TRF2 INTERACTING PROTEINS AND TELOMERE MAINTENANCE

FUNCTIONAL STUDIES OF TRF2 AND ITS INTERACTING PROTEINS IN MAINTAINING TELOMERE LENGTH AND INTEGRITY

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Abstract

The ends of eukaryote chromosomes, a region called the telomeres, plays a critical role in maintaining genome stability. With each round of mitotic division the telomeres erode until a critically short length is reached, which signals the cell to permanently stop dividing. This is recognized as a contributing factor to ageing and the onset of age related diseases. Telomere repeat binding factor 2 (TRF2), is an important telomere DNA binding protein that has an essential role in protecting telomeres from being recognized as DNA breaks, however it has also been implicated in other aspects of telomere maintenance, such as telomere replication and telomere transcription. TRF2 acts as a protein hub for the recruitment of a number of telomere associated proteins involved in telomere maintenance, and it has been shown to be heavily modified by numerous types of post-translational modification. We demonstrate that TRF2 is methylated on arginine residues in its N-terminal region by protein arginine methyltransferase 1 (PRMT1) and that this arginine methylation is important for proper telomere maintenance. We further demonstrate that methylated TRF2 is a component of the nuclear matrix and has a distinct staining pattern in senescent cells. The importance of telomeres to ageing is exemplified by previously reported observations that defects in telomere maintenance are a common characteristic to numerous premature ageing disorders. We show that the premature ageing disorder, Cockayne Syndrome has an underlying defect in telomere maintenance. Approximately 80% of Cockayne Syndrome patients have mutations in the Cockayne syndrome group B (CSB) protein. We identified a novel interaction between TRF2 and CSB. The work presented in this thesis characterizes these novel interactions and gives new insight into the function of TRF2 in telomere maintenance.

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Table of Contents

List of Figures

List of Abbreviations

53BP1	p53 binding protein 1
A	Adenine
ADMA	asymmetric dimethylarginine
ALT	Alternative lengthening of telomeres
APB	ALT-associated PML bodies
AT	Ataxia telangiectasia
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia Rad3-related
BER	Base excision repair
Bp	Base Pair
BRCA1	Breast cancer 1
BRCT	BRCA1 C terminus
C	Cytosine
ChIP	Chromatin immunoprecipitation
Chk	Checkpoint kinase
CLL	Chronic lymphocytic leukemia
CPD	Cyclobutane pyrimidine dimers
CS	Cockayne syndrome
Cs	Cesium
CSB	Cockayne syndrome group B protein
CSA	Cockayne syndrome group A protein
DAPI	4', 6-diamidino-2-phenylindole
DC	Dyskeratosis congenita
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DSB	Double strand break
dsDNA	Double stranded DNA
D-loop	Displacement loop
ERCC1	Excision repair cross-complementation group 1
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
G	Guanine
G4	G quadruplexes
GAR	Glycine arginine rich
GFP	Green fluorescent protein
GGR	Global genome repair
Gy	Gray
HGPS	Hutchinson Gilford progeria syndrome
HP1	Heterochromatin protein 1
HR	Homologous recombination
hTERT	Human telomerase reverse transcriptase
hTR	Human telomerase RNA

IF	Immunofluorescence
IP	Immunoprecipitation
IR	Ionizing radiation
K5	Keratin 5 promoter
kb	Kilobase
LMNA	Lamin A
MDC1	Mediator of DNA damage checkpoint protein 1
MEF	Mouse embryonic fibroblast
MMA	Monomethylarginine
MRE11	Meiotic recombination 11
MRN	MRE11-RAD50-NBS1
NBS1	Nijmegen Breakage syndrome 1
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NLS	Nuclear localization signal
OB	Oligosaccharide/oligonucleotide binding
OIS	Oncogene induced senescence
ORC	Origin of replication complex
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly ADP-ribose polymerase
PD	Population doubling
PIP1	POT1 interacting protein 1
PML	Promyelocytic leukemia
PNA	Peptide nucleic acid
POT1	Protection of telomeres 1
PRMT	Protein arginine methyltransferase
PTOP	POT1 and TIN2 organizing protein
Rap1	Repressor/ Activator protein
RCT	RAP1 C-terminal
RE1	Repressor element 1
REST	Repressor element 1 silencing transcription factor
RIF1	Rap1 interacting factor 1
RNA	Ribonucleic Acid
RNF	Ring finger
RPA	Replication protein A
SAM	S-adenosyl methionine
SDS	Sodium dodecyl sulfate
sh	Short hairpin
Siah1	Seven in absentia homolog 1
SDMA	Symmetric dimethylarginine
ssDNA	Single stranded DNA
SUN1	Sad1p UNC84 domains 1 protein
SV40	Simian virus 40
T	Thymine

