Mechanisms of Telomere Protection and Deprotection in Human Cells

By
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Date defended: 7/16/2009

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Abstract

Telomeres, the nucleo-protein complexes at the ends of linear chromosomes, have critical roles in genome stability, cancer, and aging. Early work by B. McClintock and H.J. Muller demonstrated that eukaryotic chromosome ends contain specialized structures that prevent recognition and processing by the DNA repair machinery. The importance of these structures is illustrated by studies showing that loss of chromosome end protection results in massive genome instability and cell death. Although Muller and McClintock's initial observations were made several decades ago, little progress has been made in understanding the molecular markers that distinguish naturally occurring chromosome ends from *de novo* DNA double strand breaks, especially in humans. Using a novel system to specifically target proteins of interest to human telomeres, we have uncovered a role for hRAP1 in protecting telomeres from non-homologous end joining (NHEJ). We find that telomeric DNA containing hRAP1, but not TRF2, is protected from NHEJ *in vitro.* Furthermore, we show that telomeres containing TRF2 but not hRAP1 can be fused by NHEJ *in vivo,* and we also demonstrate that targeting hRAP1 to telomeres *in vivo,* even when TRF2 is not detected, is sufficient to protect telomeres from NHEJ. These results identify hRAP1 as a critical mediator of telomere protection and genome stability in humans. Related to this work, we have also identified a new type of telomere dysfunction associated with semi-conservative replication stress at human telomeres. This new type of telomere dysfunction is telomerase and NHEJ-independent and may require the RecQ helicase WRN for its formation, suggesting

that it is related to telomere entanglements observed upon induction of replication stress in fission yeast. The finding that this type of dysfunction is conserved from yeast to man is a testament to the underappreciated role of semi-conservative DNA synthesis in maintaining telomere structure and function.

Acknowledgements

The preparation of this document provides an opportunity to reflect upon the past four years and to recall and appreciate the essential contributions of many individuals to the completion of my dissertation. Though I can not acknowledge each and every person who has made these past four years enjoyable, memorable, and life-changing, the following people deserve special recognition:

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Introduction to Telomeres and Significance of Research

I.1: The Eukaryotic Genome

All organisms are comprised of cells, which are considered to be the fundamental unit of life on Earth. The first description of cells is from the 17th century polymath Robert Hooke. While inspecting cork bark at high magnification using the newly invented microscope, Hooke famously noted the similarity in shape between mysterious structures in cork bark and the cells in which cloistered monks reside [1]. Since their initial description three hundred years ago, much has been learned about cells including their mechanisms for survival, propagation, and communication. To date, though, the most important discovery about cells was the realization that they represent the fundamental unit of life on Earth.

A prerequisite for being the basic unit of life is the ability to faithfully transmit information from the parental cell to its progeny. To facilitate faithful transmission of information, cells store information in the form of highly stable deoxyribonucleic acid (DNA) polymers. In eukaryotes, these polymers are tightly spooled around histones, creating protein:DNA complexes termed nucleosomes. Nucleosomes are then further packaged into structures called chromosomes, and the collection of chromosomes within the cell is referred to as the genome (Figure 1.1). Cells have numerous mechanisms to safeguard information contained within the genome, including mechanisms to prevent and repair damage to DNA, and a

complex machinery to ensure that each daughter cell receives and maintains the appropriate numbers and types of chromosomes.

Chromosomes can be maintained in two states, circular and linear. Though most bacteria and archaea have circular chromosomes, naturally occurring eukaryotes invariably rely upon linear chromosomes for genome maintenance[2]. Each of these types of chromosomes has its own benefits and limitations. For example, circular chromosomes are easy to replicate and segregate, but their maximum size is quite small. On the other hand, linear chromosomes are inherently difficult to fully replicate and segregate, but can handle much larger amounts of DNA[3]. To accommodate their complexity, eukaryotes are forced to rely upon linear chromosomes for genome maintenance.

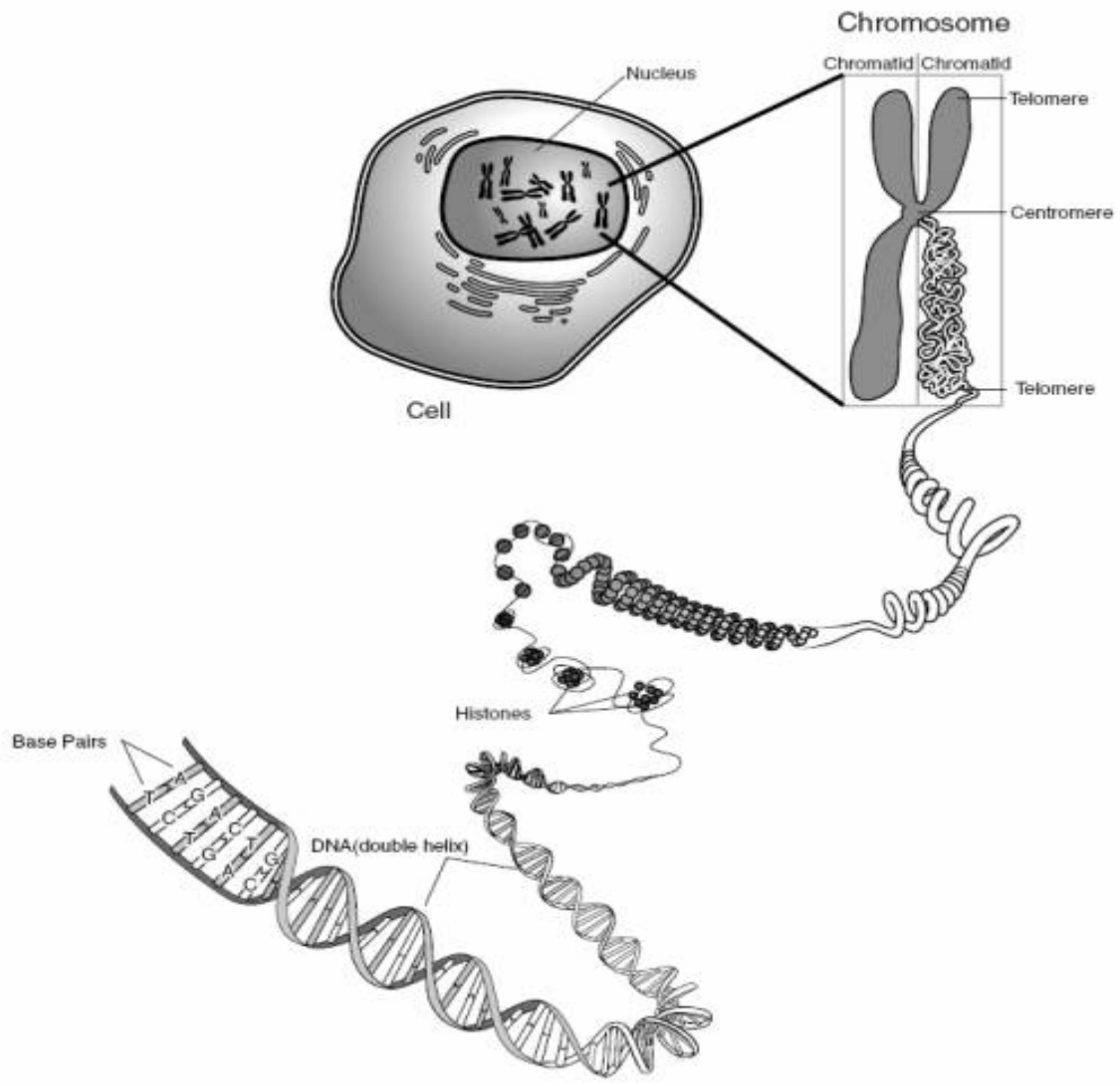


Figure 1.1. Schematic of the compaction and storage of DNA within the eukaryotic cell. Uncopyrighted image reprinted from National Human Genome Research Institute.

Linear chromosomes pose multiple challenges for the organisms that contain them. Each of these problems has been met with a plethora of creative and efficient solutions that demonstrate both the difficulty in maintaining linear chromosomes and

the power of evolution to cope with them. Without the ability to circumvent the inherent difficulties of carrying linear chromosomes, genome complexity would be substantially constrained and the eukaryotic cell in its current form would not exist. Thus, a discussion of the fundamental mechanisms that promote genome stability in eukaryotes, as well as a description of the problems associated with maintenance of linear chromosomes are necessary before further exploration of this topic.

I.2: Distinguishing chromosome ends from DNA breaks

Cells encounter a diverse array of mutagenic insults including UV light, genotoxic chemicals, radiation, and reactive oxygen species. Each of these insults can damage DNA, potentially leading to deleterious changes in DNA sequence that may ultimately impact RNA and protein sequences, structures, and functions. To avoid accumulation of potentially deleterious mutations, cells have numerous mechanisms for both preventing DNA damage and for repairing damage once it has occurred.

I.2a Why are DNA damage and double strand breaks so harmful?

One type of damage that is particularly harmful to cells is the DNA double strand break (DSB). DSBs occur when phosphodiester bonds on complementary strands are broken. DSBs can be disastrous to the cell for several reasons. First, a DSB removes the connection between the centromere and at least one region of a chromosome. Because centromeres provide the physical link between DNA and the

chromosome segregation machinery, the estrangement of one region of a chromosome from its centromere would result in daughter cells not receiving their full complement of genetic material. If an essential gene was present on a chromosome fragment that was not properly allocated to the daughter cell, then the daughter would not be viable. Another problem associated with DSBs is that transcription of mRNA from DNA can not occur across a broken phosphodiester bond. The inability to properly transcribe genes can have disastrous results for the cell, including sterility and death. Due to the potential lethality of unrepaired DSBs, cells actively and effectively resist harboring such insults.

I.2b: Sensing a DSB

Since DSBs can be deleterious to cells, every cell, from the simplest bacteria to syncytial invertebrate gonads, contains a mechanism for quickly sensing and repairing *de novo* DSBs. In eukaryotes, when two phosphodiester bonds are broken on opposing DNA strands, changes in the local chromatin environment occur[4]. During this process, Ser139 on histone H2A (called γ-H2AX) at the site of the DNA break is phosphorylated by DNA damage sensing kinases, including the PI3K-related kinases ataxia-telangietscia mutated (ATM) and ATM-related kinase (ATR) (reviewed in [5]). This phosphorylation can extend to neighboring histones several hundred kilobases away [6]. It is not yet known how the broken DNA ends or chromatin environment signal for H2A phosphorylation to occur[5]. However, it is known that this phosphorylation event leads to a complex and poorly understood

signaling cascade that engages a 5' to 3' exonucleolytic complex consisting of MRE11, Rad50, and Nbs1 (MRN complex) to process DNA ends into a format that is amenable to repair[4]. After processing by the MRN complex, either of two highly-conserved pathways is used to repair the DNA break. Remarkably, the time from when a cell experiences a break until H2A phosphorylation occurs is on the order of seconds[7]. The speed with which a DSB is detected and the amplification in signal that occurs following DSB recognition are a testament to the catastrophic consequences that an unrecognized DSB can have on a cell.

I.2c: Repairing a DSB

Perhaps the best characterized mechanism for DSB repair, non-homologous end joining (NHEJ) relies upon the reformation of juxtaposed broken phosphodiester bonds (Figure 1.2b). In mammals, this pathway is used to repair DSBs incurred by mutagenic insults including those sustained during V(D)J recombination. The mammalian NHEJ machinery found in most cells is composed of the Ku heterodimer (Ku70 and 86 subunits), DNA-PKcs, Artemis, Polynucleotide Kinase (PNK), Cernunnos, XRCC4, and Ligase IV, (reviewed in [8, 9]). The first event in NHEJ is the binding of the Ku heterodimer to broken DNA ends. After Ku binds DNA, it recruits the kinase DNA-PKcs to the broken DNA ends. The contributions of DNA-PKcs to NHEJ are unclear but may relate to its ability to bridge DNA ends [10, 11]. Its significance in NHEJ is clear, however, and is best illustrated by DNA-PKcs mutations that result in severe combined immunodeficiency (SCID) due to the

inability to perform NHEJ during V(D)J recombination [12]. The DNA-PKcs:Ku complex then recruits the Artemis exonuclease and PNK to create ligatable DNA ends. Finally, DNA Ligase IV and its stimulatory cofactors XRCC4 and Cernunnos perform the final step of forming new phosphodiester bonds [13]. During NHEJ it is possible for a few nucleotides to be lost from the free ends due to nucleolytic degradation. Since NHEJ simply religates free ends and does not have the ability to sense loss of information, the process is sometimes error prone. Thus, cells have a second error free pathway that can be used to repair DSBs when sister chromatids are available [14].

Best known for its role in the repair of DSBs induced during meiotic recombination, homologous recombination provides an error-free alternative to NHEJ for DSB repair (Figure 1.2a). In homologous recombination, the double-strand break is resected by the MRN complex (Mre11-Rad50-Nbs1), yielding two single-stranded overhangs. These overhangs are then bound by the RecA homologue Rad51 and associated proteins, including Rad52, Rad54, Rad57, BRCA1 and BRCA2 (reviewed in [15]). These proteins work together through a complicated and poorly understood sequence of events to ensure that the single-stranded 3' overhangs invade and identify of homologous regions on the sister chromatid [15]. After a region of homology common to the DNA break and sister chromatid has been identified, base-pairing between complementary strands allows for stabilization of this structure. Critically, the 3' end of the invading strand can now be used to initiate DNA synthesis with the complementary intact strand as a template. DNA synthesis at this

stage results in 4-way intermediates called Holliday Junctions (HJ). These junctions are resolved by an HJ resolvase[16], and after further processing the end result is restoration of genetic information within the previously damaged chromosome. This type of repair is considered error-free because no information is lost after repair is completed, and occurs during S and G2 phases of the cell cycle when sister chromatids available for use as a template [14].

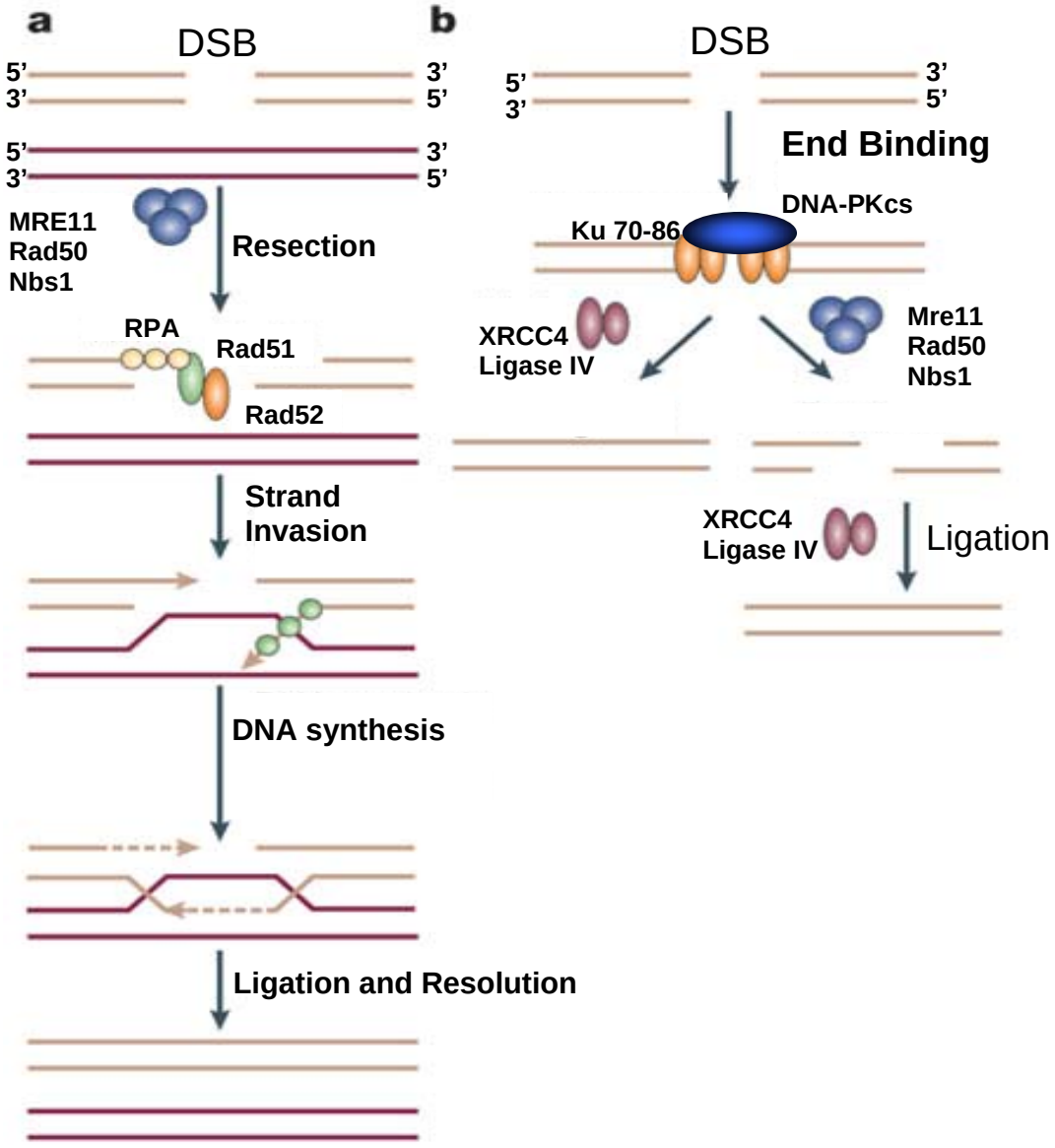


Figure 1.2. DNA double strand break repair pathways in mammals. (a) Homologous Recombination Repair Pathway. (b) Non-Homologous End Joining Pathway. Adapted from [17] with permission from Nature Publishing Group.

I.3: Naturally occurring chromosome ends are shielded from DNA repair

Studies of transposition events in maize and irradiated *Drosophila melanogaster*, pioneered by Barbara McClintock and H.J. Muller, respectively, showed that double strand breaks are efficiently repaired by the cell. Thus, each of these scientists were surprised by the observation that, although DSBs could be joined to other DSBs efficiently creating myriad combinations of translocations, naturally occurring chromosome termini never participated in this process[18, 19]. Since naturally occurring chromosome ends, which Muller called "Telomeres", were hypothesized to look to the cell like de novo double strand breaks, it was surprising that new breaks were not joined to the native ends. This observation led McClintock and Muller to hypothesize that telomeres contain some mechanism that makes them refractory to DNA repair.

I.3a Repetitive DNA at Chromosome Termini

Since these initial studies, modest progress has been made in understanding how mammalian telomeres prevent the DNA repair machinery from acting on them. Much of this progress has depended upon the identification of elements that are unique to telomeres. The first element to be recognized as essential for telomere function is the short, repetitive sequence of DNA found at the ends of most eukaryotic chromosomes. Repetitive DNA at chromosome ends was first identified in the ciliate *Tetrahymena thermophila*, and has since been found in all eukaryotes studied to date [20]. In vertebrates, telomeres consist of double-stranded 5'

TTAGGG 3' perfect repeats that terminate in a 3' single-stranded overhang of the same sequence [21, 22]. The double-stranded regions of telomeres range in size from ~1-100 kb, while the single-stranded regions are usually a ~10-500bp. However, neither the telomeric sequence itself nor the single-strand overhang are intrinsically refractory to DNA repair, as telomeric DNA and telomeres containing intact overhangs can be processed by the DNA repair machinery both *in vitro* and *in vivo* [23-25]. These results are important, as it has been shown that the G-rich strand of telomeres can adopt a special conformation called a G-quadruplex, involving non-Watson-Crick base pairing between four juxtaposed guanine residues coordinated by monovalent cations [26]. Data indicating that telomeric DNA can be joined by the DNA repair machinery suggests that either telomeres do not adopt G-quadruplex conformations regularly, or that this structure by itself does not inhibit repair.

It is important to note that the telomeric sequence is critical for telomere function. Evidence of stringent sequence-specific requirements for telomere end protection comes from organisms that have been manipulated to produce telomeres with mutated sequences [27, 28]. In these organisms, which include yeasts, ciliates, mice and humans, mutated telomeres invariably result in telomere deprotection [27-30]. Therefore, the question becomes: how does the sequence specificity of telomeric repeats contribute to telomere protection?

I.3b: Telomeric Proteins bind DNA in a Sequence-Specific Manner

The ability of DNA-binding proteins to bind DNA in a sequence specific manner is ubiquitous in biology, with examples from the simplest bacteriophage to the most complex cell [31, 32]. Thus, early models of telomere protection suggested that unknown factors may bind in a sequence-specific manner to telomeres and prevent DNA repair proteins from acting aberrantly at chromosome termini. This hypothesis provided the impetus for the isolation of the first telomere binding protein, the *Oxytricha nova* Telomere End Binding Protein (TEBP-α), in the 1980s [33]. TEBP was shown to bind the single-strand overhang at telomeres and protect it from nucleolytic degradation. Studies of TEBP and homologues such at the Protection of Telomeres-1 (POT-1) protein indicated that end binding proteins are essential for normal telomere maintenance, as loss of function of TEBP homologues leads to telomere deprotection, dysfunction, and fusion [34-36]. The discovery of TEBP also signaled a shift from the study of telomeric DNA to telomere binding proteins

The identification of the first vertebrate telomere binding protein, telomere repeat binding factor 1 (TRF1)[37], was one of the biggest fruits of this change in emphasis. TRF1 was identified based on a DNA binding activity in human cell extract that has high affinity and specificity for double-stranded TTAGGG repeats. TRF1 has an acidic stretch of ~100 amino acids at its N-terminus whose contribution to telomere maintenance is unknown (Figure 1.3e). The C-terminus contains a well-characterized Myb-like domain, which is structurally related to homeodomain

transcription factors [38]. Unlike its homeodomain-containing family members, the TRF1 Myb domain exhibits a strong preference for the sequence 5'YTAGGGTTR3', providing the specificity necessary to localize to and bind telomeres [39]. The middle region of TRF1 contains the TRF homodimerization domain (TRFH), and both the TRFH and Myb have been crystallized and studied in great detail. The TRF homodimerization domain binds another TRF1 molecule to promote formation of a DNA-binding competent homodimer, since Myb domains require dimerization for DNA binding [40].

The binding of TRF1 to telomeres is thought to regulate telomere length maintenance, as overexpression of TRF1 leads to telomere shortening while inhibition leads to longer telomeres [41]. Importantly, it has also been shown that recruitment of TRF1 to lacO sites embedded within an engineered telomere results in shortening of the targeted telomere. This result demonstrates that additional domains besides the DNA binding domain within TRF1 contribute to cis-regulated functions, a theme that is common among telomere binding proteins [42]. Finally, TRF1 has been implicated in the cohesion of sister telomeres during and after DNA replication [43, 44]. Recent studies have identified telomere-specific cohesion complexes that pair sister telomeres during mitosis [43, 45]. Aberrant regulation of sister telomere cohesion was shown to result in NHEJ-dependent sister telomere fusion, suggesting that TRF1 may indirectly contribute to telomere protection by ensuring proper telomere separation [45]. The ribosylation of TRF1 was shown to facilitate separation of sister telomeres, suggesting that TRF1 contributes to genome stability

and telomere protection by ensuring that telomeres are segregated properly during mitosis [44]. However, loss of TRF1 function is not associated with an increase in DNA repair at telomeres or growth delay [46], indicating that its contribution to telomere protection may be minor or is masked by other telomere binding proteins.

Two years after the discovery of TRF1, two groups independently identified TRF2 [47, 48]. One of the most studied telomere binding proteins identified to date, TRF2 has roles in numerous processes including telomere protection, DNA repair, and transcriptional activation[49-51]. Similar to TRF1, TRF2 has a C-terminal Myb domain and a TRFH domain between amino acids 89-200 (Figure 1.3e). TRF2 binds telomeric DNA with high affinity and specificity *in vitro*, and resides at telomeres *in vivo [48, 52]*. However, unlike TRF1, TRF2 has a basic region at its N-terminus. This region has gained notice recently due to the presence of several glycine and arginine residues, which are common in RNA binding proteins, suggesting that TRF2 may bind RNA.

The roles of TRF2 in telomere protection were demonstrated in a series of groundbreaking experiments from the laboratory of T. de Lange [53, 54]. In these experiments, the de Lange group expressed truncated versions of TRF2 and observed distinct types of telomere dysfunction resulting from deletion of either the basic or Myb domains. Amazingly, TRF2 fragments lacking the basic or Myb domains resulted in increased HR and NHEJ at telomeres, respectively, suggesting that TRF2 has multiple roles at telomeres [53, 54]. Deletion of the N-terminus of TRF2 leads to homologous recombination-dependent telomere hyper-recombination and hinders

replication fork progression through telomeres, which identifies a role for TRF2 in facilitating telomere replication that is discussed in detail later in this thesis [54-56]. Exactly how this domain promotes telomere replication is not known, and is an exciting and unexplored area of telomere biology.

In contrast to the telomere-HR phenotype associated with TRF2ΔB expression, expression of Myb-domain deletions of TRF2 result in NHEJ-dependent telomere fusions [25, 53]. This allele is hypothesized to work by dimerizing with endogenous TRF2, creating a DNA-binding incompetent heterodimer. In accordance with this hypothesis, expression of TRF2 fragments lacking the Myb domain lead to a reduction in the amount of endogenous TRF2 at telomeres [53, 57]. Further supporting a role for TRF2 in telomere protection, loss of TRF2 from telomeres is associated with increased ATM kinase signaling and DNA repair processing at telomeres, and another report has shown that TRF2 can directly bind and inhibit the ATM kinase [58, 59]. Finally, a recent study has shown that TRF2 binds unphosphorylated Chk2, which is a downstream target of ATM, potentially further inhibiting damage signaling at telomeres [60]. Despite these results, the mechanism or mechanisms TRF2 employs to inhibit NHEJ at telomeres is still not known.

The idea that telomere binding proteins work not just by binding telomeres, but by carrying out functions using other domains within these proteins suggested that TBPs may recruit other proteins to telomeres. The search for TRF1 and TRF2-interacting factors has yielded a long list of interacting factors including exonucleases[61], poly-ADP-ribosylases [62], transcription factors [49], DNA repair

proteins [59, 63], and other telomere maintenance factors [64, 65]. The exact contributions of these accessory proteins to telomere protection are still not known.

Although TRF2 has been shown to interact with myriad different factors, perhaps the strongest interaction between an interacting factor and TRF2 is the RAP1 protein, which is supported by both biochemical and genetic data [65, 66]. hRAP1 was first identified in a yeast two hybrid screen for TRF2 interacting factors, and was named based upon limited sequence homology to the budding yeast Repressor-Activator Protein 1 [65]. Initial studies of the 399 amino acid protein indicated that it contains several domains, including an N-terminal BRCT domain that is expected to bind an unknown phosphorylated protein, as well as a single internal Myb domain (Figure 1.3e). Studies of this Myb domain suggest that it is not capable of binding DNA since the electrostatic surface of this domain is neutral, while Myb domains from DNA-binding competent proteins are positively charged allowing them to interact with the negatively charged DNA backbone [67]. hRAP1 also contains a coiled-coil domain of unknown function, and a C-terminal region that is conserved between RAP1 homologues. This region is an important protein-protein interaction domain for RAP1, as it mediates the interaction of hRAP1 and hTRF2 in humans, and ScRAP1 and ScRIF1, ScRIF2, and ScSIR4 in budding yeast [65, 68]. In support of the idea that the C-terminus of RAP1 homologues is critical for its function, deletion of 60 amino acids near the C-terminus (271-330) resulted in an hRAP1 fragment that did not localize to telomeres [66, 69]. When combined with data demonstrating that loss of TRF2 from telomeres results in concomitant

delocalization of hRAP1 as well, it is clear that hRAP1 requires TRF2 for its localization.

