Functional analysis of candidate phosphorylation sites of telomere repeat binding factor 2 (TRF2)
FUNCTIONAL ANALYSIS OF CANDIDATE PHOSPHORYLATION SITES OF
TELOMERE REPEAT BINDING FACTOR 2 (TRF2)

By KYLE REINSCHLID-LINDSAY, B.Sc
TITLE: Functional analysis of candidate phosphorylation sites of telomere repeat binding factor 2 (TRF2)

AUTHOR: Kyle Reinschild-Lindsay, B.Sc

SUPERVISOR: Dr. Xu-Dong Zhu

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Abstract

TRF2 is a multifunctional protein implicated in telomere length maintenance, DNA double strand break repair and telomere protection. TRF2 undergoes extensive post-translational modification including phosphorylation. Mass spectrometry analysis has identified two candidate TRF2 phosphorylation sites: T317 and S323. In this study, the roles of these two potential phosphorylation sites were examined for their role in cell growth, telomere length maintenance and DNA damage response. Through retroviral infection, HT1080, HeLaII and GM847 cell lines stably expressing the vector alone, Myc-tagged wild type TRF2, Myc-tagged TRF2 carrying a nonphosphorylatable mutation of either T317A or S323A and Myc-tagged TRF2 carrying a phosphomimic mutation of either T317D or S323D were generated. Overexpression of TRF2 mutant alleles has no effect on cell growth and proliferation as well as TRF2 association with ALT-associated PML bodies. On the other hand, the effect of TRF2 mutant alleles on DNA damage response and telomere length maintenance is inconclusive and requires further investigation.
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<th>Description</th>
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<tr>
<td>53BP1</td>
<td>p53 binding protein 1</td>
</tr>
<tr>
<td>A</td>
<td>Alanine</td>
</tr>
<tr>
<td>ALT</td>
<td>Alternative lengthening of telomeres</td>
</tr>
<tr>
<td>APB</td>
<td>ALT-associated promyelocytic leukemia body</td>
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<td>CTBP-interacting protein</td>
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<td>Aspartic Acid</td>
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<td>Oligosaccharide/oilonucleotide binding</td>
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<tr>
<td>O/N</td>
<td>Overnight</td>
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<td>Telomere-sister chromatid exchange</td>
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CHAPTER 1

1 Introduction

1.1 Telomeres

1.1.1 Telomere Structure

Telomeres are highly repetitive, DNA sequences found at the ends of linear chromosomes (de Lange, 1990; McEllingott & Wellinger, 1997; van Steensel et al., 1998). Their main function is to protect linear chromosome ends from being recognized as damaged DNA. Telomeres are composed of double stranded TTAGGG tandem repeats that end in a G-rich 3’ overhang (Cheng et al., 1989; de Lange et al., 1990; de Lange et al., 2005; Henderson & Blackburn, 1989; McEllington & Wellinger, 1997; Wright et al., 1997). The G-rich 3’ overhang is generated through a combination of the removal of the last RNA primer during DNA synthesis, as well as processing of telomeric DNA through the actions of Exo1 and Apollo nucleases (Olovnikov, 1973; Watson, 1972; Wu et al., 2012). Since DNA polymerase utilizes RNA primers to initiate DNA synthesis, telomeric DNA naturally shortens after each cellular division, as DNA polymerase is unable to fill in the gap following the removal of the last RNA primer on the lagging strand (Olovnikov, 1973; Watson, 1972). Within different species, telomere length can range dramatically, however, human tissue telomeres typically range between 10 to 15 kilobases (kb) in length (de Lange et al., 1990; de Lange, 2005; Harley et al., 1990; Hastie et al., 1990).

Shelterin, a six-protein subunit complex consisting of TRF1, TRF2, hRap1, POT1, TPP1 and TIN2, binds to telomeric regions of DNA (Diotti & Loayza, 2001; de Lange, 2005). Shelterin influences the state of telomeres in a number of ways, including
telomere protection, function and maintenance (Palm & de Lange, 2008). This complex aids in the formation of a higher order telomeric structure referred to as the t-loop, which prevents telomeres from being recognized as DNA damage (Griffith et al., 1999; Stansel et al., 2001). In addition to aiding in t-loop formation, shelterin plays an important role in telomere protection, as well as telomere length maintenance, as it inhibits telomerase-dependent telomere elongation (Diotti & Loayza, 2001; de Lange, 2005; Palm & de Lang, 2008).

The G-rich 3’ overhangs that are found at the ends of each chromosome are important for the formation of a higher order structure, referred to as the t-loop (Amiard et al., 2007; de Lange, 2005; Palm & de Lange, 2008; Stansel et al., 2001). The t-loop occurs when the 3’ single strand telomeric overhang loops back and invades duplex telomeric DNA (Griffith et al., 1999). The formation of this t-loop is thought to act as a telomere protecting structure, as it provides a physical barrier to prevent the ends of linear chromosomes from being recognized as DNA damage (Griffith et al., 1999; Palm & de Lange, 2008; Stansel et al., 2001). T-loop formation is facilitated by TRF2, one of the proteins that make up the shelterin complex (Amiard et al., 2007; Stansel et al., 2001).

1.1.2 Telomeres and Genome Stability

Telomeres have a number of important functions within the cell, from preventing events of DNA damage at the ends of linear chromosomes, as well as acting as a molecular clock, to limit cell proliferation in the absence of any telomere length maintenance mechanism (Allsopp et al., 1992; de Lange, 1998; Hayflick, 1965; Karlseder, 1999; McEllingott & Wellinger, 1997). The most prominent function of
telomeres is its role in telomere protection, as the ends of linear chromosomes would be recognized as sites of DNA damage without the telomere region, which would elicit a DNA damage response by the cell (Karlseder et al., 1999; van Steensel et al., 1998). This functions is aided by the shelterin complex, and more specifically TRF2, which facilitates the formation of the t-loop as well as inhibiting the ATM-dependent DNA damage response at telomeres (Amiard et al., 2007; Karlseder et al., 2004; Stansel et al., 2001). The loss of this highly repetitive DNA sequence can result in telomere abnormalities, such as end-to-end fusions, which can lead to induction of cellular senescence (de Lange, 1998; Karlseder et al., 1999; van Steensel et al., 1998).

1.1.3 End Replication Problem

Another crucial role of telomeric DNA is its function as a molecular clock, also known as the Hayflick limit (Hayflick, 1965). The Hayflick limit is believed to act as a tumor suppressing mechanism, limiting the number of division a cell can perform before entering senescence (Palm & de Lange, 2008). This is due to the continual shortening of telomeric regions of DNA after each cell division. This phenomenon is referred to as the ‘end replication problem,’ in part due to the inability of DNA polymerase to fill the gap following the removal of the last RNA primer (Olovnikov, 1973; Watson, 1972).

The enzyme DNA polymerase, which synthesizes DNA from a template strand, facilitates DNA replication. Unfortunately, telomeric regions of linear chromosomes present challenges to this mechanism, which result in their continual shortening over time (Olovnikov, 1973; Watson, 1972). DNA polymerase is restricted to synthesize DNA in a 5’ to 3’ direction, which leads to both leading and lagging strand synthesis. Leading
strand synthesis is a continual process while lagging strand synthesis takes advantage of small Okazaki fragments, which are ligated together following replication (Olovnikov, 1973). During replication, DNA polymerase utilizes short RNA primers in order to begin synthesizing Okazaki fragments. Unfortunately, during replication of the lagging strand, a gap along the template strand is created, as the final RNA primer is unable to bind. This gap is unable to be filled and the end of the chromosome cannot be replicated, leading to continual shortening of the lagging strand (Olovnikov, 1973; Watson, 1972). Due to this phenomenon, telomeres shorten roughly 100 nucleotides every cell division (Chow et al., 2012). Once telomeres reach a critically short threshold, about 1kb in length, cells become senescent and are unable to divide further (Baird & Kilping, 2004; Counter et al., 1992). This phenomenon plays an important role in preventing uncontrollable cell proliferation, leading to the formation of a tumor. Unfortunately, once cancer cells arise, they are able to increase their replicative capacities through the use of a number of telomere lengthening mechanisms.

1.1.4 Mechanisms for Telomere Lengthening

Once a cell has transitioned into a cancerous state, a number of cellular changes occur. One such change is the establishment of cellular mechanisms in order to counteract the end replication problem, ultimately allowing cancer cells to bypass their Hayflick limit. Roughly 90% of cancer cells utilize a mechanism involving the ribonuclearprotein telomerase, a reverse transcriptase enzyme, in order to counteract the end replication problem (Greider & Blackburn, 1985, 1987, 1989). As previously described, telomeric DNA ends in a 3’ G-rich single stranded tail, which acts as a
substrate for telomerase that adds TTAGGG repeats to the end of telomeric DNA (Greider & Blackburn, 1985, 1987, 1989). Telomerase is composed of two subunits, hTR, and hTERT (Cong et al., 2002; Feng et al., 1995; Harrington et al., 1997). hTR, the RNA subunit, acts as a template telomere strand with the complimentary telomere sequence of CCCUAA. This subunit binds to the 3’ G-rich single strand overhang of telomeres in order for hTERT, the reverse transcriptase subunit of telomerase to synthesize telomeric repeats at the ends of linear chromosomes (Cong et al., 2002; Feng et al., 1995; Harrington et al., 1997; Meyerson et al., 1997). This allows cells to counteract the telomere loss that occurs after each cell division to extend their replicative capacity. Telomerase is not only up regulated in cancer cells. A variety of cell types such as germline and stem cells express telomerase, giving them an elevated proliferative capacity compared to normal somatic cells that do not have a detectable telomerase activity. While the vast majority of cancer cells rely on telomerase to maintain their telomere length, about 10-15% of cancer cells maintain their telomere length through a homologous recombination-based mechanism, referred to as the alternative lengthening of telomeres (ALT) (Bryan et al., 1995).