TCAB1	Telomerase cajal body protein 1
TCR	Transcription coupled repair
TD	Telomere doublets
TDM	Telomere double minute
TERRA	Telomeric repeat containing RNA
TEL	TPP1 glutamate (E) and leucine (L)-rich patch
TFE	Telomere free end
TIF	Telomere dysfunction induced foci
TIN2	TRF1 interacting nuclear protein 2
TINT1	TIN2 interacting protein
T-loop	Telomeric loop
T-SCE	Telomere sister chromatid exchange
TPP1	TINT1/ PTOP/PIP1
TRF1	Telomere repeat binding factor 1
TRF2	Telomere repeat binding factor 2
TRITC	Tetramethyl rhodamine isothiocyanate
UBD	Ubiquitin binding domain
UV	Ultraviolet
WCE	Whole cell extract
WRN	Werner
WS	Werner syndrome
WT	Wild type
XP	Xeroderma pigmentosum

Preface

The work presented herein is centered on the telomere protein TRF2 and the characterization of its novel interacting proteins involved in telomere maintenance. The work has been divided into three chapters. Chapters 2 and 4 have been published in peer reviewed journals and Chapter 3 has been submitted for publication. As such, each of these publications has a referencing style that conforms to the journal in which they were submitted. Complementary data have been supplied at the end of Chapters 2 and 3 that were not included in the publications, however I felt was relevant to my findings. The referencing style for the complementary data matches the style used for the rest of the thesis. Chapter 1 is an extensive literature review that is meant to complement the introductions that are already included in the publications. Lastly, Chapter 5 will discuss the findings and supply future direction.

Each publication contains its own section of materials and methods. For the complementary data only materials and methods that are not previously mentioned in publications have been detailed. All the experiments were done by me except where indicated in the prefaces that prelude each individual chapter.

Chapter 1

Introduction

1.1 Aging

Aging is the time dependent decline in the overall health of an organism, which coincides with an increase risk in disease and death (Longo, Mitteldorf & Skulachev 2005). A decline in health over time is observed in most organisms from yeast to humans, and aging at the cellular level maintains many conserved characteristics (Burtner, Kennedy 2010). It is well accepted that the rate of aging, and an individual's longevity, is determined principally by environmental, and to a lesser degree, by genetic factors (Barzilai et al. 2012). However, aging is still poorly understood at the molecular level and many hotly debated theories exist as to the true cause. With the greying of the population and the increasing average life expectancy, the need for a better understanding of the aging process has become paramount for medical, social and economic reasons (Freitas, de Magalhaes 2011).

The work done throughout the 1960's by Hayflick and Moorhead demonstrated that explanted human somatic cells have a finite replicative capacity known as the Hayflick limit (Hayflick 1965, Hayflick, Moorhead 1961). The permanent exit from the cell cycle due to an exhausted replicative capacity while maintaining cell viability and metabolic function is commonly referred to as cellular senescence, replicative senescence, or cellular aging (Kuilman et al. 2010). The interpretation of the importance of cellular senescence initially led to two seemingly contrasting views of its role *in vivo*. Firstly, unlike normal fibroblasts, cancer cells have unlimited replicative capacity. This led to the

idea that cellular senescence is a beneficial anti-tumour mechanism that halts cell growth at a pre-malignant stage (Dimri et al. 1995). Conversely, Hayflick hypothesized that the limited replicative capacity of our cells explained the degeneration of our tissues as we age, implicating senescence to be a negative process contributing to age related ailments such as heart disease and liver failure (Hayflick 1965). These differences are reconciled by the antagonistic pleiotropy theory of aging, which suggests that the benefits early in life (cancer prevention), outweighs the cost to our bodies later in life (aging) (Wright, Shay 1995).

Shortly after the mechanism of DNA replication was discovered, Watson and Olovnikov independently realized that conventional replication machinery cannot fully synthesize the terminus of the lagging strand of DNA replication (Olovnikov 1973, Olovnikov 1971, Watson 1972). This is because DNA synthesis is catalyzed off of a 3'-hydroxyl group made available from an RNA primer, and DNA synthesis therefore can only occur in the 5'-3' direction. The leading strand of DNA synthesis can continue unimpeded to the chromosome end as DNA is unwound. However in the lagging strand direction *de novo* DNA synthesis requires newly formed RNA primers to be laid down as DNA is unwound. Therefore, the final RNA primer and any template sequence situated on its 5' end will not be replicated, resulting in the gradual loss of approximately 70-100 nucleotides per round of cellular division depending on the position of the last RNA primer (Chow et al. 2012, Diotti, Loayza 2011). Watson termed this the "end replication problem" and Olovnikov had the foresight to suggest this may be a contributing factor to

replicative senescence (Kuilman et al. 2010). We now know that the shortening of the chromosome ends, a region known as the telomere, plays a critical role in determining the replicative capacity of a cell (Harley, Futcher & Greider 1990, Martinez, Blasco 2010).

