incubated on ice for 30 minutes, and centrifuged at 13,000 rpm for 20 minutes at 4°C. Supernatants were isolated and assessed for protein concentration using the Bradford assay, then snap frozen in liquid nitrogen and stored at -80°C. Telomerase activity was measured using the TRAPEze kit (Millipore) per manufacturer’s directions. Extracts were pre-incubated with telomeric templates for 30 minutes at 30°C and then phenol-chloroform-extracted to remove endogenous PCR inhibitors. After the PCR
reaction, products were separated by 10% non-denaturing PAGE and $^{32}$P-labeled TRAP products were exposed to a phosphor-screen overnight and imaged on a Typhoon Imaging system (GE) at The Ohio State University Nucleic Acids Shared Resource. Each sample was independently analyzed at least five times. Positive control immortalized cell lines HeLa and WI-38 VA-13/2RA were purchased from ATCC and lysates prepared per TRAPeze kit manufacturer’s directions.

**TRF Southern blot.**

Frozen tumor samples were mechanically homogenized in Buffer ATL (Qiagen). DNA was extracted using the DNeasy kit (Qiagen) per manufacturer’s directions and the concentration was measured on a NanoDrop spectrophotometer. Two micrograms of DNA were digested overnight at 37°C with 10U each *Hinf*1 and *Rsa*I restriction enzymes in 1X Buffer 4 (NEB). DNA fragments were separated on a 0.6% agarose gel in 1X TBE for 3 hours at 60V. DNA was transferred by capillary action to a charged nylon membrane (Hybond) overnight in 20X SSC at room temperature. DNA was UV cross-linked to the membrane using a Stratalinker 3400 (Stratagene) and the membrane was hybridized overnight at 42°C in UltraHyb-oligo (Ambion) with a $^{32}$P-labeled (CCCTAA)$_3$ probe. The membrane was washed twice in 2X SSC/0.5% SDS for 30 minutes each at 42°C. Products were exposed to a phosphor-screen overnight and imaged on a Typhoon Imaging system (GE) at The Ohio State University Nucleic Acids Shared Resource. Each sample was independently analyzed at least three times.
**Immunohistochemistry.**

Tumor samples were formalin-fixed, embedded in paraffin blocks, and cut in 4 micron-thick sections onto glass slides at The OSU Pathology Core Facility. Automated antibody staining was performed on a Ventana Benchmark XT with protease 1 pre-treatment (Ventana). Manual immunohistochemistry and combined fluorescent *in situ* hybridization (FISH) were performed following deparaffinization, rehydration, and antigen retrieval by steaming tissues in 10 mM sodium citrate buffer pH 6 for 15 minutes. Tissues were cooled for 20 minutes to room temperature and telomeres were hybridized with a Cy3-(TTAGGG) peptide nucleic acid probe (Panagene) at 800 ng/mL in 1X *in situ* hybridization buffer (Enzo Life Sciences) for 1 hour at 37°C after denaturation for 5 minutes at 80°C. After hybridization, excess probe was washed off in 2X SSC at 55°C. Immunohistochemistry was then performed by blocking nonspecific binding sites in 10% goat serum for 30 minutes and subsequent primary antibody incubation overnight at 4°C in 1% BSA in 1X TBS. For immunohistochemistry, antibodies used were: anti-TRF2 at a dilution of 1:50 (IMG-124A; Imgenex), anti-PML at a dilution of 1:150 (ab53773; Abcam), anti-PML at a dilution of 1:50 (PG-M3; Santa Cruz), and anti-telomerase at a dilution of 1:250 (Ab-2; Calbiochem). Tissues were washed in 0.1% Tween in 1X TBS and incubated with secondary antibody in 1% BSA in 1X TBS for 1 hour at room temperature. Alexa Fluor (Invitrogen) secondary antibodies were used at a dilution of 1:2000. Blinded image capture and analysis were performed on a Nuance multispectral imaging system (CRi) as previously described (Nuovo, 2010) and staining was confirmed on an Olympus FV 1000 spectral confocal microscope. At least 200 cells (when
possible) from at least three geographic locations within each tumor were analyzed for cell percentages. Positive control immortalized cell lines HeLa and WI-38 VA-13/2RA were purchased from ATCC and stained with standard immunocytochemical techniques as above.

III. Results

*Some human sarcomas are mosaic for telomere maintenance mechanism.*

The observation of tumors with characteristics of both TMMs suggested questions of whether tumors could contain some cells expressing telomerase and other cells expressing characteristics of ALT, or whether the same cell could express both types of telomere elongating characteristics. We examined TMM within human sarcomas, due to their high prevalence of ALT, using a computationally enhanced imaging system capable of analyzing three immunohistochemical stains per tumor cell. We analyzed four tissue microarrays that included 57 high-grade sarcomas: 26 chondrosarcomas, 4 osteoclastomas and 27 osteosarcomas (Table 2). Microarrays were analyzed for ALT characteristics using colocalization of PML with the telomere/telomeric proteins to identify APBs. APBs were identified *in situ* by two techniques: TRF2 and PML chromogenic immunohistochemistry using an automated stainer, and manual PML fluorescent immunohistochemistry combined with telomere FISH. Microarrays were also stained with antibodies specific to the reverse transcriptase subunit of telomerase (TERT). All staining was validated in human immortalized cell lines with known
TMMs: telomerase-positive HeLa cells and ALT-positive WI-38 VA-13/2RA cells (Appendix F). Spatial localization of all three signals was analyzed using a Nuance multispectral imaging system, which allows management of a threshold to exclude background staining. Cells with TRF2/telomere and PML colocalization excluded telomerase; however, in some tumors, separate populations of cells within the same tumor stained positive for telomerase and negative for TRF2/telomere and PML colocalization.
Table 2. Analysis of telomere maintenance mechanism in human sarcoma tissue microarrays (TMA)