1.2 Shelterin Complex

Shelterin is a six-protein subunit complex composed of TRF1, TRF2, hRap1 TIN2, POT1 and TPP1 (Figure 1) (de Lange, 2005; Diotti & Loayza, 2001). This complex helps regulate telomere integrity, length maintenance and t-loop formation (de Lange, 2005; Diotti & Loayza, 2001; Palm & de Lange, 2008; van Steensel et al., 1998; Xin et al., 2008). Three of the six shelterin proteins interact with telomeric DNA. TRF1 and TRF2
bind to double stranded telomeric DNA as homodimers while POT1 directly interacts with the 3’ G-rich single strand overhang regions of telomeres (Baumann & Cech, 2001; Bianchi et al., 1997; Bilaud et al., 1997; Loayza et al., 2004; Shen et al., 1997; van Steensel & de Lange, 1997; Xin et al., 2008). Both TRF1 and TRF2 proteins contain a Myb-like DNA binding domain at their C-terminus and a central TRFH domain responsible for the formation of homodimers (Broccoli et al., 1997; Chen et al., 2009; Fairall et al., 2001). TRF1 and TRF2 are negative regulators of telomere lengthening (Smogorzewska et al., 2000; van Steensel & de Lange, 1997). TIN2 interacts with both TRF1 and TRF2 and is often referred to as “the linchpin” of the shelterin complex (Houghtaling et al., 2004; Kim et al., 2004; Ye et al., 2004). TPP1 interacts with POT1 through its OB-fold domain and plays a role in telomerase recruitment to telomeres (Corriveau et al., 2013; Zhang et al., 2012; Zhong et al., 2012). POT1, which is bound to the shelterin complex through an interaction with TPP1, is only bound to the 3’ single stranded region of telomeric DNA (Baumann & Cech, 2001; Taylor et al., 2011). POT1 is implicated in telomere protection and telomere length regulation. Not only does POT1 prevent ATR from recognizing telomeric regions as sites of DNA damage, but also inhibits the binding of telomerase to the G-rich 3’ single strand overhang (Denchi & de Lange, 2007; Loayza & de Lange, 2003). hRap1 binds to TRF2 and has been implicated in preventing non-homologous end joining (Bae & Baumann, 2007; Janouskova et al., 2015).

1.3 Telomere Repeat Binding Factor 2 (TRF2)
Telomere repeat binding factor 2 (TRF2) is a 500 amino acid, 55 kDa protein that is transcribed from the TERF2 gene on chromosome 16. It contains four conserved domains: the N-terminal basic domain, the TRFH homodimerization domain, the linker region and the C-terminal Myb-like DNA binding domain (Figure 2) (Bilaud et al., 1997; Broccoli et al., 1997; de Lange, 2005; Fairall et al., 2001; Palm & de Lange, 2008). TRF2 binds to duplex telomeric DNA as a homodimer (Broccoli et al., 1997; Fairall et al., 2001). TRF2 has been shown to be a negative-regulator of telomere length. Overexpression of TRF2 causes the shortening of telomeres in both telomerase-positive and telomerase-negative cells, indicating that the effect of TRF2 on telomere length maintenance is independent of telomerase (Ancelin et al., 2002; Karlseder et al., 2002; Munoz et al., 2006; Smogorzewska et al., 2000; van Steensel et al., 1998). TRF2 has a number of additional functions, which will be discussed in the following sections. These include roles of telomere protection, t-loop formation and DNA damage repair.

1.3.1 TRF2 and Telomere Protection

TRF2 is mainly known for its role in telomere protection. It has been suggested that TRF2 facilitates the formation of the t-loop and blocks the activation of ATM at telomeres (Celli & de Lange, 2005; Denchi & de Lange, 2007; Karlseder et al., 1999; Stansel et al., 2001; Stansel et al., 2002; van Steensel et al., 1998).

Overexpression of a dominant negative allele of TRF2 lacking its basic and Myb-like DNA binding domains leads to the loss of TRF2 from telomeres, resulting in the formation of telomere end-to-end fusions (Karlseder et al., 1999; van Steensel et al., 1998). Loss of TRF2 activates the ATM-dependent DNA damage response pathway and
promotes the recruitment of DNA damage factors to dysfunctional telomeres, giving rise to the formation of telomere dysfunction induced foci (TIF) (Takai et al., 2003). DNA repair factors such as XPF/ERCC1, KU70/80 and DNA ligase IV have been implicated in the processing of the exposed 3’ overhangs, and the subsequent ligation of telomere ends (Celli, 2006; Smogorzewska et al., 2002; van Steensel et al., 1998; Zhu et al., 2003).

TRF2 directly interacts with ATM, which may contribute to the inhibition of ATM activation at telomeres (Karlseder et al., 2004, Denchi & de Lange, 2007). Additionally, if a DNA damage response is triggered at telomeres, TRF2 is able to inhibit the E3 ubiquitin ligase RNF168, which prevents the spread of the ubiquitylation signal required to facilitate a functional repair process (Okamoto et al., 2013). TRF2 has also been shown to interact with Ku70, a protein required for non-homologous end joining (NHEJ) and this interaction may prevent the ligation of telomere ends (Ribes-Zamora et al., 2013).

1.3.2 TRF2 and DNA Damage Response

Even though TRF2 is a well-defined telomere binding protein, it has been shown to promote the DNA damage response at non-telomeric regions of DNA. Following induction of DNA double strand breaks, TRF2 influences the repair pathway choice between homologous recombination (HR) and non-homologous end joining (NHEJ), the two main DNA repair mechanisms utilized by the cell (Mao et al., 2007). Overexpression of TRF2 promotes the repair mechanism HR, while suppressing NHEJ (Mao et al., 2007). Knockdown of TRF2 impaired HR efficiency while having no affect on the NHEJ repair mechanism (Mao et al., 2007). In addition, TRF2 is rapidly phosphorylated following the
induction of DNA double strand breaks (Huda et al., 2009; Tanaka et al., 2005). This phosphorylated form of TRF2 is not bound to telomeric DNA, instead, it is recruited to sites of DNA damage, facilitating the fast pathway of DNA double-strand break repair through the formation of early recombination intermediates (Bradshaw et al., 2005; Huda et al., 2009; Tanaka et al., 2005). These findings suggest that the telomere binding protein TRF2 has influential roles outside of telomere biology, aiding in DNA double strand break repair.

1.3.3 Post-Translational Modifications of TRF2

As previously described, TRF2 has a wide rage of functions, from its role in telomere protection to its involvement in DNA damage response following the induction of double strand breaks. The post-translational modifications of TRF2, including phosphorylation, ubiquitylation, SUMOylation, PARsytlation and arginine methylation, have been shown to promote its function (Figure 2).

Following the induction of DNA double strand breaks through ionizing radiation, TRF2 has been shown to be phosphorylated at T188 by the ATM kinase (Bradshaw et al., 2005; Huda et al., 2009; Tanaka et al., 2005). This phosphorylation promotes the repair process HR (Tanaka et al., 2005). TRF2 phosphorylated at T188 does not associate with telomeric DNA but is quickly recruited from telomeric regions to sites of DNA double strand breaks (Tanaka et al., 2005). The role of T188 phosphorylation at sites of DNA damage was examined through the use of mutant TRF2 alleles, in which the T188 residue was mutated to alanine (T188A), simulating the lack of phosphorylation at this site (Huda et al., 2009). It was discovered that cells expressing the mutant T188A allele were
sensitive to laser-induced DNA double strand breaks, due to an impairment in the fast pathway of DNA double strand break repair (Huda et al., 2009).

TRF2 has also been shown to interact with the DNA damage checkpoint protein Chk2 through its N-terminus (Buscemi et al., 2009). This interaction prevents the phosphorylation of Chk2 at T68 by ATM, which is believed to repress the DNA damage response at telomeres, supporting the role of TRF2 in telomere protection (Buscemi et al., 2009). Interestingly, following the induction of DNA double strand breaks, Chk2 has been seen dissociating from telomeres (Buscemi et al., 2009). This dissociation of Chk2 promotes the phosphorylation of TRF2 at S20, impairing its binding ability to telomeric DNA (Buscemi et al., 2009). This supports the idea that the phosphorylation of TRF2 at S20 and subsequent loss of telomere binding, is important for the response of TRF2 to DNA damage sites at non-telomeric regions of DNA (Buscemi et al., 2009).

In addition to the previously described phosphorylation events, TRF2 is found, through mass spectrometry analysis, to undergo additional phosphorylation at residues T317 and S323 (Chi et al., 2008; X.-D. Zhu, unpublished date). The roles of these phosphorylation events in the cell will be further explained in later sections.

Aurora C has also been identified as a kinase responsible for a phosphorylation event on TRF2 at T358 through a yeast-two hybrid assay (Spengler, 2007). Even though much is unknown about this kinase, it is believed to play a role in regulating cell morphology, mitosis and cell growth. Just like the relatively unknown function of the Aurora C kinase, its resulting phosphorylation of TRF2 at T358 has yet to show functional relevance (Spengler, 2007).
Ubiquitylation is another crucial post-translational event affecting TRF2. Ubiquitylation is utilized by the cell to target proteins for proteasome-dependent degradation as well as serving as a signaling event (Al-Hakim et al., 2010; Komander, 2009; Mukhopadhyay & Riezman, 2007). TRF2 has been shown to be ubiquitylated on residues K173, K180 and K184, mainly through the action of the E3 ubiquitin ligase SIAH1, which has been shown to physically interact with TRF2 (Fujita et al., 2010). The ubiquitylation of TRF2 is believed to function as a mechanism promoting cellular senescence in cells with critically short telomeres. The SIAH1 ubiquitin ligase is transcriptionally induced by p53, one of the main proteins involved in stimulating cellular senescence (Matsuzawa & Reed, 2001). Cells that have reached the end of their proliferative capacity possess critically short telomeres, which would activate p53 and in turn up-regulate the E3 ubiquitin ligase SIAH1, promoting TRF2 for degradation (Fujita et al., 2010). This degradation of TRF2 further promotes telomere instability, leading to the induction of cellular senescence and apoptosis (Fujita et al., 2010). This positive feedback loop between p53 and TRF2 is an additional layer of protection, minimizing the chances of cells possessing critically short telomeres from replicating.

An additional post-translational modification that affects the role of TRF2 is SUMOylation, which is similar to the process of ubiquitylation. SUMOylation plays an important role in regulating protein cellular localization, protein-protein interactions, DNA damage response and repair pathways (Bekker-Jensen & Mailand, 2011; Dou et al., 2011; Muller et al., 2001). The E3 SUMO-protein ligase MMS21 has been shown to SUMOylate members of the shelterin complex including TRF2, TRF1, TIN2 and hRap1 (Potts & Yu, 2007). The SUMOylation of both TRF1 and TRF2 is required for telomere
length maintenance in telomerase-negative (ALT) cancer cells (Dunham et al., 2000; Potts & Yu, 2007). The SUMOylation of TRF1 and TRF2 is important for the formation of APBs, the subnuclear domains where homologous recombination-based telomere length maintenance takes place.