Studying aging in humans is difficult due to ethical reasons, complex genetics and long life spans. However, a number of diseases have supplied clues about the aging process, such as Hutchinson-Gilford Progeria, Werner Syndrome, Cockayne Syndrome, Xeroderma Pigmentosum and Ataxia Telangiectasia. In these diseases the affected individual progresses through the aging process at an accelerated rate (Navarro, Cau & Levy 2006, Collado, Blasco & Serrano 2007). These are collectively known as premature aging syndromes and are often used as models for studying normal aging. A common characteristic often seen in these disorders is a defect in telomere maintenance (Collado, Blasco & Serrano 2007, Cao et al. 2011, Benson, Lee & Aaronson 2010, Crabbe et al. 2004, Kong, Lee & Wang 2013, Callen, Surralles 2004, Metcalfe et al. 1996). Evidence suggests that telomere dysfunction can be a driving force for both cellular aging and malignancy (Kong, Lee & Wang 2013). Thus, understanding the molecular mechanisms of telomere maintenance will shed light on the black box of aging, as well as provide potential therapeutic targets against age related diseases.

1.2 Telomeres

1.2.1 Telomere Structure

Telomeres are the DNA/protein complexes that cap the ends of linear chromosomes. On average humans are born with 10-15 kb of telomeric DNA consisting of the repetitive duplex sequence, 5'-TTAGGG/3'-AATCCC (de Lange et al. 1990, Moyzis et al. 1988). The duplex telomeric DNA is followed by a 3' G rich overhang that is essential for the two main functions of telomeres in the cell; telomere length regulation and chromosome end protection (Makarov, Hirose & Langmore 1997, Greider, Blackburn 1985, Griffith et al. 1999). The 3' overhang acts as a substrate for the ribonucleoprotein, telomerase (Blackburn 2005). Telomerase is a reverse transcriptase that adds *de novo* TTAGGG DNA sequences to the 3' overhang (Greider, Blackburn 1985). Conventional DNA replication can then fill in the complementary strand to allow for telomere lengthening. In humans, the expression of telomerase is restricted to germ cells, stem cells, and is often re-activated in cancer cells. All of these cell types experience extended proliferative capacity compared to the aforementioned somatic cells that are subject to the Hayflick limit (Gunes, Rudolph 2013).

The 3' overhang aids in chromosome end protection by allowing the formation of a higher order structure of telomere chromatin (Griffith et al. 1999, Palm, de Lange 2008). The chromosome end folds back on itself to form a large lasso structure. This allows the 3' G rich overhang to invade the internal duplex telomere tract and form complementary base pairing with the C rich strand. This stable structure is called the

telomere loop (t-loop) and the short sequence of displaced G rich DNA is the displacement loop (D-loop) (Griffith et al. 1999). This higher order structure aids in chromosome protection by warding off nucleolytic attack from exonucleases and preventing the natural chromosome end from being mistaken as an aberrant DNA break (Griffith et al. 1999, Palm, de Lange 2008). The formation of a t-loop is an evolutionarily conserved mechanism that has been visualized *in vivo* in numerous species from yeast to humans (Griffith et al. 1999, Murti, Prescott 1999, Munoz-Jordan et al. 2001, Cesare et al. 2003, Cesare et al. 2008, Raices et al. 2008). The size of the t-loop can vary drastically from 500 bp to 18 kb, however in mammals they are typically a few kilobases smaller than the length of the telomere (Griffith et al. 1999, Wei, Price 2003). Although the size of the t-loop is not stringently regulated the sequence at the 5' terminus is. The C-rich strand is processed so that it terminates in the sequence ATC more than 80% of the time (Sfeir, Shay & Wright 2005).

The presence of telomeric DNA at all chromosome ends acts as a buffer that prevents the loss of coding DNA with each round of cell division. Once the telomeric DNA reaches a critically short length, senescence signalling is activated. The critical length seems to be cell specific and telomere lengths as low as 0.3 kb have been recorded, however telomere lengths are typically between 2-3 kb when senescence is activated (Britt-Compton et al. 2006). A correlative link exists between long telomere length and increased life expectancy (Cawthon et al. 2003), which has supported a view that

telomeres are an internal molecular clock that can tell the time on cellular age (Mitteldorf 2013).

Although it is important to ensure that chromosome ends are always protected, they also need to be dynamic. Telomeres must be accessible to replication machinery, they are subject to both telomere lengthening and telomere trimming mechanisms, and they are transcribed into non-coding RNA that is itself important for proper telomere maintenance (Palm, de Lange 2008, Azzalin et al. 2007, Schoeftner, Blasco 2008, Pickett et al. 2011, Martinez et al. 2009, Sfeir et al. 2009). These and virtually all aspects of telomere metabolism are controlled by the multi protein complex, shelterin (de Lange 2005).