Though similarities exist between hRAP1 and ScRAP1, as noted above, numerous differences are readily apparent. Most importantly, while hRAP1 can not bind DNA directly, budding yeast RAP1 directly binds DNA, alleviating the need for a TRF2 homologue to recruit it to telomeres. In addition to differences in DNA binding ability, hRAP1 localizes exclusively to telomeres while ScRAP1 is present at telomeres and in the promoters of ~5% of budding yeast genes [70]. Also, ScRAP1 interacts with many different proteins, including ScRAP1 interacting factors ScRif1 and ScRIF2, and ScSIR4 [71, 72]. No homologues of these proteins have been identified in humans, further demonstrating the divergence between hRAP1 and ScRAP1. This data suggests that ScRAP1 and hRAP1 have diverged significantly since their last common ancestor and that their functions may not necessarily be conserved.

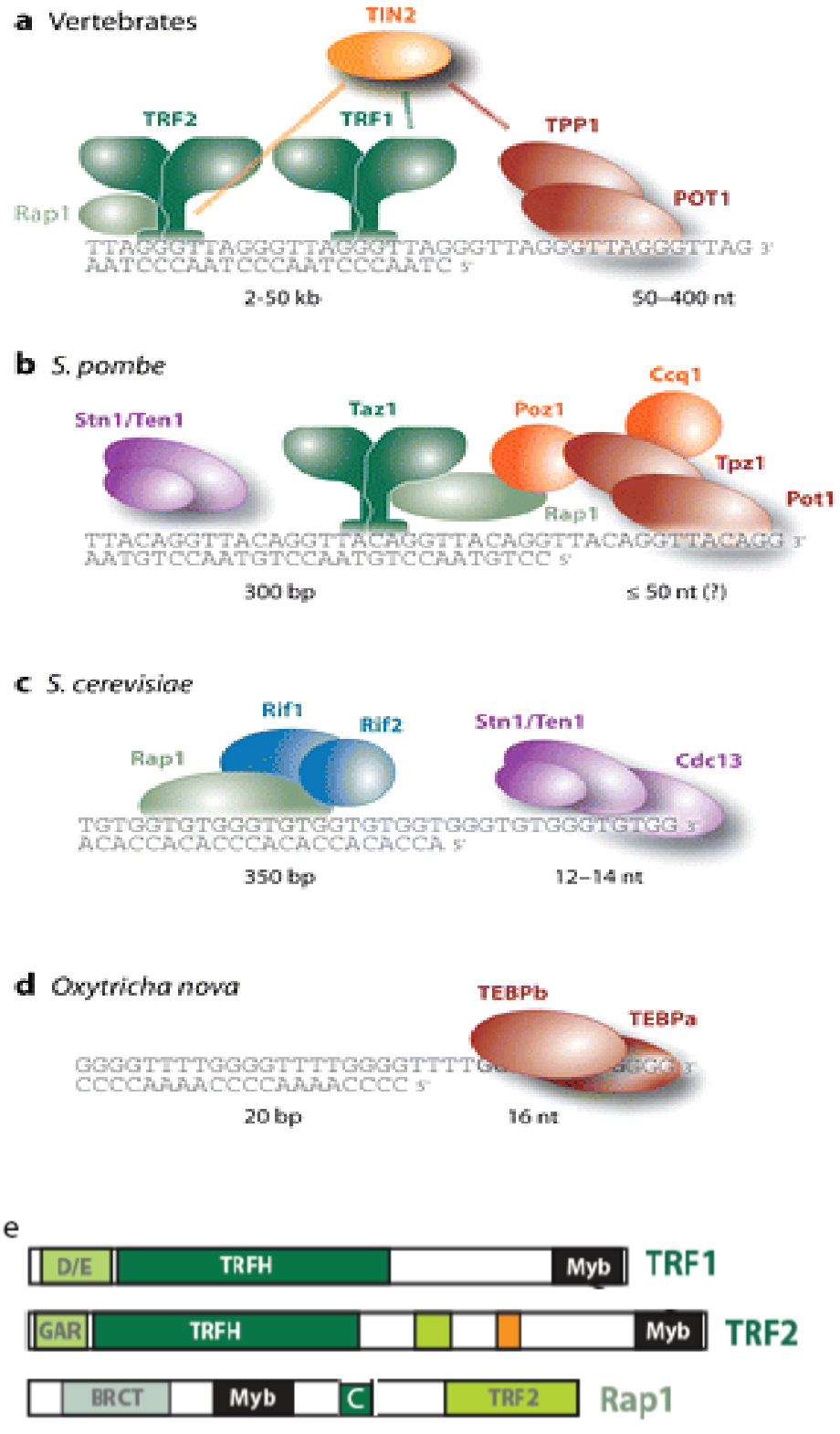

a Vertebrates
TIN2
TRF2
TRF1
TPP1
POT1
Rap1
TTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAG 3'
AATCCCAATCCCAATCCCAATC 5'
2–50 kb
50–400 nt
b S. pombe
Ccq1
Stn1/Ten1
Taz1
Poz1
Tpz1
Pot1
Rap1
TTACAGGTTACAGGTTACAGGTTACAGGTTACAGGTTACAGG 3'
AATGTCCAATGTCCAATGTCCAATGTCC 5'
300 bp
≤ 50 nt (?)
c S. cerevisiae
Rif1
Rif2
Stn1/Ten1
Rap1
Cdc13
TGTGGTGTGGGTGTGGTGTGGTGGGTGTGGGTGTGG 3'
ACACCACACCCACACCACACCA 5'
350 bp
12–14 nt
d Oxytricha nova
TEBPb
TEBPa
GGGGTTTTGGGGTTTTGGGGTTTTGGGG 3'
CCCCAAAACCCCAAAACCCC 5'
20 bp
16 nt
e
D/E
TRFH
Myb
TRF1
GAR
TRFH
Myb
TRF2
BRCT
Myb
C
TRF2
Rap1

Figure 1.3. (a-d) Telomeric DNA sequence and associated proteins in different species of eukaryotes. (e) Domain structures of three human shelterin components. Reprinted, with permission, from the *Annual Review of Genetics,* Volume 42 © 2008 by Annual Reviews www.annualreviews.org

I.3c: Do ScRAP1 and hRAP1 carry out functions that are conserved between species?

Interestingly, ScRAP1 is a critical mediator of NHEJ inhibition at telomeres in budding yeast, and RAP1 loss from the distantly related fission yeast S. pombe also results in NHEJ-dependent telomere fusions, further implicating RAP1 in protecting telomeres from NHEJ [24, 68, 73]. Only ScRAP1's role in NHEJ inhibition has been characterized, though. Recent data suggests that ScRAP1 serves as a protein hub that recruits two factors that work independently to inhibit NHEJ at telomeres, ScRIF2 and ScSIR4 [68]. Each of these factors inhibits NHEJ using unknown mechanisms, as concomitant deletion of ScRIF2 and ScSIR4 results in more telomere fusions than deletion of either gene alone. ScRAP1 may be more than just a protein hub, however, since a central region in ScRAP1 also contributes to NHEJ inhibition at budding yeast telomeres independently of ScRIF2 and ScSIR4 [68]. The tripartite model for NHEJ inhibition at telomeres has yet to be demonstrated in other eukaryotes and may not be relevant to metazoans since no ScRIF2 or ScSIR4 homologues have been identified in these species.

Because loss of RAP1 function in the distantly related budding and fission yeasts leads to telomere deprotection, it was surprising that hRAP1 has not been shown to play such a role at human telomeres. Previous work failed to effectively

disrupt hRAP1 function, leaving its role in telomere protection unaddressed [66, 69]. Moreover, gene targeting in mice is complicated by the presence of a bidirectional promoter that also transcribes a putatively essential lysyl tRNA synthetase [74]. Overexpression studies with full length hRAP1 and a series of hRAP1 truncations suggested that it plays a role in establishing telomere length since hRAP1 overexpression led to an increase in telomere length heterogeneity[66]. Although this is the only reported function for hRAP1, hRAP1's exact contribution to telomere length maintenance is not known. Surprisingly, a TRF2/hRAP1 complex was demonstrated to be required for protecting telomeric DNA ends from end-joining in an *in vitro* NHEJ assay, suggesting that hRAP1 may contribute directly to telomere protection [23]. To date, no evidence for hRAP1 in NHEJ inhibition at telomeres *in vivo* exists, though the ascertainment of such evidence is of high interest to the telomere field [75]. Therefore, the search for hRAP1's function *in vivo*, including its potential contribution to telomere protection, remains an important area of research in the telomere field and is the subject of Chapter Three of this thesis.

I.3d: TBPs and telomeric sequence may work in concert to protect chromosome ends

Numerous studies have reported loop-like structures at chromosome ends in electron micrographs prepared from different eukaryotes, including humans (Figure 1.4) [76-78]. These loops are hypothesized to occur when the terminal single-stranded overhang invades the adjacent double-stranded region, causing displacement of the G-rich strand into a structure called a D-loop. Such structures are

reminiscent of HR intermediates. The entire loop structure has been termed a t-loop (telomere loop), and has been hypothesized to contribute to telomere protection because the end of the 3' overhang would be protected against nucleolytic attack when buried within the t-loop. TRF2 has been shown to promote t-loop formation *in vitro* [79, 80]. Also, the basic domain of TRF2, which may inhibit HR at telomeres, was shown to be necessary for TRF2-mediated t-loop formation [79]. Thus, loss of TRF2 from telomeres may compromise t-loop formation *in vivo.* This observation may explain the telomere fusion phenotype observed upon loss of TRF2 from telomeres. It is important to note that no data on the protective function of t-loops at telomeres has been reported, even though ample opportunity for the ascertainment of such data exists given that t-loops have been found in many species including trypanosomes, humans, yeasts, plants, and ciliates [77, 78, 81, 82]. Also noteworthy is the fact that t-loops have only been observed under conditions that are not amenable to life, since t-loop visualization requires either protein:DNA or DNA inter-strand crosslinking for stabilization [76, 78]. The artificial stabilization required to preserve and visualize t-loops suggests that they may be ephemeral structures *in vivo,* which would not be commensurate with a major role in telomere protection.

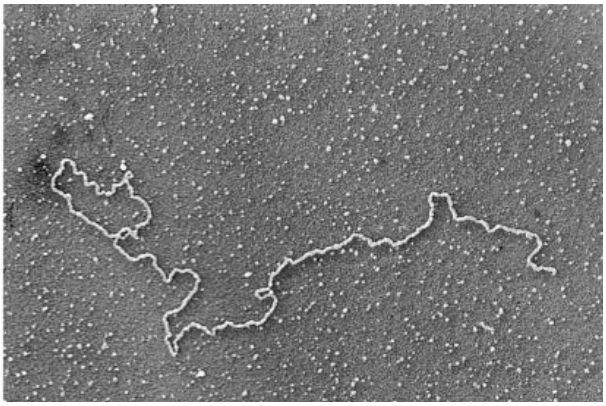


Figure 1.4: T-loop observed in electron microscopy samples prepared from HeLa I.2.11 DNA [76]. Copyright Elsevier 1999.

Though the identification of single-and double-stranded TBPs and t-loops suggest intriguing models for telomere protection, neither addresses a poorly understood conundrum; why loss of double-stranded telomere binding proteins leads to NHEJ at telomeres. As stated previously, telomeres terminate in a single-stranded region that is bound by TEBP homologues including POT-1. Because the NHEJ machinery needs free DNA ends on which to load, the presence of POT-1 on telomeric overhangs may block the MRN and Ku complexes from accessing DNA ends. It is possible that TRF2 regulates or modulates POT1 binding to DNA [83, 84]. However, since POT-1 knockout mice display a very mild telomere fusion phenotype, the roles of POT-1 and associated factors in NHEJ inhibition at telomeres remain unknown [85, 86].

Despite the single-strand overhang, the loss of double-stranded telomere binding proteins from telomeres results in NHEJ-dependent telomere fusions in a variety of species. In addition, other reports have demonstrated that very short

telomeres with few TBP binding sites can become fusogenic, leading to covalent telomere fusions [87-89]. Because pre-neoplastic cells display chromosomal fusions with critically short telomeres at the fusion site[87], understanding how telomeres shorten and what distinguishes a critically short, fusogenic telomere from a functional one are of utmost interest to the telomere field and cancer biology.

I.4: Telomere Replication

The machinery that executes semiconservative DNA replication is highly conserved among eukaryotes. In eukaryotic DNA replication, an origin recognition complex consisting of origin recognition complex (ORC) subunits 1-6 binds origins of replication on chromosomes in the G1-phase of the cell cycle. The binding of ORC to replication origins mediates recruitment of other factors, including Cdc6, Cdc45, Cdt1, and MDM 2-7, which all work together to license the replication origin for firing (reviewed in [90]). It is important to note that while budding yeast has sequence-specific recruitment of ORC to replication origins, ORC recruitment in humans appears to be sequence independent [90]. The factors that mediate ORC recruitment to human replication origins remain unknown. Upon recruitment of DNA polymerase α, primase, RPA, and associated factors, replication fork progression occurs in the 5' to 3' orientation. This polarity is due to the ability of DNA polymerases to catalyze phosphodiester bond formation between nucleotide triphosphates with phosphate groups attached to the 5' position on sugars and DNA

polymers with an available hydroxyl group at the 3' position on the sugar for phosphdioester bond formation.

The anti-parallel configuration of complementary DNA strands renders symmetrical DNA synthesis impossible. To circumvent this issue, cells employ asymmetric DNA replication. This means that at replication origins the strand to be synthesized continuously in the 5' to 3' direction is copied first. The displacement of the complementary strand by the newly synthesized "leading" strand allows for initiation of 5' to 3' DNA synthesis of the "lagging" strand, which is synthesized discontinuously in segments called Okazaki fragments.

I.4a: The end-replication problem

Since DNA polymerases use an RNA primer to initiate synthesis, and must synthesize DNA in the 5' to 3' direction, excision of the RNA primer at the 5' end yields a gap in the DNA that is usually filled in by initiating synthesis from adjacent Okazaki fragments. However, when the terminal 5' RNA primer is removed, no upstream lagging strand DNA is available to initiate "fill in" synthesis from, yielding a single-stranded DNA end. Thus, the lagging strand ends of each chromosome are shorter than their templates. Eventually, this shortening due to incomplete end replication would lead to loss of critical regions in chromosomes such as essential genes or structural elements that are necessary for cell function. If unchecked, this problem would result in the extinction of any species—eukaryotic or prokaryotic—with linear chromosomes. The "end replication problem" (Figure 1.5), as was

independently hypothesized by Jim Watson and A. Olovnikov in the 1970s, is one of the most fundamental and interesting problems in biology [91, 92].

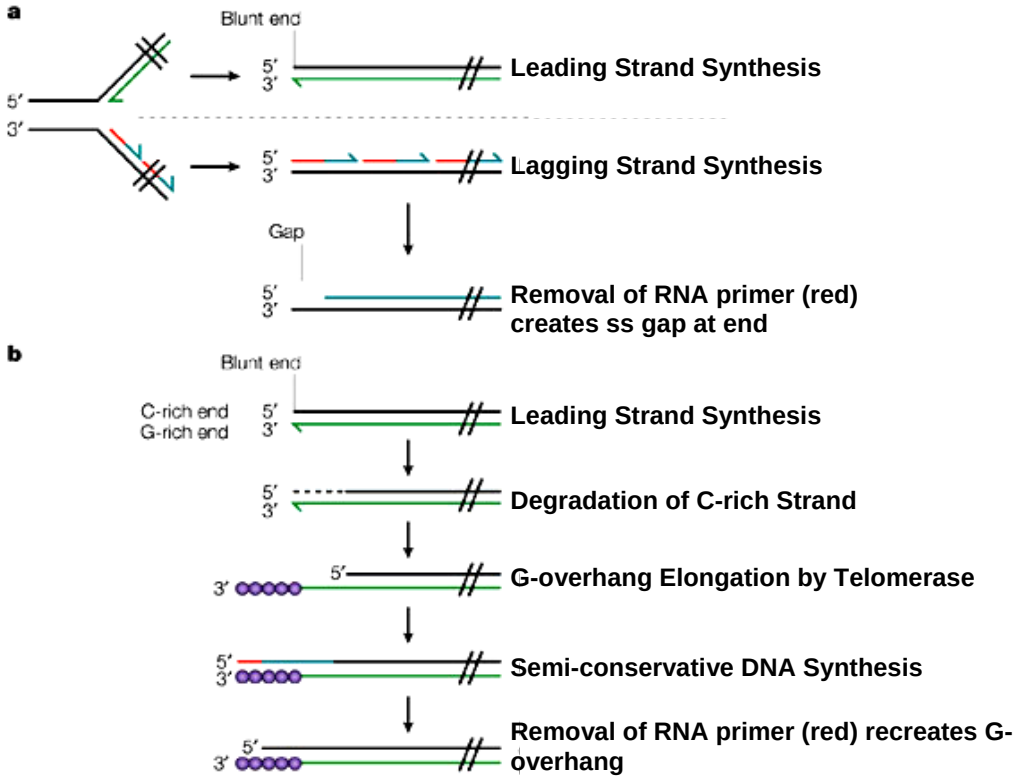


Figure 1.5: (a) Representation of the "end replication problem". (b) Generation of single-stranded overhangs and elongation by telomerase counteracts the "end replication problem". Adapted from [93] with permission from Nature Publishing Group.

I.4b: Telomerase counteracts the end-replication problem

Of course, since all eukaryotes have linear chromosomes, these cells must contain a mechanism to counteract the end replication problem. The ingenious solution in most cases is provided by a reverse-transcriptase enzyme called telomerase [94]. This enzyme is comprised of both protein and RNA components,

and uses an RNA primer contained within the RNA component to add nucleotides to the ends of chromosomes during S-phase. In vertebrates, for example, telomerase invariably adds repeats of the sequence TTAGGG to chromosome ends, which extends the template that can be used for synthesis of the complementary strand and allows for fill-in synthesis of the lagging strand. Thus, telomerase counteracts the loss of DNA associated with lagging strand synthesis that would normally occur with each division.

The impact of telomerase on individual cells was illustrated in an important set of experiments by Shay, Wright, Harley and colleagues in the 1990s. It had been previously shown that telomerase-negative primary fibroblasts divided a definite number of times before ceasing to proliferate [95]. This phenomenon, ascribed to dysfunctional telomeres resulting from the end replication problem associated with each round of cell division, has been termed the "Hayflick Limit". Shay, Wright and Harley demonstrated that reconstitution of telomerase activity in primary fibroblasts extended the lifespan of primary cells indefinitely [96], suggesting that telomere shortening was responsible for the finite lifespan of primary cells. This data also supports a role for telomeres in organismal aging, whereby cells in tissues can only divide a certain number of times before succumbing to the effects of a critically short telomere [97].

The importance of telomerase to organisms has been demonstrated in a wide range of species from budding yeast to man. In every species that relies upon telomerase-mediated telomere lengthening, loss of telomerase activity results in loss

of organismal viability [98-101]. Most importantly, the loss of viability is anticipated by the average telomere length of the affected individual. For example, telomerase null mice with telomere length distributions of ~10-80kb are indistinguishable from their wildtype littermates, while telomerase null mice with much shorter telomere length distributions (~0-50kb) are not viable [100]. Because mice with longer telomeres have plenty of telomeric DNA in reserve to counteract the end replication problem, these mice survive without telomerase for many generations. Remarkably, this trend is also observed in the human progeroid disease Dyskeratosis Congenita (DKC), which can be caused by mutations in telomerase [101, 102]. DKC patients demonstrate a phenomenon called "anticipation", whereby the onset of the disease is anticipated by telomere length of the diseased individual [102].

Though the end-replication problem seems like quite a challenge to surmount in order to have larger genomes, it does have at least one benefit. In complex multicellular organisms such as mammals, most differentiated cells do not divide. Instead, these cells carry out their functions and then eventually die, without giving rise to a progenitor. In addition, these cells do not express enough telomerase to counter-act the end-replication problem [103]. An example of such cells is neurons, which do not divide, express little if any telomerase, and carry out their specified function for as long as possible before succumbing to any of a variety of insults [104]. However, in rare cases, cells such as differentiated neurons receive aberrant signals to start dividing uncontrollably. These precancerous cells extinguish themselves if they do not reactivate telomerase [105], as with each aberrant cell

division their chromosome ends become shorter, until they are no longer functional. This has been observed in mice lacking telomerase, providing evidence for the end-replication "problem" as a tumor suppressor mechanism [106]. Related to this, telomerase inhibition may also prevent uncontrolled proliferation of cancerous cells, and numerous pharmaceutical companies are developing anti-telomerase therapeutics.

I.4c: Some transformed cells can divide indefinitely without telomerase

This question was first postulated by Reddel and colleagues in 1996 [107], when they made the observation that certain immortalized human cancer cell lines do not express telomerase. Since their observation, studies in organisms ranging from yeast to worms to human cells have identified alternative pathways of telomere maintenance [107-109]. In human cells, one pathway of telomerase-independent telomere length maintenance has been identified, called alternative lengthening of telomeres (ALT) [110]. Though it is not known how cells engage this pathway, human cancers can use ALT-related mechanisms to counteract the end-replication problem[111]. Remarkably, in some cancer types such as leiomyosarcomas, approximately 77% of tumors rely on ALT, where as in rhabdomyosarcomas, only 6% of cells use this pathway [111]. It is still unclear why different cell types display very different propensities for engaging this pathway. However, numerous studies have identified events that are associated with the transition to ALT status, as well as genes important for ALT[110, 112-114]. One important factor that may be involved

in ALT development is the pro-myeloid leukemia (PML) body, as its association with telomeres in ALT cell lines has been well documented [115-117]. Though these bodies are poorly understood, they may function to facilitate recruitment and stimulate activities of certain proteins involved in homologous recombination, which is the very same pathway used for DNA DSB repair and described earlier. In fact, ALT appears to rely on homologous recombination-directed DNA synthesis to facilitate lengthening of critically short telomeres. Such a pathway for telomere maintenance is a demonstration of the intricate relationship between telomere maintenance and the DNA repair machinery.

I.4d: Replication through repetitive DNA that can form non-canonical structures

Another problem posed by telomeres is their ability to adopt structures that have been shown, either *in vitro* or *in vivo*, to hinder DNA replication (Figure 1.6). For example, the guanine residues in TTAGGG repeats can form non-Watson Crick base pairs, leading to G-quadruplex structures that have been observed *in vivo [26]*. It is thought that these structures disrupt replication fork progression due to intra-strand base pairing [118]. To counteract this problem, vertebrates contain highly conserved RecQ-like helicases, including WRN and BLM, that can relax such structures. WRN localizes to telomeres in S phase, and promotes replication of G-rich lagging strands [119, 120]. In addition, helicase-dead mutants of WRN had an increase in stalling of replication forks at telomeres, and loss of G-rich lagging strands, further implicating WRN in this process. Remarkably, mutations in WRN

have been linked to the progeroid disease Werner's syndrome, suggesting that defects in telomere replication play a role in aging [121]. This disease further illustrates the importance of proper telomere replication in both cellular and organismal viability

G-quadruplexes are just one potential hindrance to telomere replication. Telomeres also consist of densely packed heterochromatin, which means that telomeric nucleosomes are packaged together more tightly than other nucleosomes within the cell, which could restrict access of the replication machinery to telomeres [122, 123]. Recent research suggests that double-stranded telomere binding proteins, including TRF2 in mammals and Taz1 in fission yeast, may recruit the origin recognition complex to telomeres [55, 124, 125]. If TRF2 indeed recruits ORC to telomeres, this recruitment would be independent of nucleosomes, since TRF2 interacts with DNA directly, therefore abrogating the difficulties associated with ORC finding appropriate replication origins buried in telomeric DNA.

As was stated previously, telomeres can adopt specialized structures called t-loops. Though it is possible that these structures could shield telomeres from recognition by the DNA damage machinery, they would undoubtedly also block replication fork progression. Thus, they would need to be resolved during S-phase for telomere replication to occur. How these structures are resolved in S-phase is not known, but may relate to the ability of TRF2 to recruit RecQ helicases like BLM and WRN to t-loops [126, 127], where they could be unwound before a replication fork encounters them. It is possible that the telomere dysfunction associated with

expression of a helicase dead mutant of WRN could be linked to the inability of the helicase dead mutant to unwind t-loops[120], though that model is purely speculative at this time.

I.4e: Consequences of Improper Telomere Replication

While the search for telomere dysfunction phenotypes associated with improper telomere replication in mammals has been limited to the identification of telomere recombination with subsequent production of t-circles, work in fission yeast has suggested that dynamic interchromosomal telomere associations can result from improper telomere replication [128]. Because stalled replication forks are efficiently repaired by homologous recombination using the newly synthesized sister chromatid as a template, most stalled forks are not particularly harmful to the cell. On the contrary, when telomeric DNA is present in a stalled replication fork, two problems occur. First, since all chromosome ends contain telomeric DNA, any chromosome end could be used as a template for repair, and any homologous region within that chromosome end could be used, leading to the unequal exchange of DNA between these two chromosomes. Second, the special structures that telomeres adopt, including G-quadruplexes, may be aberrantly processed by RecQ helicases during HR, yielding unrepaired joint molecules that persist through the cell cycle [128]. The end result of this process is that telomeres can become "entangled", as has been observed in fission yeast [129]. Interestingly, these telomere entanglements have been linked to both the ability of the TRF2 homologue taz1 to promote proper

telomere replication, as well as the WRN homologue rqh1 to improperly process telomeric Holliday junctions [125, 128]. These results strengthen the idea that telomere replication poses unique challenges to the replication machinery and illuminate the importance of proper telomere replication in the life cycle of a cell. Chapter Four describes the identification of a novel phenotype associated with replication stress at mammalian telomeres.

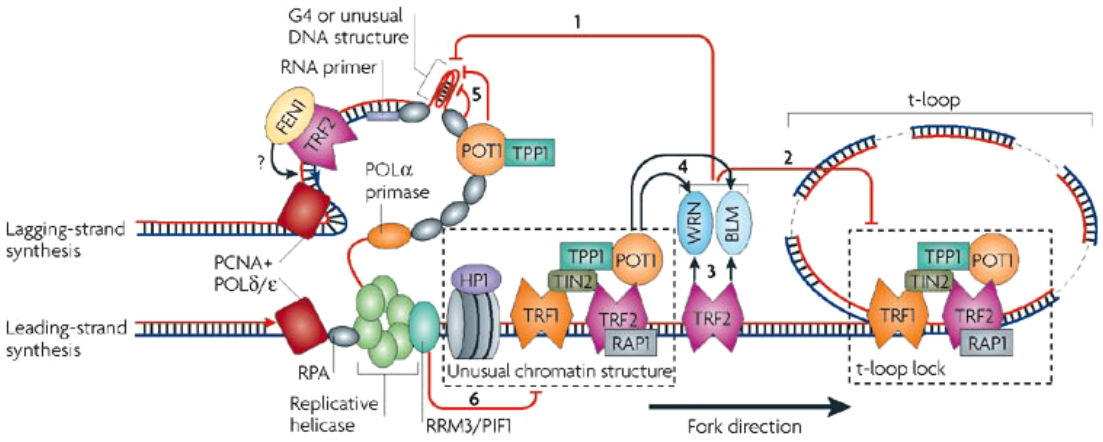


Figure 1.6: Problems associated with replication of telomeres during DNA synthesis. Reprinted with permission from Nature Publishing Group.