<table>
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<th>Tumor</th>
<th>Site</th>
<th>Stage</th>
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(TNM=tumor, lymph node, metastasis staging; % ALT=percent of cells positive for alternative lengthening of telomeres; % Tel=percent of cells positive for telomerase)

Four of 57 tumors contained only ALT-positive cells, 23/57 contained only telomerase-positive cells, and 26/57 contained mixed populations of ALT- and telomerase-positive cells; 4 tumors exhibited neither APBs nor telomerase staining. Those tumors with both TMMs displayed a wide variability in the proportion of each cell...
type, ranging from 1% to 47% ALT-positive and 2% to 95% telomerase-positive (Table 2). Each tumor varies, in that not all cells within a tissue section were classified as ALT- or telomerase-positive. This may be due to incomplete staining due to the osseous nature of these tumors, methodological detection limits or varying protein expression levels. Within asynchronous cultures of ALT cells, APBs are not observed in all cells because their presence is cell cycle-regulated (Grobelny et al., 2000). This could explain the incomplete characterization of all tumor cells in our samples and would underestimate the proportion of ALT-positive cells. Neither ALT- nor telomerase-positive cells were detected in normal bone and cartilage tissues, indicating the staining specificity (data not shown). Since ALT has not been detected in colorectal cancers (Henson and Reddel, 2010; Heaphy et al., 2011), a human colon cancer tissue microarray was stained as a negative control. This array included 40 adenocarcinomas, all of which displayed telomerase expression and no colocalization of TRF2 and PML signals (Appendix G, H). Normal thymus tissue also stained positive for telomerase and negative for ALT colocalization (Appendix H).

**Tumors that are mosaic for telomere maintenance mechanism exhibit variability in whole tumor assays.**

Our findings using in situ colocalization and immunohistochemistry were validated with solution-based biochemical analysis and evaluation of an independent set of five frozen human osteosarcomas. Telomere characteristics of each tumor were assessed using whole tumor lysates and single cell assays. Whole tumor lysate assays
characterized telomere length and telomerase activity as a general representation of many cells within the tumor. Telomere length was measured with telomere restriction fragment (TRF) Southern blotting (Figure 16A) and telomerase activity was measured with the telomere repeat amplification protocol (TRAP) assay (Figure 17). The relative average telomere lengths of each tumor were independently confirmed using quantitative real time PCR (Cawthon, 2009) (Figure 16B). Long heterogeneous telomeres identified by TRF Southern blotting and a lack of telomerase activity by TRAP suggest ALT mechanisms; conversely, telomerase-positive staining and shorter, more homogenous telomere lengths suggest telomerase-associated mechanisms. The telomerase-positive human immortalized cell line HeLa and the ALT-positive human immortalized cell line WI-38 VA-13/2RA were used as positive controls for qualitative characteristics of each TMM. Each of the five osteosarcomas contained telomeres that closely corresponded with characteristics of either HeLa or WI-38 VA-13/2RA cells. One of five human osteosarcomas (A) displayed short and homogeneous telomere lengths via TRF Southern blotting (Figure 16A) and telomerase activity via TRAP assay (Figure 17). Two tumors (C and D) exhibited only ALT phenotypes (Figure 16A and Figure 17). Two tumors (B and E) provided ambiguous results with weak and variable telomerase activity (Figure 17) and telomere lengths (Figure 16A) that clearly suggested telomerase- (B) or ALT- (E) maintained telomeres.
Figure 19: Characterization of telomere lengths of human osteosarcoma lysates.

(A) Average telomere length of DNA from five frozen human osteosarcomas was measured by TRF Southern blotting. Signal in each lane represents telomeric DNA fragments liberated from digested genomic DNA. HeLa and WI-38 VA-13/2RA are positive controls for telomerase and ALT cells, respectively; telomerase-positive cells have shorter homogeneous telomere lengths, while heterogeneous lengths characterize ALT-positive cells. DNA-minus and lambda DNA represent negative controls. (B) Telomere length of each frozen osteosarcoma sample was independently confirmed using quantitative real time PCR. Delta Ct (cycle threshold) represents the difference between Ct values for a telomere PCR reaction and a single copy gene (albumin) PCR reaction. Values are relative average telomere length.
Figure 20: Telomerase activity in the same five human osteosarcoma lysates by the TRAP assay.
A 36 bp internal control (IC) band is present in each lane, with laddered bands beginning from 50 bp upwards signifying positive telomerase activity. The presence of only the internal control band suggests telomerase-negative ALT mechanisms. Heat denaturation serves as a negative control for each extract. HeLa and WI-38 VA-13/2RA cell lysates are positive controls for telomerase-positive and ALT-positive cells, respectively; other positive and negative controls are controls provided by the manufacturer.