1.2.2 The shelterin complex

Shelterin, a protein complex found at telomeres throughout the cell cycle, contains six unique polypeptides (Figure 1.1A) (Palm, de Lange 2008, Verdun et al. 2005). Two of the shelterin components, telomere repeat binding factors 1 and 2 (TRF1 and TRF2) bind directly to duplex telomere DNA (Bilaud et al. 1997, Bilaud et al. 1996, Broccoli et al. 1997). These two factors do not interact with each other directly but are rather held together by Terf1 interacting nuclear factor 2 (TIN2), which can interact simultaneously with TRF1, TRF2 and a third component, TPP1 (TINT1/PTOP/PIP1) (Broccoli et al. 1997, Ye et al. 2004a, O'Connor et al. 2006). Recruited through TPP1 to the complex is the single strand telomeric DNA binding protein, protection of telomeres 1 (POT1)

(Hockemeyer et al. 2007, Baumann, Cech 2001). Lastly, repressor/activator protein 1 (RAP1) is seen in a stoichiometric ratio of 1:1 with its recruitment factor TRF2 (Figure 1.1A) (Zhu et al. 2000, Takai et al. 2010). Shelterin is required for the formation and the stability of the t-loop (Figure 1.1B) (Griffith et al. 1999, Stansel, de Lange & Griffith 2001). The amount of shelterin recruited to telomeres is limited by the number of TRF1 and TRF2 binding sites (Takai et al. 2010), and subcomplexes (TRF1-TIN2-TPP1/POT1 and TRF2-RAP1) of these proteins have been identified, adding an additional layer of functional regulation and complexity (O'Connor et al. 2006, Takai et al. 2010, Liu et al. 2004a).

1.2.3 TRF1 (telomere repeat binding factor 1)

TRF1 is transcribed from the *TERF1* gene, located on chromosome 8 at position q21.11. It was the first identified human telomere DNA binding protein (Chong et al. 1995). TRF1 plays important roles in telomere length homeostasis, sister chromatid separation, and telomere replication (Sfeir et al. 2009, van Steensel, de Lange 1997, Canudas et al. 2007). TRF1 has a C-terminal SANT/Myb-like domain, TRFH domain, a linker region, and an N-terminal acidic domain (van Steensel, de Lange 1997). TRF1 is the least conserved shelterin component and only contains 65% sequence similarity between mouse and human (Broccoli et al. 1997).

When TRF1 is overexpressed, telomerase is prevented from extending the chromosome ends leading to gradual telomere shortening (van Steensel, de Lange 1997,

Smogorzewska et al. 2000, Ancelin et al. 2002). In contrast, the expression of a dominant negative allele of TRF1 or the loss of endogenous TRF1 leads to rapid telomere elongation in a telomerase dependent manner (van Steensel, de Lange 1997). In this model of action, TRF1 acts in cis to inhibit telomerase from elongating telomere ends (Smogorzewska et al. 2000). A long telomere will have more TRF1 bound which will render the chromosome end inaccessible to telomerase, whereas a shorter telomere will have less TRF1 and will be more accessible to telomerase (van Steensel, de Lange 1997).