I.5: Scope of Dissertation

The primary goal of the research conducted during my tenure as a graduate student is to understand the intricate relationship between DNA repair and telomere maintenance. Since the DNA repair machinery consists of multiple complex pathways that are difficult to analyze in concert, we sought to establish a new

paradigm for studying the relationship between telomeres and DNA repair by investigating the mechanisms that telomeres employ to locally inhibit the NHEJ machinery. By dissecting the precise contributions of several telomere binding proteins and associated factors to NHEJ inhibition at telomeres, we have uncovered novel roles for hRAP1 and TRF2 in telomere protection in humans. The results of this research are reported in Chapter Three of this thesis.

In addition, during the course of our research on requirements for NHEJ inhibition at telomeres, we identified a previously unreported phenotype associated with telomere dysfunction in human cells. Characterization of this phenotype suggests that it is very different from previously reported phenotypes associated with telomere dysfunction, especially the well-characterized covalent NHEJ-dependent telomere fusions observed upon loss of TRF2 from telomeres [25, 53, 58, 130, 131]. Instead, we present evidence demonstrating that this new type of telomere dysfunction is associated with replication stress at telomeres, and may result from aberrant homologous recombination. The results and implications of its significance are presented in Chapter Four of this thesis.

Chapter Two of this dissertation contains a comprehensive list of the materials and methods used to execute the studies described in subsequent chapters. Chapter Five provides a summary of the results presented in Chapters Three and Four. In addition, Chapter Five also provides a synthesis of the information presented in Chapters Three and Four with current literature. Finally, this chapter

describes numerous future directions for research that build upon the studies reported in Chapters Three and Four.

Chapter Two:

Materials and Methods

II.1: Polymerase Chain Reaction (PCR)

High-fidelity DNA polymerase HotStart PfuTurbo (Stratagene) was used for all reactions since PCR was only used for the purposes of cloning cDNAs into vectors for expression in either bacterial or mammalian systems. The components of the 50μL PCR reactions were as follows: 0.5μM of each DNA oligonucleotide (Integrated DNA Technologies, IDT) (usually containing 24-27bp of complementary bases); 0.2mM dNTPs; 5μL of 10X Pfu Buffer (Stratagene); 1μL of template (DNA mini or maxipreps diluted ~1:100, or 1μL of first-strand cDNA from fission yeast provided by K. Trujillo); 1μL of PfuTurbo Hot Start Polymerase (Stratagene); ddH_20 to 50μL. The PCR reactions were carried out in either an ABI 2400 thermocycler or an Eppendorf EP mastercycler as follows: after 3min at 94°C, 30 cycles of 94 °C for 30s, 55 °C for 45s, 72 °C (time=60s for every kilobase to be amplified, such that a 2kb fragment was incubated for 120s) were performed. Following the 30 cycles, an extra extension of 72 °C for 7min was performed, regardless of the length of the anticipated fragment.

II.2: Purification of PCR fragments

For gel purification, 50μL of the PCR reaction was run on an agarose gel, excised with a razor under UV light (280nm), and placed in a 2.0mL tube. The fragment was then weighed and processed using the Qiagen Gel Extraction Kit according to the manufacturer's instructions, except that a 5 minute incubation in buffer PE was always performed. For direct purification of the PCR product without gel purification, the Qiagen PCR Purification Kit was used according to the manufacturer's instructions, except that a 5 minute incubation with buffer PE was always employed.

II.3: Restriction Enzyme Digestion

All restriction enzymes used in these studies were purchased from New England Biolabs. For analytical restriction digestion of miniprep-purified plasmid DNA, 2μL of DNA (~200-300ng/μL) was incubated with the appropriate NEB buffer (10X stock), restriction enzyme(s), and if necessary 100μg/mL bovine serum albumin (BSA), in a final reaction volume of 15μL for 1-18hr at 37 ^{o}C. Digests incubated <3hr were placed in a water bath, while longer digests were incubated in a dry incubator. The volume of total restriction enzyme(s) added to the reaction was always 10%, such that 1.5μL of one enzyme was added to a single digest, or 0.75μL of two enzymes, or 0.5μL of three enzymes were added. BSA was added from a 10X stock such that 1.5μL was added to the reaction. For preparative digests using either

gel-extracted or PCR-purified DNA, 30μL of these reactions were digested with 5μL of the appropriate 10X NEB buffer, 100μg/mL BSA if necessary, and either 5μL of one restriction enzyme of 2.5μL of each of two restriction enzymes in a final reaction volume of 50μL. Reactions were incubated identically to the conditions listed for analytical scale digests. Finally, for preparative restriction digests of plasmids, 2μg of plasmid was digested in a 50μL final volume, otherwise the other components of the reaction are identical to those listed for preparative digest of PCR products. When necessary, 1μL of Calf Intestinal Phosphatase (New England Biolabs) was added to the restriction digest for 30 minutes to dephosphorylate DNA ends, thereby preventing self-ligation.

II.4: Ligation of DNA

For T4 DNA Ligase ligation, reactions were carried out in a final volume of 10μL. In these reactions, 3μL of the DNA fragment to be inserted (obtained either by PCR followed by Gel or PCR purification, or a DNA fragment obtained by digestion of another plasmid) was incubated with 1μL of the receiving vector, 1μL of hand-thawed 10X T4 DNA ligase buffer (NEB), and 4μL of ddH_20. After a 2 minute incubation at 37 ^oC, 1μL of T4 DNA ligase (NEB) was added, and the reaction was incubated at room temperature for 2-4hr, followed by overnight incubation at 15^oC if directional cloning (cloning of fragments with complementary single-stranded

overhangs) was being performed. Negative controls simply replaced the 3μL of "insert" DNA with Qiagen EB.

For quick ligation, the amounts of DNA used for this reaction are the same as those used for T4 DNA ligation and the final reaction volume of 10μL is unchanged, also. 5μL of 2X Quick Ligase buffer was also used in this reaction, and after incubating the buffer and DNA at 37°C for 2 minutes, 1μL of Quick Ligase was added. The reaction was then incubated at room temperature for at least 5 but not more than 10 minutes. For negative controls, the 3μL of inserted DNA was replaced with an equivalent volume of Qiagen EB.

Ligation of blunt-ended PCR products into pCR4-Blunt vector (Invitrogen) was performed as follows: 1μL of vector was incubated with 2μL of the insert to be cloned, 1μL of TOPO Salt Solution, and 2μL of ddH_20 were gently mixed by pipetting and incubated for 20minutes at room temperature.

II.5: Transformation

Transformation of DNA into E. coli was performed using either of two methods: electroporation or heat shock.

II.5a: Electroporation

For electroporation, 0.8 μL of DNA (1X from a ligation or 1:100 from a plasmid prep) was added to a pre-chilled 1.0mM electroporation cuvette (VWR),

taking care to not touch the metal surfaces of the cuvette. Next, 40μL of electroporation-competent XL-1 blue cells were added to the cuvette, and the cuvette was recapped. Electroporation using 1.8kV with an infinite resistance and capacitance of 25 uF was carried out in accordance with the manufacturer's directions. After electroporation, 250μL SOC media was added to the cuvette, and cells were removed from the cuvette, placed in a 15mL plastic tube, and incubated at 37 ^{o}C and 250rpm for 1hr, after which they were spread (1 plate had 15μL of cells, the other had 200μL) onto a Petri dish containing Luria Broth agar with the appropriate antibiotic. The concentration of carbenicillin in LB agar was 50μg/mL, while kanamycin was used at 25μg/mL.

II.5b: Heat-Shock

Heat-shock transformation for subcloning was performed with either library efficiency DB3.1 or OneShot TOP10 (Invitrogen). After slowly thawing cells on ice, 1-2μL (between 1 and 100ng) of DNA sample was added, and cells were incubated with DNA on ice for 5min. Next, the sample was incubated at 42 ^{o}C for 30s in a water bath. After cooling the cells on ice for 2min, 250μL of SOC was added to the tube, and the tube was placed on a rotating wheel for 1hr at 37 ^{o}C. Cells were then spread onto an appropriate LB-agar plates with relevant antibiotic, such that one plate contained 20μL of the cell mix, while the other had 200μL. For protein

expression, either BL21 DE3(pLysS), or Rosetta cells were transformed with 10ng DNA in the same manner as DB3.1 or OneShot TOP10 cells, except that a 45second heat shock at 42 °C was used.

II.6: Preparation of Plasmid DNA

For small-scale preparation of DNA, 2mL cultures were inoculated with 1 colony from a freshly prepared bacterial plate 12-18hr before processing. Plasmids were purified using the Qiagen SpinMiniprep Kit. For DNA to be used for transfection, 110mL cultures were inoculated with 1 colony from a freshly streaked plate 15-20hr before harvesting. The harvested pellet was spun at 3000 rpm in a Beckman Allegra 6R centrifuge for 20min, and the pellet was then processed using the Qiagen EndoFree Maxi Kit according to the manufacturer's directions, except that a JS-13.1 rotor (with Beckman tube 357003) was used for centrifugation, 500μL of EndoFree TE was used to resuspend DNA, and DNA was incubated at 65 °C for 1min to facilitate resuspension.

II.7: Agarose gel electrophoresis

Agarose gels were poured by addition of between 0.6 to 1.8g of SeaKem GTG Agarose (Lonza) to 100mL of 0.5X Tris-Borate EDTA (TBE) in a 500mL Erlenmeyer flask, which after addition of the agarose and TBE, was weighed. After heating for 120s, ddH_20 was readded to the original mass of the flask plus its contents. The slurry was then reheated for 45s, capped with aluminum foil, and

incubated at 42 °C for 10min before addition of 2.5μL of ethidium bromide (10mg/mL stock). The gel solution was then poured into a casting tray and allowed to cool for 1hr. The gel was run for 60min at 80V in 0.5X TBE with 15μL of ethidium bromide (10mg/mL stock) added to the running buffer.

II.8: Cell culture

All mammalian cells used in this study were grown at 37 °C in a humidified incubator with 5% CO_2 and ambient O_2. For HeLa S3 (ATCC), cells were grown in media containing DMEM supplemented with 2mM GlutaMAX (Invitrogen), 0.1mM non-essential amino acids (Invitrogen), and 10% fetal bovine serum (JRH), and passaged at a 1:10 dilution every 3 days. GM00558 were purchased from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ) and grown in RPMI supplemented with 15% FBS and 2mM L-glutamine. GM16147 (XRCC4 null) cells were grown in Ham's F12 supplemented with 10% FBS, and were split 1:7.5 every 3 days. The WRN deficient cell line AG11395 and the normal transformed fibroblast VA-13 were grown in media containing DMEM supplemented with 2mM GlutaMAX (Invitrogen), 0.1mM non-essential amino acids (Invitrogen), and 10% fetal bovine serum (JRH), and passaged at a 1:10 dilution every 3 days.

II.9: Transfection

For transfection, HeLa S3, GM16147, VA13, and AG11395 were seeded at between 500,000 and 1,000,000 cells/per well of a 6 well dish 24hr before

transfection. Transfections followed this general format for 6 well dish: Lipofectamine2000 (10μL per well for HeLa S3 and GM16147, 5μL per well for AG11395 and VA13) was diluted into 240μL of OptiMEM and incubated for 5 min at room temperature. DNA (4μg for HeLa S3 and GM16147, 2μg for AG11395 and VA13) was diluted with OptiMEM to a final volume of 250μL. After the 5 minute lipofectamine incubation, the DNA and lipofectamine were mixed and incubated for an additional 20min before addition directly to each well of a 6 well dish, such that 500μL total lipofectamine:DNA mix was added per well. Media was changed 24hr after transfection, and when necessary antibiotics were added at this time (3.3μg/mL Puromycin (Sigma) or 250μg/mL Hygromycin (Gibco)).

II.10: Cloning TebDB and constructing Venus-TebDB fusion cDNA

The cDNA encoding amino acids 32-227 of the S. pombe ORF SPAC13G7.10 was obtained by PCR amplification with the forward primer BLoli 1070 5'TATGGATCCCCAAAGAAGAAGCGTAAGGTTGAAATGTCTAAAAGGGAGGTAGCTCAAGATGTTCCAGG3' containing a BamHI site for directional cloning, and reverse primer BLoli 1071 5'TATGCGGCCGCCTATAGTTGTAGAACTATCGTTCGGGGTCGTAGC3' with a NotI site, using S. pombe cDNA prepared from reverse-transcription of S. pombe total RNA with an oligo $dT_{(12-18)}$ primer performed and generously provided by K.

Trujillo. BLoli 1070 contains the cDNA encoding the amino acids for the nuclear localization sequence from SV40 large T antigen (PKKKRKVE), which facilitates import of proteins containing this sequence to the nucleus in mammalian systems. This PCR product was then cloned into pCR4-blunt for subcloning and sequencing. After successful sequencing of miniprep DNA, this product was then digested and ligated with T4 DNA ligase into pIRESneo2 that already contained the cDNA for the fluorescent protein Venus (purchased from the Stowers Institute Molecular Biology Facility), where Venus was inserted between NheI and BamHI sites. TebDB with the SV40 NLS was then inserted between BamHI and NotI sites in the pIRESneo2 vector containing Venus, creating a Venus-TebDB fusion protein. For cloning Venus-TebDB into pIRESpuro2 and pIREShyg2, the Venus-TebDB fragment from Venus-TebDB-pIRESneo2 was excised with NheI and BstXI and cloned into these sites in the pIRESpuro2 and pIREShyg2 plasmids.

II.11: Cloning Rap1-TebDB fusion proteins

Human RAP1 in pGEX-4T was a gift from Z. Songyang. A cDNA encoding TebDB (amino acids 32-227 of Teb1, SpX, SPAC13G7.10) was cloned from cDNA generously provided by K. Trujillo. TebDB was inserted into pGEX-4T in frame with an N-terminal GST tag and thrombin cleavage site using BamHI and NotI sites, creating pJSCTeb. To create a GST-TebDB-RAP1 fusion protein (pJSC3), TebDB with flanking AscI sites was obtained by PCR with primers BLoli 1173 5' TATGGCGCGCCATCTAAAAGGGAGGTAGCTCAAGATGTTC 3' and BLoli

1174 5’TATGGCGCGCCTGTAGAACTATCGTTCGGGGTCGTAGCATC3’. This fragment was inserted into the AscI site between the thrombin cleavage site and RAP1 in RAP1-pGEX-4T. GST-tagged proteins were expressed in *E. coli* and bound to glutathione-coupled beads in batch. After elution with glutathione, thrombin (Amersham) cleavage was performed on GST-TebDB-RAP1 containing eluates. TebDB-RAP1 and GST-TebDB were further purified by mono Q ion-exchange chromatography. Protein concentrations were determined by Bradford assay (BioRad).

To clone an hRAP1-Venus-TebDB fusion protein, the cDNA for RAP1 was PCR amplified with BLoli 1072 5’TATGCTAGCATGGCGGAGGCGATGGATTTGGGCAAAG3’ containing an NheI site, and BLoli 1080 5’GTGTTTTTCTTTCGAAATTCAATCCTCCGAG3’, and cloned into pIRESneo2 using NheI and StuI sites. TebDB with the SV40 NLS was inserted in between the BamHI and NotI sites was obtained by PCR using the BLoli 1070 and BLoli 1071 and cloned into pIRESneo2 as indicated previously. Venus was then inserted between the HpaI and BamHI sites creating a Rap1-Venus-TebDB fusion protein. The cDNA for this fusion protein was then cloned from pIRESneo2 into pIREShyg2 using NheI and BstXI. RAP1ΔCT with an N-terminal FLAG tag was purchased from Addgene (plasmid 13252)[66] and cloned into pIREShyg2 containing RAP1-Venus-NLS-TebDB using SpeI and EcoRV.

II.12: Cloning TRF2ΔBΔM and TRF2ΔB

TRF2ΔBΔM, which is a TRF2 fragment that lacks a.a. 1-43 and 446-499, was created from a PCR using BLoli 1008A 5'GAGGCACGGCTGGAAGAGGCAGTC3' and BLoli 1009 5'TTGAGCGGCCGCTCACTTTTTTGTTATATTGGTTGTAC3' with a TRF2 cDNA-containing plasmid from Addgene (plasmid 12299)[48]. The PCR product was then cloned into pIND-V5 (Invitrogen), which contained an N-terminal FLAG tag, using EcoRV and NotI sites. TRF2ΔBΔM was then excised from this vector using NheI and NotI, and cloned into pIRESpuro2. TRF2ΔB was cloned in the same manner, except that BLoli 1010 5'TTGAGCGGCCGCTCAGTTCATGCCAAGTCTTTTCATG3' was used in place of BLoli 1009 so that the last 53 amino acids of TRF2 would be retained in the PCR product.

II.13: Cloning mCherry-TRF1 and Venus-TRF1 fusions

A plasmid containing TRF1 in pBluescript SK +/- (Addgene plasmid 12303)[37] was used to clone TRF1 as a C-terminal fusion with mCherry into pIREShyg2. TRF1 with EcoRI and BamHI sites engineered by PCR using BLoli 1083 5'TGGGAATTCATGGCGGAGGATGTTTCCTCAG3' and BLoli 1084 5'TGGGGATCCTCAGTCTTCGCTGTCTGAGGAAATC3' was inserted into pIRESbleo using EcoRI and BamHI sites. An mCherry PCR product was inserted

upstream in between NotI and EcoRI sites following PCR with BLoli 1081 5’TGGGCGGCCGCAATATGGTGAGCAAGGGCGAGGAGGATAAC3’ and BLoli 1082 5’TGGGAATTCCTTGTACAGCTCGTCCATGCCGCCGGTG3’ using mCherry cDNA obtained from the Stowers Institute Molecular Biology facility. The mCherry-TRF1 fusion was then cloned from pIRESbleo into pIREShyg2 using EcoRV and BamHI sites in pIRESbleo, and StuI and BamHI sites in pIREShyg2. For creation of pIRESbleo with a Venus-TRF1 fusion, mCherry was excised with EcoRV and EcoRI, and Venus was excised from RAP1-Venus-TebDB with HpaI and EcoRI and cloned into the gap created by removal of mCherry. Venus-TRF1 was then amplified by PCR with BLoli 1450 5’TAAGCTAGCACCATGGTGAGCAAGGGCGAGGAGCTGTTC3’ and BLoli 1451 5’AGTGGATCCTCAGTCTTCGCTGTCTGAG3’ that contained NheI and BamHI sites, respectively, and these sites were then used to clone Venus-TRF1 into pIREShyg2.

II.14: Electrophoretic Mobility Shift Assays (EMSAs)

DNA substrates were generated by annealing complementary oligo nucleotides. The G-rich strand for the telomeric substrate is 5’ACGTGGTCAAAGTCTGGAAC$(TTAGGG)_{10}$-3’, and 5'ACGTGGTCAAAGTCTGGAAC$(TGAGTG)_{10}$-3' for the scrambled substrate. The G-rich DNA oligonucleotides were labeled with [γ-^{32}P]ATP using polynucleotide kinase and annealed with a 1.5 fold excess of the unlabelled complementary oligo.

The annealed products were purified over G-25 sepharose columns, and used for EMSA reactions at 0.4 nM. Substrates were incubated with recombinant GST-TebDB or TebDB-RAP1 at 225:1, 450:1, 900:1, and 1800:1 molar ratios of protein-to-substrate in EMSA buffer (50mM TEA, pH7.5; 40mM KCl, 0.5mM DTT; 100 μg/mL BSA) at room temperature for 10 minutes. DNA and DNA-protein complexes were resolved by electrophoresis on 6% polyacrylamide gels in 0.5X TBE buffer at 200V for 5 minutes followed by 90 minutes at 90 V. The gels were dried and subjected to PhosphorImager analysis. Apparent dissociation constants $Kd_{[app]}$ were calculated from electrophoretic mobility shift assays as described above except that the DNA substrate was used at a final concentration of 0.09 nM. (Protein concentrations ranged in two-fold steps from 2.25nM to 36nM for GST-TebDB and in two-fold steps from 4.5nM to 72nM for TebDB-Rap1 for Kd.)

II.15: *In Vitro* Non-Homologous End Joining Assays

10μl reactions were carried out as follows: 6μl of deionized water was added to a 0.65mL microcentrifuge tube, and 1μl of NHEJ extract (prepared as described in [132]) and 2ul of 5X End-Joining Buffer (250mM TEA/OAc, pH 8.5, 300mM KOAc, 2.5mM Mg(OAc)2, 5mM ATP, 5mM DTT, 0.5mg/mL BSA) were then added to the tube. After incubation for 5 minutes at 37^0C, 1μl of DNA was then added, and the reaction was incubated at 37^0C for 1hr. The reaction was then stopped by addition of 2μl Stop Buffer (100mM Tris-HCl, pH7.5, 200mM EDTA, 10mg/mL proteinase K, 2.5% SDS) followed by a 15 min incubation at 42^0C. Products were

electrophoresed on 0.6% TBE gels and analysed using standard autoradiography methods.

II.16: Cell culture and transfection

HeLa S3 cells (ATCC) were grown in media containing DMEM supplemented with 2mM GlutaMAX (Invitrogen), 0.1mM non-essential amino acids (Invitrogen), and 10% fetal bovine serum (JRH). For transfection of cells for immunofluorescence, metaphase-spread FISH, or Western Blotting as performed in Chapter Three, cells were seeded at $5x10^5$ cells per well in a 6 well dish and transfected with 4μg DNA using Lipofectamine 2000. After 4hr, cells were reseeded into two wells of a 6 well dish. 24hr after transfection, antibiotic selection was performed using media containing 3.3μg/mL puromycin (Sigma Aldrich), and 250μg/mL Hygromycin B (Invitrogen). Dual antibiotic selection was employed to ensure that all cells analyzed by immunofluorescence, metaphase spread-TeloFISH, and Western blotting expressed the relevant proteins, thereby negating any effects due to differences in transfection efficiency. The effectiveness of antibiotic selection was assayed by quantifying the percentage of cells expressing either Venus-TebDB or Rap1ΔCT-Venus-TebDB 3 days after transfection. Fluorescence microscopy with these cells indicated that Venus was expressed in all cells.

II.17: Immunofluorescence

Cells were seeded onto 22x22mm glass coverslips 4hr post transfection. After 48hr of selection in media containing puromycin (3.3μg/mL) and hygromycin (250μg/mL), cells were washed once with PBS, and incubated with Triton extraction buffer (300mM Sucrose, 20mM HEPES pH 7.9, 50mM NaCl, 3mM $MgCl_2$, and 0.5% Triton X-100) at 4^{o}C for 2min. Cells were then washed twice with PBS, and fixed with 3% paraformaldehhyde and 2% sucrose in PBS for 10min. After fixation, cells were washed with PBS and repermeabilized with Triton extraction buffer for 10min. Cells were then washed twice for 5 min with PBS, and blocking was performed for 45min in PBS containing 1% BSA. The TRF2 monoclonal antibody 4A794 (Cat# 05-521, Millipore) and RAP1 polyclonal antibody (Cat# A300-306A, Bethyl Labs) were diluted 1:1000 in PBS with 1%BSA. Coverslips were incubated with primary antibodies for 2 hours followed by three 5 min washes in PBS with 1% BSA. Coverslips were then incubated with goat anti-mouse antibodies labeled with AlexaFluor 594 and goat anti-rabbit antibodies labeled with AlexaFluor 488 diluted 1:1000 in PBS + 1% BSA for 1hr. Coverslips were washed twice in PBS with 1% BSA, stained with DAPI (200ng/mL) for 5 min, and mounted on slides with Fluoromount G mounting media. Microscopy was performed using an AxioPlan microscope with a 100X, 1.4 NA Plan-APOCHROMAT objective (Zeiss) and AxioVision software.

II.18: Metaphase Spread preparation and Telomere-FISH

Cells growing in media containing puromycin (3.3μg/mL) and hygromycin (250μg/mL) were treated with colcemid (0.1μg/mL) for 4hr and harvested by trypsin treatment 72hours after transfection. After hypotonic swelling in 10mL of 0.075M KCl at 37 ^{o}C for 7 min, cells were pelleted by centrifugation at 1000rpm for 5minutes at room temperature in a Beckman Allegra 6R centrifuge. Next, the cell pellet was resuspended in 10mL 3:1 MeOH:CH_3COOH, and cells were fixed in this solution for 10min. Cells were respun at 1000rpm for 5min at 4^{o}C, and resuspended in 10mL of 3:1 MeOH:CH_3COOH. This process was repeated an additional two times. Cells were dropped onto coverslips from a height of ~12 inches, washed once with 2mL of 3:1 MeOH:CH_3COOH, and heated to 75 ^{o}C for 1min. After drying coverslips for 1hr at room temperature, individual coverslips were placed into wells of a 6 well dish and incubated in 3mL PBS for 10min to rehydrate the sample, and were then incubated at 37 ^{o}C for 30min in 3mL PBS with 0.5mg/mL RNAse A. Coverslips were then washed twice and fixed with 3mL of 4% paraformaldehyde in PBS for 10min. Fixative was then removed, and cells were washed twice with PBS-A before addition of freshly dissolved and filtered Pepsin (Sigma) at a final concentration of 1mg/mL in pH2.0 ddH_20. Pepsin treatment proceeded for 10min at 37^{o}C, followed by washing and re-fixing as was performed after RNAse A treatment. After the last PBS wash, cells were dehydrated in a 70%, 90%, 100% ethanol dehydration series (incubate in alcohol for 5 minutes per step), and allowed to dry for 30-60min.

Hybridization of the sample was performed with 7.5μL of hybridization buffer (70% formamide, 0.25% blocking reagent (NEN Life Sciences, now PerkinElmer), 10mM Tris pH 7.0-7.5, 4.1 mM Na2HPO4, 0.45 mM citric acid, 1mM MgCl2, 100nM BLoli 1517 (Alexa 546-labeled 5'CCCT+AA+CC+CT+AA+CC+CT+AA3', where + is a locked nucleic acid) or 100nM BLoli 1265 (Alexa 488-labeled 5'TTAGGGTTAGGGTTAGGG3' with locked nucleic acids) being added to a large microscope slide, and the dried coverslip containing the sample was then gently lowered onto the droplet of probe. The sample was heated at 85^{o}C for 3min, and then incubated for 80min at room temperature. The sample was then removed from the coverslip under PBS heated to 65^{o}C, and was washed twice for 15min with 3mL of wash buffer (70% formamide, 10mM Tris, 0.1% BSA, pH=7.0-7.5), followed by three more washes with 3mL of buffer containing (0.1 M Tris, 0.15 M NaCl, 0.08% Tween 20, pH=7.0-7.5). The sample was then stained with DAPI (2μg/mL) for 5min before mounting with Fluoromount G.

Samples were imaged on an AxioPlan microscope with a 100X, 1.4 NA Plan-APOCHROMAT objective (Zeiss) and AxioVision software. Quantification of telomere fusions was performed on blinded samples to remove experimenter bias. A chi-square test for independence was applied to the incidence of telomere fusions observed by metaphase spread analysis. Expected values were calculated based on the percentage of fused telomeres in cells expressing only TRF2ΔBΔM. Analyses were performed using GraphPad Software.