Single cells within each frozen tumor sample were then examined for ALT-associated protein colocalization and telomerase by in situ immunohistochemistry. After fixing tumor sections in 10% buffered formalin, in situ based co-expression analyses were done blinded to the solution-phase biochemistry data. Tumor sections were stained with antibodies to PML, TRF2 and telomerase, and analyzed using the Nuance multispectral imaging system. Colocalization of TRF2 and PML, denoting an ALT cell,
appeared as distinct nuclear foci consistent with the appearance of APBs. Colocalization
was confirmed using confocal microscopy to ensure that signals were overlapping in the
same focal plane. Examples of confocal micrographs of individual representative tumor
cells are shown in Figure 18. Cells scored ALT-positive demonstrated colocalization of
PML and TRF2 and minimal or no TERT staining; scored telomerase-positive
demonstrated no colocalization of PML and TRF2 but were positive for TERT staining.
Forty-eight percent of cells from tumor A were telomerase-positive, while none exhibited
TRF2/PML co-localization (Figure 19); these results corroborate those from TRF
Southern blotting and TRAP assay. Conversely, tumors C and D contained 31% and
65% of cells, respectively, with co-localization of TRF2/PML, corroborating whole
tumor lysate assays. Tumor D completely lacked cells with telomerase expression, while
tumor C contained some telomerase-positive cells (7%) (Figure 19). However, tumor C
was geographically heterogeneous, containing distinct areas of weak or robust staining;
this dimorphism could explain the negative TRAP results for this tumor even though
some telomerase-positive cells were present. Finally, tumors B and E, which had mixed
results in whole tumor assays, displayed two separate cell populations: one staining
positive for ALT protein colocalization (32% and 34%, respectively) and one staining
positive for telomerase (2% and 6%, respectively) (Figure 19). TMM results from all
three assays are summarized in Table 3. Independent and blinded histopathological
analyses were carried out to confirm that ALT-positive and telomerase-positive cells
likely represented two distinct populations of tumor cells, rather than infiltrating normal
cells.
Figure 21: Confirmation of immunohistochemistry for telomerase and ALT characteristics in human tumors.
High-power magnification (100x) confocal micrographs demonstrate PML (red) and TRF2 (green) signals (top) and TERT (white) signal (bottom) in DAPI-stained (blue) nuclei of representative tumor sections. ALT-positive tumors (left) exhibit colocalization (yellow) of PML and TRF2 signals in discrete nuclear foci (top) and a lack of TERT staining (bottom). Telomerase-positive tumors (right) exhibit both PML and TRF2 nuclear signals without colocalization (top) and TERT staining (bottom).
Figure 22: Immunohistochemistry of five frozen human osteosarcomas.
Slides were prepared from five frozen human osteosarcomas after formalin fixation and paraffin embedding and analyzed for TRF2/PML colocalization and for telomerase (TERT) expression using *in situ* immunohistochemistry. Hematoxylin and eosin (H&E) stained reference sections are shown in the top row, along with an analysis of PML/TRF2 colocalization (pseudo-colored yellow) in the center row and telomerase expression (pseudo-colored white) in the bottom row. Cells were counterstained with hematoxylin for visualization and visualized at 40X magnification. For PML/TRF2 colocalization and telomerase expression, images were pseudo-colored and analyzed using Nuance software.
Table 3. Analysis of telomere maintenance mechanism in frozen human osteosarcomas

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</table>

IV. Discussion

Our studies with an enhanced imaging system explicitly demonstrate that some human sarcomas are mosaic for two types of cells—those that use telomerase and those that use ALT. The presence of both cell types highlights a novel type of tumor heterogeneity and suggests that telomere maintenance may not be a static characteristic of all tumors. In addition, the presence of more than one TMM confounds accurate tumor assessment using a single method and indicates a need for precise tumor characterization. Our results also highlight the question of whether individual cells can switch TMM or whether a cell commits to telomerase-dependent or –independent telomere maintenance. Furthermore, we speculate that tumor types in addition to sarcomas may also contain mosaic populations of cells with regard to TMM.

Previous studies have reported a lower percentage of tumors that display characteristics of both TMMs than determined in our studies, which is 50%. While those studies have used an either-or approach for the determination of TMM, we have
combined both immunohistochemical and biochemical analyses to gain a more comprehensive view of the TMM status of some of these tumors. Some tumors contain a small percentage of ALT-positive or telomerase-positive cells and would likely be classified into one category or another with assays that analyze a tumor extract, such as the TRF Southern blot or the TRAP assay. In these solution-phase assays, tumor cells are combined into a quasi-homogeneous solution, in which a small percentage of one particular cell type would not be measured. Combining the results of these assays with a cell-by-cell analysis by immunohistochemistry has identified multiple TMM cell types within one tumor. Because some of these cell type percentages are small, further experimentation will be necessary to determine precisely the identity and origin of each cell type (which was not feasible with our samples). Immunohistochemical staining of TERT protein does not confirm telomerase activity, although it is highly correlated with telomerase activity (Kanaya et al., 1998; Wu et al., 1999) and implies that those cells are not utilizing ALT. TERT staining correlates well with TRAP activity in the subset of four homogeneously-stained osteosarcomas where immunohistochemical and biochemical assays were possible.

TMM can be a prognosticator for patient survival: ALT-positive glioblastoma multiforme tumors are associated with a better prognosis (Henson et al., 2005; Hakin-Smith et al., 2003), while ALT-positive liposarcomas are associated with a worse prognosis (Costa et al., 2006), than telomerase-positive tumors of each type. Recent clinical trials of telomerase-targeted therapies also underscore the clinical importance of telomere maintenance. Mosaicism of TMM within human tumors may impact the choice
of therapeutic approaches, as tumors with cells that maintain telomeres via more than one mechanism may require combination therapies to target each type of cell. In addition, recent work in mouse models demonstrates that inhibition of telomerase activity following tumor formation promotes the survival of tumors via the ALT pathway (Hu et al., 2012). A similar scenario is likely to occur in mosaic human tumors following telomerase inhibitor treatment, stressing the value of precise tumor TMM characterization before treatment and/or treatment with combination therapies that target both telomerase and ALT.
Chapter 5: Thesis Summary

Telomeres protect the ends of DNA and shorten with each round of cell division. Because of this perpetual shortening, telomere maintenance is critical for tumor cell immortalization. Tumor cells use expression of the telomerase enzyme or telomerase-independent ALT pathways to maintain telomere lengths. While extensive research has investigated telomerase, understanding of ALT is incomplete. This work aimed to characterize ALT by testing the hypothesis that the recQ-like WRN helicase is required for telomerase-independent telomere maintenance. It also asked whether ALT-positive tumors can contain cells using ALT and cells using telomerase in human tumor sections.