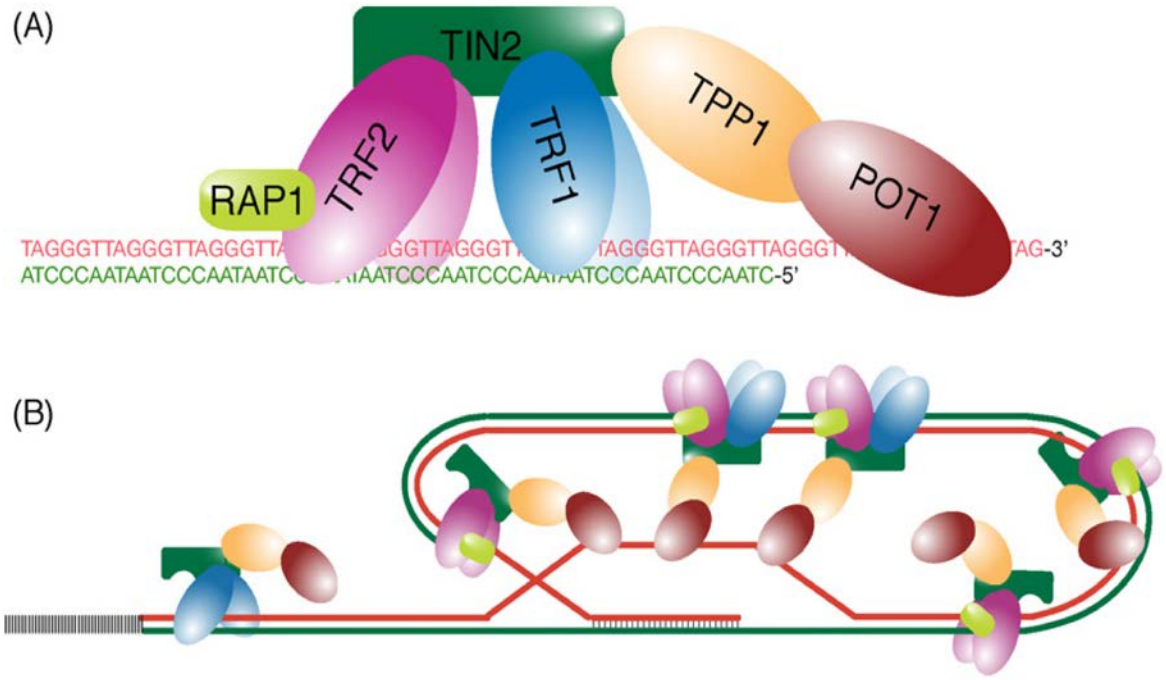

Fig. 1.1. The shelterin complex. (A) The human shelterin complex consists of six unique polypeptides (TRF1, TRF2, RAP1, TIN2, TPP1 and POT1). (B) The higher order t-loop structure of telomere DNA. Shelterin binding to telomeric DNA promotes the t-loop formation which caps the chromosome end. The 3' G-rich overhang of telomere DNA invades the duplex tract causing displacement of the internal G-rich strand, which is then bound by the single strand telomere binding protein POT1. Modified from (Denchi, 2009)

TRF1 null mice die at E6, demonstrating an essential role for TRF1 in embryonic development (Karlseder et al. 2003). However, the necessity of TRF1 for survival was independent of the presence of telomerase, itself dispensable in mice with long telomeres, which was the first indication that TRF1 has other important function beyond its predominant role in telomere length regulation (Karlseder et al. 2003, Blasco et al. 1997).

The establishment of mice with a conditionally floxed TRF1 allele identified an essential role for TRF1 in telomere replication (Martinez et al. 2009, Sfeir et al. 2009). Fragile sites are regions that represent significant difficulty for DNA replication machinery (Durkin, Glover 2007). In the absence of TRF1 telomeres resemble fragile sites, which results in the increased occurrence of replication fork collapse, causing DNA breaks and the activation of the DNA damage signalling kinase, ataxia telangiectasia and Rad3-related (ATR) (Martinez et al. 2009). Repetitive guanines, like those found in telomeric DNA, can form a stable G-quartet (G4) structure, called G-quadruplexes, through Hoogsteen hydrogen bonding (Maizels 2006). G-quadruplexes are predicted to commonly form in telomeric DNA and would likely represent a replication block. TRF1 acts in an epistatic pathway with the helicases RTEL and BLM to prevent telomere fragility by preventing fork collapse and resolving G-quartets (Martinez et al. 2009, Vannier et al. 2012). TRF1 is also important for proper mitotic progression. TRF1 localizes to the mitotic spindle and colocalizes with the mitotic checkpoint proteins BubR1 and Mad2 (Nakamura et al. 2001, Nakamura et al. 2002, Munoz et al. 2009). TRF1 interacts with the cohesin SA1 and is heavily phosphorylated in mitosis by CDK1,

which is required for proper sister chromatid separation (Canudas et al. 2007, Canudas, Smith 2009, McKerlie, Zhu 2011).

Recently, TRF1 was shown to localize to sites of DNA damage following ionizing radiation (McKerlie et al. 2013). TRF1 phosphorylated on T371 is recruited to DNA breaks in an ATM dependent manner where it promotes the homologous recombination (HR) repair pathway (McKerlie et al. 2013). The expression of a TRF1 mutant that cannot be phosphorylated at this site prevents its recruitment to sites of DNA breaks and decreases repair efficiency, thereby decreasing cell survival following DNA damage (McKerlie et al. 2013).