Chapter Three:

Human RAP1 Protects Telomeres from Non-Homologous End Joining *in vivo*

III.1: Abstract

Telomeres, the nucleo-protein structures found at the ends of linear chromosomes, promote genome stability by distinguishing chromosome termini from DNA double-strand breaks (DSBs). Cells possess two principal pathways for DSB repair, homologous recombination and non-homologous end joining (NHEJ). Several studies have implicated TRF2 in the protection of telomeres from NHEJ, but the underlying mechanism remains poorly understood. Here, we show that TRF2 promotes NHEJ inhibition by recruiting human RAP1 to telomeres. Heterologous targeting of hRAP1 to telomeric DNA was sufficient to bypass the need for TRF2 in protecting telomeric DNA from NHEJ *in vitro*. Furthermore, recruitment of hRAP1 to telomeres in cells alleviated the uncapping defect caused by the loss of TRF2/hRAP1 from chromosome ends upon expression of dominant negative TRF2. These results provide the first evidence that hRAP1 inhibits NHEJ at mammalian telomeres and identify hRAP1 as a mediator of genome stability in human cells.

III.2: Introduction

The prescient work of Barbara McClintock in the 1940s demonstrated that an essential function of telomeres is to prevent fusion of chromosome ends to *de novo* DNA breaks [19]. In order to fulfil this role, telomeres must locally inhibit the DNA

damage response; a feat that involves TRF2 and POT1 repressing DNA damage signalling through the ATM and ATR kinases [58]. Telomere dysfunction can result from a variety of events including structural changes at telomeres, loss of a telomere binding protein, or the gradual shortening of the telomeric repeat tract. Proteins that specifically bind to telomeric repeats play a critical role in chromosome end protection, and their deletion results in telomere fusions in a broad range of species[24, 34, 73, 133, 134]. Early models of telomere protection predicted that telomere binding proteins outcompete the non-sequence specific binding of DNA repair factors near chromosome ends. However, it has become apparent that key factors involved in DNA DSB repair are present at chromosome termini without triggering end-to-end fusions[135, 136]. The molecular mechanism underlying this phenomenon has remained elusive, but may relate to the t-loop, a structure in which the 3′ overhang of the telomere loops back and invades internal telomeric repeats on the same chromosome arm[76]. Such structures have been visualized by electron microscopy in DNA samples from a variety of species[77, 78, 82, 137].

A number of proteins have been identified that specifically localize to mammalian telomeres, including three factors that directly bind telomeric DNA:, POT1, TRF1, and TRF2, and three associated proteins TIN2, RAP1 and TPP1[138]. A truncated version of TRF2 (TRF2ΔBΔM) acts as a dominant negative mutant by forming heterodimers with the endogenous protein that are unable to bind to DNA[53]. Cells expressing TRF2ΔBΔM show reduced TRF2 at telomeres and chromosome ends are subject to NHEJ[25]. A requirement for TRF2 in chromosome

capping is further supported by the dramatic telomere fusion phenotype observed in mouse embryonic fibroblasts following deletion of TRF2[133].

Using an *in vitro* assay for telomere capping we have previously shown that both hRAP1 and TRF2 are required to protect telomeric DNA ends from NHEJ[23]. By targeting hRAP1 to telomeric DNA in the absence of TRF2 we now demonstrate that protection from NHEJ can be mediated by hRAP1 alone. Our results suggest that hRAP1 provides the functional interaction that blocks NHEJ at telomeres with TRF2 serving to recruit hRAP1 to chromosome ends. Consistent with these biochemical studies, targeting hRAP1 to telomeres in human cells independent of TRF2 provides efficient protection from the effects of TRF2ΔBΔM.

III.3 Results

III.3a RAP1 inhibits NHEJ in vitro

In an attempt to define the specific functions of hRAP1 and TRF2 in protecting telomeric DNA ends from NHEJ we sought to bestow hRAP1 with the ability to bind vertebrate telomeric DNA independently of TRF2. In this context, we examined the DNA binding domain of the *S. pombe* Teb1protein (also known as SpX). Teb1 was initially identified through computational approaches as a possible telomere binding protein[139], but biochemical experiments failed to demonstrate high affinity binding to fission yeast telomeric repeats and no function in telomere maintenance has been reported[140, 141]. Instead, Teb1 preferentially binds TTAGGG repeats and may function as a transcription factor for numerous *S. pombe*

genes containing this sequence motif in their promoters [141]. As TTAGGG corresponds to the vertebrate telomeric repeat we were intrigued by the possibility of utilizing Teb1 to target proteins of interest to human telomeres.

To further characterize the Teb1 DNA binding domain (from hereon referred to as TebDB), we expressed and purified a 195 amino acid fragment of Teb1 fused to glutathione-S-transferase (GST). TebDB showed robust and specific binding to vertebrate telomeric repeats (Figure 3.1a) with an apparent binding constant of 25 nM. The reported K_d for TRF2 binding to telomeric DNA is 180 nM[52], suggesting that TebDB has similar affinity for human telomeric repeats to an endogenous telomere binding protein. Fusing hRAP1 to the N-terminus of TebDB did not diminish its affinity or specificity for binding to TTAGGG repeats (Figure 3.1b, $K_{d[app]}$=15nM).

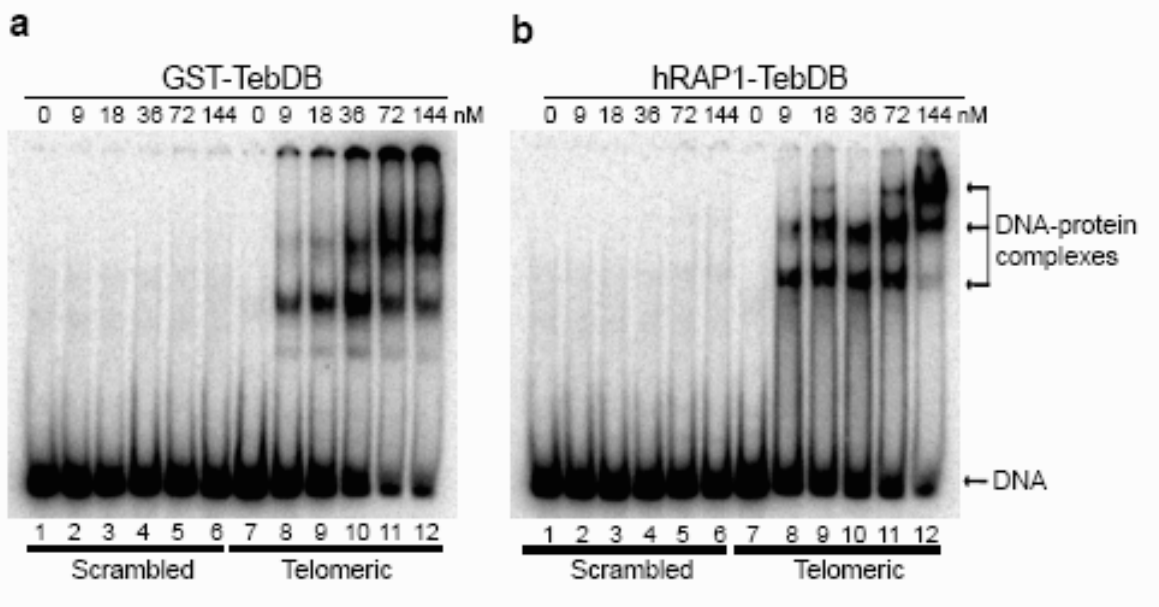


Figure 3.1: RAP1-TebDB binds vertebrate telomeric DNA. **(a)** Electrophoretic mobility shift assay (EMSA) of double-stranded scrambled and telomeric DNA oligonucleotides incubated with indicated amounts of GST-TebDB. No protein was added in lanes 1 and 7. **(b)** EMSA of DNA substrates incubated with hRAP1-TebDB.

Having demonstrated that TebDB fusions to GST and hRAP1 bind human telomeric DNA in gel mobility shift assays, we tested the ability of these proteins to protect telomeric DNA from NHEJ *in vitro*. We have previously shown that telomeric DNA ends are protected from double-strand break repair activities when incubated with NHEJ-competent human lymphocyte extract [23]. After immunodepleting the extract of TRF2 and hRAP1 (Figure 3.2), end protection was lost but could be restored by adding back recombinant TRF2 and hRAP1, whereas addition of either protein alone was insufficient[23]. We now wanted to test whether TebDB-mediated recruitment of hRAP1 to telomeric DNA bypasses the need for TRF2. Addition of GST-TebDB to TRF2/hRAP1 immunodepleted extract had little effect on NHEJ at telomeric ends indicating that high affinity binding of this exogenous protein fails to recapitulate end protection (Figure 3.3a, lanes 3-6). Instead, a modest increase in end joining activity was observed at the lower concentrations of GST-TebDB (Figure 3.3a, lanes 3 and 4). In contrast, hRAP1-TebDB inhibited end joining in a concentration dependent manner (lanes 7-10) with a five-fold reduction in end joining products being observed at a concentration where GST-TebDB had no effect (Figure 3.3a, compare lanes 5 and 9).

To verify that the NHEJ-inhibiting activity of hRAP1-TebDB is due to recruitment of hRAP1 to telomeric DNA ends by TebDB, non-telomeric DNA ends

were also incubated with hRAP1/TRF2-immunodepleted extract. Though GST-TebDB exhibited a very slight impact on end-joining (Figure 3.3b, lanes 2-4), hRAP1-TebDB had no impact on NHEJ of non-telomeric ends (Figure 3.3b, lanes 5-7). This result demonstrates that both the telomeric DNA binding ability of TebDB and the NHEJ-inhibiting activity of hRAP1 are responsible for telomeric end protection observed in this assay (Figure 3.3a). Because immunodepletion had removed most of TRF2 (Figure 3.2), these results suggest that TRF2 contributes to NHEJ inhibition at telomeres by recruiting hRAP1, which in turn blocks end joining. To test this model *in vivo*, we proceeded to evaluate ways of TRF2-independent recruitment of hRAP1 to telomeres in cells.

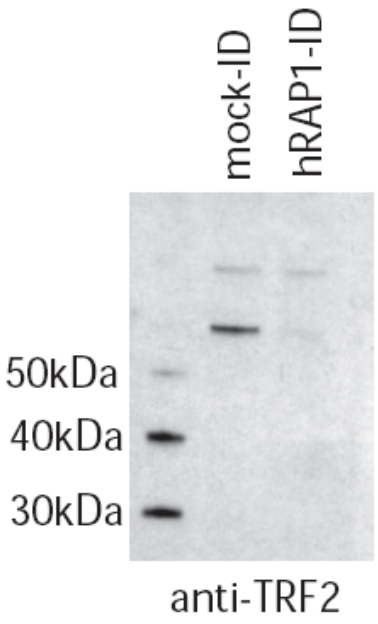


Figure 3.2: Immunodepletion of hRAP1 results in loss of TRF2 from NHEJ-competent extract. Mock (Rabbit IgG) or hRAP1-immunodepleted NHEJ-competent

extract was subjected to SDS-PAGE followed by immunoblotting analysis with a monoclonal TRF2 antibody (4A794) at 1:2000 and an HRP-conjugated goat anti-mouse antibody at 1:5000 (Pierce).

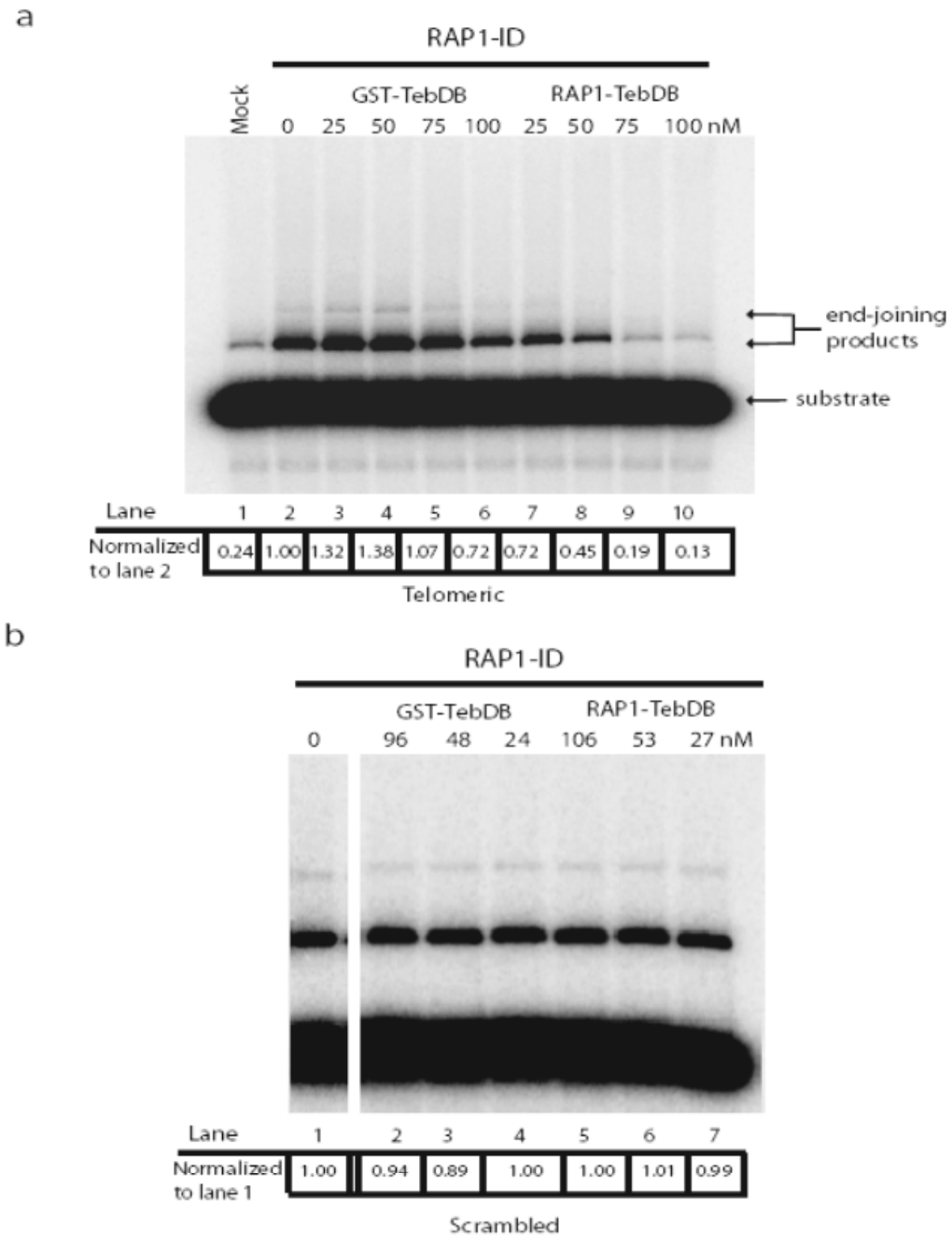


Figure 3.3: Inhibition of end joining by hRAP1-TebDB specifically at telomeric DNA ends. (a) Linear plasmid DNA containing twelve 5'-TTAGGG-3' repeats at

one end was incubated with GM00558 cell-free extract that was either mock depleted (lane 1), or immunodepleted (ID) of hRAP1 and TRF2 with anti-hRAP1 (lanes 2-10). GST-TebDB (lanes 3-6) and hRAP1-TebDB (lanes 7-10) were added at the indicated concentrations prior to incubation with DNA substrates. As each DNA substrate contains one telomeric (head) and one non-telomeric (tail) end, the presence of tail-to-tail fusions serves as an internal control for the presence of NHEJ activity in the extract (lane 1). End joining products were quantified by densitometry and were normalized to hRAP1-immunodepleted extract (lane 2).(b) Linear plasmid DNA containing twelve 5'-TGAGTG-3' repeats at one end were incubated with GM00558 cell-free extract that was immunodepleted (ID) of hRAP1 and TRF2 with anti-hRAP1 (lanes 1-7). GST-TebDB (lanes 2-4) and hRAP1-TebDB (lanes 5-7) were added at the indicated concentrations prior to incubation with DNA substrates. End joining products were quantified by densitometry and were normalized to hRAP1-immunodepleted extract (lane 1). Gel was spliced to remove intervening lanes that were not pertinent to this experiment.

III.b TebDB localizes to human telomeres in vivo

Encouraged by the high affinity and specificity of TebDB for vertebrate telomeric DNA (Figure 3.1a), we examined the subcellular localization of TebDB fused to the GFP variant Venus. To ensure efficient nuclear import, the nuclear localization signal from SV40 large T antigen was included in Venus-TebDB and all other TebDB-containing fusion constructs used in this study. Fluorescence microscopic analysis of HeLa S3 cells expressing Venus-TebDB revealed punctate nuclear staining that largely colocalized with endogenous TRF2 (Figure 3.4a). However, a minor fraction of Venus-TebDB foci lacked a corresponding TRF2 signal. This could reflect Venus-TebDB localization to non-telomeric sites in addition to telomeres. Alternatively, the fluorescent fusion protein may simply visualize telomeric loci more efficiently than the TRF2 antibody. To distinguish between these possibilities we generated a fusion of TRF1 to the fluorescent protein mCherry and examined the extent of co-localization with endogenous TRF2.

Consistent with fluorescent fusion proteins labeling telomeres more efficiently, all TRF2 foci colocalized with mCherry-TRF1, with a few additional mCherry-TRF1 foci in places with weak or non-detectable TRF2 signal (Figure 3.4b). Finally, we co-expressed mCherry-TRF1 and Venus-TebDB and observed widespread co-localization of the two proteins (Figure 3.4c). We concluded that the TebDB is capable of mediating localization of fusion proteins to human telomeres *in vivo*.

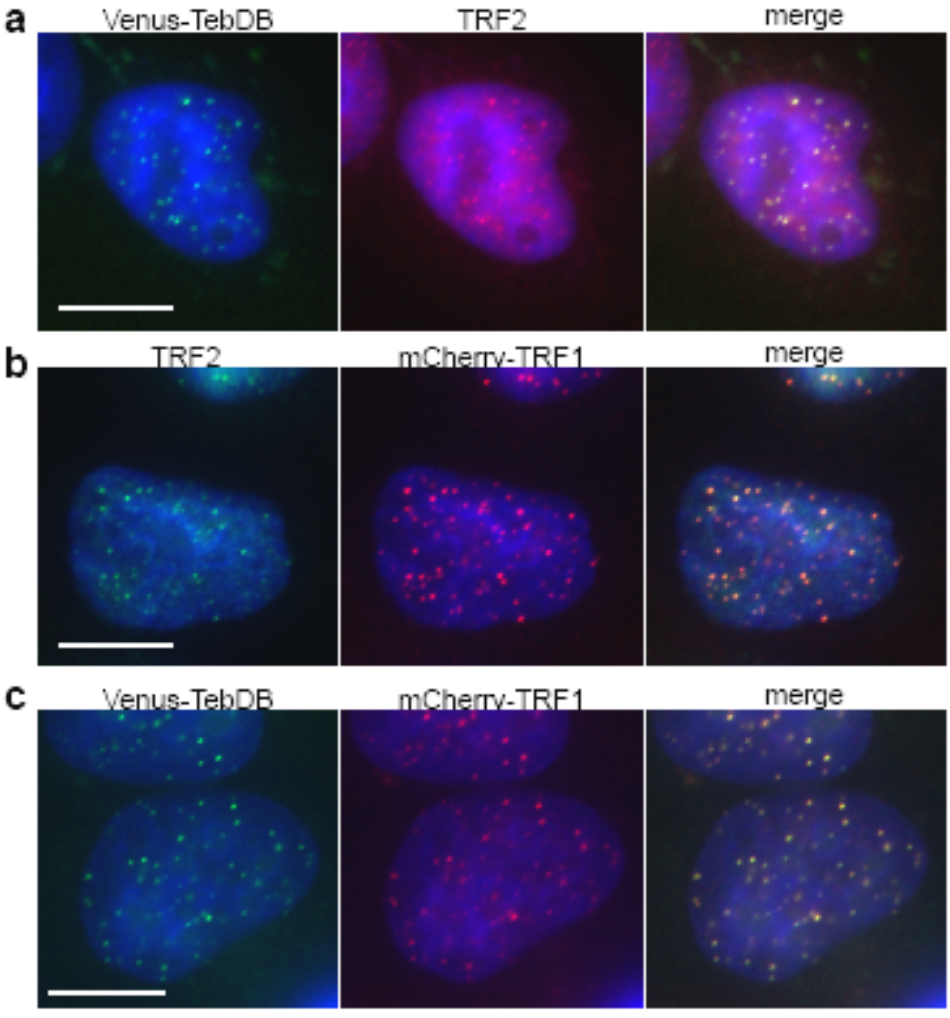


Figure 3.4: Telomeric localization of TebDB in human cells. **(a)** HeLa S3 cells transfected with Venus-TebDB (green) were stained with a mouse monoclonal

antibody for TRF2 followed by an AlexaFluor 594-conjugated secondary antibody (red). Nuclei were visualized by counterstaining with DAPI (blue). Cells were subjected to nucleoplasmic extraction so that only chromatin-associated proteins remain within nuclei. **(b)** Cells expressing mCherry-TRF1 (red) were stained with a mouse monoclonal antibody for TRF2 and AlexaFluor 488 secondary antibody (green). **(c)** Visualization of Venus-TebDB (green) and mCherry-TRF1 (red) in co-transfected cells. All scale bars correspond to 10 μm.

III.3c Expression of TRF2ΔBΔM results in preferential loss of hRAP1 from telomeres

High-level expression of TRF2ΔBΔM is thought to drive endogenous TRF2 into heterodimeric complexes that fail to bind telomeric DNA, thereby reducing the association of endogenous TRF2 with telomeres [53, 142]. Interestingly, several studies have indicated that telomeric TRF2 foci remain detectable in cells expressing TRF2ΔBΔM [53, 57, 143]. In addition, ChIP analysis from cells expressing Trf2ΔBΔM showed a 14-fold increase in 53BP1 levels at telomeres, while Trf2 was only reduced by 50% [57]. In light of the absence of haploinsufficiency in $TRF2^{+/-}$ heterozygous murine cells[58] and modest phenotypes observed in TRF2 knockdown experiments[144, 145] (our unpublished data), these observations suggest that TRF2ΔBΔM may mediate telomere uncapping by acting on multiple targets. A likely candidate for such a target is hRAP1, which interacts with a region of TRF2 present in the dominant negative fragment[65]. As TRF2ΔBΔM is expressed at much higher levels than endogenous TRF2, the majority of TRF2ΔBΔM is expected to form homodimers which lack the ability to bind telomeric DNA and have no effect on endogenous TRF2. However, endogenous hRAP1 may be sequestered by

TRF2ΔBΔM homodimers thus preventing its recruitment to telomeres. To investigate the possibility that preferential loss of hRAP1 contributes to telomere fusions when TRF2ΔBΔM is expressed, we introduced TRF2ΔBΔM into HeLa S3 cells and analyzed the localization of endogenous hRAP1 and TRF2. A nucleoplasm extraction procedure[65] was used to ameliorate the high nucleoplasmic background associated with TRF2ΔBΔM expression when probing with an antibody against TRF2. While TRF2 and hRAP1 foci were prominent in cells transfected with empty vectors (Figure 3.5a), no telomeric hRAP1 was detected in TRF2ΔBΔM-expressing cells (Figure 3.5b). In contrast, and consistent with previous results[53, 65, 143], TRF2 foci were reduced but readily detectable in these cells (Figure 3.5b). We verified that the remaining TRF2 foci were telomeric in origin by co-expressing TRF2ΔBΔM and fluorescently-tagged TRF1 as a telomeric marker that is unaffected by TRF2ΔBΔM expression[53]. Following antibiotic selection to eliminate untransfected cells, telomeric TRF2 was observed in the majority of cells whereas hRAP1 was not detected (Figure 3.5c and d). Despite the presence of telomeric TRF2 in these cells, metaphase spreads confirmed widespread uncapping as 29.3% of telomeres had participated in fusions (see below). In summary, these experiments revealed extensive telomere uncapping under conditions where TRF2 was detectable at telomeres while telomeric hRAP1 was not observed. We cannot rule out the possibility that differences in antibody affinity contribute to the apparent loss of hRAP1 but retention of telomeric TRF2. However, together with the *in vitro* work described above these results indicate that loss of hRAP1 may be primarily

responsible for telomere uncapping. A corollary to this hypothesis predicts that hRAP1 inhibits NHEJ at telomeres.

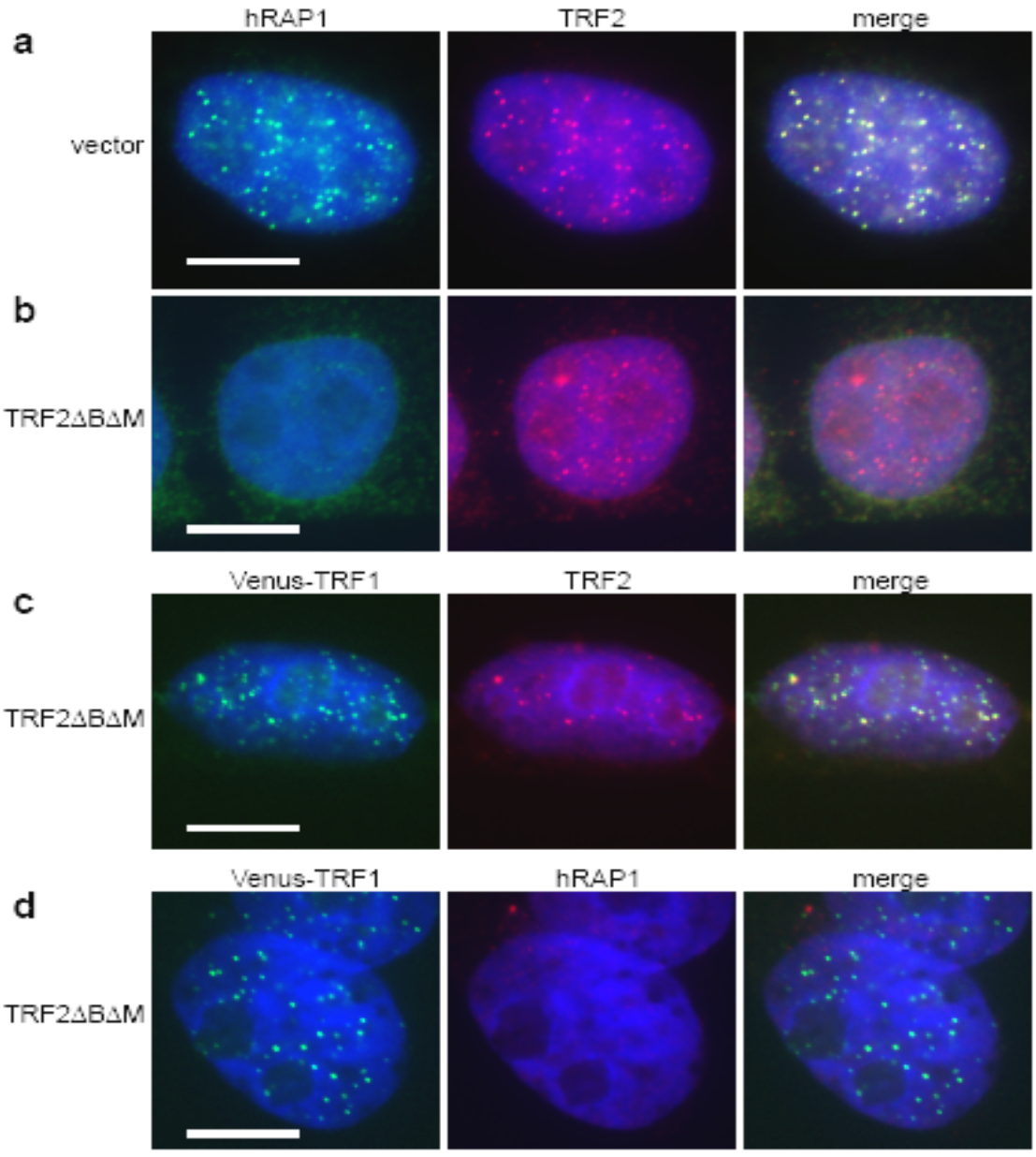


Figure 3.5: Dominant negative TRF2 (TRF2ΔBΔM) preferentially removes hRAP1 from telomeres. **(a)** Immunostaining of hRAP1 (AlexaFluor 488, green) and TRF2 (AlexaFluor 594, red) in HeLa S3 cells transfected with and selected for the presence

of the vector controls. DNA was stained with DAPI (blue). **(b)** Visualization of TRF2 and hRAP1 as in (a) using cells expressing TRF2ΔBΔM. **(c)** Cells expressing TRF2ΔBΔM and Venus-TRF1 (green) were stained with an anti-TRF2 (AlexaFluor 594, red) and DAPI (blue). **(d)** Cells as in (c) were stained with anti-hRAP1 (AlexaFluor 594, red). All scale bars correspond to 10 μm.