The WRN helicase is mutated in those with the autosomal dominant Werner syndrome (WS) and localizes to telomeres (Johnson et al., 2001; Opresko et al., 2004) and APBs in ALT cells (Johnson et al., 2001). WRN also interacts with telomeric proteins (Johnson et al., 2001; Opresko et al., 2002). The characterization of a WS cell line with ALT features (Fasching et al., 2005) led to the assumption that WRN is dispensable for ALT. Due to structural and functional similarities to the BLM helicase, mutated in Bloom syndrome (BS), and its requirement for ALT (Bhattacharyya et al., 2009), this work asked whether WRN is required for ALT in some immortalized human cancer cell lines. Three cell lines that use ALT and two cell lines that use telomerase
were examined for telomeric characteristics in the absence of WRN. Pooled siRNAs that target WRN and knock down protein expression were transiently transfected into cells, as stable expression of a plasmid encoding WRN shRNA could not be established. Stable transfection of cells with WRN shRNA would have been a preferred technique to ask whether WRN is required for telomere maintenance but, due to unclear reasons, did not generate stable cells. siRNA transfection was used as an alternative as greater than 90% knockdown was seen after transient transfection of WRN siRNAs. Persistent transfection of immortalized cells may raise concerns about cell health and viability; therefore, scrambled control siRNAs were transfected into each cell type as a control for nonspecific siRNA or transfection effects. siRNAs were repeatedly transfected into cultured cells to permit time for telomere length changes to occur, as telomeres are estimated to shorten an average of 50-100 base pairs per cell division (Harley et al., 1990; Allsopp et al., 1992; Vaziri et al., 1993, 1994).

The cell lines chosen for study included the telomerase-positive MCF7 and HeLa, and the ALT-positive VA-13, Saos-2, and U-2 OS. The breast cancer cell line MCF7 was the most sensitive cell line of those tested to siRNA transfection. MCF7 cells transfected with either WRN or SC siRNAs died in culture after 32 PD. This response was likely due to the stress of transfection as similar outcomes were observed in both WRN siRNA- and SC siRNA-transfected cells but not in untreated controls. No other cell line tested exhibited sensitivity to transfection and SC siRNA-transfected controls of those cells remained healthy throughout experiments. Due to differences in baseline telomere length and PD time, each cell line was maintained in culture for a varying
number of PD: VA-13 for 54 PD, MCF7 for 32 PD, U-2 OS for 77 PD (in the presence of p53 siRNAs), HeLa for 58 PD, and Saos-2 for 124 PD. Each length of time was calculated to be sufficient for telomere length changes to be detectable. Cells were maintained in culture in the presence of siRNAs and DNA samples were collected for telomere length measurement using qRT-PCR. This PCR-based technique allows a quantitative measure of average telomere repeat length from a small amount of DNA (30 ng used here) and gives a quantitative measure. It is limited by detecting only the average telomere repeat length of a sample and does not provide estimates of telomere length heterogeneity, which can be better assessed by TRF Southern blotting. Furthermore, PCR amplification originates from both or either chromosomal and ECTR DNA. Nonetheless, average telomere repeat length decreased in two ALT cell lines, VA-13 and U-2 OS, after WRN knockdown. U-2 OS cells required an accompanying p53 knockdown, as intact p53 in these cells elicited an apoptotic response to WRN knockdown alone. The telomerase-positive HeLa and MCF7 cells and the ALT-positive Saos-2 cells did not show any change in telomere length in response to WRN knockdown. These results show that some but not all ALT cells require WRN to maintain telomere lengths. In addition, WRN is not required to maintain telomeres in the presence of telomerase.

A reduction of ALT characteristics was observed in VA-13 and U-2 OS cells after WRN knockdown in addition to a reduction in average telomere repeat length. APB formation was significantly impaired following WRN knockdown in VA-13 and U-2 OS cells, but not in Saos-2 cells. Saos-2 cells were confirmed to lack telomerase activity,
ruling out the possibility of telomerase activation after *WRN* knockdown and confirming that ALT is functional in Saos-2 cells without WRN. No change in circular ECTR was detected after *WRN* knockdown in any ALT cell line tested, suggesting that WRN does not mediate the formation/degradation of C-circles and that the presence of C-circles alone is insufficient for ALT in VA-13 and U-2 OS cells. The C-circle assay is sensitive to changes in ALT activity (Henson *et al.*, 2009); however, the assay depends on the processivity of a bacteriophage polymerase during rolling circle amplification and may not be sensitive to small changes in ECTR levels. VA-13 and U-2 OS cells have low levels of C-circles in comparison to Saos-2 cells (Henson *et al.*, 2009), suggesting that ECTR changes may be difficult to detect. Alternatively, different ALT cell lines may use different mechanisms of ALT, only some of which are dependent upon ECTR.