1.2.4 TRF2 (Telomere Repeat Binding Factor 2)

TRF2 is a 500 amino acid protein transcribed from the *TERF2* gene located on chromosome 16 at position q22.1. TRF1 and TRF2 are distant homologs that share a similar domain structure (Broccoli et al. 1997). TRF2 contains a C-terminal SANT/Myb-like DNA binding domain, a flexible linker region, a TRF homology (TRFH) domain, and a short N-terminal basic domain (Broccoli et al. 1997). TRF2 has diverged at an expected rate and shares 82% sequence similarity between mouse and human (Broccoli et al. 1997). The linker region, which is the least conserved domain, separates the TRFH and DNA binding domains and is required for several important protein interactions (Broccoli et al. 1997, Okamoto et al. 2013, Chen et al. 2011). TRF2 homodimerizes via the TRFH domain which is required to bring together two Myb-like

domains, allowing for its high affinity binding to duplex telomeric DNA (Bilaud et al. 1997, Bilaud et al. 1996, Broccoli et al. 1997, Bianchi et al. 1997). The N-terminal basic domain has been shown to bind DNA in a sequence independent manner and has an affinity for DNA junctions, which is a characteristic that may be important for the formation and stability of the t-loop (Fouche et al. 2006, Amiard et al. 2007, Wang, Smogorzewska & de Lange 2004).

The most well characterized function of TRF2 is in telomere end protection; however TRF2 is also involved in telomere length regulation (Smogorzewska et al. 2000, van Steensel, Smogorzewska & de Lange 1998). TRF2 prevents an ATM dependent DNA damage response from recognizing the naturally occurring chromosome ends as aberrant DNA double strand breaks (van Steensel, Smogorzewska & de Lange 1998, Denchi, de Lange 2007, Celli, de Lange 2005). TRF2 has evolved multiple mechanisms to monitor and control the DNA damage response at telomeres which will be discussed in greater detail in chapter 1.3, along with the other functions of TRF2 and its associated factors.

1.2.5 TIN2 (Terf1 Interacting Nuclear Factor 2)

TINF2, located at 14q12 gives rise to the TIN2 protein. TIN2 was identified by a yeast two hybrid assay with TRF1 as bait (Kim, Kaminker & Campisi 1999). It is also capable of simultaneously interacting with TRF2 and TPP1, thereby allowing it to act as a bridge between the double strand telomere binding proteins, TRF1 and TRF2, and the

single strand end binding protein complex, TPP1/ POT1 (Ye et al. 2004a, Houghtaling et al. 2004, Kim et al. 2004, Ye et al. 2004b). The overexpression of TIN2 negatively regulates telomere length, whereas an N-terminal deletion mutant promotes robust telomere elongation (Kim, Kaminker & Campisi 1999). Mutations in TIN2 can cause dyskeratosis congenita (DC), a disease whose primary phenotypes are the manifestation of a defect in telomerase activity (Savage et al. 2008, Walne et al. 2008, Tsangaris et al. 2008, Mitchell, Wood & Collins 1999). Most individuals with DC will succumb to bone marrow failure or their predisposition to cancers (Mitchell, Wood & Collins 1999). The mutations in TIN2 that lead to DC are clustered around a PTVML motif which is an HP1γ binding site (Canudas et al. 2011). The authors determined this to be an important interaction for sister chromatid cohesion and telomerase dependent telomere elongation (Canudas et al. 2011).

Besides its role in telomere length regulation, TIN2 is also important for telomere end protection. Without TIN2, the recruitment of TPP1/POT1 to telomeres is strongly abrogated (O'Connor et al. 2006, Houghtaling et al. 2004, Ye et al. 2004b, Liu et al. 2004b, Takai et al. 2011). This results in the activation of ATR in response to the exposed 3' overhang of telomeric DNA, whereas there is only minimal ATM activation since TRF2 binding is only mildly decreased in the absence of TIN2 (Takai et al. 2011).

1.2.6 RAP1 (Repressor Activator Protein 1)

The *RAP1* gene is found at 16q23.1. It was first identified in budding yeast as a positive regulator of gene transcription and later found to be a double strand telomere binding protein essential for telomere length regulation, telomere position effect, gene silencing and telomere end protection (Huet et al. 1985, Shore 1994). RAP1 is the only mammalian shelterin component that is also found in budding and fission yeast (Chen et al. 2011). In budding yeast RAP1 contains two Myb domains which are used for DNA binding, however the mammalian RAP1 has a single Myb domain and hence cannot bind telomeric DNA, causing RAP1 to rely on its interaction with TRF2 for recruitment to telomeres (Li, Oestreich & de Lange 2000). The Myb domain in human RAP1 is used for protein-protein interactions. RAP1 also has an N-terminal BRCT domain, which like the Myb domain, is implicated in protein-protein interactions, and both domains affect telomere length heterogeneity (Li, de Lange 2003). The RAP1 C-terminal (RCT) domain interacts with the linker region of TRF2 (Takai et al. 2010, Hanaoka et al. 2001). Previous reports have implicated a role for mammalian RAP1 in telomere length regulation and the protection of the chromosome ends against non-homologous end joining (NHEJ) (Li, de Lange 2003, Sarthy et al. 2009, Bae, Baumann 2007, O'Connor et al. 2004, Sfeir et al. 2010). However, in the mouse knockout there was no activation of ATM or its downstream targets, indicating TRF2 alone was sufficient to repress a damage response and NHEJ. On the other hand, the authors observed a novel role for RAP1 in inhibiting HR (Sfeir et al. 2010). The authors observed an increase in telomere sister chromatid exchange (T-SCE) in RAP1 null MEFs (Sfeir et al. 2010). An increase in T-

SCE would explain the previous findings that RAP1 affects telomere length since uneven crossover events would produce heterogeneous telomere length (Li, de Lange 2003, O'Connor et al. 2004, Sfeir et al. 2010).