Poly(ADP-ribose) polymerases are able to change the properties of protein acceptors through the generation of ADP-ribose polymers on glutamic acid residues. TRF2 has been shown to be poly(ADP-ribose)ylated by PARP1 and PARP2 at its Myb-like DNA binding domain following induction of DNA damage (Dantzer et al., 2004; Gomez et al., 2006). PARP1 and PARP2 have been implicated in roles relating to telomere maintenance. Deletion of PARP1 and PARP2 has been shown to lead to telomere end-to-end fusions and telomere loss (Dantzer et al., 2004; Gomez et al., 2006). The ability of TRF2 to bind to telomeric DNA has been shown to be negatively influenced by the poly(ADP-ribosyl)ation, which is thought to promote DNA repair at telomeres (Gomez et al., 2006).

TRF2 undergoes arginine methylation, which is carried out by protein arginine methyltransferase 1 (PRMT1) (Mitchell et al., 2009; Mitchell & Zhu, 2014). PRMT1 is implicated in DNA repair, transcription regulation, RNA processing and protein trafficking (An et al., 2004; Boisvert et al., 2005; Mitchell et al., 2009; Smith et al., 2004). PRMT1 methylates TRF2 at arginine residues within its N-terminal basic domain (Mitchell et al., 2009). Overexpression of TRF2 alleles carrying amino acid substitutions of arginines to lysines results in the recruitment of DNA damage factors to telomeres, leading to induction of cellular senescence (Mitchell et al., 2009). In addition, arginine methylation of TRF2 regulates its cellular localization. Methylated TRF2 is found to be
tightly associated with the nuclear matrix and has been suggested to serve as a biomarker for cellular senescence in an ATM-dependent manner (Mitchell & Zhu, 2014).

1.4 ATM Kinase

The ATM kinase is a Ser/Thr protein kinase mainly known for its role in the DNA damage response of a cell. ATM is made up of very few distinct domains, which include a C-terminal active site, HEAT domain, and a few known interacting sites. The C-terminal active site is the most important domain in terms of its role in the DNA damage response of the cell (Lempianinen & Halazonetis, 2009; Lovejoy & Cortez, 2009). This domain occupies roughly 10% of the protein and is responsible for the kinase activity of ATM.

ATM is a master regulator of the DNA damage response pathway following the induction of DNA double strand breaks. This response is initiated seconds after DNA double strand break induction, as ATM is recruited to sites of damage (Andegeko et al., 2001). Mre11, Rad50 and Nbs1, members of the MRN complex, are recruited to site of DNA damage. The recruitment of the MRN complex is required to facilitate optimal ATM recruitment (Falck et al., 2005; Shiloh & Ziv, 2013). This early recruitment is essential for the actions of downstream proteins in order to ensure the proper repair of DNA damage. In addition, ATM phosphorylates a number of downstream proteins including Chk2 and p53, leading to the activation of the DNA damage checkpoints (Smith et al., 2010). This is a crucial process since the cell must be given time to repair their damaged DNA before dividing. If the damaged DNA cannot be repaired,
phosphorylation of p53 by ATM leads to an accumulation of p53, promoting cell cycle arrest or apoptosis (Choi et al., 2012; Rashi-Elkeles et al., 2011).

The crucial role of ATM in the cell can clearly be visualized in ataxia-telangiectasia patient cell lines. Ataxia-telangiectasia is an autosomal recessive genomic instability disorder, which can arise from mutations of ATM. Ataxia-telangiectasia patient cell lines are characterized by increased chromosomal breakage, premature senescence and sensitivity to DNA-damage agents.

1.5 Cyclin-dependent Kinase

Cyclin-dependent kinases (CDK) are serine/threonine kinases that are well established to regulate cell cycle progression through a network of phosphorylation events. CDKs are composed of two subunits, the catalytic subunit and the regulatory subunit, cyclin, which are regulated in a cell-cycle dependent manner. In addition to controlling cell cycle progression, CDKs have also been shown to play a role in transcription, DNA damage response and telomere maintenance (Aylon et al., 2004; Frank et al., 2006; Ira et al., 2004; Liu et al., 2014; Loyer et al., 2005; Zhang et al., 2009)

As previously stated, telomeres mainly function to protect linear chromosome ends from being recognized as DNA damage (Karlseder et al., 1999; van Steensel et al., 1998). Unfortunately, due to the end-replication problem, telomeres shorten after each round of replication. Cdk1 has recently been shown to facilitate telomerase-dependent telomere elongation in budding yeast (Liu et al., 2014). Cdc13 is phosphorylated by Cdk1, facilitating the recruitment of telomerase to telomeric DNA, allowing for telomerase-dependent telomere elongation (Lui et al., 2014; Weinert et al., 1993). Loss of this
protein has been shown to result in excessive telomere loss leading to the activation of DNA damage checkpoints (Weinert et al., 1993). Cdk1 is also important for the early stages of telomerase dependent telomere elongation. Cdk1 is required for the formation of the 3’ single strand overhangs at telomeres (Frank et al., 2006). In human cells, Cdk1 has also been shown to play a role in telomere maintenance through the phosphorylation of TRF1 at T371 upon mitotic entry (Mckerlie & Zhu, 2011). This phosphorylation event keeps TRF1 free from telomeres, inducing temporal telomere de-protection, which promotes the resolution of sister telomeres (Mckerlie & Zhu, 2011).

Cdk1 has also been implicated in the DNA damage repair pathway HR. During the process of HR, Cdk1 has been shown to influence the formation of 3’ single strand overhangs, which are required for strand invasion during HR (Aylon et al., 2004; Ira et al., 2004, Zhang et al., 2009). This in turn suppresses the error prone DNA damage pathway non-homologous end joining. In addition to aiding in the formation of 3’ single strand overhands, Cdk1 has also been shown to influence the recruitment of Rad51 and RPA to sites of IR induced DNA double strand breaks, which in turn affects the activation of the G2/M cell cycle checkpoint (Caspari et al., 2002). Recently, Cdk1 has been shown to phosphorylate unbound fractions of TRF1 at threonine 371 following the induction of DNA double strand breaks, where it is subsequently recruited to sites of DNA damage (McKerlie et al., 2013). The recruitment of (pT371)TRF1 to sites of DNA damage aids in the repair process by facilitating DNA end resection and the activation of the G2/M checkpoint (McKerlie et al., 2013).

1.6 Alternative Lengthening of Telomeres
Approximately 90% of cancers utilize a telomerase-mediated telomere lengthening mechanism to counteract the end-replication problem in order to extend their proliferative capacity. The remaining 10% of cancers utilize a homologous recombination-based mechanism to elongate their telomeres in the absence of the ribonuclear protein telomerase (Bryan et al., 1995; Dunham et al., 2000; Lundblad & Blackburn, 1993). This homologous recombination-based mechanism is referred to as alternative lengthening of telomeres (ALT). There are a number of characteristics of ALT cells, which distinguish them from telomerase-positive cells. In addition to utilizing homologous recombination as a means to elongate their telomeres, ALT cells possess a heterogeneous-telomere length, the presence of ALT-associated promyelocytic leukemia bodies (APBs), extrachromosomal telomeric DNA and an elevated occurrence of telomere-sister chromatin exchange events (Cesare & Griffith, 2004; Cesare & Reddel, 2010; Henson et al., 2009; Londono-Vallejo et al., 2004; Murnane et al., 1994; Wang et al., 2004; Yeager et al., 1999). The majority of ALT cancer cell lines display all of the above characteristics, however, it has been shown that cells can be classified as ALT while missing one or more of the ALT characteristics (Chung et al., 2012). ALT cells that possess all of the main characteristics are thought to follow the canonical ALT pathway, while those lacking one or more defined characteristics are considered to belong to the non-canonical ALT pathway (Chung et al., 2012).

ALT cancers unfortunately have a poor prognosis, with the mean survival time ranging from several months to several years after diagnosis (Darefsky et al., 2012; Hakin-Smith et al., 2003). Fortunately, with ongoing research, the treatments for ALT cancers have substantially improved, leading to improved odds for individuals suffering
from such cancers. Cancers of neuroepithelial and mesenchymal origin, such as soft tissue sarcomas and osteosarcomas, utilize the ALT pathway in order to maintain telomere length (Henson et al., 2005).

1.6.1 Extrachromosomal Telomere Repeats

Extrachromosomal telomere repeats (ECTR) refers to the presence of telomere repeats, in either circular or linear forms, which are not associated with the chromosome. ECTRs in ALT cells are found mainly in the form of circular DNA, including T-circles, C-circles and G-circles. T-circles, which are double stranded circles of telomeric DNA, are thought to arise from the resolution of t-loop junctions, or the trimming of telomere regions to shorten extremely long telomeres (Ceasare & Reddel, 2010; Henson et al., 2009; Pickett et al., 2009; Wang et al., 2004). C- and G-circles are defined as being partially single-stranded, self-priming circles, with either a high abundance of C or G nucleotides, depending on which telomeric strand, template or non-template strand, they were derived from (Cesare & Griffith, 2004). While the abundance of t-circles is elevated in ALT cell compared to other cell types, C-circles are utilized to assess ALT activity (Ceasare & Reddel, 2010). Even though the origin of these circles is unclear, there is an established relationship between the abundance of C-circles and overall ALT activity within a given population of ALT cells (Henson et al., 2009).

1.6.2 ALT associated Promyelocytic Leukemia Bodies (APBs)
One of the main characteristics of ALT cells is the presence of ALT-associated promyelocytic leukemia bodies (APBs) (Yeager et al., 1999). This refers to a unique subnuclear body that contains telomeric chromatin, telomere-binding proteins and recombination mediating proteins, which is housed within a promyelocytic leukemia (PML) nuclear body. PML nuclear bodies are found in both telomerase positive and ALT cancer cells, where they play a role in transcriptional activation, cellular stress response and cellular senescence (Vallian et al., 1997; Wang et al., 1998; Yang et al., 2002). In ALT cells however, it is believed that APBs are sites of homologous recombination based telomere elongation, as they contain a high abundance of telomeric chromatin and recombination mediating proteins (Draskovic et al., 2009). Also, following the inhibition of APB formation in ALT cells, telomere shortening occurs (Jiang et al., 2005). APBs can be seen at a higher frequency in cell within the S and G2 phase of the cell cycle, when sister chromatin are highly abundant, leading to elevated recombination (Grobelny et al., 2000).