VA-13 telomeres shorten without WRN and stabilize lengths when WRN is no longer reduced. Telomere lengths of recovered cells do not reach those measured before *siRNA* transfection. This observation led to the hypothesis that WRN may function as a telomere capping protein. The shelterin complex is a group of six proteins that normally protects the telomere end by acting as a protein cap, hiding the end from DNA damage sensors and maintaining the integrity of telomere structure. It is possible that shelterin associates with or is composed of variations of proteins in different cell types (i.e. ALT versus telomerase). *WRN* knockdown did not significantly alter DNA damage signaling at the telomere in TIFs. This suggests that although WRN maintains telomeres in VA-13 and U-2 OS cells, it does not cap the telomere end.
A reduction in telomere length and APBs was observed after *WRN* knockdown in VA-13 and U-2 OS cells, as well as a novel protein interaction between WRN and BRCA1 in these cells. Both immunofluorescence and immunoprecipitation experiments have identified WRN-BRCA1 interactions in VA-13 and U-2 OS ALT cells and have not been able to do so in Saos-2 ALT cells. WRN interacts with BRCA1 in response to interstrand crosslinks (Cheng et al., 2006) and after resveratrol treatment (Rusin et al., 2009), but an ALT-specific interaction has not been previously reported. The interaction between WRN and BRCA1 reported here correlates with the requirement for WRN in ALT, suggesting this protein interaction may be important for the function of WRN in ALT. This hypothesis is supported by the alterations of BRCA1 localization to APBs after *WRN* knockdown in VA-13 and U-2 OS cells and suggests that WRN may facilitate the organization of APBs or protein localization to APBs. Saos-2 cells exhibit very little colocalization of BRCA1 and PML, suggesting different functions for both WRN and BRCA1 in Saos-2 cells. These descriptive findings also suggest the presence of different ALT mechanisms in VA-13, U-2 OS, and Saos-2 cells. These data support a model whereby WRN facilitates the formation of APBs in a subset of ALT cells that require APBs for telomere maintenance.

The WS cell line AG11395 maintains telomeres by ALT, although it has type I-like telomeres with SV40 sequences interspersed in telomere repeats and lacks APBs. Expression of WRN in AG11395 cells results in loss of SV40 sequences, increased telomere signals, and the appearance of APBs (Siddiq et al., 2012). These results
confirm that WRN can promote the formation of APBs and imply that different ALT mechanisms can elongate telomeres in the presence or absence of WRN.

The formation of both PML bodies (present in non-ALT cells) and APBs (ALT cell-specific) are regulated by post-translational protein modifications to PML and related proteins by small ubiquitin-related modifiers (SUMO). The attachment of SUMO to proteins, known as sumoylation, regulates protein localization and protein interactions. Sumoylation is critical for the formation and stabilization of PML bodies (Zhong et al., 2000; Nacerddine et al., 2005). WRN itself interacts with SUMO1 and UBC9 (Kawabe et al., 2000), which transfers SUMO to its target protein. Furthermore, p14ARF interacts with WRN to promote WRN sumoylation, controlling localization of the helicase (Woods et al., 2004). Sumoylation may promote localization of WRN to APBs and the interaction between WRN and UBC9 may promote sumoylation of additional proteins, such as BRCA1, and their localization to APBs (Figure 20).
Figure 23. Proposed model of WRN localization and promotion of APBs.
Sumoylation of WRN through interactions with UBC9, SUMO1, and p14ARF influence nuclear localization and may promote the localization of WRN to APBs. WRN interaction with UBC9, a SUMO transporter, may also influence the sumoylation of other proteins such as BRCA1, and their localization to APBs for telomere maintenance.

The function of APBs is debatable. Some evidence suggests that APBs are sites of telomere elongation: APBs are sites of DNA synthesis (G. Wu et al., 2000); APBs dynamically associate with chromosome ends (Molenaar et al., 2003; Jegou et al., 2009); APB levels decrease when ALT is inhibited (Perrem et al., 2001); APB presence correlates with ALT activity (Yeager et al., 1999); and APB levels increase during G2 phase of the cell cycle (Grobelny et al., 2000; G. Wu et al., 2000). Alternatively, other
evidence suggests that APBs are not sites of telomere elongation and instead serve other purposes, such as protein sequestration: APBs are found in tumor cells lacking other characteristics of ALT cells (Slatter et al., 2010); small colocalized foci of telomeres and PML are found in some non-neoplastic cells (Slatter et al., 2012); and large APBs are induced in ALT cells in response to cell cycle arrest or senescence (Fasching et al., 2007; Jiang et al., 2009). Both scenarios may occur in different ALT cell lines or in cells in vivo.

WRN functions in DNA recombination and the resolution of DNA intermediates, which suggests participation in recombination to maintain telomeres of ALT cells. WRN is required for DNA damage responses at exposed telomere ends, most likely through the degradation of the 3’ telomere overhang (Eller et al., 2006). Synthetic telomere overhang homologues (T-oligos) induce DNA damage responses at the telomere when introduced into immortalized cell lines. These responses are blunted in the absence of WRN in experiments using a WS cell line or in fibroblasts treated with WRN siRNAs. Degradation of the T-oligos is required for this response, suggesting that WRN-mediated exonucleolytic degradation of the telomere overhang induces a damage response at exposed telomeres. This process could initiate telomere unwinding for recombination and elongation to occur, thus placing WRN in a position to initiate ALT.