III.3d: TebDB neither uncaps nor protects human telomeres

Telomeric localization of TebDB allowed us to target hRAP1 to chromosome ends independent of TRF2 and analyze whether such recruitment would ameliorate the effects of expressing TRF2ΔBΔM. Prior to proceeding with this experiment we had to examine whether TebDB binding alone affected telomere function *in vivo*. To address this issue, we performed telomere-FISH on metaphase spreads prepared from cells transfected with empty vector, Venus-TebDB, or TRF2ΔBΔM. While chromosome structure was normal in cells harbouring the vector control (Figure 3.6a), abundant chromosome fusions were observed in cells expressing TRF2ΔBΔM giving rise to long trains of fused chromosomes (Figure 3.6b). In contrast, no telomere fusions were observed in metaphases of cells expressing TebDB (Figure. 3.6c). TebDB expression did result in an apparent increase in telomere length heterogeneity, suggesting some effect on telomere structure that may facilitate telomere recombination.

Although TebDB alone was insufficient to protect telomeric DNA ends from NHEJ-mediated fusions *in vitro* (Figure 3.3), it was critical to test whether TebDB expression would partially or completely negate the effect of TRF2ΔBΔM *in vivo*. Analysis of metaphase spreads from cells co-expressing TebDB and TRF2ΔBΔM

revealed abundant telomere fusions (Figure 3.6d). In fact, co-expression with TebDB appeared to exacerbate the TRF2ΔBΔM phenotype. This synergistic effect may be related to the increased telomere length heterogeneity caused by TebDB sensitizing telomeres to the loss of TRF2 and its interaction partners. However, as TebDB expression alone neither induced nor inhibited NHEJ-mediated telomere fusions, we proceeded to utilize this telomere binding domain to target hRAP1 to chromosome ends independent of TRF2.

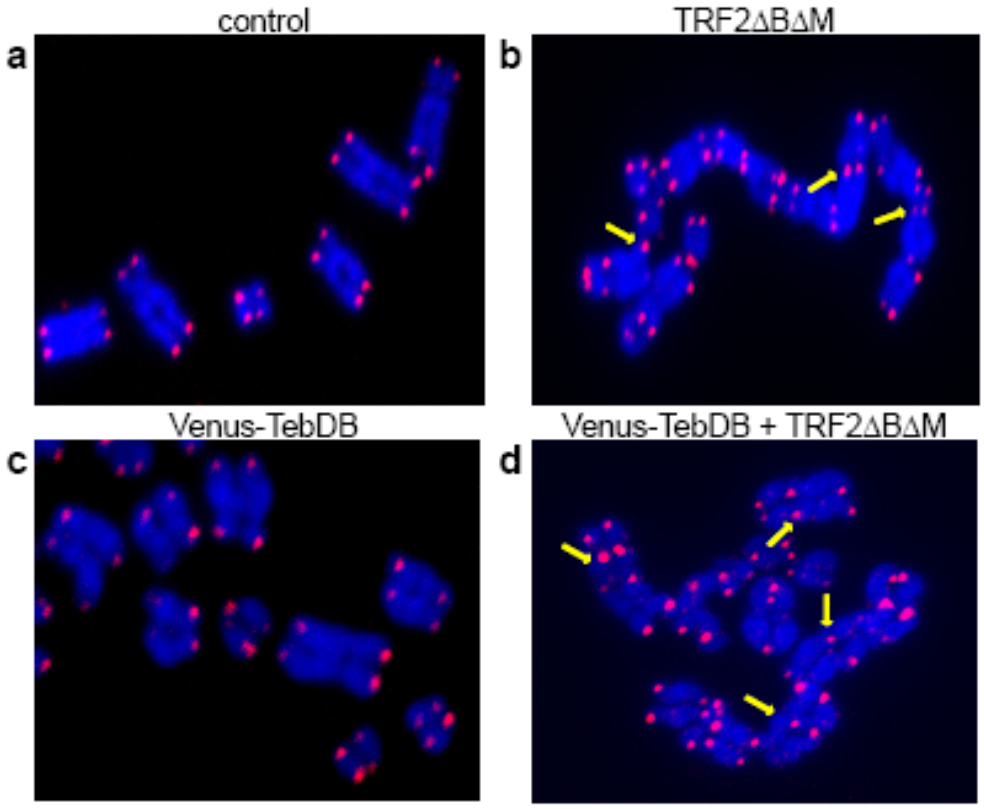


Figure 3.6: TebDB neither induces nor protects against NHEJ-dependent telomere fusions. Telomere-FISH was performed on metaphase spreads from cells transfected with and selected for vector control **(a)**, TRF2ΔBΔM **(b)**, Venus-TebDB **(c)**, or Venus-TebDB and TRF2ΔBΔM **(d)**. Telomeres were visualized with an AlexaFluor

543-labeled locked nucleic acid probe complementary to the G-rich strand (red). Chromosomes were counterstained with DAPI (blue). Representative chromosomes from the respective samples are shown. Some telomere:telomere fusions are highlighted with yellow arrows.

III.3e: Telomeric hRAP1 counteracts uncapping by TRF2ΔBΔM

To restore hRAP1 at telomeres following loss of TRF2, we fused Venus-TebDB to a previously characterized hRAP1 fragment lacking the homodimerization and TRF2 interaction domains hRAP1ΔCT[65, 66]. The hRAP1ΔCT fragment was chosen as it cannot interact with either endogenous TRF2 or TRF2ΔBΔM and will therefore neither be recruited to telomeres by TRF2, nor will it interfere with the ability of TRF2ΔBΔM to remove endogenous hRAP1 and TRF2 from telomeres[69]. In contrast, a full length hRAP1-TebDB fusion efficiently recruited TRF2ΔBΔM to telomeres (Figure 3.7). As expected, hRAP1ΔCT-Venus-TebDB accumulated in foci that colocalized with TRF1 even when TRF2ΔBΔM was expressed (Figure 3.8a). We noted that co-expressing TebDB fusion proteins with TRF2ΔBΔM caused a further reduction in endogenous TRF2 foci, thereby providing us with a system in which TRF2 is not detected at telomeres but the hRAP1 fusion protein localises efficiently to chromosome ends (Figure 3.8b).

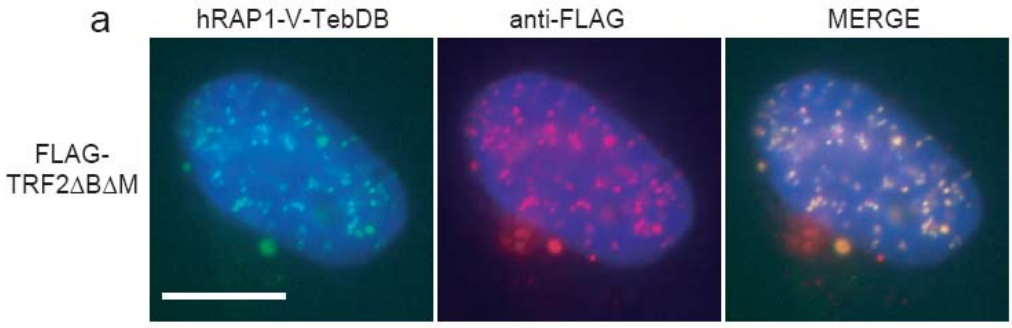


Figure 3.7: TRF2ΔBΔM can be recruited to telomeres by an hRAP1-Venus-TebDB fusion protein. (a). Cells transfected with FLAG-TRF2ΔBΔM and hRAP1-Venus-TebDB were selected for expression of these proteins by treatment with puromycin and hygromycin for 48 h. Cells were then subjected to triton extraction, fixation, and immunostaining with a monoclonal anti-FLAG antibody (M2) at 1:1000, and a AlexaFluor 594-conjugated goat anti-mouse secondary antibody at 1:1000. Scale bar represents 10 μm.

Next, we assayed the incidence of telomere fusions in metaphase spreads of cells co-expressing hRAP1ΔCT-Venus-TebDB and TRF2ΔBΔM. For comparison, telomere fusions were also scored in cells expressing only TRF2ΔBΔM and cells co-expressing TRF2ΔBΔM and hRAP1ΔCT not fused to the TebDB domain. The prevalence of telomere fusions observed in metaphase spreads of cells expressing hRAP1ΔCT and TRF2ΔBΔM (Figure 3.8c) contrasted sharply with cells co-expressing hRAP1ΔCT-Venus-TebDB and TRF2ΔBΔM (Figure 3.8d). Scoring of several thousand telomeres revealed that TRF2-independent recruitment of hRAP1 to telomeres caused a 10-fold reduction of end fusions when compared to cells expressing TRF2ΔBΔM alone ($p<0.0001$, Figure 3.9). Importantly, expression of hRAP1ΔCT not fused to TebDB did not provide such protection, as the incidence of

telomere fusions was 7-fold higher in these cells ($p<0.0001$, Figure 3.9). Immuno blotting confirmed that modulation of TRF2ΔBΔM expression was not the mechanism by which hRAP1ΔCT-V-TebDB protects telomeres as the dominant negative form of TRF2 was expressed at similar levels in all samples (Figure 3.10a). Furthermore, the hRAP1 fusion protein was expressed at similar levels to endogenous hRAP1, confirming that protection is not due to gross overexpression (Figure 3.10b). Taken together with the inability of Venus-TebDB to protect telomeres from NHEJ, our data strongly suggest that hRAP1 mediates protection of telomeres from NHEJ in human cells.

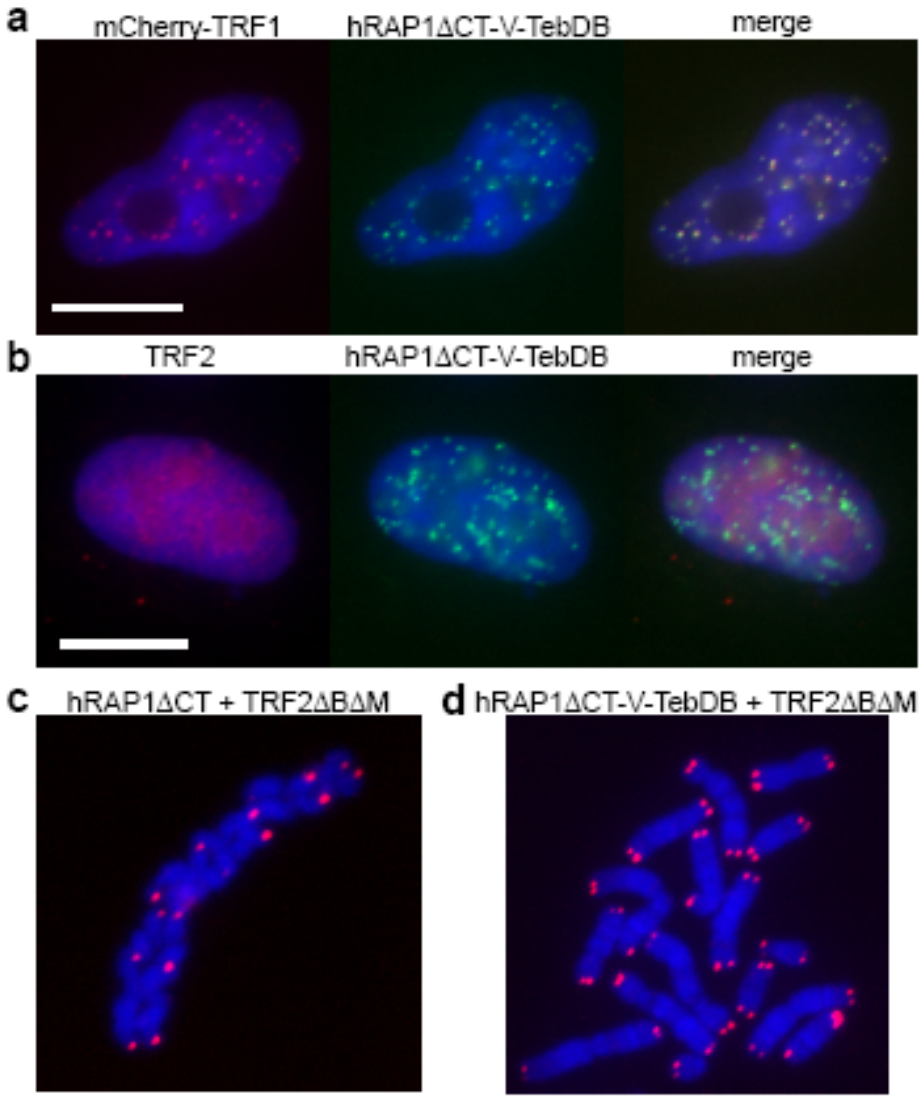
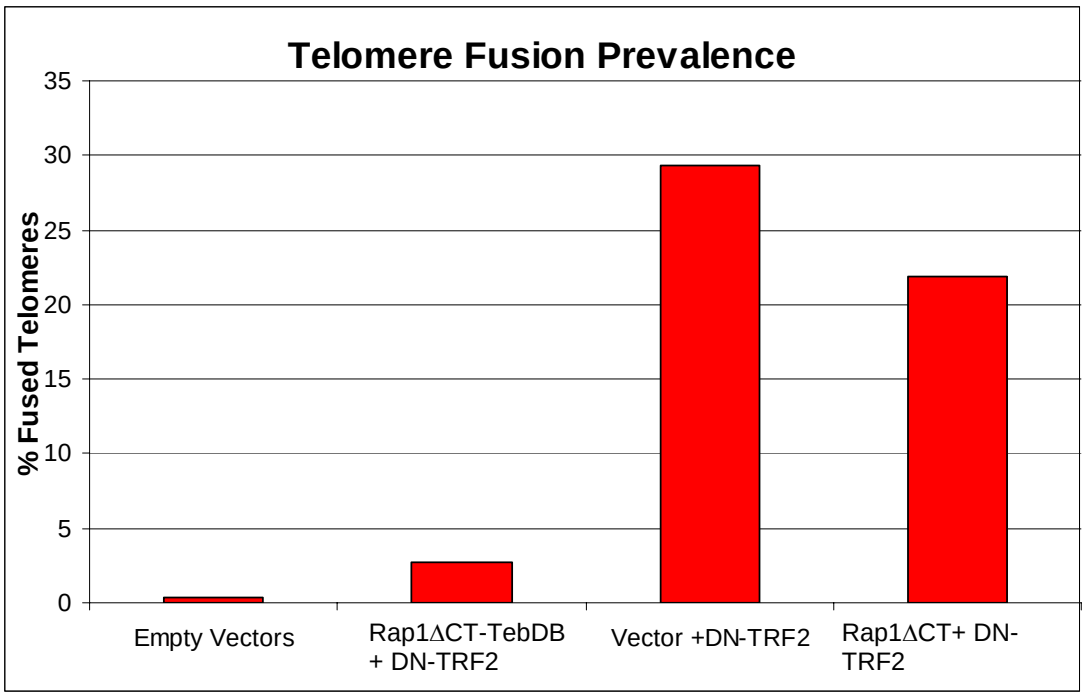


Figure 3.8: RAP1ΔCT-Venus-TebDB localizes to and protects telomeres in the absence of TRF2. **(a)** Colocalization of mCherry-TRF1 and hRAP1ΔCT-Venus-TebDB at telomeres in cells expressing TRF2ΔBΔM. **(b)** Cells expressing TRF2ΔBΔM and hRAP1ΔCT-Venus-TebDB were stained with anti-TRF2 and AlexaFluor 594 conjugated secondary antibody (red). Nucleoplasmic extraction was employed to limit visualization to chromatin-associated TRF2. hRAP1ΔCT-Venus-TebDB was visualized by virtue of Venus fluorescence. Scale bars correspond to 10 μm. **(c, d)** Telomere-FISH performed on metaphase chromosomes transfected with and selected for expression of the indicated proteins. Telomeres were detected with an AlexaFluor 543-labeled locked nucleic acid probe that detects the G-rich strand (red). Chromosomes were stained with DAPI (blue).



Vector	TRF2ΔBΔM +/-	Telomeres analyzed	Fused Telomeres	% Fused Telomeres
pIREShyg2	-	1024	4	0.4
pIREShyg2	+	4597	1349	29.3
hRap1ΔCT-V-TebDB	+	4353	120	2.8
hRap1ΔCT	+	2691	588	21.9

Figure 3.9: Quantification of telomere fusions in metaphase spreads of cells transfected with and selected for the indicated constructs. Telomere fusions were quantified in images of metaphases from cells harvested 72 h after transfection.

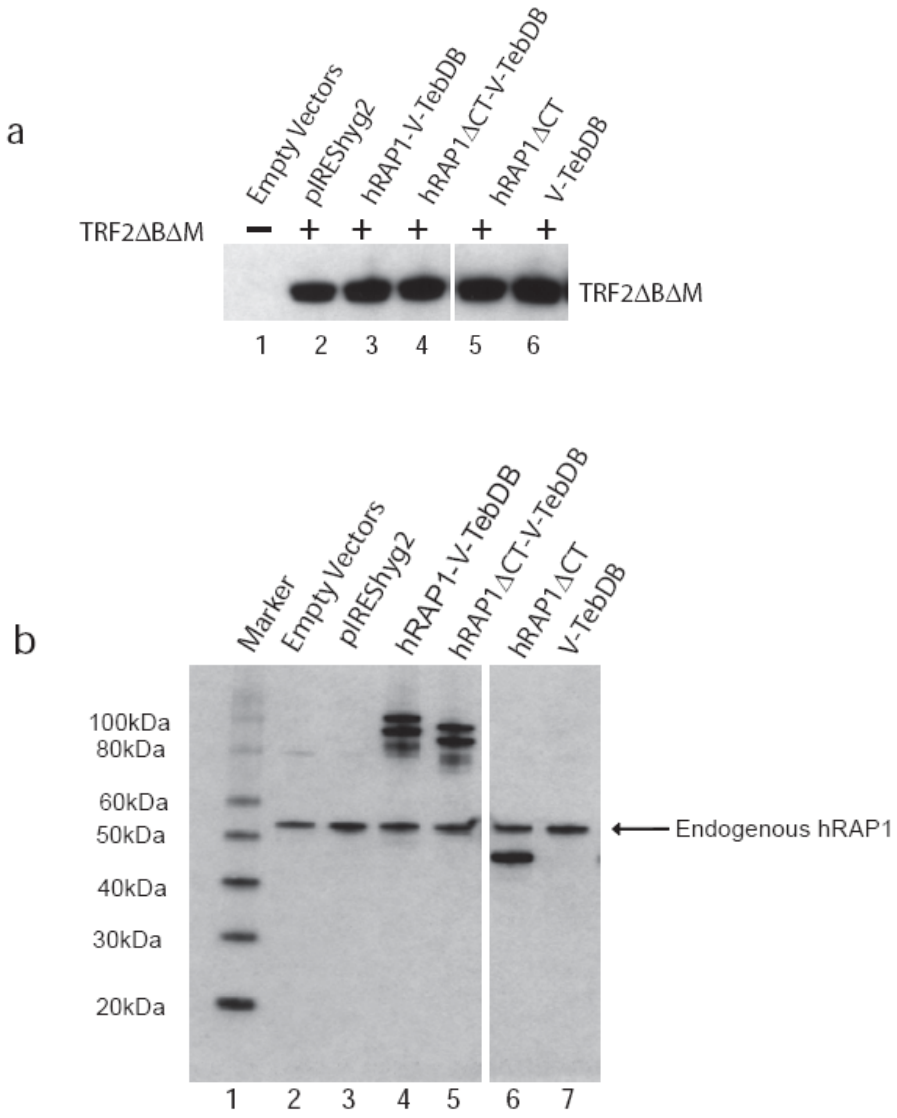


Figure 3.10: TRF2ΔBΔM is expressed at similar levels independent of which constructs are co-transfected. (a) Transfected cells were selected by treatment with puromycin and hygromycin and lysed 72 h post-transfection. Proteins were separated by SDS-PAGE and subjected to immunoblotting analysis using anti-TRF2 (4A794) at 1:2000 and HRP-conjugated goat anti-mouse at 1:5000 (Pierce). hRAP1-containing constructs are expressed at similar levels (b). Cells from (a) were separated by SDS-PAGE and subjected to immunoblotting analysis using a polyclonal hRAP1 antibody at 1:1000 and HRP-conjugated goat anti-rabbit at 1:5000 (Pierce). Endogenous hRap1 is indicated as a loading control for (b) and (a) since identical amounts of extract were loaded in both experiments.

III.4: Discussion

The isolation of a protein complex containing TRF1, TRF2, RAP1, TIN2, TPP1 and POT1[146, 147] has led to the proposal that all six telomeric proteins form a functional unit in chromosome capping. In reference to this putative role the six member complex has been named shelterin[148]. Efforts at elucidating shelterin function(s) have been hampered by both the varied phenotypes associated with loss of individual shelterin components as well as the identification of shelterin-component specific subcomplexes [23, 58, 85, 149-152]. Delineating the roles of individual telomere proteins in telomere maintenance will shed light on both telomere subcomplex and shelterin functions. To this end, we have developed a system that targets proteins of interest to telomeres independently of their own capacity for DNA binding. The ability of the DNA binding domain from fission yeast Teb1 to bind vertebrate telomeric repeats with high specificity and affinity *in vitro* (Figure 3.1a), and to localize to mammalian telomeres *in vivo* (Figure 3.4a and b) provided us with a unique opportunity to investigate the functions of individual telomere-associated proteins in telomere protection. We found that NHEJ of telomeric termini was specifically inhibited upon TRF2-independent recruitment of hRAP1 to telomeric DNA *in vitro* (Figure 3.3), whereas TRF2 alone was insufficient to mediate protection. Extending these results *in vivo,* we demonstrate that heterologous targeting of hRAP1 to telomeres is sufficient to avert the uncapping phenotype associated with expression of dominant negative TRF2 (Figure 3.9). The

convergence of biochemical and cell biological data presented here identify hRAP1 as a critical mediator of telomere protection and genome stability in human cells.

Uncovering roles for hRAP1 in telomere protection has proven challenging due in large part to the intricate relationship between hRAP1 and TRF2[138]. Previous studies have reported chromosome uncapping in response to removal of TRF2 from telomeres by various means including siRNA-mediated knockdown[144, 145], expression of dominant negative forms of TRF2 [53, 131] and TIN2 [153] and conditional knockout of TRF2 in mouse embryonic fibroblasts[133]. Furthermore, the stability of hRAP1 in cells depends on TRF2, as hRAP1 levels are drastically reduced in TRF2-/- MEFs[133]. Since removal of TRF2 from telomeres elicits the concomitant loss of hRAP1 [133], it has not been possible to distinguish the contributions of TRF2 and hRAP1 to telomere protection. Importantly, the poorly understood end-joining phenotypes observed in TRF2 loss-of-function experiments are explained by the results presented here demonstrating that hRAP1 can protect telomeres from NHEJ, as concomitant loss of the NHEJ-inhibiting hRAP1 is anticipated to result in telomere deprotection.

The mechanisms that telomeres employ to evade the DNA damage response are not clear, and subsequently the contribution of hRAP1 to telomere protection is not known[138]. Our experiments provide novel insight into hRAP1's role in this process. Surprisingly, we find that hRAP1-dependent telomere protection is unlikely to operate through modulation of TRF2 DNA binding, as an hRAP1 fragment that is incapable of interacting with TRF2 efficiently protects telomeres from NHEJ (Figure

3.9). Our results also indicate that hRAP1 alone is sufficient to inhibit NHEJ (Figure 3.2 and 3.9), showing that the interface between TRF2 and hRAP1 is not required for telomere protection. These results suggest a model whereby TRF2 protects telomeres by recruiting hRAP1 which in turn distinguishes telomeres from sites of *de novo* DNA damage (Figure 3.11). This model contrasts with current models of NHEJ inhibition at mammalian telomeres, which are largely based on the finding that a telomeric 3' overhang can invade internal sequences on the same telomere thereby forming a t-loop that renders the end inaccessible to degradation or fusion [76]. Since TRF2 can promote t-loop formation *in vitro*[80], it has been suggested that chromosome fusions caused by the loss of TRF2 from telomeres are ultimately due to the dissociation or resolution of t-loops in the absence of TRF2. However, this assertion remains to be tested. At least *in vitro*, t-loops are not required for NHEJ inhibition as 12 telomeric repeats are too short to form a t-loop but are sufficient to mediate protection of a DNA terminus from NHEJ[23]. Interestingly, similarly short but stable telomeres have been observed *in vivo* as well [87, 154]. An attractive explanation for this seeming discrepancy is that multiple pathways protect telomeric ends. For example, t-loops may protect long telomeres that are capable of forming such structures while very short telomeres that can not form t-loops are protected by hRAP1.

The idea that multiple pathways protect telomeres in humans is supported by an emerging literature[23, 143, 152]. Recently, both phosphatase nuclear targeting subunit (PNUTS) and microcephalin (MCPH1) were shown to interact with a

domain in TRF2 that is separate from the hRAP1 interaction domain[143]. In addition, mutation of the PNUTS/MCPH1 binding site in DN-TRF2 reduced its ability to elicit a DNA damage signal at telomeres, suggesting that PNUTS and/or MCPH1 may also contribute to telomere protection. Taken together with our results showing that hRAP1 at telomeres can protect DNA ends from TRF2 loss, this combination strongly suggests that TRF2 mediates telomere protection through multiple distinct and possibly redundant pathways. Further analysis will be required to elucidate the links between hRAP1, PNUTS/MCPH1, t-loops and NHEJ-inhibition at telomeres.