APBs are considered to be the sites of homologous recombination based telomere maintenance. A number of proteins involved in the process of homologous recombination can be found to localize within APBs, including members of the MRN complex (Nbs1, Mre11, Rad50), BRCA1, RecQ helicases WRN and BLM, Rad51 and RPA (Henson et al., 2002; Wu et al., 2003; Yeager et al., 1999). Other DNA damage factors such as 53BP1 and γH2AX have been seen within PML nuclear bodies that contain telomeric chromatin (Cesare et al., 2009). The localization of these proteins and DNA damage markers within APBs supports the idea that APBs facilitate homologous-recombination based telomere elongation in ALT cancer cells.
1.6.3 Telomere-sister chromatid exchange (T-SCE)

It has been suggested that telomere length maintenance can occur through the use of telomere-sister chromatid exchange (T-SCE) events, which is defined as the recombination of telomeric DNA between sister telomeres. Due to the high concentrations of telomeric DNA located within APBs, T-SCE events have been considered as an additional means by which telomere length is controlled (Cesare & Reddel, 2010). During T-SCE, telomeric sequences are exchanged from one sister telomere to another, ultimately leading to one sister telomere containing an increased amount of telomeric sequences compared to the other. This may leave one resulting daughter cell with a critically short region of telomeric DNA, which could lead to cellular senescence. However, the resulting daughter cell with increased telomere sequences now has an increased replicative capacity, contributing to continued cell proliferation.

1.6.4 TRF2 and ALT Activity

Similar to telomerase-positive cells, when TRF2 is overexpressed in ALT cells, there is a gradual shortening of telomere length (Ancelin et al., 2002). This indicates that TRF2 is a negative regulator of telomere length in ALT cells. Within APBs, the saturation of TRF2 on telomeric regions of DNA has been shown to substantially decrease when compared to telomeres free from APBs (Osterwald et al., 2015). This decrease in telomere bound TRF2 within APBs is thought to be regulated by the SUMOylation of TRF2 by MMS21 (Osterwald et al., 2015). This reduction of telomere bound TRF2
within APBs is suggested to trigger the activation of ATM at telomeric regions (Osterwald et al., 2015). It has been suggests that the lack of TRF2 within APBs, and the subsequent activation of ATM, promote the homologous recombination based telomere-elongation mechanism of ALT cells. Overexpression of TRF2 however, has been shown to partially suppress the DNA damage response (DDR) at telomeres in ALT cells, while the overall DDR of ALT cells remained unchanged (Cesare et al., 2009). Following the knockdown of TRF2 from U2OS cells, a well-known ALT cell line, the level of apoptosis increased when compared to control cells lines (D’Alcontres et al., 2007). However, U2OS cells expressing a dominant negative allele of TRF2, lacking its basic and Myb-like DNA binding domains, presented telomere end-to-end fusions at a substantially reduced level when compared to telomerase positive cells (D’Alcontres et al., 2007; van Steensel et al., 1998). Knockdown of TRF2 also results in impaired APB formation, p53 activation and the loss of telomeric DNA (D’Alcontres et al., 2007).

1.7 DNA Damage Response

DNA damage occurs when there is an alteration in the structure of DNA, which can be a result of either endogenous or exogenous sources, such as ionizing radiation, ultraviolet radiation, reactive oxygen species or mutagenic chemicals, or from the harmful byproducts of cellular metabolism. DNA damage can come in many forms, such as single strand breaks, double strand breaks, pyrimidine dimers and mismatched bases (Houtgraaf, 2006). All forms of DNA damage can be extremely hazardous to the cell if not repaired, leading to cell death or tumorigenesis. Fortunately, organisms have evolved mechanisms in order to detect and to repair DNA damage to prevent any adverse effects.
from occurring. If the cell cannot properly repair the damaged DNA, mechanisms have evolved to activate apoptosis or cell cycle arrest. The DNA repair pathways, homologous recombination (HR) and non-homologous end joining (NHEJ), are utilized by the cell to repair DNA double strand breaks. Defects in these repair pathways have been shown to lead to genome instability, leading to increased chances of cancer (Wiesmuller et al., 2002).

### 1.7.1 Homologous Recombination

Homologous recombination is one of the two main DNA repair pathways used to repair DNA double strand breaks. These breaks can be extremely dangerous to the cell and can lead to tumorigenesis and cell death if not properly repaired. HR is mainly restricted to the S and G2 phases of the cell cycle as it utilizes sister chromatids as a template for repair (Liang et al., 1998; Moynahan & Jasin, 2010; San Filippo et al., 2008). However, it has recently been suggested that this repair mechanism is also able to repair double strand breaks in the G0 and G1 phase of the cell cycle, where it takes advantage of transcript RNA as a template for repair (Keskin et al., 2014).

HR utilizes a number of proteins to recognize and repair DNA double strand breaks. Following the induction of double strand breaks, the MRN complex is recruited to the damage site (Carson et al., 2003; Petrini, 1999; Petrini & Stracker, 2003). The MRN complex is composed of three proteins, Mre11, Rad50 and Nbs1. This protein complex is necessary for the recruitment and activation of ATM (Falck et al., 2005; Jazayeri et al., 2006; Lee & Paull, 2004; You et al., 2005). ATM phosphorylates H2AX at S139, which is subsequently referred to as γH2AX (Burma et al., 2001; Paull et al.,
The phosphorylation of H2AX leads to the recruitment of additional repair proteins involved in the HR repair pathway, such as MDC1. MDC1 recruits the ubiquitin E3 ligase RNF8, resulting in the ubiquitylation of H2A and H2AX histones, which promotes chromatin relaxation around the break (Huen et al., 2007; Mailand et al., 2007). Subsequently, 53BP1 and BRCA1 are recruited. This recruitment is crucial for appropriate repair pathway choice, as 53BP1 promotes the repair pathway NHEJ while BRCA1 promotes HR (Daley & Sung, 2014). The wrong choice in repair pathway can result in genome instability and cell death. Following proper repair choice, CTBP-interacting protein (CtIP) is phosphorylated by CDK at S327, which enhances its interaction with BRCA1 (Polato et al., 2014; Yu et al., 2006). This interaction allows the two proteins, in addition to the MRN complex, to generate single stranded DNA overhangs at the double strand break (Chen at al., 2008; Huertas & Jackson, 2009; Sartori et al., 2007). Replication protein A (RPA) then binds to the single stranded DNA in order to protect it from degradation and to inhibit the formation of bulky secondary structures (Alani et al., 1992). Rad51 then binds to the single stranded DNA, where it displaces RPA and forms the nucleoprotein filament. This Rad51 coated nucleoprotein filament is then able to search for the homologous sequence on the sister chromatid, which will be used as the template for DNA repair (Baumann & West, 1998; Liu et al., 2010; New et al., 1998). Once homology is found, the nucleoprotein filament invades the complimentary strand, producing a displacement loop (D-loop) (Baumann & West, 1998; Radding, 1978; San Filippo et al., 2008). By utilizing the sequence in the template strand, DNA polymerase δ is able to synthesis DNA in order to replace the nucleotides lost as result of the damage, as well as the additional nucleotides that were removed during the
formation of the single strand overhangs (Li et al., 2013; Li et al., 2009). Once DNA replication is complete, the two holiday junctions are resolved through the actions of DNA helicases and resolvase (Ip et al., 2008; Liu et al., 2004; Mimitou & Symington, 2009).

1.7.2 Non-Homologous End Joining

Non-homologous end joining can be utilized by the cell to repair DNA double strand breaks in any phase of the cell cycle. Even though NHEJ is not limited to specific stages of the cell cycle, it mainly occurs during the G1 phase, as it does not require the use of a template strand to repair damaged DNA (Burma et al., 2006; Mao et al., 2008). Unfortunately, this causes the NHEJ pathway to be error prone when compared to HR, which takes advantage of the homologous sequence of sister chromatids (Burma et al., 2006; Lieber, 2010; Mao et al., 2008; Moore & Haber, 1996).

The repair pathway of NHEJ begins in a similar manner as HR, as H2AX is phosphorylated through the actions of ATM and the MRN complex. 53BP1 is then recruited to DNA damage sites where it promotes the NHEJ pathway by inhibiting 3’ end resection (Zimmermann et al., 2013). DNA-dependent protein kinase (DNA-PK) is then recruited to the site of DNA damage. DNA-PK is composed of two subunits, the large catalytic subunit termed DNA-PKcs and a regulatory factor made up of the heterodimer Ku70/80 (Gottlieb & Jackson, 1993). The regulatory factor Ku70/80 is utilized to hold the two DNA ends together as well as activating the catalytic subunit of DNA-PK, DNA-PKcs (Gottlieb & Jackson, 1993). Artemis is then recruited to the DNA ends where its 5’ to 3’ endonuclease activity is activated by DNA-PKcs in order to eliminate any DNA
overhangs which would interfere with the ligation of the two ends (Gottlieb & Jackson, 1993; Ma et al., 2002; Moshous et al., 2001). If unnecessary end resection occurs through the actions of Artemis, DNA polymerase is utilized to fill the gaps in order to produce blunt DNA ends, which can then be successfully ligated together by DNA ligase IV (Burna et al., 2006; Grawunder et al., 1997).

1.7.3 Checkpoint Activation

In addition to DNA repair pathways such as homologous recombination and non-homologous recombination, checkpoint activation following DNA damage is crucial for cell survival following induction of DNA damage. Checkpoints reside at the G1/S boundary, S phase and the G2/M boundary of the cell cycle. Activation of any can be initiated in order to inhibit the progress of a cell through the cell cycle.