BRCA1 also functions in telomere maintenance. Tissue-specific deletion of Brca1 in mouse T-cells results in telomere shortening and end-to-end fusions (McPherson et al., 2006). In GM847 ALT cells, BRCA1 localizes to a small subset of APBs and its loss induces anaphase bridges, most likely through its homologous
recombination function, but does not change telomere length (French et al., 2005), suggesting that BRCA1 is not required to maintain telomere ends by ALT in these cells or that its loss induces another mechanism of telomere elongation. BRCA1 does bind to telomeric DNA; its siRNA-mediated inhibition in the T47D cell line reduces the length of the telomere overhang (Ballal et al., 2009), implying BRCA1 function at the telomere overhang. BRCA1 stimulates both helicase and exonuclease activities of WRN using forked telomeric substrates (Cheng et al., 2006), further suggesting a functional role for their interactions at telomeres. BRCA1 also influences the expression of telomerase. BRCA1 binds the TERT promoter and BRCA1 overexpression inhibits both TERT expression and activity (Xiong et al., 2003). Transfection of BRCA1 siRNAs into telomerase-positive cell lines increases telomerase expression and activity and lengthens telomeres (Ballal et al., 2009).

Future experiments to explore the interaction between WRN and BRCA1 could map the WRN domain that interacts with BRCA1; previous work has defined the WRN interaction to amino acids 452-1079 of BRCA1 (Cheng et al., 2006). Sub-mapping the interaction domain could then lead to studies testing whether the interaction between WRN and BRCA1 facilitates ALT to maintain telomeres in VA-13 and U-2 OS cells. Stable generation of WRN shRNA clones in these cell lines could be reconstituted with wildtype WRN or WRN with a mutated BRCA1 interaction domain to determine the ability to recover ALT characteristics. Helicase assays could also be used to examine the ability of deleted BRCA1 to influence enzymatic activity of WRN on telomeric overhang substrates and those that mimic the T-loop.
Experiments measuring telomere length and assessing telomere characteristics of ALT cells after \textit{WRN} knockdown suggest that different ALT cells have different mechanisms of ALT. ALT cells all maintain telomeres in the absence of telomerase, but there are specific differences in ALT characteristics of these cell lines (\textbf{Table 4}). Specifically, VA-13 and U-2 OS share high levels of APBs, lower levels of C-circles, lower levels of T-SCE, and absent TERC; these differences set them apart from Saos-2 cells and may be correlated with the different ALT mechanisms used by each cell line. The templates used for telomere recombination could vary among ECTR, sister chromatids, non-homologous chromosomes, and the T-loop. The use of different telomeric templates may correspond to different mechanisms of ALT in telomere maintenance. It is interesting to speculate that VA-13 and U-2 OS cells require APBs for ALT, as these cells contain much larger and more numerous protein foci; Saos-2 cells have much higher levels of C-circles and may instead require their presence for rolling circle amplification to elongate telomeres from ECTR.
Table 4. Summary of differences in ALT cell lines used in these studies

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>$p53$ status</th>
<th>APB level</th>
<th>TIF level</th>
<th>c-circle activity</th>
<th>T-SCE /100 ch</th>
<th>TERC</th>
<th>WRN knockdown</th>
</tr>
</thead>
<tbody>
<tr>
<td>WI-38 VA-13/2RA</td>
<td>lung fibroblast (SV40)</td>
<td>Inhibited by SV40</td>
<td>High</td>
<td>High</td>
<td>12.7$^1$</td>
<td>$\sim3^5$</td>
<td>No$^6$</td>
<td>Telomere shortening and loss of APBs (54 PD)</td>
</tr>
<tr>
<td>Saos-2</td>
<td>Osteosarcoma</td>
<td>Null$^{1,2}$</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>244$^4$</td>
<td>$\sim6^5$</td>
<td>Yes$^6$</td>
<td>No effect</td>
</tr>
<tr>
<td>U-2 OS</td>
<td>Osteosarcoma</td>
<td>Wild type$^3$</td>
<td>High</td>
<td>Intermediate</td>
<td>67.6$^4$</td>
<td>$\sim3^5$</td>
<td>No$^5$</td>
<td>$p53$-mediated apoptosis (17 PD); telomere shortening and loss of APBs with $p53$ knockdown (77 PD)</td>
</tr>
</tbody>
</table>

$^1$Chen et al., 1990; $^2$Iijima et al., 1996; $^3$Landers et al., 1997; $^4$Henson et al., 2009; $^5$Tilman et al., 2009; $^6$Bryan et al., 1997

The existence of multiple ALT mechanisms in ALT cells suggests that it would be interesting to test all ALT cell lines for a WRN requirement in telomere maintenance. Three ALT cell lines have been examined here, but at least 40 different ALT cell lines exist (reviewed in Henson and Reddel, 2010). A more efficient strategy for WRN knockdown would be necessary to avoid lengthy experiments and financial restrictions. Defining the requirement for WRN would separate cell lines into two ALT categories, although an understanding of how these ALT mechanisms differ will be needed. One way to explore this is to characterize ALT cell lines using microarray analysis and cluster the gene expression profiles based upon ALT mechanisms. Recent gene expression analyses using ALT cell lines and human tumors have identified a specific gene signature.
associated with ALT (Lafferty et al., 2009); further experiments may identify each ALT mechanism.

The work summarized here elucidates how telomeres are maintained by ALT by asking if WRN is required for telomere maintenance and investigating its functions. These results demonstrate that ALT mechanisms are variable in immortalized human cell lines and that different mechanisms require different protein complexes. In addition, this thesis also addressed ALT variability on a macroscopic scale by analyzing human tumors in situ for the presence of one or both TMMs, in this case ALT and telomerase. Previous studies (reviewed in Henson and Reddel, 2010) have analyzed whether ALT or telomerase is present in different human tumor types. Almost every study identified a subset of tumors that do not readily exhibit characteristics of only ALT-positive or only telomerase-positive cells. Immunohistochemical staining and analyses using the Nuance enhanced imaging system allowed the examination of human sarcomas for the presence of both telomerase and ALT characteristics. These analyses demonstrated that both cells with ALT characteristics and telomerase-positive cells can co-exist within the same tumor. This had been speculated in previous work, but not demonstrated precisely.