In addition to its telomere function, RAP1 also plays a role in NF-kB signalling and gene regulation (Martinez et al. 2010, Teo et al. 2010). RAP1 was identified in a gain of function screen looking for positive regulators of the NF-kB pathway (Teo et al. 2010). Mouse knockouts for RAP1 have early onset obesity due to changes in gene regulation (Martinez et al. 2013, Yeung et al. 2013). The loss of RAP1 leads to a deficiency in PGC1α and PPARα, two genes involved in nutrient homeostasis and mitochondrial function (Martinez et al. 2013). Data from ChIP-sequencing identified $(TTAGGG)_2$ as the highest ranking motif for non telomere localization of RAP1, suggesting that the recruitment of RAP1 to these sites may be through its interacting partner, TRF2 (Martinez et al. 2010). However, using a separation of function allele that could not bind TRF2, it was found that the expression of 87% of the deregulated genes identified were rescued (Yeung et al. 2013). How RAP1 is recruited to these sites is yet to be determined.

1.2.7 POT1 (Protection of Telomeres 1)

The POT1 gene is located at 7q31.33. POT1 is important for both telomere length and telomere end protection (Denchi, de Lange 2007, Kibe et al. 2010, Wu et al. 2006, Loayza, De Lange 2003). It contains two highly conserved

oligonucleotide/oligosaccharide-binding (OB) folds which are used to bind with high affinity to single strand telomeric DNA (Palm, de Lange 2008). Mice have two POT1 genes, POT1a and POT1b, which have distinct roles in telomere maintenance. POT1a prevents the activation of the ATR kinase signalling pathway. In the absence of POT1a the deprotected 3' G-rich overhang resembles a single strand break, which is then coated by another single strand DNA binding protein, RPA as part of the upstream signalling events in ATR activation (Figure 1.2) (Denchi, de Lange 2007, Kibe et al. 2010). POT1b regulates the length of single strand telomeric DNA at the chromosome end and does so in a telomerase independent manner (Hockemeyer et al. 2006). Both of these functions are played by a single human POT1 protein (Palm et al. 2009).

POT1 was recently identified as the second most frequently mutated gene (3.5% of cases) in a study of chronic lymphocytic leukemia (CLL) (Ramsay et al. 2013). Analysis by clonal and subclonal studies indicates that POT1 is mutated during the early stages of cancer and may be a driver mutation in CLL. POT1 is the first shelterin component identified to be associated with cancer development (Chang 2013). Strikingly, 9 of the 12 mutations identified occurred in the OB folds and prevented *in vitro* binding to single stranded telomeric DNA (Ramsay et al. 2013). These findings support the hypothesis that telomere dysfunction can cause genome instability that leads to cancer progression.