Following the induction of DNA damage, the ATM and ATR kinases are recruited to sites of damage where they initiate the DNA damage checkpoint pathway (Jazayeri et al., 2006; Lee & Paul, 2004; Smith et al., 2010). Following their recruitment and subsequent activation, ATM and ATR phosphorylate a number of downstream proteins depending on the phase of the cell cycle. Within the G1 phase of the cell cycle, ATM and ATR activate p53, which leads to the upregulation of p21. p21 is a cyclin-dependent kinase inhibitor mainly acting on Cdk2 which impairs the cell from progressing through the G1/S phase boundary (Agarwal et al., 1995; Bartek & Lukas, 2001). If the DNA damage occurs within the S phase of the cell cycle, ATM and ATR phosphorylate Chk2 and Chk1 respectively, leading to the phosphorylation and subsequent degradation of phosphatase Cdc25A (Finn et al., 2012; Houtgraaf et al., 2006; Smith et al., 2010). The degradation of
Cdc25A impairs its phosphatase action leading to the inactivation of Cdk2 and preventing the progression through the S phase (Finn et al., 2012; Houtgraaf et al., 2006). Finally, if the DNA damage occurs within the G2 phase of the cell cycle, ATM and ATR phosphorylate Chk2 and Chk1 in similar fashion, which in turn phosphorylate Cdc25 (Fernandez-Capetillo et al., 2002; Finn et al., 2012; Houtgraaf et al., 2006). This phosphorylation event inhibits the phosphatase function of Cdc25, where it is then unable to dephosphorylate the Cdk1-cyclin B complex, preventing the cell from progressing into M phase.

Cell cycle checkpoints are a crucial mechanism in place to allow the cell to repair DNA damage before the cell divides. If DNA damage checkpoints were not activated, the cell would be able to replicate the damaged DNA and divide, passing along DNA mutation to daughter cells.

1.8 Objectives and Significance

TRF2 has been shown to play an important role in a number of functions within the cell, including telomere maintenance, telomere protection, and DNA damage response. Previously, it has also been shown that the post-translational modifications of TRF2 regulate its functions. Based on this, I asked whether the candidate phosphorylation sites T317 and S323 of TRF2 played a role in regulating the function of TRF2 in cellular proliferation, DNA damage response, telomere length maintenance and ALT activity.

Serine 323 of TRF2 has been identified as a potential cyclin dependent kinase phosphorylation site as it fits consensus S/TP (Chi et al., 2008). Cyclin dependent kinases have been shown to have an extensive role within the cell, from regulating cell cycle
progression, to its involvement in the early stages of telomerase-mediated telomere elongation in yeast. Threonine 317 of TRF2 has also been identified as a potential ATM phosphorylation site through mass spectrometry since it fits the consensus S/TQ phosphorylation sequence of ATM (X.-D. Zhu, unpublished date).

Major findings presented in this thesis include the investigation of candidate phosphorylation sites T317 and S323 of TRF2 in cellular proliferation, telomere length maintenance, DNA damage response and ALT activity.
**Figure 1:** The Shelterin Complex. Schematic representation of the six-protein subunit shelterin and its interaction with telomeric DNA (de Lange, 2010).
Figure 2: Domain Structure and Post-translational Modifications of TRF2. Schematic representation of the major domains of TRF2. The first and last amino acids of each domain are indicated. Phosphorylation sites are indicated in black, ubiquitylation sites in red, SUMOylations sites in green and arginine methylation sites in blue. Diagram is not drawn to scale.
Chapter 2

2 Methods and Materials

2.1 Plasmids and Antibodies

Expression constructs for various mutant TRF2 (T317A, T317D, S3232 and S323D) alleles were generated by John Walker through site directed mutagenesis using a wild-type TRF2 template. Antibody against TRF2 was a gift from Titia de Lange, Rockefeller University. Antibodies obtained commercially are as followed, c-Myc (9E10, Calbiochem), Rabbit anti-PML (Abcam), Mouse-anti-PML (Abcam), γ-tubulin (GTU88, Sigma), H3-pS10 (Cell Signaling).

2.2 Cell Culture and Retroviral Infections

Parental cell lines, including Phoenix A, HeLaII, HT1080, and GM847 were grown in Dulbecco’s modified Eagle medium containing 5% fetal bovine serum, 1% non-essential amino acids, 20mM L-glutamine, 100U/mL penicillin and 0.1 mg/mL streptomycin. Cells expressing stably integrated pLPC constructs were grown in media containing 2μg/ml puromycin. Cells were grown at 37°C, 5% CO2 and 100% humidity.

Plasmid DNA transfection was performed with Liptofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Retroviral infection was completed as previously described (Karlseder et al., 2002). In belief, 2x10^6 phoenix cells were seeded on 6-cm plates 24 hours prior to transfection. The recipient cell lines GM847 (7x10^5) cells, HeLaII (8x10^5) and HT1080 (2.5x10^5) cells were seeded on 10-cm plates on the same day of transfection. Retroviral infections were carried out at 36, 48, 54, and 60 hours post transfection as previously described (McKerlie & Zhu, 2011; McKerlie et al., 2012).
For HeLaII long term growth curves, Beckman Z1 Counter © Particle Counter was utilized and 5x10^5 cells were seeded on 10-cm plates every four days in selection media. For HT1080 long term growth curves, 2.5x10^5 cells were seeded on 10cm plates every three days in selection media. Population doublings (PDs) were calculated from the formula 2^n a = b, where “a” is the initial number of cells seeded, “b” is the final number of cells following 3 day incubation period and “n” is the number of PD.

### 2.3 Protein Extracts and Immunoblotting

Protein extracts were obtained as previously described (McKerlie et al., 2013). In brief, cells were trypsinized, washed, collected and counted utilizing the Beckman Z1 Counter © Particle Counter. Cells were subsequently spun at 1000rpm for 5 minutes at 4°C in Beckman Coulter Allegra Z-15R Centrifuge and re-suspended in 1ml of cold 1xPBS and transferred to eppendorf tubes. Cells were then washed twice in 1ml of cold 1xPBS and spun down at 3000 rpm for 2 min at 4°C in microcentrifuge (Eppendorf 5415C). Cell pellets were re-suspended in buffer C-420 [20mM HEPES buffer (pH7.9), 25% glycerol, 5 mM MgCl, 0.2% Nonidet P-40, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 µg/mL aprotinin, 10 µg/mL pepstatin, 1 µg/mL leupeptin and 420mM KCl] in a final concentration of 2x10^4 cells/µl. Cell mixture was incubated on ice for 30 minutes. Cells were then spun at 14,000 rpm for 10 min at 4°C in microcentrifuge (Eppendorf 5415C). Supernatants were collected and re-suspended in 2xLaemmlli buffer for a final concentration of 1x10^4 cells/µl. Protein extracts were stored at -20°C.

Immunoblotting was performed as previously described (McKerlie et al., 2013).
Protein extracts were separated on 8% SDS-PAGE gels, followed by the transfer of protein to nitrocellulose membranes, which were immunoblotted with indicated antibodies.

### 2.4 Immunofluorescence

Immunofluorescence was performed as described previously (Batenburg et al., 2012; McKerlie et al., 2013; Mitchell et al., 2009). In brief, cells were grown on coverslips and fixed in PBS-buffered 3% paraformaldehyde for 10 minutes at room temperature (RT), washed three times in PBS for 5 min and permeabilized in 0.5% Triton X-100 in PBS for 10 minutes at RT. Coverslips were incubated for 30 min in blocking buffer containing 0.2% cold water fish gelatin (Sigma) and 0.5% Albumin (Sigma) in PBS. Coverslips were then incubated in primary antibody for 2 hours at RT, followed by three PBS washes for 5 min each at RT. Coverslips were then incubated in secondary antibody for 45 minutes at RT followed by 3 washes in PBS for 5 min at RT. DNA was stained utilizing 4, 6-diamidino-2-phenylindole (DAPI; 0.2 µg/ml) and embedded on glass slides in 90% glycerol/10% PBS containing 1mg/ml p-phenylene diamine (Sigma). Cells were visualized on a Zeiss Axioplan 2 microscope. Images were captured utilizing a Hammamatsu C4742-95 camera and processed with Openlab software package.

### 2.5 Genomic DNA Isolation and Digestion

Cells were collected from two confluent 10cm plates by scrapping and spun at 1000rpm for 5 min at 4°C in Beckman Coulter Allegra Z-15R Centrifuge. Cell pellets (50-100ul) were stored at -80°C until further processing. Phenol-chloroform was used to
isolate genomic DNA. Cells pellets were first resuspended in 1ml 1xTNE buffer and then mixed with 1 ml freshly-made TNES/proteinase K buffer. The mixture was incubated O/N at 37\(^{0}\)C in 15-ml phase lock gel heavy tubes (Eppendorf). An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was then added, followed by gentle shaking until phases were completely mixed. Samples were then spun at 3000rpm for 10 min at 4\(^{0}\)C in Beckman Coulter Allegra Z-15R Centrifuge. The aqueous phase was transferred to a new phase lock tube where the previous step was repeated. Genomic DNA was fished out following mixing the aqueous phase with 2ml iso-propanol and 220µl 2 M NaAc (pH5.5). Genomic DNA was resuspended in 300µl 1xTNE buffer containing 100µg/ml RNase A and incubated for 30 min at 37\(^{0}\)C. Genomic DNA was mixed again with cut blue pipette tip and incubated for an additional 2 hours at 37\(^{0}\)C. Following the addition of 300µl 1xTNES/proteinase K buffer, genomic DNA mixture incubated for an additional 1 hour at 37\(^{0}\)C. Genomic DNA was extracted with 600µl phenol/chloroform/isoamyl alcohol (25:24:1) and isolated with 600µl isopropanol and 66µl 2M NaAC (pH5.5). Isolated genomic DNA pellets were resuspended in TE buffer (pH8.0) at 37\(^{0}\)C for 30 min.

Isolated Genomic DNA was digested with RsaI and Hinf1 and the DNA concentrations were measured by fluorimeter using Hoechst dye. Digested DNA with a range of concentrations between 100µg/µl and 500µg/µl was used for further analysis.

### 2.6 In-Gel Hybridization

Four micrograms of digested genomic DNA was run on a 0.7% agarose gel in 0.5xTBE buffer. The gel was run first at 30V for one hour and then at 40V O/N. Gels
were then dried for 2 hours at 50°C. For detection of double stranded telomeric DNA, gels were denatured in denaturing solution (3M NaCl, 0.3M Sodium Citrate) for 30 min and subsequently neutralized by washing in neutralizing solution (3M NaCl, 0.5M Tris-HCl, pH 7.0) twice for 15 minutes each at RT. Gels were rinsed in H₂O for 3 minutes and pre-hybridized in Churchmix (0.5 M NaPi (pH7.2), 1mM EDTA (pH 8.0), 7% SDS, 1% BSA) for 1 hour at 55°C. Gels were then hybridized in Churchmix containing TelC₄ y-32PdTTP (CCCTAA₄) radioactive probe O/N at 55°C. On the following day, gels were washed twice with 4x SSC for 30 min with a final wash of 4x SSC with 0.1% SDS for 30 min. Gels were then exposed to PhosphorImager screens O/N. Gels were scanned using STORM 820 molecular dynamics PhosphorImager. Median length of telomeres was determined using ImageQuant and Microsoft Excel software programs.