Earlier work has shown that the other shelterin components continue to associate with telomeres following loss of TRF2/hRAP1[53, 57, 83, 84]. Our experiments do not exclude the possibility that TRF1, TIN2, TPP1 and/or POT1 may contribute to the hRAP1-dependent telomere capping reported here. However, a key role for hRAP1 is demonstrated by the observation that telomeric TIN2, TPP1 and POT1 are insufficient to prevent widespread telomere fusions, whereas recruitment of hRAP1 has a potent inhibitory effect on chromosome fusions. Previous studies suggest that any putative role for POT1 in inhibiting NHEJ at telomeres is minor since knockdown experiments show only modest increases in telomere fusions[155, 156] and MEFs lacking Pot1a and b do not display the dramatic telomere fusion phenotype seen in cells lacking TRF2 [85, 157].

Although hRAP1 was named based on limited domain and sequence similarity with the budding yeast repressor and activator protein (*RAP1*), the two

proteins have diverged substantially [65]. Unlike human RAP1 which has a single myb domain of unknown function, budding yeast RAP1 has tandem myb-like domains which mediate DNA binding critical for its functions in transcriptional regulation and telomere maintenance[158]. In *S. cerevisiae* recruitment of ScRIF2 and ScSIR4 by the C-terminal domain of RAP1 is required for NHEJ inhibition at telomeres[68]. The C-terminal domain (CT) of hRAP1 mediates homodimerization and interaction with TRF2[65]. Consistent with divergent modes of inhibition, the CT of hRAP1 was not required for NHEJ inhibition in our experiments and homologues of yeast ScRIF2 and ScSIR4 appear to be absent from mammalian genomes. The divergence between ScRAP1 and hRAP1 may be explained by the realization that ScRAP1 binds telomeric DNA and inhibits NHEJ through multiple pathways, suggesting either that it may actually be a functional orthologue of TRF2 rather than hRAP1, or that it has integrated TRF2 and hRAP1 functions into one protein.

If ScRAP1 has indeed integrated the functions of TRF2 and hRAP1 into one module, then what functions of ScRAP1 and hRAP1 are conserved? Interestingly, a minor ScRAP1- dependent, but ScRIF2 and ScSIR4-independent NHEJ inhibition pathway has also been identified in yeast[68]. This pathway is not mediated by the conserved BRCT or CT domains in ScRAP1, but requires the central region of the protein. It is tempting to speculate that hRAP1 relies predominantly on this mode of NHEJ inhibition. Further supporting the idea of an evolutionarily conserved mechanism of NHEJ inhibition common to diverse RAP1 homologues is the

observation that loss of fission yeast SpRAP1 leads to telomere fusions[73], since no ScRIF2 or ScSIR4 homologues have been identified in this organism either. Several DNA repair factors including the Ku heterodimer, Mre11, Rad50, and PARP1 copurify with hRAP1[69] raising the intriguing possibility that hRAP1 contains a domain that directly binds and prevents these proteins from executing DNA repair. Ku and DNA-PKcs associate with telomeres[159, 160] and at least *in vitro* hRAP1 does not appear to inhibit the assembly of NHEJ factors at telomeric ends under conditions where it inhibits fusions (NSB, PB; unpublished data). We expect that uncovering the physical and functional interactions between hRAP1 and the NHEJ machinery will now be critical to elucidating the mechanism by which chromosome ends are protected from unsolicited repair events.

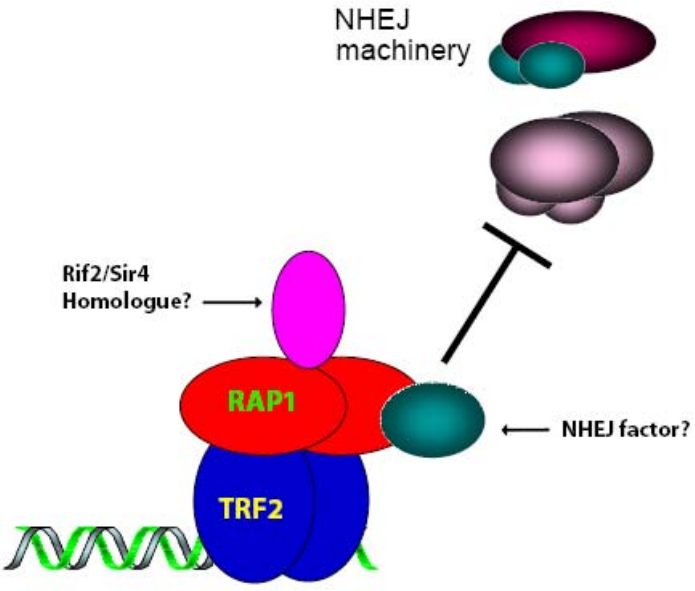


Figure 3.11: Model of the roles for hRAP1 and TRF2 in protecting telomeres from NHEJ.

Chapter Four:
Replication Stress at Telomeres Results in "t-wrecks", a New Type of Highly Conserved Telomere Dysfunction

IV.1: Abstract

Telomeres, the nucleoprotein structures that cap the ends of chromosomes, pose unique challenges to the DNA replication machinery. These challenges include telomeres' repetitive nature, high guanine content, location at the terminus of chromosomes, and slough of factors that bind them with high affinity and specificity. Little is known about the strategies that the conventional DNA replication machinery employs to deal with these challenges. As much has been learned in biology through the study of mutant phenotypes, the identification of phenotypes associated with replication stress at telomeres may help to shed light on the process of normal telomere replication. To this end, we have identified the first example of dynamic telomere fusions caused by replication stress in metazoans. This novel type of fusion differs substantially from the previously reported NHEJ-dependent fusions associated with loss of TRF2 function, as they are NHEJ independent, contain large amounts of telomeric DNA and telomere binding proteins, and are not covalent. Most importantly, these fusions are induced by insults that cause replication stress at telomeres, and also appear to be dependent upon the RecQ helicase WRN for their formation. These characteristics indicate that telomere associations described here may be similar to the replication stress-induced "telomere entanglements" observed in fission yeast. The conservation of telomere entanglements between yeast and man

is illustrates the importance of the underappreciated role of semi-conservative DNA synthesis in telomere maintenance.

IV.2: Introduction

The highly repetitive nature of telomeres, their guanine-rich composition, the array of factors that specifically localize to them, their location at the termini of chromosomes, and the multiple conformations they can adopt makes these regions among the most difficult to replicate in eukaryotic genomes (Figure 1.6) [118, 161]. To counteract the difficulties associated with telomere replication, eukaryotic cells contain specialized machineries and mechanisms that facilitate initiation and progression of DNA synthesis through these challenging regions. However, most of the components of these machineries, as well as the mechanism of DNA synthesis through telomeres, remain elusive. Furthermore, what little is known about telomere replication comes primarily from budding yeast[162], whose mode of DNA replication differs substantially from other eukaryotes. A striking example of the difference between budding yeast DNA replication and DNA replication in other eukaryotes is the sequence-dependent initiation of replication at defined origins in budding yeast, which contrasts strongly with sequence-independent replication origins in other eukaryotes[163].

The origin recognition complex (ORC) is responsible for recognizing and licensing origins for DNA synthesis in all eukaryotes, however, requirements for ORC recruitment to mammalian origins independently of sequence context are still

not known [164]. Previous work has shown that the telomere binding protein TRF2 binds the origin of replication in the Epstein Bar Virus (EBV) F1' episome, which contains a triplex nonamer of TTAGGGTTA, and that this binding is essential for viral replication [165-167]. Related to this work, TRF2 has been shown to physically interact with ORC [55, 124], and it has also been shown that this interaction is necessary for the recruitment of ORC to the F1 origin of replication[167]. Finally, multiple groups have reported that TRF2 recruits ORC to mammalian telomeres, and that expression of a fragment of TRF2 that can not interact with ORC, TRF2ΔB, significantly reduces the amount of ORC at telomeres [55, 124]. The reduction in ORC at telomeres causes replication fork stalling through telomeric repeats and induces homologous recombination between sister telomeres presumably during repair of stalled telomeric forks [54, 55]. These results demonstrate that in addition to its role in telomere protection, TRF2 is a critical mediator of ORC recruitment and conventional DNA synthesis at telomeres. These results also identify TRF2ΔB as a potent inducer of replication stress at telomeres.

Here, we report a novel phenotype associated with replication stress at telomeres. In transformed cells expressing the telomere-specific inhibitor of replication TRF2ΔB, we observed chromatin bridges between sister nuclei in interphase. Characterization of these telomere associations indicates that they contain shelterin components and massive amounts of telomeric DNA. Surprisingly, these telomere associations do not require non-homologous end joining and are not covalent, explaining why they have been overlooked previously[54, 165]. These

telomere associations were observed in cell lines that maintain telomeres either through telomerase-mediated mechanisms or the alternative lengthening of telomeres pathway (ALT), suggesting that telomerase is not essential for their development. It also appears that the RecQ helicase WRN is essential for their formation, as TRF2ΔB-expressing fibroblasts derived from Werner syndrome patients do not display this phenotype. The finding that replication stress at telomeres appears to trigger dynamic telomere associations suggests that they may be similar to the "telomere entanglements" previously observed in fission yeast and that TRF2 and SpTaz1 may play similar roles in promoting telomere replication by the conventional DNA replication machinery [125, 128, 129]. The data presented here are also the first demonstration of replication stress at mammalian telomeres resulting in genome instability, strengthening the connection between telomeres, genome instability, and cancer.

IV.3: Results

4.3a: TRF2ΔB induces chromatin bridges between sister nuclei that contain large amounts of telomeric DNA

During the course of studies on NHEJ-dependent telomere fusions induced by expression of TRF2ΔBΔM, as a putatively negative control we expressed TRF2ΔB in HeLa S3 cells. TRF2ΔB was employed as a negative control because previous work showed that it caused telomere dysfunction without producing covalent telomere fusions [89]. Unexpectedly, expression of TRF2ΔB in HeLa S3

yielded numerous chromatin bridges between sister interphase nuclei (Figure 4.1a and c). This result appeared to be inconsistent with previous reports since expression of TRF2ΔB was shown to cause telomere recombination without yielding telomere fusions, and TRF2ΔB expression does not result in loss of shelterin components [89]. Quantification of the prevalence of chromatin bridges in cells expressing TRF2ΔB indicated that 74 out of 200 exhibited this phenotype, while 80 out of 207 cells displayed interphase bridges in cells expressing TRF2ΔBΔM.

Previous work has shown that TRF2 can localize to non-telomeric DNA breaks and may contribute to the repair process[50, 51, 168], suggesting that the chromatin bridges we observed may be due to dysfunctional DNA repair at non-telomeric sites with subsequent induction of genomic instability. To address this issue, we investigated whether these chromatin bridges contained telomeric DNA. To accomplish this, HeLa S3 cells were transfected with either empty vector, TRF2ΔBΔM, or TRF2ΔB, and subjected to fluorescence *in situ* hybridization (FISH) with a telomere-specific locked nucleic acid probe against the G-rich telomeric DNA strand. In this assay, we noted that telomere fusions in cells expressing TRF2ΔB invariably contained strikingly large amounts of telomeric DNA in all bridges observed in that sample, while TRF2ΔBΔM induced bridges did not display this phenotype (Figure 4.1 a and b). Recent reports of telomeric RNA transcripts (TERRA) suggested that the telomeric signal observed in these bridges may result from an increase in telomere transcription [169]. However, since the FISH procedure

employed here contains an RNAse treatment step, it is unlikely that TERRA are primarily responsible for the telomeric signal seen in these bridges.

Because there appeared to be more telomeric DNA in these bridges than is normally observed within the cell (Figure 4.1a), we hypothesized that these bridges may contain single-stranded DNA arising from uncontrolled DNA replication. To determine whether the DNA in the TRF2ΔB-induced chromatin bridges is double-stranded, we probed these bridges with an LNA oligonucleotide against the C-rich telomeric strand (Figure 4.1c). As was observed with the probe against the G-strand, the C-strand probe specifically labeled chromatin bridges induced by TRF2ΔB, but not those induced by TRF2ΔBΔM (Figure 4.1 c and d). This result indicates that the chromatin bridges induced by TRF2ΔB contain large amounts of double-stranded DNA. It is also possible that some single-stranded telomeric DNA is present in these bridges, however, potential differences in labeling efficiency of the C-and G-strand probes prevent us from addressing this issue. The massive accumulation of telomeric DNA in TRF2ΔB-induced bridges demonstrates that these bridges are cytologically distinct from the previously characterized NHEJ-dependent chromatin bridges caused by loss of TRF2 from telomeres [25, 53, 58, 130, 131, 133, 170].

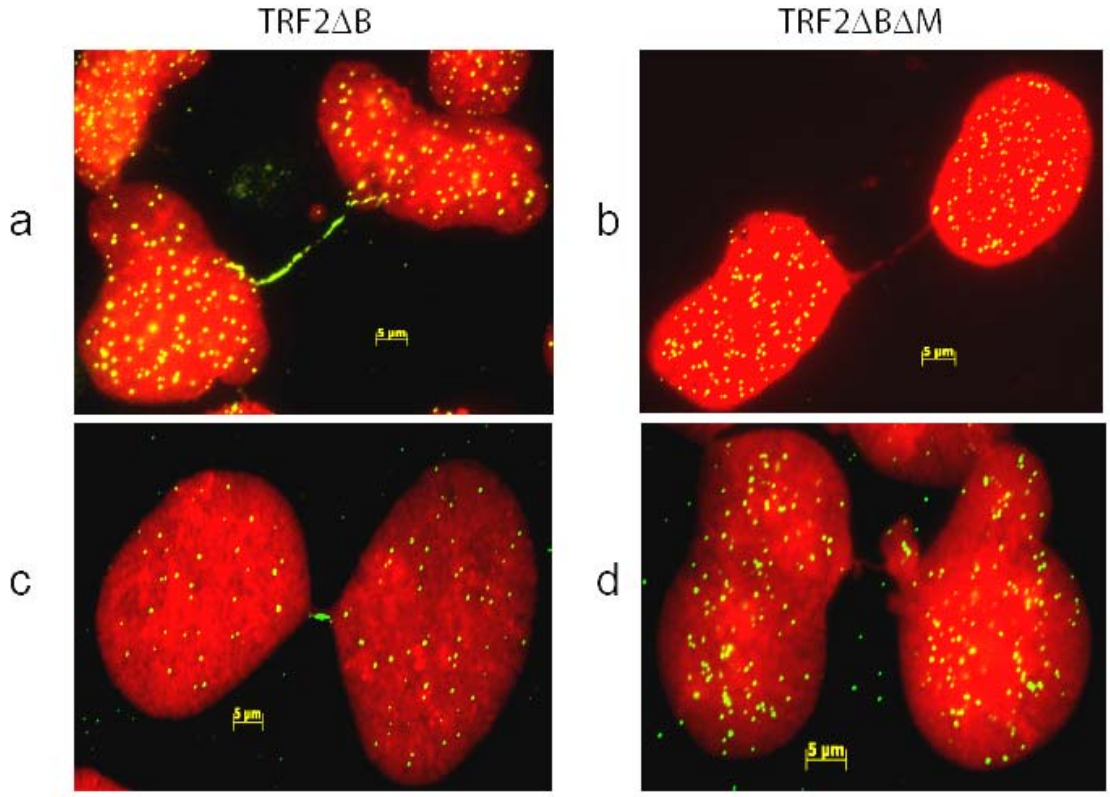


Figure 4.1: TRF2ΔB induces chromatin bridges that contain large amounts of double-stranded telomeric DNA. (a,b) HeLa S3 expressing TRF2 fragments were stained with an AlexaFluor 543-labeled LNA probe detecting the G-rich telomeric DNA strand (green). DNA is stained with DAPI (red). (c,d) HeLa S3 expressing TRF2 fragments were stained an AlexaFluor488-labeled LNA probe detecting the C-rich telomeric DNA strand (green). DNA is stained with DAPI (red).

IV.3b: TRF2ΔB does not induce covalent telomere fusions

In an attempt to further characterize TRF2ΔB-induced telomere associations, we transfected cells with either empty vectors, TRF2ΔB, or TRF2ΔBΔM and prepared metaphase spreads for telomere-FISH. In this assay, covalent non-sister telomere fusions give rise to chromosomes with telomere signals at their ends as well as internal telomeric DNA that represents the site of fusion. Fusions induced by loss

of TRF2 function such as those observed upon TRF2ΔBΔM expression and expression of Cre in TRF2 -/flox MEFs display this phenotype [53, 58]. In contrast, it was previously reported that cells expressing TRF2ΔB did not display covalent telomere fusions [54], which is surprising in light of the chromatin bridge phenotype reported here (Figure 4.1 a and c).

While cells containing empty vector had normal chromosome structure (Figure 4.2a), chromosomes from cells expressing TRF2ΔB displayed increased telomere length heterogeneity (TLH), telomeric DNA free ends (TFEs), and sister telomere fusions, all of which have been reported previously and are hallmarks of telomere recombination (Figure 4.2b) [54]. Critically, telomeres in these cells did not display covalent telomere:telomere fusions (Figure 4.2b), which is in agreement with previous work [54, 55]. To ensure that our metaphase spread protocol did not disrupt covalent telomere fusions, we analyzed telomere fusions in cells expressing TRF2ΔBΔM. In accordance with published results, these cells had massive chromosome fusions with telomeres at the fusion points (Figure 4.2c). This data suggests that the version of TRF2ΔB expressed here acts in a similar manner to the versions of TRF2ΔB employed in previous reports and that TRF2ΔB induces non-covalent telomere associations [56, 89, 165].

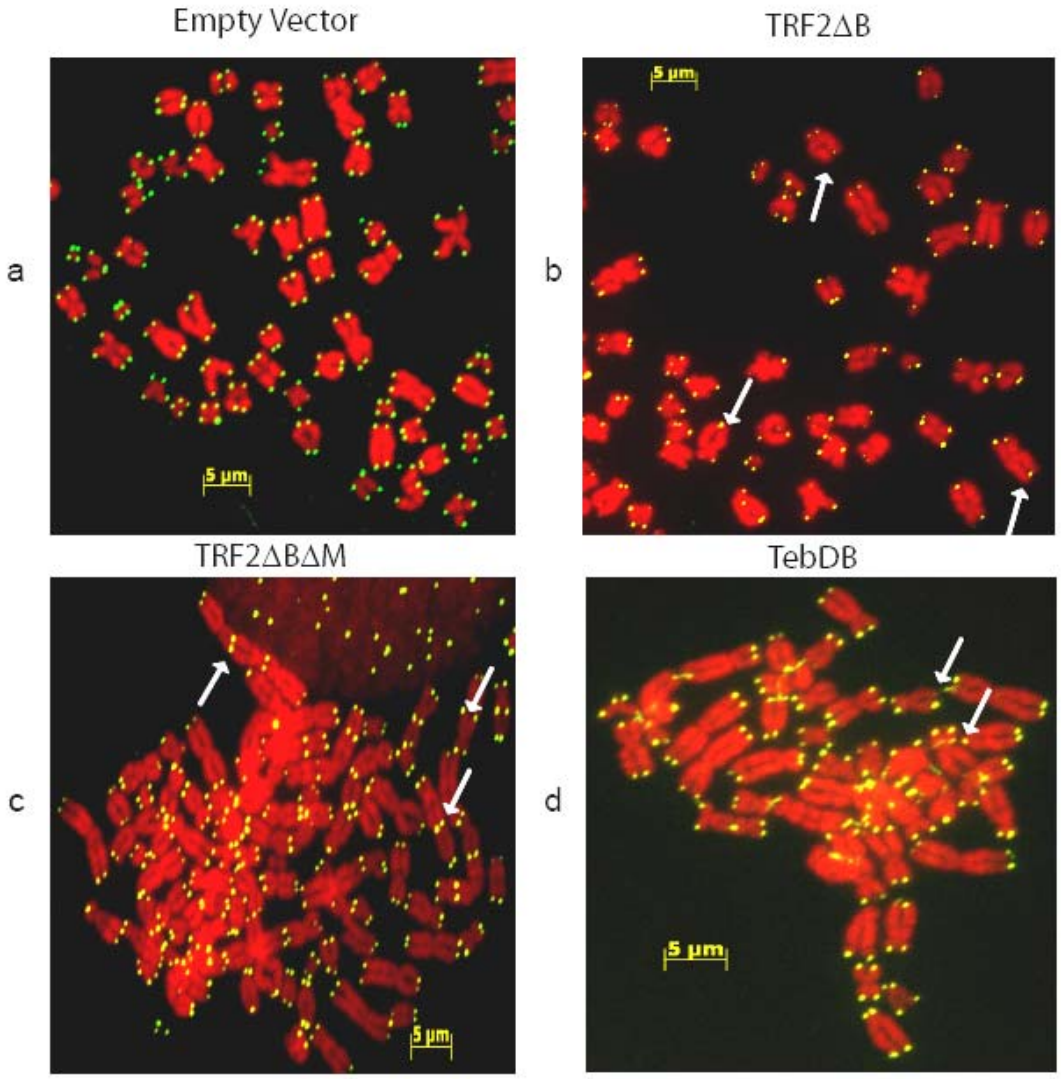


Figure 4.2: Telomere FISH on metaphase spreads from HeLa S3 transfected with the indicated constructs. LNA probe detecting the G-rich telomeric DNA strand was labeled with AlexaFluor 543 (green) to visualize telomeres. DNA stained with DAPI (red). (a) HeLa S3 transfected with empty vector. (b) HeLa S3 transfected with TRF2ΔB. Telomere dysfunction including telomere free ends and sister chromatid fusions indicated by white arrows. (c) HeLa S3 transfected with TRF2ΔBΔM. White arrows show telomere:telomere fusions. (d) HeLa S3 transfected with TebDB. White arrows show telomere free ends and sister telomere recombination events.

The dramatic increase in TLH, sister telomere recombination, and TFEs induced by TRF2ΔB is similar to the phenotype displayed by human cells heterologously expressing the DNA binding domain from the fission yeast protein SpTeb1. This protein was initially identified in a bioinformatics search for fission yeast homologues of TRF1/2 based on its highly conserved Myb domain [139]. Though SpTeb1 does not function in fission yeast telomere maintenance, we and others have previously shown that a fragment of SpTeb1 carrying the DNA binding domain, TebDB, binds the vertebrate telomeric repeat with high affinity and specificity, and that it can localize to mammalian telomeres (Figures 3.1, 3.4). Furthermore, TebDB expression in HeLa S3 leads to increased TLH, TFEs, and sister telomere recombination (Figure 4.2d). Suspecting that TebDB may induce telomere fusions that are similar to those induced by TRF2ΔB, we expressed a Venus-TebDB fusion protein in HeLa S3 cells and performed telomere-FISH. These cells displayed a dramatic accumulation of double-stranded telomeric DNA within chromatin bridges (Figure 4.3 a and b), appearing identical to those observed upon expression of TRF2ΔB. This result clearly demonstrates that Trf2ΔB and TebDB elicit similar telomere dysfunction resulting in non-covalent telomere associations.

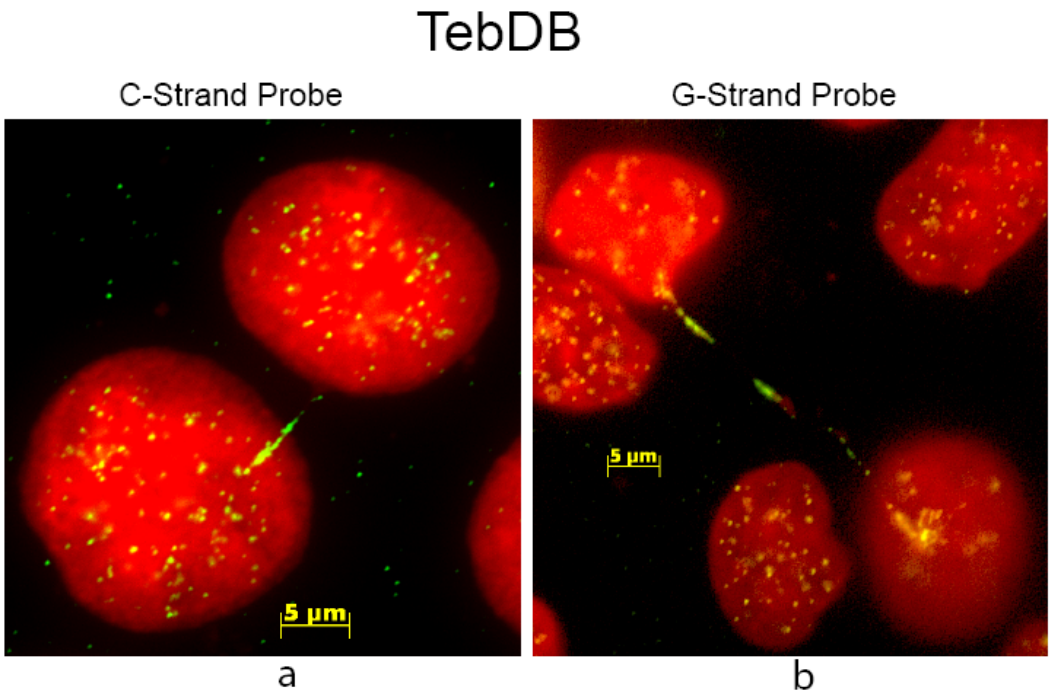


Figure 4.3: TebDB induces non-covalent telomere associations. HeLa S3 expressing TebDB were stained with an LNA oligonucleotide probe detecting the C-rich strand (a, green), or G-rich strand (b, green). DNA was stained with DAPI (red).

IV.3c: Double-stranded telomere binding proteins localize to TRF2ΔB-induced chromatin bridges

The fusion of TebDB to the yellow fluorescent protein variant Venus also provided an opportunity to examine whether TebDB localized not only to telomeres, but also to chromatin bridges containing telomeric DNA. This experiment would further demonstrate that these bridges contain double-stranded telomeric DNA, since TebDB specifically binds the double-stranded TTAGGG sequence, and not its single-stranded counterpart[141]. Cells expressing Venus-TebDB exhibited numerous chromatin bridges between cells, and the Venus-TebDB fusion protein localized to these structures (Figure 4.4a). The finding that Venus-TebDB, a protein

that localizes to mammalian telomeres (Figure 3.4), also localized to chromatin bridges suggested that other telomere binding proteins, including TRF1 and TRF2, may localize to these structures. If so, these telomere association would be the first example of telomere dysfunction with an accumulation of telomere binding proteins at the site of fusion. This finding would also help to shed light on the mechanism by which these fusions arise, as it would appear unlikely that DNA damage signaling through the ATM kinase would be responsible for these fusions since the ATM inhibitor TRF2 would be present at the site of dysfunction[58, 59] .

To investigate the localization of human telomere binding proteins at sites of telomere fusion, we stained HeLa S3 expressing TRF2ΔB and TRF2ΔBΔM with an antibody against TRF2. TRF2ΔBΔM mediated fusions are caused by loss of TRF2 and associated factors from telomeres, leading to NHEJ dependent telomere fusions. As expected, TRF2ΔBΔM removed TRF2 from telomeres, and the chromatin bridges between sister nuclei did not contain TRF2 (Figure 4.4c). In contrast, the TRF2 antibody brightly labeled the telomere bridges induced by TRF2ΔB (Figure 4.4b). It is important to note that the TRF2 antibody used here can not distinguish endogenous TRF2 from TRF2ΔB, therefore the localization of endogenous TRF2 in these bridges is unknown. Since previous reports have demonstrated that TRF2ΔB does not displace and in fact robustly colocalizes with endogenous TRF2 [54], and that these bridges contain large amounts of double-stranded telomeric DNA (Figure 4.1 a and c), it is likely that both TRF2 alleles reside in the chromatin bridges.