Immunohistochemical analyses were validated with whole tumor assays—telomere length analyses and telomerase activity assays—that correlated well with immunohistochemical results and demonstrated that the presence of both types of telomere maintenance can confound accurate analysis with whole tumor assays. Blinded histopathological analyses suggested that both populations of cells represent tumor cells, although future studies will need to determine the origin and identity of each population.
Other possible cell types are infiltrating immune cells, stromal cells, or other non-tumor cell types of the extracellular matrix. Laser capture microdissection could be used to separate these cells after immunohistochemical analyses and would allow for subsequent analyses of genetic identity.

Both telomerase-positive cells and cells with characteristics of ALT have been observed in single human sarcomas. As many previous studies examining TMM in other tumor types have identified a subset of tumors with characteristics of both or neither TMM, it is likely that other tumor types also exhibit TMM mosaicism. Future work could use similar methods to determine TMM mosaicism. Although this work has demonstrated characteristics of cells with either TMM in the same tumor, future work will need to determine if both TMMs are active in these cell populations, although these questions will be complex to answer and to interpret. In situ TRAP assays could be performed on frozen tumor sections to determine telomerase activity within tumor cells. A definitive ALT activity assay would also be useful to assess tumor cells.

The ability of ALT to enhance tumor progression is not equal to that of telomerase. Oncogenic H-RAS expression in immortalized GM847 ALT fibroblasts does not confer transformation, as these cells cannot form tumors following injection into immunocompromised mice; expression of telomerase in these cells, however, allows the formation of tumors (Stewart et al., 2002). Late generation mTerc<sup>-/-</sup>, Ink4a/Arf<sup>+</sup> mouse embryonic fibroblasts using ALT can form tumors following injection into immunocompromised mice, but these tumors lack metastatic potential; reconstitution of mTerc promotes the formation of metastatic lesions (Chang et al., 2003). Faithful
expression and activity of telomerase are required for progression of epithelial tumors in vivo. Murine prostate tumors in mTert\textsuperscript{-/-} and mTert\textsuperscript{+/-} mouse models with conditional prostate-specific Pten/p53 knockouts show that telomere dysfunction constrains the ability of tumors to progress beyond initiation, as only mTert\textsuperscript{+/-} tumors develop into invasive adenocarcinomas. Expression of an inducible mTert knockin allele in mTert\textsuperscript{-/-} mice after prostate tumor initiation decreases telomere damage signaling, increases tumor weight and confers tumor invasiveness (Ding et al., 2012). Telomerase may offer some advantage in tumor progression, although metastatic potential is not mutually exclusive with ALT. Human metastases from bone tumors (Sotillo-Piñeiro et al., 2004), soft tissue sarcomas (Henson et al., 2005), and liposarcomas (Costa et al., 2006) more frequently express telomerase than characteristics of ALT. Human tumors can also show differential telomerase expression, as one example of a lung squamous cell carcinoma lacked telomerase but its hilar lymph node metastasis showed strong telomerase expression (Hiyama et al., 2001). These studies suggest that telomerase expression may offer an advantage for tumor metastases.

Confirmation that tumors do not use each TMM in an either-or manner is important for the understanding of tumorigenesis and tumor progression. These data suggest that: 1) some tumors may not arise clonally; 2) each tumor cell can activate either ALT or telomerase independently of its lineage; or 3) tumor cells can actively switch between ALT mechanisms and telomerase (Figure 21). It is currently unknown whether tumor cells can spontaneously switch TMM in situ or whether tumor cells commit to a particular TMM upon immortalization. Recent in vivo studies from mouse models (Hu et
*al., 2012* demonstrate that tumor cells exhibit flexibility of TMM and can actively switch among the mechanisms depending on genetic and epigenetic changes.
Figure 24. Revised model for activation of a telomere maintenance mechanism. Neoplastic mutations allow relaxation of cell cycle controls with increased proliferation and telomere shortening (red circles represent telomeres on green chromosomes). Once critically short, telomeres signal the activation of telomerase or ALT to permit tumor cell immortalization (top) or the cell will exit the cell cycle through senescence and/or apoptosis (bottom, shaded cell). Once activated, telomere maintenance mechanisms may be able to switch back and forth within a single tumor cell.
Telomere maintenance is a critical characteristic of tumor cells. Because most human adult somatic cells do not maintain their telomeres, telomerase and ALT are attractive therapeutic targets and their inhibition would be predicted to inhibit tumor cell growth specifically and efficiently while minimizing off-target effects. Telomerase inhibitors are currently in clinical trials for a number of cancer types and have shown limited success. Mouse models demonstrate that inhibition of telomerase after telomerase-mediated tumor formation pressures tumor cells to activate ALT (Hu et al., 2012), suggesting that combination therapies to inhibit both telomerase and ALT may be required to inhibit tumors. Recent work has demonstrated that ALT cells have upregulated mitochondrial function and are sensitive to the inhibition of mitochondrial regulator PGC-1β (Hu et al., 2012), elucidating a potential therapeutic target of the ALT pathway.

This work has added to current understanding of ALT pathways by demonstrating the variability of human tumor cells in regards to telomere maintenance. The current designation of ALT likely represents more than one mechanism for the elongation of telomeres in the absence of telomerase, some of which require the WRN helicase. Human sarcomas also demonstrate variability in telomere maintenance and can exhibit tumor cell mosaicism for ALT or telomerase within the same tumor. These results suggest great variability and flexibility in immortalization and imply that sophisticated therapies will be required to challenge the survival of tumor cells.
Chapter 6: References


formation and recruits the PML-interacting protein DAXX to this nuclear structure when modified by SUMO-1. J Cell Biol 147, 221-234.