2.7 Clonogenic Survival and G2/M Checkpoint Assay

Clonogenic survival and G2/checkpoint assays were performed as previously described (McKerlie & Zhu, 2013). For the clonogenic survival assay, HeLaII cells stably expressing various mutant TRF2 alleles were collected, counted, and irradiated with 1, 3 or 5Gy. IR was delivered by a Cs-137 source at McMaster University (Gammacell 1000). 120 cells (0-3Gy) or 720 cells (5Gy) were then seeded on 6-cm plates, followed by replacement with fresh media 24 hours post seeding. Cells were allowed to grow for ten days before they were fixed and stained with a solution containing 50% methanol, 7% acetic acid and 0.1% Coomassie blue at RT for 10min. Colonies of greater then 32 cells were then scored as positive.
To perform the G2/M checkpoint assay, cells were seeded on cover slips 24 hours prior to the treatment with 12Gy IR. Following ionizing radiation, cells were allowed to recover in incubator for 1 hour. Cells were subsequently washed in PBS and fixed with 3% paraformaldehyde and processed for IF with anti-H3-pS10 antibody.
Chapter 3

3 Results

3.1 Analysis of the role of candidate phosphorylation sites T317 and S323 of TRF2 in the regulation of cell proliferation

TRF2 plays an important role in telomere protection and DNA damage response. Lack of TRF2 causes DNA abnormalities such as end-to-end fusions, which can lead to cellular senescence (van Steensel et al., 1998). Mass spectrometry analysis has suggested that TRF2 may be phosphorylated at T317 and S323 (Chi et al., 2008; X.-D. Zhu, unpublished data). T317 of TRF2 is thought to be a potential ATM kinase phosphorylation site as it fits the consensus TQ, whereas, S323 of TRF2 is believed to be a potential CDK phosphorylation site, matching the consensus SP (X.-D. Zhu, unpublished date). Mutant alleles for both phosphorylation sites included a nonphosphorylatable and phosphomimic mutant, in which the amino acids at position 317 or 323 were mutated to alanine or aspartic acid. To investigate if the candidate phosphorylation sites T317 and S323 of TRF2 are important in the regulation of cellular proliferation, HT1080 and HeLaII cell lines stability expressing the vector alone, wild-type TRF2 or TRF2 mutant alleles were subjected to analysis of long-term growth. Multiple cell lines were utilized during this experiment to verify initial results and to confirm that any change in growth was not cell type specific.

As seen in figure 3A, HT1080 cells stably expressing mutant and wild-type TRF2 alleles (Figure 3C) were grown for over 160 population doublings with no significant change in growth rates between the nonphosphorylatable (TRF2-323A/TRF2-317A),
phosphomimic (TRF2-323D/TRF2-317D) and control cell lines. Following these results, new HT1080 and HeLaII cell lines stably expressing mutant TRF2-S323 alleles were utilized to complete additional long-term growth curves (Figure 3D and 4B). As seen in figure 3B and 4A, no change in growth rates were seen over numerous population doublings in both HT1080 and HeLaII cell lines. These results suggest that the candidate phosphorylation sites T317 and S323 of TRF2 are not involved in the regulation of cellular proliferation.
Figure 3: Candidate phosphorylation sites T317 and S323 of TRF2 are not involved in the regulation of cellular growth in HT1080 cells. (A-B) Long-term growth curves of HT1080 cells stably expressing various TRF2 alleles as indicated. (C) Western analysis of HT1080 cells expressing various alleles of TRF2 utilized in (A). Immunoblotting was performed with anti-Myc antibody. The γ-tubulin blot was utilized as loading control. (D) Western analysis of HT1080 cells expressing various alleles of TRF2 utilized in (B). Immunoblotting was performed with anti-Myc antibody. The γ-tubulin blot was utilized as loading control.
An M. Sc.

$\gamma$ Tubulin

Vector

TRF2

317A

317D

323A

323D

A

B

C

D

Vector TRF2 317A 317D 323A 323D

Anti Myc

Anti $\gamma$-Tubulin

Vector TRF2 323A 323D

Anti Myc

Anti $\gamma$-Tubulin
Figure 4: Candidate phosphorylation site S323 of TRF2 is not involved in the regulation of cellular growth in HeLaII cells. (A) Long-term growth curve of HeLaII cells expressing various TRF2 alleles as indicated. (B) Western analysis of HeLaII cells expressing various alleles of TRF2. Immunoblotting was performed with anti-Myc antibody. The γ-tubulin blot was utilized as loading control.
A

![Graph showing population doubling vs passage number for different groups: Vector, TRF2, 323A, 323D.](image)

B

![Western blots for Anti Myc and Anti γ-Tubulin showing expression levels for different groups: Vector, TRF2, 323A, 323D.](image)
3.2 Analysis of the role of candidate phosphorylation sites T317 and S323 of TRF2 in telomere maintenance

As previously stated, TRF2 is a negative regulator of telomere length since overexpression of TRF2 causes a reduction in telomere length in both telomerase-positive and telomerase-negative cells (Karlseder et al., 2002; Munoz et al., 2006; Smogorzewska et al., 2000; van Steensel et al., 1998). To further evaluate the potential role of candidate phosphorylation sites T317 and S323 of TRF2 in telomere length maintenance, telomere length dynamics were analyzed for HT1080 and HeLaII cells stably expressing the vector alone, Myc-tagged wild-type TRF2, and Myc-tagged TRF2 carrying a T317A, T317D, S323A or S323D mutation.

Initial results suggest that the candidate phosphorylation site T317 of TRF2 does not promote telomere elongation (Figure 5A-C). However, HT1080 cells expressing the phosphomimic TRF2-S323D allele showed a slight telomere elongation phenotype when compared to cells expressing the nonphosphorylatable TRF2-S323A allele (Figure 6A-C). This suggests that the candidate phosphorylation site S323 of TRF2 might be involved in a mechanism promoting telomere elongation.

In order to confirm initial results and to ensure that the observed increase in telomere length seen in HT1080 cell expressing the phosphomimic TRF2-S323D allele was not cell type specific, genomic DNA from new HT1080 and HeLaII cells expressing wild-type and mutant TRF2-S323 alleles (Figure 6F and 7C) was utilized to perform additional in gel-hybridizations (Figure 6D and 7A). Following the quantification of median telomere length, expression of the phosphomimic TRF2-S323D allele in both HT1080 and HeLaII cells did not induce telomere elongation (Figure 6E and 7B). These results
suggest that the previously observed telomere elongation phenotype within HT1080 cells expressing the phosphomimic TRF2-S323D allele may have been caused by the phosphorylation event itself.

Unfortunately, the TRF2 overexpressing cell lines, which acted as the wild-type control within these experiment did not display the well-characterized phenotype of telomere shortening (Ancelin et al., 2002; Karlseder et al., 2002; Munoz et al., 2006; Smogorzewska et al., 2000). This prevents me from drawing firm conclusions from these experiments, which suggested that the candidate phosphorylation sites T317 and S323 of TRF2 might not be involved in telomere length maintenance in telomerase-positive cells. Additional experiments will be required to definitively conclude the role of TRF2 candidate phosphorylation sites T317 and S323 in telomere length maintenance.

Potential explanations for the lack of telomere shortening in TRF2 overexpressing cells as well as further experiments to definitively conclude whether the candidate phosphorylation sites T317 and S323 of TRF2 play a role in telomere length maintenance will be further discussed in later sections.
**Figure 5:** Analysis of the role of TRF2 candidate phosphorylation site T317 in the regulation of telomere elongation in HT1080 cells. (A) Genomic blot of telomeric restriction fragments from HT1080 cells expressing various TRF2 alleles as indicated. Population doublings are indicated above the lanes, whereas DNA molecular weight markers are shown to the left of the blot. (B) Median telomere length of indicated cell lines was plotted against PDs. (C) Western analysis of HT1080 cells expressing various mutant alleles of TRF2 at PD 2 and 60. Immunoblotting was performed with anti-Myc antibody. The γ-tubulin blot was utilized as loading control.
A

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B

Median Telomere Fragment Length (kb)

Population Doubling

Vector  TRF2  317A  317D

C

Anti Myc

Anti γ-tubulin

Vector PD2  Vector PD60  TRF2 PD2  TRF2 PD60  317A PD2  317A PD60  317D PD2  317D PD60
Figure 6: Analysis of the role of TRF2 candidate phosphorylation site S323 in the regulation of telomere elongation in HT1080 cells. (A) Genomic blot of telomeric restriction fragments from HT1080 cells expressing various TRF2 alleles as indicated above the lanes. PD is indicated above the lanes, whereas DNA molecular weight markers are shown on the left of the blot. (B) Median telomere length of indicated cell lines was plotted against PD. (C) Western analysis of HT1080 cells expressing various mutant alleles of TRF2 at PD 2 and 60. Immunoblotting was performed with anti-Myc antibody. The γ-tubulin blot was utilized as loading control. (D) Genomic blot of telomeric restriction fragments from HT1080 cells expressing various TRF2 alleles as indicated above the lanes. PD is indicated above the lanes, whereas DNA molecular weight markers are shown on the left of the blot. (E) Median telomere length of indicated cell lines was plotted against PD. (F) Western analysis of HT1080 cells expressing various mutant alleles of TRF2 at PD 2 and 60. Immunoblotting was performed with anti-Myc antibody. The γ-tubulin blot was utilized as loading control.
**Figure 7:** Analysis of the role of TRF2 candidate phosphorylation site S323 in the regulation of telomere elongation in HeLaII cells. (A) Genomic blot of telomeric restriction fragments from HeLaII cells expressing various TRF2 alleles as indicated above the lanes. PD is indicated above the lanes, whereas DNA molecular weight markers are shown on the left of the blot. (B) Median telomere length of indicated cell lines was plotted against PD. (C) Western analysis of HeLaII cells expressing various mutant alleles of TRF2 at PD 2 and 60. Immunoblotting was performed with anti-Myc antibody. The γ-tubulin blot was utilized as loading control.
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B

C

Median Telomere Fragment Length (kb)