We also assayed TRF1 localization in cells expressing TRF2ΔBM, TRF2ΔB, and Venus-TebDB. To do this, we used a mCherry-TRF1 fusion protein that we have previously shown colocalizes with endogenous TRF2 (Figure 3.4). mCherry-TRF1 was co-transfected with either TRF2ΔBΔM, TRF2ΔB, or Venus-TebDB, and cells were visualized 48hr after transfection. Figure 4.4 (a,d,e) clearly shows that bridges in cells expressing Venus-TebDB, TRF2ΔB, and TRF2ΔBΔM are all labeled with mCherry-TRF1. Recent work demonstrating a role for TRF1 in the resolution of sister telomeres during mitosis provides an explanation for the localization of mCherry-TRF1 to bridges induced by Venus-TebDB, TRF2ΔB, and TRF2ΔBΔM [43-45]. While the accumulation of double-stranded telomeric DNA in bridges induced by Venus-TebDB and TRF2ΔB is sufficient to explain mCherry-TRF1 localization to these structures, it is possible that fused telomeres of dicentric chromosomes in cells expressing TRF2ΔBΔM accumulate TRF1 since they can not be resolved by normal mitotic processes.

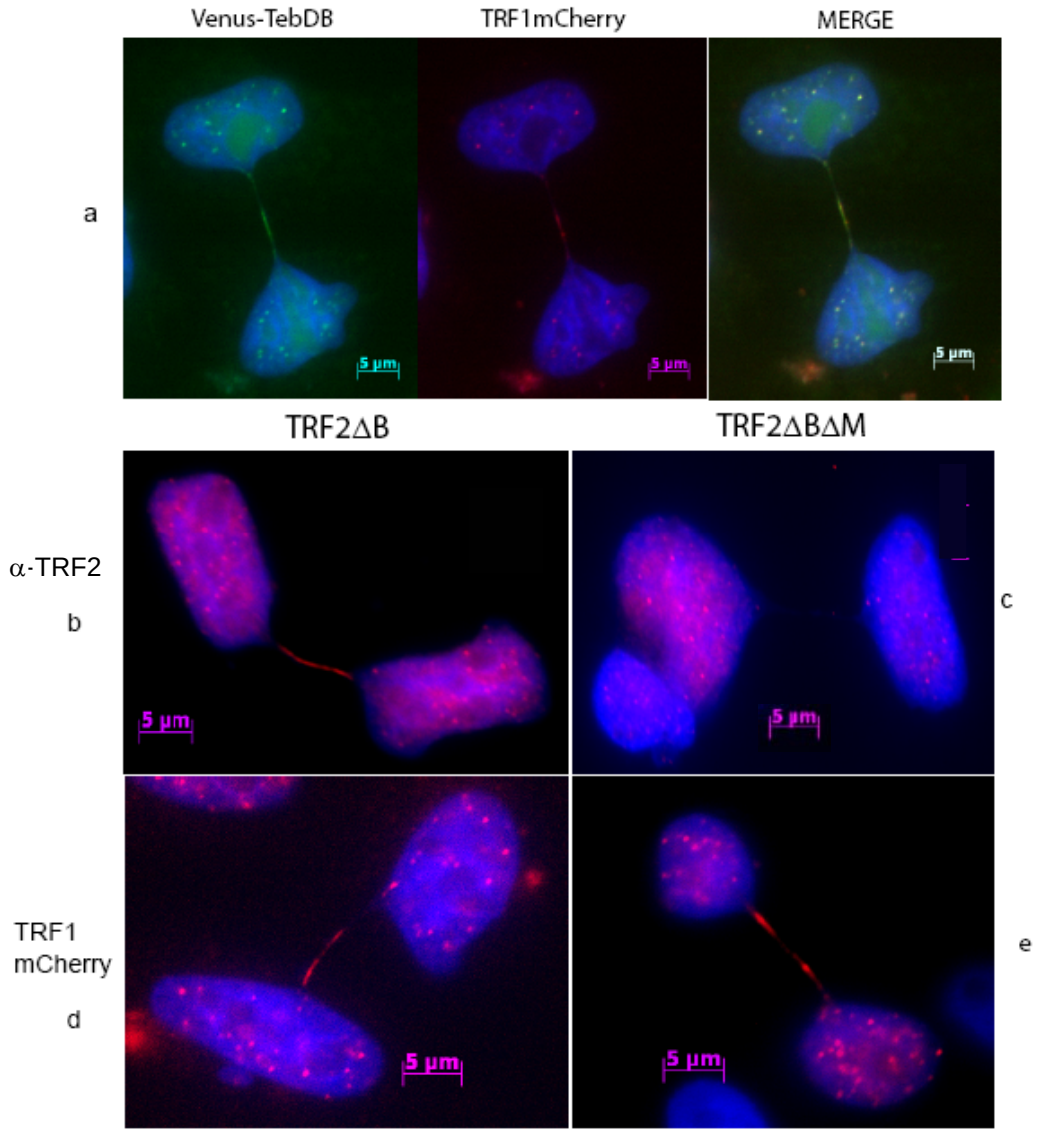


Figure 4.4: TRF2 and mCherry-TRF1 localize to chromatin bridges induced by telomere dysfunction. (a) HeLa S3 expressing Venus-TebDB (green) and mCherry-TRF1 (red). TRF2 staining (red) in cells expressing TRF2ΔB (b) or TRF2ΔBΔM (c). DNA was visualized with DAPI (blue). (d) HeLa S3 expressing TRF2ΔB and mCherry-TRF1 (red). (e) HeLa S3 expressing TRF2ΔBΔM and mCherry-TRF1 (red).

IV.3d: TRF2ΔB can induce telomere associations in ALT cells

Transformed cell lines can maintain their telomeres by multiple mechanisms, including telomerase-mediated telomere extension and a homologous recombination-based mechanism called alternative lengthening of telomeres (ALT) [110]. Because telomeres in ALT positive cells display increases in TLH, TFEs, and sister chromatid recombination events[54], we wondered whether ALT cells also display non-covalent telomere fusions at a low frequency. In addition, it is also possible that ALT cell lines have developed mechanisms to counteract the formation of telomere entanglements, thus rendering them immune to the effects of TRF2ΔB or Venus-TebDB expression. Moreover, since ALT cell lines do not express telomerase, the contribution of telomerase to these novel telomere associations can be addressed. To answer these questions, we transfected the ALT cell line VA-13 with either empty vector, TRF2ΔB, or Venus-TebDB, and performed telomere-FISH on these cells 48hr after transfection. Although examination of 250 cells transfected with empty vectors did not yield telomeric-DNA containing-chromatin bridges (Figure 4.5a), 78 out of 250 cells expressing TRF2ΔB displayed numerous telomere associations, and VA-13 expressing TebDB displayed these bridges as well (Figure 4.5 b and c). Despite rigorous attempts to identify telomere entanglements in ALT cells transfected with empty vector, none were observed. Thus, it appears that these entanglements do not arise spontaneously in ALT cells in spite of the TLH, TFEs, and sister telomere recombination events. However, it is clear that ALT cell lines do not contain a mechanism to prevent the formation of such entanglements, as

TRF2ΔB and Venus-TebDB elicited telomere entanglements in VA-13. It is also important to note that telomerase is not required for the formation of these structures, since VA-13 do not contain telomerase. Because telomeres are synthesized by only two means, telomerase and the conventional DNA replication machinery, DNA synthesis by the conventional DNA replication machinery must be responsible for this phenotype.

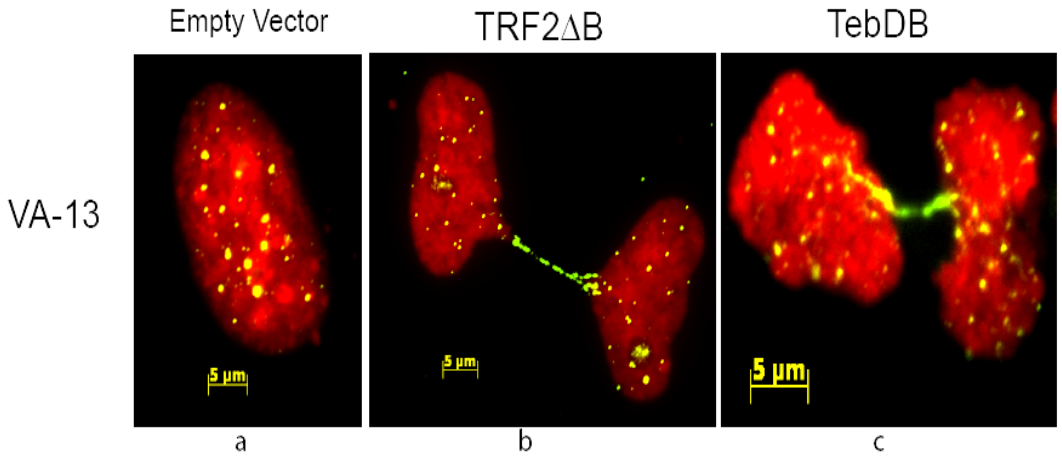


Figure 4.5: TRF2ΔB and TebDB induce chromatin bridges in ALT cells. (a,b,c) VA-13 were transfected with the indicated constructs and stained with an AlexaFluor 543-labeled LNA probe that detects the G-rich strand of telomeric DNA (green). DNA was stained with DAPI (red).

IV.3e: Telomere associations are NHEJ-independent

Non-homologous end joining is one pathway used by cells to repair DNA double strand breaks. The NHEJ machinery consists of the DNA end binding proteins Ku 70 and Ku86, the DNA-dependent protein kinase catalytic subunit DNA-PKcs, DNA ligase IV, and its stimulatory factor XRCC4 [8]. All of these proteins are

required for NHEJ, as loss of any single component renders the conventional NHEJ machinery inactive. This pathway is primarily active in the G1-phase of the cell cycle, and is responsible for fusion of dysfunctional telomeres caused by loss of TRF2 and associated factors from chromosome ends, including TRF2ΔBΔM-induced telomere fusions [14, 25]. Because this pathway was recently implicated in the covalent fusion of sister telomeres during G2 [45], we investigated whether covalent sister telomere fusion is responsible for the chromatin bridges observed upon expression of TRF2ΔB or Venus-TebDB. To this end, we expressed Venus-TebDB in the NHEJ-deficient Chinese hamster cell line GM16147, an XRCC4 null cell line that is devoid of NHEJ activity [171]. If chromatin bridges containing telomeric DNA are indeed found in this cell line, they are by definition NHEJ-independent. In GM16147 expressing Venus-TebDB and mCherry-TRF1, we observed chromatin bridges similar to those seen in HeLa S3 (Figure 4.6a and b). The presence of telomeric DNA in these bridges (Figure 4.6b) demonstrates that they are indeed identical to those observed in HeLa S3 upon expression of either TebDB or TRF2ΔB. In contrast, in GM16147 transfected with empty vector, no such structures were found (data not shown). Thus, telomere entanglements do not require NHEJ for their formation.

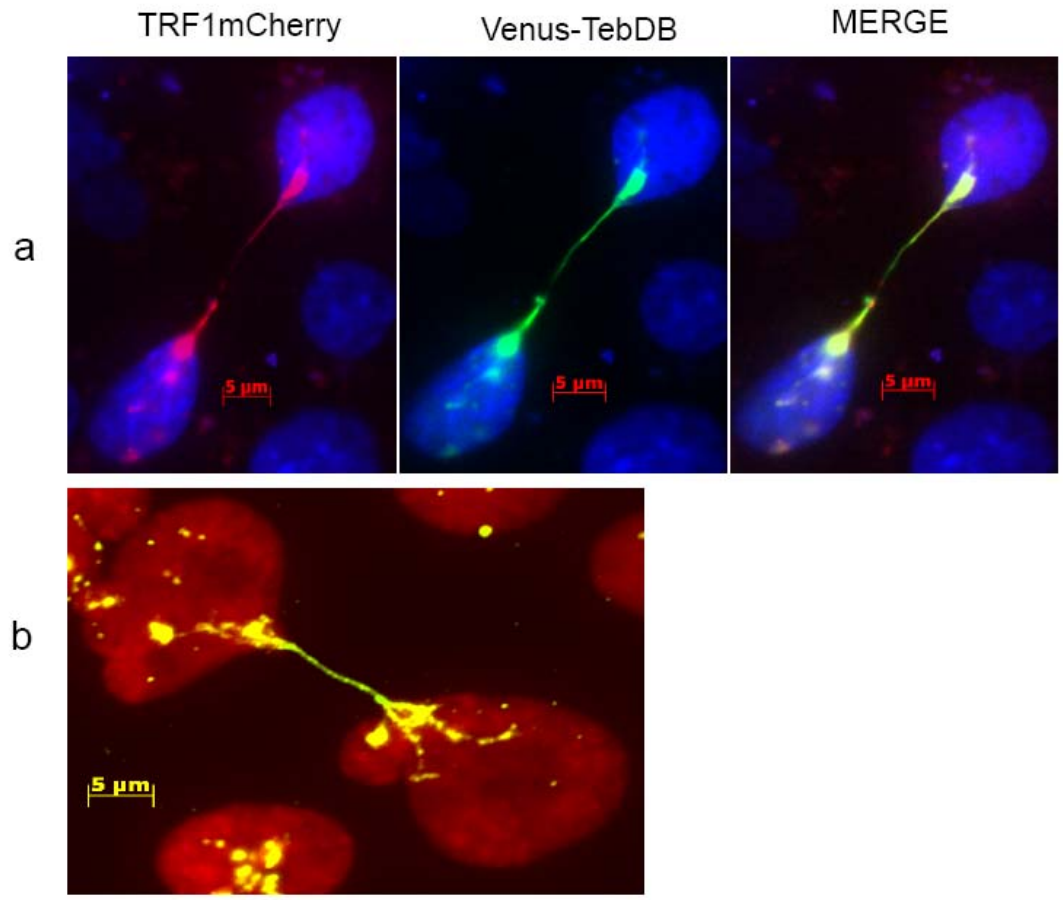


Figure 4.6: Telomere associations induced by TebDB in an NHEJ-deficient cell line. (a) The NHEJ-deficient XRCC4 null cell line GM16147 was transfected with mCherry-TRF1 (red) and Venus-TebDB (green). DNA was visualized with DAPI (blue). (b) The same cells in (a) were subjected to telomere-FISH with an LNA probe that detects the G-rich strand of telomeric DNA (green). DNA was visualized with DAPI (red).

IV.3f: WRN may be required for telomere association formation

The finding that NHEJ was not required for entanglement formation, coupled with data demonstrating that TRF2ΔB causes replication fork stalling at telomeres, suggests a model where compromised telomere replication leads to aberrant homologous recombination between both sister and non-sister telomeres. Previous

reports have shown that replication stress-induced telomere entanglements in fission yeast required the RecQ helicase SpRqh1 acting inappropriately at telomeres for their formation [128]. Interestingly, a recent report also showed that telomere recombination induced by TRF2ΔB is mediated by the SpRqh1 homologue WRN in humans [56]. WRN is a helicase best known for its role in the pathogenesis of Werner's syndrome, a progeroid disorder [121]. This helicase/nuclease plays important but poorly understood roles in DNA repair particularly during S-phase, the phase during which telomere entanglements are thought to occur [125, 128]. Because the aberrant function of SpRqh1 at dysfunctional telomeres led to telomere entanglements in fission yeast, we hypothesized that fibroblasts from patients with Werner's syndrome should actually be spared from telomere entanglements induced by TRF2ΔB and Venus-TebDB.

To address the role of WRN in creating telomere entanglements, we used a transformed WRN fibroblast cell line (AG11395) that does not contain WRN activity due to the presence of a homozygous mutation in exon 9 (C>T) that creates a protein with a premature stop codon (Arg368Stop) [172]. The mRNA that encodes this truncated protein is expected to be degraded by the non-sense mediated decay machinery, and this cell line has been shown to be deficient in DNA mismatch repair [173]. After transfection with either TRF2ΔB, Venus-TebDB, or empty vector, cells were subjected to either telomere-FISH or fluorescence microscopy to observe telomere entanglements. In AG11395 expressing either TRF2ΔB or Venus-TebDB, no telomere entanglements were found in 250 cells examined, suggesting that

telomere entanglements require WRN for their formation (Figure 4.7 a and c). Because we did not have either WRN cDNA or an isogenic WRN-positive counterpart for AG11395, we employed VA-13 as a positive control because it possesses WRN activity but is also an SV40-transformed fibroblast cell line that maintains its telomeres by ALT, similar to AG11395. Analysis of 250 VA-13 cells expressing TRF2ΔB yielded 78 telomere fusions, demonstrating that VA-13 are susceptible to telomere entanglements (Figure 4.5 b and c and Figure 4.7b), suggesting that WRN deficiency in AG11395 is responsible for protection against telomere entanglement. It is important to note that the Venus-TebDB fusion protein seems to label not only the chromatin bridges between cells, but also labels filament-like protrusions from these bridges that appear to arise from the mechanical stress associated with chromosomes pulling apart during anaphase and telophase (Figure 4.7b).

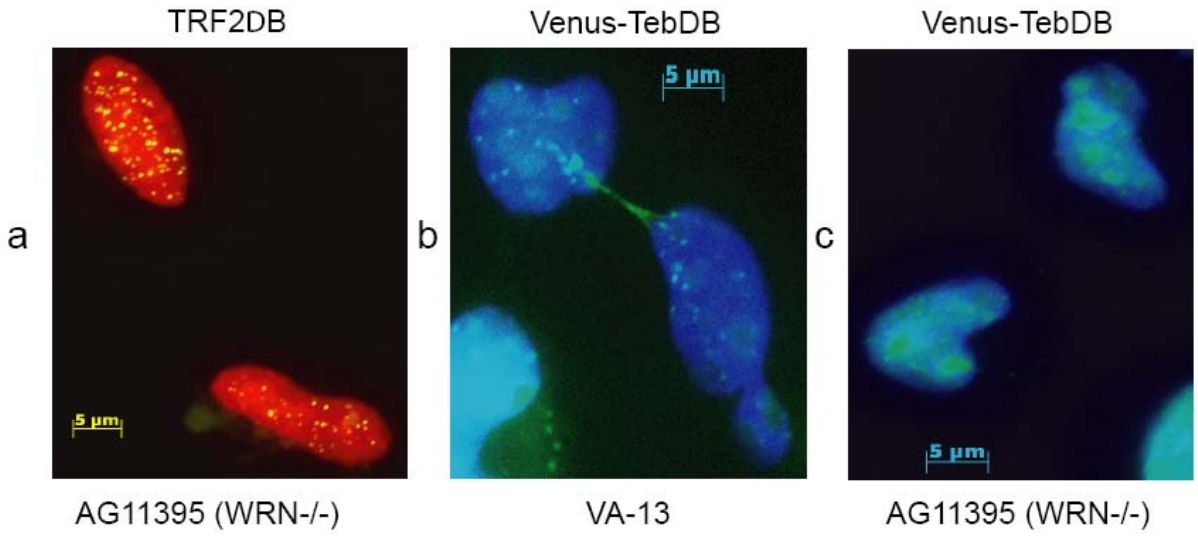


Figure 4.7: WRN may be required for formation of chromatin bridges caused by TRF2ΔB and TebDB expression. (a) The WRN deficient ALT cell line AG11395 was transfected with TRF2ΔB and stained with an LNA probe that detects the G-rich telomeric DNA strand (green). DNA was visualized with DAPI (red). (b) VA-13 were transfected with Venus-TebDB (green). DNA was stained with DAPI (blue). (c) AG11395 were transfected with Venus-TebDB(green). DNA was stained with DAPI (blue).

IV.4: Discussion

An essential and overlooked aspect of telomere maintenance is the replication of telomeric DNA carried out by the conventional DNA replication machinery. Although the end-replication problem associated with lagging-strand synthesis has garnered much publicity due to its contributions to aging and cancer [101, 105, 106], little attention has been paid to the machinery that is responsible for synthesizing the lion's share of telomeric DNA. Considering the inherent difficulties in replicating the G-rich strand and the fact that this process is essential for cellular proliferation, the lack of attention given to this topic is surprising. It is hoped that the identification of phenotypes caused by dysfunctional semi-conservative DNA replication at telomeres may lead to the identification of factors and mechanisms that cells employ to replicate this and other challenging DNA.

In this work, we have shown that replication stress at mammalian telomeres can result in telomere entanglements. These entanglements contain telomeric DNA, TRF1, and TRF2, demonstrating that they are cytologically distinct from previously reported mammalian telomere fusions. Importantly, these entanglements are NHEJ-independent and thus are not covalent, providing the first example of non-covalent telomere associations in metazoans. We also show that they can occur in ALT cell

lines, suggesting that they do not require telomerase for their formation. Finally, they appear to require the RecQ helicase WRN and possibly mismatch repair for their creation, as WRN deficient patient fibroblasts are immune to this phenotype. These results indicate that we have identified a novel type of mammalian telomere fusion that can occur in a wide variety of cell lines. Keeping with the tradition of naming telomere specific structures with "t-", including "t-loops", "t-stumps", and "t-complexes" [76, 154, 174], we have named this new class of telomere entanglements "t-wrecks".

How do t-wrecks form? Combining results presented here and elsewhere, the initial event in t-wreck formation is the stalling of replication forks within telomeric repeats [55, 125]. When a replication fork stalls in a telomere, repair by homologous recombination leads to either intra- or inter-telomeric recombination (Figure 4.8) [54]. These recombination events can then lead to t-circle and/or unresolved Holliday Junction formation [54, 56, 128, 174]. In the case of t-circle formation, these molecules can serve as templates for rolling circle replication, yielding large amounts of extrachromosomal telomeric DNA (Figure 4.1, 4.3, 4.5)[174, 175] (Figure 4.8). If inter-telomere recombination occurs, aberrant processing by SUMOylated RecQ helicases including SpRqh1 and WRN may create structures that can not be untangled by resolvases [128] which would lead to interchromosomal associations mediated by dynamic HJ-like structures (Figure 4.9). Since both t-circle and telomere-specific recombination intermediates depend on the activity of WRN [66, 128], either of these possibilities are consistent with our results. Furthermore,

since the HR pathway would be used in each of these cases, NHEJ would be dispensable for t-wreck formation.

Recombination events alone do not explain the t-wreck phenotype, however. The formation of telomeric DNA-containing chromatin bridges, as observed in t-wrecks (Figure 4.1, 4.3, 4.5), could be the result of homo or heterotypic protein:protein interactions between proteins that bind both t-circles and telomeres. Attractive candidates for these proteins include TRF1 and cohesins, both of which are involved in sister telomere segregation [43-45] and are consistent with the finding that TRF1 localizes to these bridges (Figure 4.4, 4.6). The segregation of centromeres to daughter nuclei during mitosis would lead to lagging t-circle/telomere structures that accumulate between sister nuclei, as observed in t-wrecks and telomere entanglements (Figure 4.9)[129]. This explanation is also intriguing because the protein:protein interactions at the heart of t-wrecks would be disrupted by the hypotonic swelling step in the metaphase spread preparation protocol [44], providing an explanation for the failure to visualize t-wrecks in metaphase spreads.

Another explanation for the inability of t-wrecks to survive metaphase spread preparation is that they result from unresolved HJs between sister telomeres (Figure 4.8). These structures would also "lag" between sister nuclei after mitosis, and the well-known requirement for salt during HJ formation[176] also explains the sensitivity to hypotonic swelling. It is also possible that both t-circles and inter-telomere recombination work together to produce t-wrecks; further investigation of t-wrecks will help to elucidate this issue. An interesting test of the potential

contribution of t-circles to t-wreck formation is to transfect circular plasmids containing several kilobases of telomeric DNA, and to assay t-wreck prevalence 48hr after transfection. The prediction is that circular plasmids containing telomeric DNA would be replicated by rolling circle replication, leading to accumulation of large amounts of telomeric DNA that become entangled during anaphase, yielding t-wrecks and demonstrating a role for t-circles in t-wreck formation.

The telomere entanglements identified here help to resolve a poorly understood issue in the telomere field. Previous work demonstrated that both cells expressing TRF2ΔB and ALT cells exhibited TLH, TFEs, SCEs, and spontaneous t-circle formation, however, it was unclear why cells expressing TRF2ΔB died given that similar dysfunction in ALT cells did not lead to their demise[54]. It appears that TRF2ΔB elicits telomere dysfunction to such an extent that telomere entanglements ensue, while the low levels of spontaneous telomere dysfunction in ALT cells are not sufficient to cause entanglements.

In addition, this report identifies TebDB as a new tool for both visualizing telomeres in species with TTAGGG-containing telomeric repeats, as well as inducing telomere dysfunction in cells undergoing DNA replication. The ability of TebDB to induce telomere dysfunction that contains the hallmarks of replication stress at telomeres suggests that TebDB causes telomere dysfunction by interfering with telomere replication. As TebDB binds telomeric DNA with extraordinarily high affinity (Kd=15nM) *in vitro* (Figure 3.1), it is unsurprising that the replication fork may halt when TebDB molecules are encountered. TebDB comes from a fission

yeast protein whose normal DNA binding regulation is unlikely to be recapitulated in mammalian cells. This situation is reminiscent of replication fork stalling induced by TRF1 and TRF2 in an *in vitro* system that recapitulates semi-conservative telomeric DNA replication [177]. It is possible that fission yeast contains a mechanism to regulate the binding of TebDB to S. pombe DNA such TebDB does not bind in S-phase, while mammalian cells have no way to regulate heterologously expressed TebDB. Furthermore, it is interesting to speculate that both TRF1 and TRF2 bind telomeres in a regulated manner, such that their binding affinity for telomeric DNA is reduced in S-phase to allow replication fork progression[124, 177]. The binding of TRF2 to telomeres in S-phase may be affected by its interaction with ORC, such that ORC binding to TRF2 weakens TRF2's affinity for DNA, allowing replication fork progression [124].

This work demonstrates that replication stress at mammalian telomeres results in t-wrecks, which are reminiscent of fission yeast telomere entanglements. The finding of t-wrecks in species ranging from fission yeast to hamster to human is a testament to the importance of conventional DNA synthesis in maintaining telomeres, and the deleterious consequences of replication stress at telomeres regardless of the telomeric repeat sequence (e.g. vertebrates are 5' TTAGGG 3' and fission yeast is 5' $G_{1-8}TTACA_{0-1}C_{0-1}$ 3'). We have identified both a novel phenotype associated with replication stress at mammalian telomeres, the presence of large amounts of telomeric DNA within chromatin bridges between sister nuclei in interphase, and a tool to induce this type of dysfunction in any vertebrate, TebDB.

The identification of this new phenotype and a tool for its induction will undoubtedly facilitate the discovery of other circumstances that lead to t-wreck formation, factors that involved in their development, including TRF2 and WRN, and their potential contributions to diseases such as Werner's Syndrome.