Appendix A: WRN in clonal cell populations transfected with *pSilencerWRN*. 
Cells were transfected with pSilencerWRN, selected for antibiotic resistance with 5 ug/ml hygromycin, and single cells were clonally expanded under constant antibiotic selection. Cell extracts were prepared from these clones, separated by SDS-PAGE, and subjected to western blot analysis with the indicated antibodies. Lamin B is a loading control.
Appendix B: Timecourse of WRN expression following *WRN siRNA* transfection into HeLa cells.
WRN knockdown in HeLa cells up to 120 hours post-transfection. HeLa cells were transfected with 50 nM WRN siRNA or mock transfected and cell extracts were collected at the indicated times post-transfection. Extracts were separated by SDS-PAGE and western blotted with the indicated antibodies. Lamin B is a loading control.
Appendix C: Quantitative real time PCR (qRT-PCR) validation.
(A) Representative amplification plots depicting both telomere PCR reactions (blue/green) and albumin control PCR reactions (pink/purple). (B) Representative dissociation curves depicting both telomere PCR reactions (blue/green) and albumin control PCR reactions (pink/purple). (C) Standard curve of Ct values versus log [DNA] for telomere and albumin reactions. Genomic DNA samples were amplified on an Applied Biosystems 7900HT Fast Real Time PCR machine.
Appendix D: Validation of telomere and γH2AX staining in immortalized cell lines.
(A) Hybridization of HeLa, MCF7, VA-13 and Saos-2 cells with a Cy3-conjugated (red) telomere peptide nucleic acid (PNA) probe demonstrates telomeric foci that overlap with the telomeric protein TRF2 (green) in DAPI-stained nuclei (blue). (B) γH2AX staining was confirmed by treating HeLa and VA-13 cells with camptothecin, a known inducer of DNA double strand breaks. Cells were mock treated or treated with 2 µM camptothecin for 24 hours, fixed, and stained with antibodies to γH2AX (red). Nuclei were labeled with DAPI (blue) for visualization.
Appendix E: WRN associates with BRCA1 during G2/M-phases of the cell cycle in ALT cells.
(A) HeLa and VA-13 cells were grown asynchronously or arrested with 1 µg/ml aphidicolin, released, and harvested at the indicated times post-release. Cell extracts were immunoprecipitated with antibodies to WRN, separated by SDS-PAGE, and immunoblotted with antibodies to BRCA1. IgG is a negative control as well as anti-WRN immunoprecipitation in AG11395 WS cells. Anti-BLM immunoprecipitation is a positive control for the interaction with BRCA1 in ALT cells. (B) VA-13 cells were grown asynchronously or arrested with 1 µg/ml aphidicolin, released, and harvested at the indicated times post-release. Cells were stained with propidium iodide and analyzed by flow cytometry. Two hours corresponds to a predominance of cells in G1-phase, 6 hours corresponds to S-phase, and 12 hours corresponds to G2/M-phases of the cell cycle.
Appendix F: Confirmation of telomerase and ALT characteristic staining in human immortalized cell lines.
One telomerase-positive cell line, HeLa, and one ALT cell line, WI-38 VA-13/2RA, were grown on coverslips, fixed, and immunofluorescently stained with anti-PML (red) and anti-TRF2 (green) antibodies (top) and an anti-TERT (red) antibody (bottom) as used for in situ immunohistochemistry. Colocalization of anti-PML and anti-TRF2 signals (yellow) indicate APBs and were observed only in ALT cells (top right); anti-TERT foci were observed only in telomerase-positive cells (bottom left). DAPI counterstain (blue) allowed visualization of cellular nuclei.
Appendix G: Analysis of telomere maintenance mechanism in a colon cancer tissue microarray
<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Tumor</th>
<th>Site</th>
<th>Grade</th>
<th>TNM</th>
<th>ALT</th>
<th>Tel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>67</td>
<td>M Adenocarcinoma</td>
<td>Colon</td>
<td>I</td>
<td>T2N0M0</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>71</td>
<td>F Adenocarcinoma</td>
<td>Colon</td>
<td>I</td>
<td>T3N0M0</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>63</td>
<td>M Adenocarcinoma</td>
<td>Colon</td>
<td>I</td>
<td>T2N0M0</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>82</td>
<td>M Adenocarcinoma</td>
<td>Colon</td>
<td>I</td>
<td>T3N0M0</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>61</td>
<td>M Mucinous adenocarcinoma</td>
<td>Colon</td>
<td>I</td>
<td>T3N0M0</td>
<td>-</td>
<td>+</td>
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<td>-</td>
<td>+</td>
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<td>+</td>
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(TNM=tumor, lymph node, metastasis staging; % ALT=percent of cell positive for alternative lengthening of telomeres; % Tel=percent of cells positive for telomerase)
Appendix H: *In situ* immunohistochemistry of control tissues demonstrates telomerase, but not ALT, staining.
(A) A colon cancer tissue microarray was stained for TRF2 and PML and for telomerase, then analyzed on the Nuance multispectral imaging system. The left panel demonstrates telomerase positive cells in white; the right panel demonstrates a lack of TRF2/PML colocalization, which would be indicated in yellow. Cells are counterstained with hematoxylin (blue).

(B) Normal thymus tissue was also analyzed for ALT- and telomerase-positive cells as above. Cells are counterstained with hematoxylin (blue). Images were analyzed and captured on a Nuance multispectral imaging system.