Population Doublings

Vector  TRF2  323A  323D

Anti Myc

Anti γ-Tubulin
3.3 Candidate phosphorylation sites T317 and S323 of TRF2 are not involved in the regulation of TRF2 association with APBs

TRF2 is required for the formation of APBs in ALT cells, as APB formation is inhibited following the knockdown of TRF2 (Jiang et al., 2007). As seen in figure 8A, three staining patterns can be visualized through immunofluorescence of GM847 cells overexpressing mutant and wild-type Myc-tagged TRF2 alleles. These include dim pan-nuclear staining, APB-like and punctate telomeric foci. To investigate if the candidate phosphorylation sites T317 and S323 of TRF2 regulate its association with APBs in telomerase-negative cells, immunofluorescence utilizing GM847 cells stably expressing wild-type and mutant TRF2 alleles was performed (Figure 8B). To analyze the recruitment of TRF2 to APBs, three categories of APB foci were established, including small telomere-like foci, medium round foci, and large, bright rounded foci. All foci could be seen within the nucleus of interphase cells. Nuclei containing one or more distinct medium or large Myc-tagged TRF2 foci fully encapsulated by a PML structure were scored as positive. Representative immunofluorescent images of large TRF2/PML foci can be seen in figure 9. Myc-tagged TRF2 and PML colocalization was not affected by the introduction of either a nonphosphorylatable or phosphomimic mutation of T317 and S323 of TRF2 (Figure 8C). These results suggest that the candidate phosphorylation sites T317 and S323 of TRF2 do not control its association to APBs in telomerase-negative cells. To investigate if the lack of change in TRF2 association with APBs in GM847 cell lines might be caused by alterations in the expression levels of Myc-tagged TRF2 mutant alleles, Myc positive cells were analyzed. As seen in figure 8D, no substantial differences in the percentage of cells positive for Myc staining was observed.
among the different cell lines. These results further support the notion that the candidate phosphorylation sites T317 and S323 of TRF2 are not involved in its association with APBs.
Figure 8: Analysis of the role of candidate phosphorylation sites T317 and S323 of TRF2 in its association with APBs. (A) Indirect Immunofluorescence of GM847 cells utilizing anti-Myc antibody. Cell Nuclei were stained with DAPI in blue. (B) Western analysis of GM847 cells expressing various mutant alleles of TRF2. Immunoblotting was performed with anti-Myc antibody. The γ-tubulin blot was utilized as loading control. (C) Quantification of GM847 cells positive for co-localization events between Myc-tagged TRF2 and PML. (D) Quantification of GM847 cells positive for Myc-tagged TRF2.
A

**Anti-Myc**

**Dapi**

Dim, Pan-Nuclear

APB-like

Telomere Like

B

**Anti-Myc**

**Anti γ-Tubulin**

C

![Graph: Myc-PML colocalization events vs Cell Type]

D

![Graph: Myc Positive Cells vs Cell Type]
Figure 9: Analysis of the localization of various Myc-tagged TRF2 alleles in GM847 cells. (A) Indirect Immunofluorescence on GM847 cells with anti-Myc and anti-PML antibodies. Cell nuclei were stained with DAPI.
A

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M.Sc. Thesis – K. Reinschild-Lindsay; McMaster University - Biology
3.4 Analysis of the role of candidate phosphorylation sites T317 and S323 of TRF2 in DNA damage response and repair

One of the main functions of TRF2 is its involvement in the DNA damage response pathway of a cell. As previously described, following the induction of DNA double strand breaks, TRF2 is phosphorylated by ATM where it is recruited to sites of damage, promoting the fast pathway of DNA double strand break repair (Bradshaw et al., 2005; Huda et al., 2009; Tanaka et al., 2005). The overexpression of TRF2 has also been shown to promote the repair mechanism homologous recombination (Mao et al., 2007). To investigate if the candidate phosphorylation sites T317 and S323 of TRF2 promote DNA double strand break repair, colony survival assays were performed utilizing HT1080 cells expressing mutant and wild-type TRF2 alleles (Figure 3C). As seen in figure 10A, HT1080 cells expressing the nonphosphorylatable, and phosphomimic T317 alleles of TRF2 did not show a significant change in cell survival when exposed to 5 Gy of ionizing radiation (IR). However, as seen in 10B, HT1080 cells overexpressing the phosphomimic S323D alleles of TRF2 displayed improved cell survival following the induction of IR-induced DNA double strand breaks when compared to cells expressing wild-type and the nonphosphorylatable S323A allele of TRF2.

Unfortunately, even though the data suggests that the candidate phosphorylation site S323 of TRF2 might be involved in promoting cell survival following induction of DNA damage, no strong conclusion can be given. This is a result of a number of circumstances. Firstly, within this experiment, wild-type TRF2 overexpressing cell lines showed sensitivity to IR induced DNA double strand breaks when compared to the vector control. This is uncharacteristic, as previous reports have shown that the overexpression of TRF2
in HT1080 cells did not cause a sensitivity in cell survival following the induction of DNA double strand breaks (Huda et al., 2009). In addition, data presented in figure 10A and 10B represents a single colony survival experiment completed in triplicate. Additional assays were performed in the hopes of reconfirming initial results, unfortunately, due to the lack of colony formation, as well as concerns about overlapping colonies, these experiments were unsuccessful.

Given the issues presented above, no definitive conclusion can be drawn regarding the role of candidate phosphorylation sites T317 and S323 of TRF2 in cell survival following episodes of DNA damage.

In addition to examining the candidate phosphorylation sites of TRF2 and their potential role in cell survival following induction of DNA damage, the effect of TRF2 phosphorylation at S323 on G2/M checkpoint activation following the induction of DNA double strand breaks was analyzed. In order to analyze G2/M checkpoint activation, immunofluorescence utilizing an antibody against H3-pS10 was performed. H3-pS10 is a marker for cells within mitosis, as H3 is phosphorylated at S10 during the onset of mitosis, where it aids in chromosome condensation (Goto et al., 1999; Gurley et al., 1978). As seen in figure 10C, cells expressing the nonphosphorylatable TRF2-S323A allele were not impaired in the activation of the G2/M checkpoint following the induction of IR-induced DNA double strand breaks. This suggests that the candidate phosphorylation site S323 of TRF2 does not affect the activation of the G2/M checkpoint following induction of DNA damage.
Figure 10: Analysis of the role of candidate phosphorylation sites T317 and S323 of TRF2 in DNA damage response and repair. (A-B) Clonogenic survival assays of IR treated HT1080 cells stably expressing various TRF2 alleles as indicated. (C) Quantification of the percent of HeLaII cells expressing wild-type and mutant TRF2 alleles stained positive for H3-pS10 staining.
Chapter 4

4 Discussion and Future Direction

4.1 Candidate phosphorylation sites T317 and S323 of TRF2 are not involved in the regulation of cell proliferation

TRF2 is widely known for its role in telomere protection and telomere length maintenance (Karlseder et al., 1999; Karlseder et al., 2002; Munoz et al., 2006; van Steensel et al., 1998). Both functions can have implications on cell growth, as telomere abnormalities, such as end-to-end fusions and critically short telomeres can trigger cellular senescence. The overexpression of TRF2 has been shown to reduce the senescence set point of cells, as TRF2 overexpressing cells continued to grow with critically short telomeres past their normal senescent set point (Karlseder et al., 2002). This overexpression of TRF2 is believed to protect critically short telomeres from end-to-end fusions, resulting in prolonged proliferative capabilities. This suggests that replicative senescence is triggered by a change in the protective status of critically short telomeres and not the loss of telomeric DNA (Karlseder et al., 2002).

Given that the post-translational modifications of TRF2 have been shown to have an influence on its function, the role of candidate phosphorylation sites T317 and S323 of TRF2 on cellular proliferation was investigated. As previously described in Results, the phosphorylation of TRF2 at T317 and S323 is not required to facilitate cell growth. Since the results throughout multiple long-term growth curves are consistent, no further action is required to evaluate the role of these candidate phosphorylation sites of TRF2 in cell growth.
4.2 Inconsistencies in control phenotypes prevents a conclusions from being drawn
in regards to the role of TRF2 candidate phosphorylation sites at T317 and S323
in telomere length dynamics

TRF2 is widely known as a negative regulator of telomere length (Karlseder et al., 2002; Munoz et al., 2006; Smogorzewska et al., 2000; van Steensel et al., 1998). However, the exact mechanism by which it regulates telomere length is unknown. In the hopes to further understand and characterize the role of TRF2 in telomere length maintenance, the affects of the candidate phosphorylation sites T317 and S323 of TRF2 on telomere length dynamics was investigated. As previously outlined in Results, the phosphorylation of TRF2 at T317 and S323 did not alter telomere length dynamics in either HT1080 or HeLaII cells. Unfortunately, concerns arose regarding these results, as cells overexpressing wild-type TRF2 did not display the well-characterized phenotype of telomere shortening. This well-characterized phenotype has been visualized within a number of cell lines, including hTERT-BJ-LT, IMR90 and HTC75 cells, as well as in TRF2 overexpressing mice (Karlseder et al., 2002; Munoz et al., 2006; Munoz et al., 2005; Smogorzewska et al., 2000; van Steensel et al., 1998, Wu et al., 2007). Even though the expression of mutant TRF2 alleles did not induce an altered telomere length phenotype when compared to wild-type cell lines, it is difficult to fully support the conclusion that the candidate phosphorylation sites T317 and S323 of TRF2 do not affect telomere length dynamics, due to the discrepancy in telomere length phenotype of previously-reported TRF2-overexpressing cell lines and TRF2 overexpressing cells in this study.
Previously, others have reported altered telomere length dynamics in TRF2 overexpressing HTC75 cells that have been cultured for extended periods of time (Smogorzewska et al., 2000). In this study, prolonged culturing of TRF2 overexpressing HTC75 cells resulted in altered telomere length dynamic, ultimately leading to an increase in telomere length in late passage cells when compared to starting telomere lengths (Smogorzewska et al., 2000). However, this was attributed to the loss TRF2 expression due to a decline in steady-state levels of TRF2 as well as a reduction in the expression levels of individual cells. In order to analyze whether this phenomenon influenced my results, additional experiments need to be completed, including immunofluorescence of the mutant HT1080 and HeLaII cell lines in order to visualize any alterations in Myc-tagged TRF2 expression between early and late passage cells.