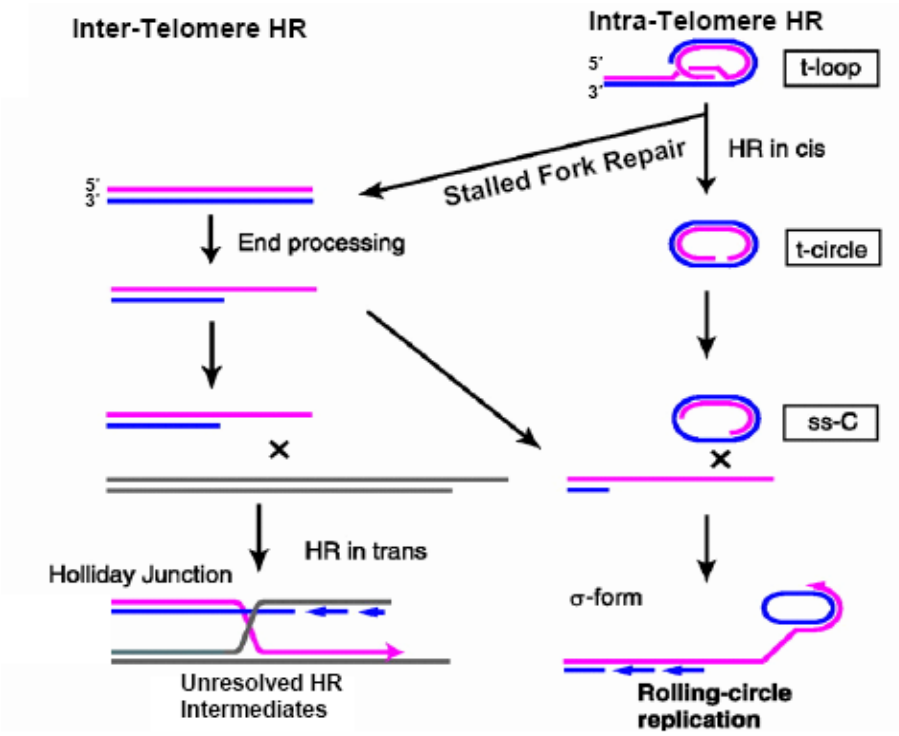


Figure 4.8: Consequences of inter-and intra-telomeric homologous recombination during the repair of stalled replication forks. Inter-telomere HR may lead to HR intermediates that are difficult to resolve, while intra-telomere HR can yield t-circles with subsequent induction of rolling-circle replication leading to accumulation of large amounts of telomeric DNA. Adapted with permission from the American Society for Microbiology from [174].

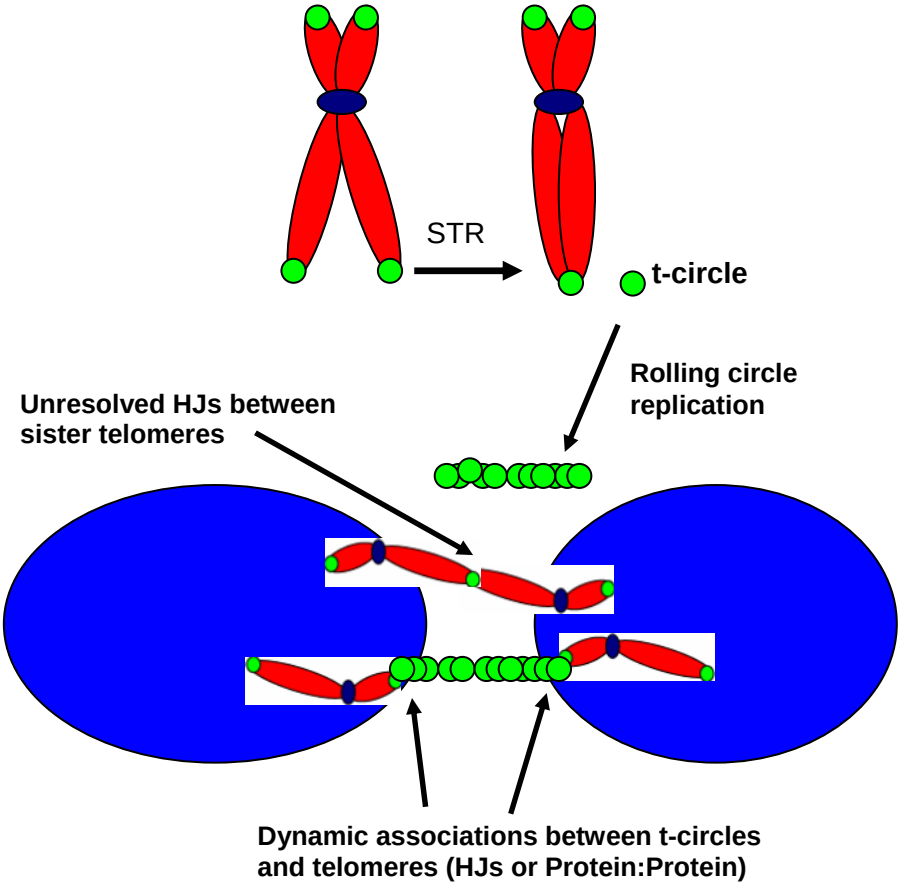


Figure 4.9. Model for t-wreck formation based on sister telomere recombination (STR) yielding t-circles and potentially unresolved Holliday Junctions (HJs).

Chapter V:
Conclusions and Future Directions

V.1: Future directions for Chapter 3

Research into cell biology and cytogenetics has been one of the most fruitful areas of science in the past 75 years. Within this field, arguably the most progress has been made in the subdiscipline of DNA damage and repair. Since initial work by McClintock and Muller, much knowledge has been gained about the types of damage DNA encounters and the complicated machineries that cells contain for repair. Although research in this area of biology has yielded key concepts and principals of DNA repair, little progress has been made in understanding how naturally occurring chromosome termini of eukaryotic genomes escapes recognition by the DNA repair machinery. The inability to dissect the intricate relationship between DNA repair and telomere maintenance is directly related to both the complexity of the DNA repair machineries and the intractable nature of highly repetitive DNA elements. Due to the complexity of this relationship, we sought to establish a more feasible paradigm for interrogating connections between DNA repair and telomere maintenance: how are telomeres protected from illegitimate processing by the NHEJ machinery?

The development of a novel system that allows for the recruitment of proteins to telomeres independently of their own capacity for DNA binding provides a new tool for investigation of the relationship between telomeres and DNA repair. We have shown that the DNA binding domain from the *S. pombe* protein Teb1, TebDB, binds vertebrate telomeric DNA *in* vitro and localizes to mammalian telomeres in

vivo. We also showed that TebDB can efficiently recruit proteins of interest to telomeres. Finally and most importantly, we showed that fusion of the TRF2-interacting factor hRAP1 to TebDB protects telomeres from the end-joining phenotype associated with loss of hRAP1/TRF2 from telomeres both *in vitro* and *in vivo*. These results identify hRAP1 as a critical mediator of telomere protection and genome stability in humans, and indicate that despite highly diverged functions, RAP1 homologues have conserved roles in telomere protection. Though both the development of a new system to tease apart the contribution of telomere binding proteins to telomere protection and the identification of a protective function for hRAP1 are important contributions to the telomere field, I believe the most important contribution of the research presented in Chapter Three are the new questions raised by these results.

The first and most pressing question put forth by the data reported in Chapter Three pertains to the mechanism by which hRAP1 protects telomeres. Loss of function experiments with hRAP1 are an obvious place to start and would help to shed light on this issue, however, mammalian RAP1 has not yet yielded its secrets to investigators wielding the standard genetics tools including knockout mice, RNA interference, and dominant-negative allele expression. Furthermore, we have expressed a fragment of TRF2 containing just the hRAP1 interaction domain (a.a. 280-446). This fragment should bind and occupy the TRF2 binding site on hRAP1, disrupting the association of endogenous hRAP1 and TRF2 thereby preventing hRAP1 from localizing to telomeres. Although we saw a dramatic reduction in

hRAP1 at telomeres in this experiment, no telomere deprotection phenotype was observed (Figure 5.1a and b). The lack of success of this and other hRAP1 loss-of-function experiments[66, 69, 144] can be explained by three possibilities, either that hRAP1 does not normally contribute to telomere protection, that very little hRAP1 is needed to protect telomeres, or that hRAP1 is one among many proteins that have redundant roles in telomere protection.

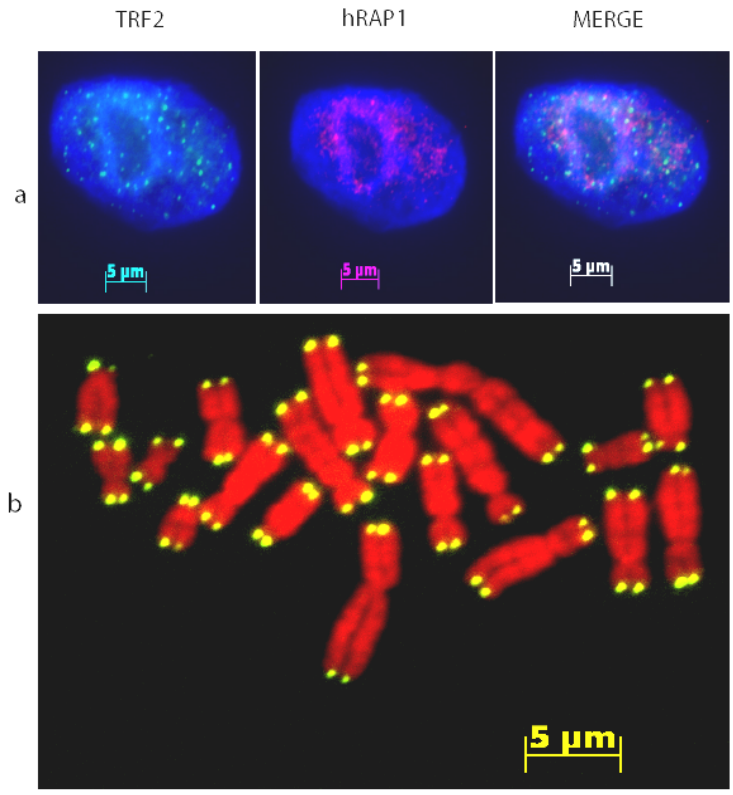


Figure 5.1: Analysis of HeLa S3 cells expressing $TRF2_{277-446.}$ (a) HeLa S3 were stained with TRF2 (green) and hRAP1 antibodies (red). DNA was visualized with DAPI (blue). (b) Telomere-FISH on metaphase spreads from HeLa S3 expressing $TRF2_{277-446}$. Telomeres are detected with an LNA probe that detects the G-rich telomeric DNA strand (green). DNA visualized with DAPI (red).

Since hRAP1 loss of function experiments have proven unwieldy to date, a more productive approach may be to further characterize the telomere protection afforded by hRAP1 in the *in vitro* and *in vivo* NHEJ assays reported in Chapter Three. For example, we have started to perform domain mapping to identify the minimum domain of hRAP1 that is sufficient to protect telomeres from the loss of TRF2/ hRAP1. Results presented in Chapter Three indicate that the TRF2 interaction domain on hRAP1 is dispensable for telomere protection and demonstrate that the domain mapping strategy can be successfully employed in this system. Concurrent deletion of other domains in hRAP1 including the BrCT, Myb, and coiled-coil domains may identify key regions of hRAP1 involved in telomere protection. The strength of this system is that it can be used both *in vitro* and *in vivo*, such that one could immunodeplete TRF2 and hRAP1 from NHEJ extract and add purified hRAP1-TebDB with the hRAP1-Myb domain deleted. Hypothetically, if this fusion protein was unable to restore telomere protection, the Myb domain would be a strong candidate for a region in hRAP1 that is responsible for telomere protection. One could then take the same hRAP1-TebDB construct with the Myb and TRF2-interaction domains removed (to prevent recruitment of TRF2ΔBΔM to telomeres) and co-express it with TRF2ΔBΔM. If telomere deprotection persisted in this sample, this data would support the model that the Myb domain is critical for telomere protection, and the convergence of *in vitro* and *in vivo* data would strongly justify pursuing this project further.

Having putatively identified regions of hRAP1 that are important for telomere protection, one could then try to discover factors that interact with that domain of hRAP1. This could be accomplished by many straightforward strategies, including yeast-2-hybrid technology[178] or immunoprecipitation of an epitope-tagged hRAP1 fragment containing only the domain of interest coupled with mass spectrometry to identify other peptides/proteins in the immunoprecipitation eluate [179]. Mass spectrometry would have the added advantage of being able to identify post-translational modifications in critical hRAP1 domains that may be necessary for end protection. It is likely that the hRAP1 associated proteins may work together with hRAP1 to ensure telomere protection, and their identification may help solve the enigma of hRAP1's role in telomere maintenance.

Another set of experiments that could help address hRAP1's role in telomere protection involves the analysis of damage signaling at telomeres containing hRAP1 but devoid of TRF2. Previous work has demonstrated that telomeres in TRF2 deficient MEFs signal for DNA damage through the ATM pathway, as deletion of ATM in TRF2 null MEFs abrogates the telomere fusion phenotype associated with TRF2 deletion [75]. In addition, other reports have suggested that TRF2 can bind and directly inhibit the autophosphorylation of ATM, a critical event in damage signaling, and that TRF2 can also bind the ATM effector Chk2 [59, 60]. The functional significance of these interactions in telomere protection is not yet known, however. It is also possible that hRAP1 inhibits damage signaling through unknown mechanisms. To address this possibility, quantification of the DNA damage response

and DNA repair both at telomeres and in whole cell extracts can be performed. For example, immunofluorescence with antibodies against different DNA damage and repair proteins, including 53BP1, phospho-H2AX, phospho-ATM, and others may show different staining patterns at hRAP1-containing and deficient telomeres [145]. Western blotting of whole cell extracts from cells expressing hRAP1-TebDB with TRF2ΔBΔM may also reveal differences in both the types and relative amounts of damage signaling in cells. These experiments would help pinpoint hRAP1's site of action in the DNA damage and repair signaling cascades.

The second question put forth by Chapter Three pertains to the idea that multiple pathways inhibit NHEJ at telomeres [68]. The experimental strategy outlined in Chapter Three provides an opportunity to capitalize on the TebDB system for the study of proteins that localize both to telomeres and other parts of the genome. PNUTS and MCPH1 are recently identified TRF2 interacting factors and, interestingly, mutation of their binding site in TRF2ΔBΔM reduced the telomere deprotection phenotype associated with expression of this allele [143]. It is possible that they play redundant roles with hRAP1 in protecting telomeres from NHEJ. To dissect any potential contribution of these proteins to telomere protection, one could fuse either PNUTS or MCPH1 to TebDB and co-express that fusion protein with TRF2ΔBΔM. If telomere protection was observed (and relevant controls such as those used in Chapter Three supported this result), the data would suggest that protein plays a role in telomere protection. To further investigate the issue, one could look for a synergistic effect of loss of function of both hRAP1 and the newly

identified telomere protection factor, perhaps by knocking down both mRNAs concurrently using RNA interference. The emergence of a telomere fusion phenotype when both hRAP1 and the other protein are knocked down, while knockdown of either alone is innocuous, would argue for the multiple pathway hypothesis and provide the first evidence for multi-modal NHEJ inhibition at telomeres in metazoans. It is worth noting that this type of experiment could be conducted by fusing any protein of interest to TebDB and assaying its ability to inhibit telomere end-joining, which is a timely discovery given recent reports suggesting that over 200 different proteins reside at telomeres [180].

V.2: Future directions for Chapter 4

While Chapter Three of this thesis provides several new avenues of investigation for telomere researchers, Chapter Four's contribution to the expanding literature of semi-conservative DNA synthesis at telomeres should not be overlooked. In Chapter Four, we show that abrogation of telomere replication by TRF2ΔB expression in transformed cell lines leads to dynamic telomere associations that are reminiscent of the telomere entanglements associated with replication stress in fission yeast [129]. Furthermore, we demonstrate that these human telomere entanglements are telomerase and NHEJ-independent and contain telomeric DNA and telomere binding proteins. Finally, we show that WRN deficient cell lines are resistant to this type of dysfunction, further linking these telomere associations to telomere replication stress and fission yeast telomere entanglements.

Chapter Four reports two important results that can be used to pursue research in the area of telomere replication. First, the identification of chromatin bridges between sister nuclei in interphase that contain large amounts of telomeric DNA provides a readily detectable phenotype for screening, particularly by automated microscopy platforms. For example, one could seed cells in a 96-well glass bottom dish, treat the cells with a siRNA library, and perform high-throughput telomere-FISH[181] on the cultured cells 72hr after transfection to look for chromatin bridges that are positive for telomeric DNA. Visualization of such structures would identify siRNAs that induce telomere replication stress. This experiment would uncover other factors associated with telomere replication, and may provide insight into how the cell deals with difficult-to-replicate regions. Another experiment that uses the same strategy would be to seed cells in a 96 well dish, transfect the cells with a siRNA library, and then transfect the cells with TRF2ΔB. If cells that are resistant to telomere entanglement formation are observed, then it is likely that the siRNA target gene in that well plays a role in the development of replication stress at telomeres, and may also shed light on the etiology of replication stress-associated at telomeres.

Although we have identified a new type of telomere-associated replication stress, the incidence and prevalence of these events in human cells is not known. To better understand telomere entanglements' etiology and contribution to pathology, multiple complementary strategies must be employed. For instance, information about the etiology of telomere entanglements may be gained by dissecting the

signaling cascade that arises when such insults are experienced by the cell. Fission yeast telomere entanglements were shown to activate mitotic spindle checkpoints, thereby providing an explanation for their ability to halt cell cycle progression [129]. In order to dissect damage signaling associated with human telomere entanglements, a reasonable approach is to compare relative levels of activated DNA damage response proteins using Western Blotting. DNA damage response kinases of interest in this regard include ATM phosphorylated on serine 1981 and ATR phosphorylated on serine 428, as well as their downstream substrates Chk1 phosphorylated on serine 296 and Chk2 phosphorylated on threonine 68. Phosphospecific antibodies for these proteins are available, allowing for molecular dissection of the signaling pathway involved in mammalian telomere entanglement formation. Replication stress at other loci such as fragile sites primarily involves signaling by ATR [182], therefore analysis of telomere entanglements may show that they are similar to other dysfunctions whose etiology is better understood. Illuminating differences between entanglements and other types of telomere dysfunction, including NHEJ-dependent fusions that signal through ATM, may also help to shed light on their origins.

Because fragile site-associated replication stress leads to genomic rearrangements often observed in cancers [183], it is also important to understand how telomere-associated replication stress may be involved in carcinogenesis. For example, if replication stress occurs at telomeres, the inability to repair such an insult is likely to result in cellular senescence-a normally irreversible cell cycle arrest that can be bypassed by oncogenic changes including p53 and Rb inactivation or Ras

activation [184, 185]. The bypass of senescence induced by telomere-associated replication stress is expected to result in telomere entanglements like those reported in Chapter Four. Since this phenotype very obviously results in telomere-mediated genome instability and telomere instability can contribute to oncogenesis [186], it is reasonable to hypothesize that the genome instability reported in Chapter Four may actually drive tumorigenesis. Thus, a new link between telomere dysfunction and oncogenesis may be uncovered, namely that telomere replication stress can result in genome instability that leads to oncogenic transformation. However, no such evidence for this model exists to date, though it has been shown that the highly oncogenic RasV12 causes cellular senescence by promoting DNA hyperreplication with concomitant induction of the DNA damage response [187]. The contribution of telomere replication to Ras-induced senescence was not addressed, though.

Dissecting the contribution of telomere replication to carcinogenesis poses a daunting challenge. However, tractable entry points do exist. Intriguing similarities between ALT cells and cells experiencing telomere-specific replication stress have been reported here and elsewhere and include homologous recombination proteins acting at telomeres, spontaneous t-circle formation, dramatic telomere length heterogeneity, telomere-free chromosome ends, and unresolved telomeric HR intermediates [54, 174, 180, 188]. Currently, it is not known how cancer cells engage the ALT pathway on their route to immortality. Thus, the identification of reagents that can promote engagement of ALT may shed light on its etiology. The similarities between ALT and replication stress at telomeres implies a causal relationship,

suggesting that telomere-specific replication stress can promote ALT. This idea is exciting because telomeres are inherently difficult to replicate, as evidenced by their exquisite sensitivity to DNA replication inhibitors[55]. To investigate this novel hypothesis, an interesting and reasonable experiment is to "pulse" untransformed cells with an inducer of telomere-specific replication stress like TebDB or TRF2ΔB during the process of transformation and then calculate the frequency of ALT in clones derived after transformation. The prediction that stimulating homologous recombination between telomeres may predispose cells to engaging ALT could therefore be directly addressed.

Chapter Four not only provides a potential link between telomere replication and oncogenesis, it also provides a tool for investigation of this relationship in any organism with telomeric repeats comprised of the sequence 5'TTAGGG3'. To address the contribution of telomere-associated replication stress to tumorigenesis, an attractive idea is to induce replication stress specifically at telomeres in a living animal and measure the incidence of cancer. Since TebDB induces telomere-specific replication stress due to its exquisite affinity and specificity for TTAGGG repeats, one could construct a transgenic animal; zebrafish, *X. laevis*, or mouse that expresses TebDB under the control of an inducible promoter, such that specific temporal induction of TebDB can be achieved. After constructing a transgenic animal, the induction of TebDB expression should lead to inhibition of telomere replication only in frequently dividing cells, such as skin, gut, and immune system cells, leaving differentiated and non-dividing cells like neurons unharmed. Assaying for an

increased incidence of carcinomas, intestinal polyps, and lymphomas would then address the contribution of telomere-associated replication stress to cancer susceptibility.

The use of non-human vertebrates to model human diseases is widespread in biology. However, due to the difficulties associated with the "anticipation" observed in diseases such as DKC and Werner's syndrome, few animal models for these diseases exist, and those that do often do not fully recapitulate the human condition [188-191]. Recently, the identification of DKC-like phenotypes in a mutant mouse engineered to undergo telomere degradation has brought renewed interest to this field [191]. However, this mouse is difficult to make as it requires knockout of two different genes, restricting the number of mice strains in which the disease can be modeled. The identification of two proteins, Trf2ΔB and TebDB, that can potently induce telomere degradation provides new reagents to make multiple mice strains in which either of these proteins are inducibly expressed. It is likely that expression of these proteins will induce telomere dysfunction and may give investigators the opportunity to study DKC and Werner's Syndrome on demand instead of having to wait many generations for diseased mice. Since transgenic mice are much easier and cheaper to make than gene targeted knockouts, this experiment should be given serious consideration. An even cheaper alternative is to produce a zebrafish that inducibly expresses these proteins. The recent identification of zebrafish mutants with premature aging phenotypes related to abrogation of telomerase function suggests that diseases such as DKC and Werner's Syndrome can be modeled in these

organisms [192]. Fish strains that inducibly express TRF2ΔB or TebDB may be able to quickly recapitulate these diseases on demand, which may open new avenues for investigation of therapeutic interventions.

Another attractive use of TebDB is in the investigation of the contribution of individual cells (and their ability to propagate) to tissue maintenance. An example of this comes from the zebrafish retina. After encountering massive photoreceptor cell death due to overexposure to light, Muller glia within the injured retina dedifferentiate into multipotent progenitor cells [193, 194]. Since injured zebrafish retinas can regenerate photoreceptors after injury, it has been hypothesized that dedifferentiated Muller glia may redifferentiate into photoreceptors to replenish the depleted supply. However, it is also possible that other retinal stem cell populations are responsible for most of the photoreceptor replacement [195]. Surprisingly, TebDB may provide a useful tool to address this question. As Muller cell-specific promoters are available [196], one could drive expression of TebDB in Muller glia of adult zebrafish and then injure the retina. Since TebDB would hinder the ability of Muller glia to divide by interfering with telomere replication, while leaving the rest of the functions of Muller glia intact, one could tease apart the contributions of proliferation and cell function to retinal repair. Thus, TebDB provides a new tool that can help to dissect the contributions of cell function and proliferation to tissue health that contrasts with standard methods such as cell ablation, in which both proliferative capacity and function of individual cells are lost upon cell death.

The development of a transgenic animal to investigate the association between replication stress at telomeres, genome instability and tumorigenesis is both expensive and risky. However, other more conventional uses of TebDB exist. Perhaps the most important of these is the use of TebDB as a tool to visualize telomeres in vertebrates for which other genomics tools are not available, including many species that are relevant to human health and disease [197]. For example, new vertebrate model systems including marmosets, naked mole rats, opossums, bats, finches, chameleons, and icefish are currently being developed to study important biological processes such as aging. Since telomere dynamics may contribute to aging-related processes [198], the creation of tools to rapidly visualize telomeres is of high interest. Although TRF1/2 homologues in many of these organisms have not been identified rendering fusion of fluorescent proteins to endogenous telomere binding proteins impossible, GFP-TebDB could be used to visualize telomeres in these species. The small size of TebDB suggests that it can be used in virus-based gene delivery systems with broad tropisms, allowing for efficient delivery in a wide variety of genetically intractable species. It is hoped that these experiments will provide new insights into the role of telomere maintenance in cancer and aging.

Appendix 1:

Publications:

Sarthy JF, Bae NS, Scrafford JD, Baumann P. Human RAP1 Inhibits Non-Homologous End Joining at Telomeres. Submitted to *Genes and Development* (June, 2009).

Sarthy JF and Baumann P. Replication Stress at Telomeres Results in t-wrecks, a New Type of Highly Conserved Telomere Dysfunction. In Preparation.

Sarthy JF and Gamblin TC (2006) A Light Scattering Assay for Arachidonic Acid Induced Tau Fibrillization Without Interfering Micellization. *Analytical Biochemistry, 353(1):150–2.*

Shieh CC, Trumbull JD, Sarthy JF, McKenna DG, Parihar AS, Zhang XF, Faltynek CR, Gopalakrishnan M. Automated Parallel Oocyte Electrophysiology Test Station (POETs™): A Screening Platform for Identification of Ligand-Gated Ion Channel Modulators. *Assay and Drug Development Technologies. October 2003, 1(5): 655-663.*

Appendix 2:

National Meetings Attended and Posters Presented:

Jay Sarthy, Kelly Trujillo and Peter Baumann. Identification of Multiple Distinct hPOT1-containing Complexes. 47th Annual Short Course on Medical and Experimental Mammalian Genetics. July, 2006. Jackson Laboratories, Bar Harbor, ME.

Jay Sarthy, Jonathan Scrafford and Peter Baumann. Teb1-A Tool for Inducing Telomere Dysfunction in Vertebrates. Workshop on Chromatin and Chromatin Modifying Enzymes. October, 2007. Stowers Institute, Kansas City, MO.

The Role of Telomeres and Telomerase in Cancer. AACR Special Topics Meeting. December, 2007. San Francisco, CA.

Jay Sarthy, Nancy Bae, Jonathan Scrafford and Peter Baumann. hRAP1 Protects Telomeres from NHEJ. Microbial Genetics and Genomics V. May, 2008. Cassis, Fr.

Molecular Biology of Aging Workshop at the Marine Biological Laboratory, Woods Hole, MA. Directed by Steve Austad and Gary Ruvkun. August, 2008.

Appendix 3:

Collaborator's Contributions:

Nancy Bae performed the electrophoretic mobility shift assays (EMSAs) in Figure 3.1, the immunoblot in Figure 3.2, and the *in vitro* non-homologous end-joining assay in Figure 3.3.

Jonathan Scrafford cloned and helped to express and purify recombinant GST-TebDB and hRAP1-TebDB. Jonathan established conditions for the electrophoretic mobility shift assays in Figure 3.1 and calculated dissociation constants for GST-TebDB and hRAP1-TebDB using EMSAs. Jonathan also played critical roles in cloning hRAP1-Venus-TebDB, Venus-TebDB and mCherry-TRF1.

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