While evaluating telomere length dynamics in the wild-type and mutant HeLaII cell lines, two subpopulations of cells were visualized, each displaying different telomere length dynamics. As seen in figure 7A, one subpopulation of cells display a telomere shortening phenotype, while a separate population of cells sustain their telomere length throughout the experiment. This phenomenon has previously been reported in both mice and human cell lines (Cabuy et al., 2004, Wu et al., 2007). The cause of these two subpopulations in regards to telomere length dynamics is unclear. One hypothesis that can be investigated is whether the candidate phosphorylation sites T317 and S323 of TRF2 lead to the development of these subpopulations. To investigate this, telomere length must be examined through flow-FISH, as southern blots are unable to establish if the bulk of elongated telomeric DNA originated from chromosomes with exceptionally long telomeres which do not necessarily belong to a different subpopulation of cells.
(Cabuy et al., 2004). However, this phenomenon could also be an artifact of the experiment, brought on by the long-term growth of the cells. During long-term growth, additional mutations could have developed due to the inherent instability of cancer cell lines. Continual cell divisions increase the likelihood mutations, which could cause an altered telomere length phenotype within a subpopulation of cells.

In summary, due to inconsistencies between work presented in this thesis and previously published results, no definitive conclusion can be presented for the role of candidate phosphorylation sites T317 and S323 of TRF2 in telomere length maintenance.

4.3 Candidate phosphorylation sites T317 and S323 of TRF2 are not involved in the regulation of TRF2s association with APBs

In order to counteract the end replication problem, cancer cells have developed mechanisms in order to elongate their telomeres, ultimately allowing them to bypass their Hayflick limit. One such mechanism, which is utilized by roughly 10% of cancer cells, is a homologous recombination based mechanism termed alternative lengthening of telomeres (ALT) (Bryan et al., 1995; Dunham et al., 2000; Lundblad & Blackburn, 1993). This homologous-recombination based mechanism of telomere maintenance is believed to occur within APBs (Draskovic et al., 2009).

TRF2 is known to localize within APBs and has been shown to be required for their formation (D’Alcontres et al., 2007; Jiang et al., 2007). The function of TRF2 at APBs is facilitated through its post-translational modification. TRF2, along with other members of the shelterin complex are SUMOylated by MMS21, promoting APB formation (Potts & Yu, 2007). Impairment of this post-translational modification results in
a reduction in APB formation and progressive telomere shortening (Potts & Yu, 2007). Telomere bound TRF2 has been shown to substantially decrease while telomeres are housed within APBs (Osterwald et al., 2015). The binding of TRF2 to telomeres within APBs is believed to be regulated through the SUMOylation of TRF2 by MMS21 (Osterwald et al., 2015). This lack of TRF2 at telomeres within APBs is believed to trigger the activation of ATM, promoting the homologous recombination based telomere elongation mechanism (Osterwald et al., 2015). Taking into account that the post-translational modification of TRF2 regulates its role at APBs, I set out to determine whether the candidate phosphorylation sites T317 and S323 of TRF2 influence its association with APBs. Unfortunately, as described in results, the candidate phosphorylation sites T317 and S323 of TRF2 are not involved in the regulation of its association with APBs in GM847 cells.

Even though the candidate phosphorylation sites T317 and S323 of TRF2 did not alter the association of TRF2 with APBs, there are additional ALT characteristics that can be evaluated to determine whether these candidate phosphorylation sites affect ALT activity. This includes studying the formation of C-circles and T-SCE events in the mutant GM847 cell lines. C-circles and T-SCE are well-defined ALT characteristics and have previously been utilized as markers for overall ALT activity. C-circle amplification assays and gels can be run to determine whether the candidate phosphorylation sites T317 and S323 of TRF2 promote C-circle formation. In addition, T-SCE levels can be assessed through CO-FISH utilizing GM847 cells overexpressing mutant and wild-type TRF2 alleles. Through these two avenues of investigation, a better understanding of the potential role of the candidate phosphorylation sites of TRF2 on ALT activity can be
4.4 Additional work is needed to assess the role of candidate TRF2 phosphorylation sites T317 and S323 in cell survival following the induction of DNA damage

One of the main functions of TRF2 is its involvement in the DNA damage response of a cell, as TRF2 is phosphorylated and recruited to sites of DNA damage where it aids in the fast pathway of DNA double strand break repair (Bradshaw et al., 2005; Huda et al., 2009; Tanaka et al., 2005). Since the phosphorylation of TRF2 promotes its function in the DNA damage response pathway, the role of candidate phosphorylation sites T317 and S323 of TRF2 in cell survival following induction of DNA damage was analyzed. As previously outlined in Results, the phosphorylation of TRF2 at S323 showed an increase in cell survival following the induction of IR-induced DNA double strand breaks when compared to the nonphosphorylatable mutant. Unfortunately, no conclusion can be supported as the reliability and reproducibility of the results have yet to be determined.

A number of issues have prevented a definitive conclusion from being presented. The first issue is the lack of consistency in regards to the wild-type control cell line, as the phenotype visualized following induction of DNA damage does not agree with that of previously published work (Karlseder et al., 2002; Munoz et al., 2006; Smogorzewska et al., 2000; van Steensel et al., 1998). Work presented in this thesis suggested the overexpression of TRF2 leads to impaired cell survival following IR-induced DNA damage when compared to the vector control. However, this is uncharacteristic, as previous reports have shown that the overexpression of TRF2 does not result in impaired
cell survival following the induction of DNA double strand breaks (Huda et al., 2009). In an attempt to substantiate such a change, a number of hypotheses can be proposed in order to rationalize the difference in cell survival dynamics of TRF2 overexpressing cells. Differences in techniques, such as culturing and irradiation protocols could account for the change in cell survival following the induction of DNA double strand breaks. In previously published work, a number of differences in protocols can be noted. Previous studies utilized cell lines that took advantage of the inducible Tet-Off expression system as well as utilizing X-rays instead of a Cs-137 source to irradiate cells (Huda et al., 2009).

TRF2 overexpression has also previously been shown to reduce the nuclear accumulation of phosphorylated ATM, H2AX and p53 within the first hour post IR treatment (Bradshaw et al., 2005). This reduction in phosphorylated ATM, H2AX and p53 would have a negative effect on the repair of DNA double strand breaks. Within TRF2 overexpressing cells, it has also been shown that TRF2 is recruited to sites of DNA damage 1-2 second’s prior to ATM (Bradshaw et al., 2005). This suggests that TRF2 competes or attenuates the ATM DNA damage response directly following double strand break induction (Bradshaw et al., 2005). These effects of TRF2 overexpression could also account for the sensitivity of TRF2 overexpressing cells to DNA damage seen in this thesis.

The second issue preventing any conclusion from being reported for the role of candidate phosphorylation sites T317 and S323 of TRF2 on cell survival following DNA damage is the overall lack reproducibility of experiments. As previously described, results from only one colony survival assay could be utilized. Many attempts were made
to duplicate this experiment, however, due to an inability to successfully evaluate colony formation, none of the repeat experiments were considered. In the majority of repeat experiments, the lack of colony formation prevented these experiments from being utilized. Even though no intentional alterations were made to the protocol, a number of reasons can be hypothesized for the lack of colony formation, including alterations in plating and irradiation techniques, as well the reagents utilized for the separate experiments. Some repeat experiments did produce colonies; however, a true representation of colony formation was unattainable, as the size of colonies on a given plate varied dramatically. This is believed to result from a colony forming from more than one cell. In order to resolve this issue, the number of cells initially seeded would need to be adjusted in order to reduce the amount of colonies being formed by multiple cells.

In the hopes to further evaluate a potential role for the candidate phosphorylation sites of TRF2 following the induction of DNA double strand breaks, additional avenues besides cell survival could be analyzed. Phosphorylated TRF2 has previously been shown to have a number of functions within the DNA damage response pathway, from regulating γH2AX kinetics, to the formation of single strand overhangs (Huda et al., 2009; Mao et al., 2007). TRF2 has also been shown to influence the repair pathway choice, as the overexpression of TRF2 promotes the repair mechanism homologous recombination (Moa et al., 2007). This provides a number of avenues that can be addressed in order to further evaluate whether the candidate phosphorylation sites T317 and S323 of TRF2 might be involved in the DNA damage response of a cell.

The formation of single strand overhangs, which is a crucial step within the repair
mechanism homologous recombination, can be assessed within the mutant TRF2 cell lines though immunofluorescence utilizing antibodies against Rad51 and RPA. These proteins are known to directly bind to the single strand overhangs created during HR, where they help stabilization and promote strand invasion. The kinetics of γH2AX can also be analyzed within the mutant HT1080 cell lines following the induction of DNA double strand breaks through western blot, utilizing an antibody against γH2AX. Given that the overexpression of TRF2 is known to promote the repair mechanism homologous recombination, it would be important to evaluate the repair pathway choice within the mutant HT1080 cells to determine whether the candidate phosphorylation sites of TRF2 promote a given repair mechanism. This can be analyzed through the use of NHEJ and HR reporter cassettes (Mao et al., 2007). In addition, a phospho-specific antibody could be raised against the candidate phosphorylation of TRF2 at T317 and S323. By utilizing the phoso-specific antibody, one could determine the level of phosphorylation of T317 and S323 in cells as well as their potential dependence on DNA damage response factors, such as ATM and the MRN complex following induction of DNA damage.
Chapter 5

5 Conclusion and Final Thoughts

The work presented in this thesis was completed in order to investigate a potential role for the candidate phosphorylation sites of TRF2 at T317 and S323 in telomerase-positive and telomerase-negative cells. Unfortunately, not all avenues investigated produced meaningful results. Results present in this thesis suggest that the candidate phosphorylation of TRF2 at T317 and S323 does not affect cell growth in telomerase-positive cells or the association of TRF2 with APBs in telomerase-negative cells. Unfortunately, no conclusions could be drawn with respect to the candidate phosphorylation sites T317 and S323 of TRF2 in respect to telomere length maintenance and DNA damage response within telomerase-positive cells. This leaves many avenues open for further investigation.

Many roadblocks have been encountered throughout the completion of this project, which limited my ability to fully conclude many of the results I collected. Even though this is unfortunate, as every scientist strives to uncover meaningful results, I believe I have used the circumstances I have encountered as a learning experience in order to develop and improve my critical thinking skills in the hopes to understand why a given experiment is not working and develop methods in order to troubleshoot and overcome these issues.
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