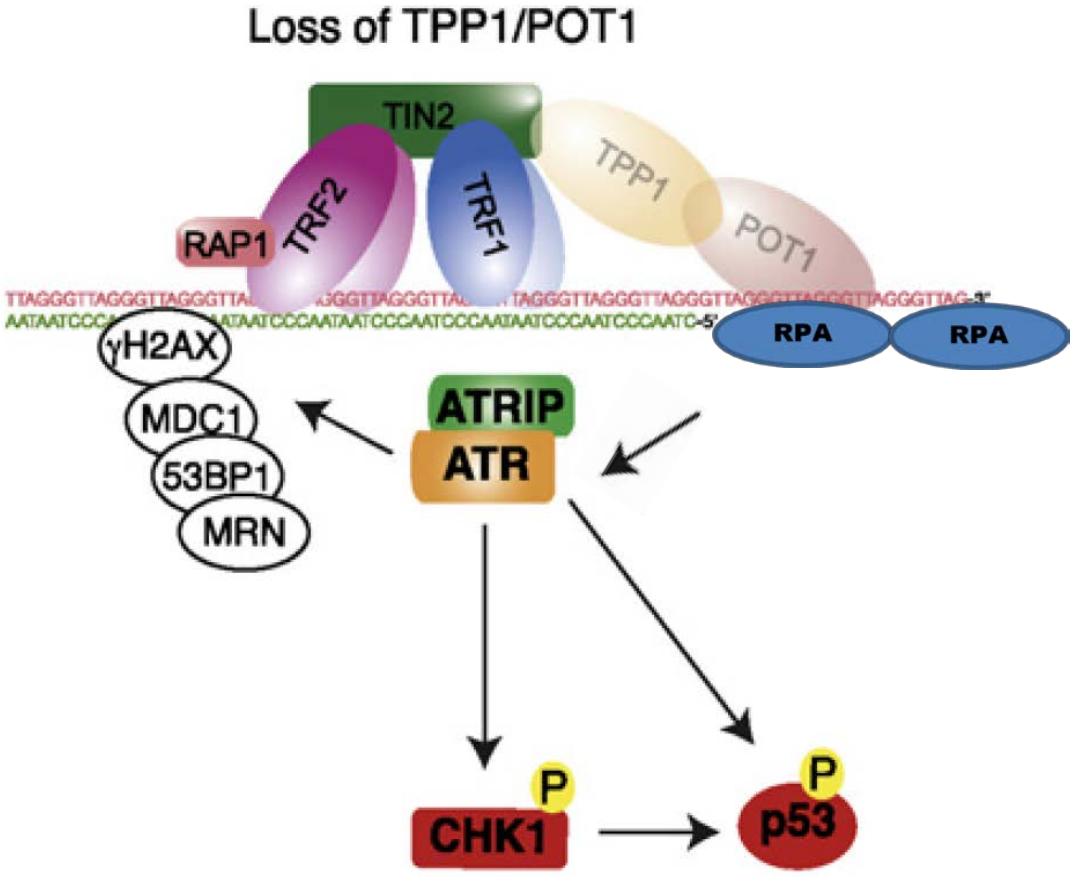

Figure 1.2. POT1 represses ATR signalling. POT1 binds to single strand telomere DNA to exclude RPA from binding to the 3' overhang. When POT1 is deleted RPA is allowed to bind and activate the kinase ATR, which initiates signalling pathways involved in senescence/apoptosis and DNA repair (MRN, 53bp1, MDC1, γH2AX) Modified from (Denchi 2009).

1.2.8 TPP1 (TINT1-PTOP-PIP1)

TPP1 is another essential component of telomere protection. The gene is located at 16q22.1 and is also referred to as adrenocortical dysplasia homolog (*ACD)*. A conditional knockout of TPP1 in MEFs causes the complete loss of the POT1 proteins while having no effect on the other shelterin components (Kibe et al. 2010). Deletion of TPP1 causes the exposure of the 3' G rich overhang which becomes increased in length, and triggers a robust ATR dependent DNA damage response (Kibe et al. 2010, Guo et al. 2007, Deng et al. 2009). The deletion of TPP1 also resulted in a low frequency of chromosome-type fusions, sister telomere fusions and an endoreduplication phenotype (Kibe et al. 2010). The chromosome fusions caused by the loss of TPP1 are through the Ku86 independent alternative non-homologous end joining pathway (Rai et al. 2010). These phenotypes are recapitulations of the loss of the POT1 proteins, which suggests one of the major functions of TPP1 is the recruitment of POT1 to telomeres (Palm et al. 2009, Wu et al. 2006).

Besides its role in telomere protection TPP1 has a role in regulating telomerase dependent telomere elongation (Xin et al. 2007). TPP1 was thought to recruit telomerase to telomeres and stimulate telomerase processivity (Xin et al. 2007, Wang et al. 2007, Zaug et al. 2010). However, this role for TPP1 *in vivo* was difficult to study since genetic manipulation also caused the loss of POT1 and resulted in telomere deprotection and instability (Hockemeyer et al. 2007, Kibe et al. 2010). Validation came with the creation of a TPP1 separation of function mutant that maintained telomere protection, but was

deficient in telomerase recruitment and processivity (Nandakumar et al. 2012, Zhong et al. 2012). The region of TPP1 that was found to interact with telomerase was termed the TPP1 glutamate (E) and leucine (L)-rich (TEL) patch and it provides a new target for cancer therapeutics that prevent telomerase dependent telomere extension (Nandakumar et al. 2012).

1.2.9 Telomeric Repeat Containing RNA (TERRA)

Telomere chromatin is enriched for histone 3 lysine 9 trimethylation (H3K9me3) and histone 4 lysine 20 trimethylation (H4K20me3) which are associated with a heterochromatic state (Schoeftner, Blasco 2009, Garcia-Cao et al. 2004). It was surprising then, when it was discovered that telomeres are transcribed into heterogeneous long non-coding telomeric repeat-containing RNA (TERRA) (Azzalin et al. 2007, Schoeftner, Blasco 2008). Although both the C-rich and G-rich strands of telomere RNA have been detected *in vivo* the 5'-UUAGGG-3' RNA molecule is substantially more abundant and biologically relevant (Azzalin et al. 2007, Schoeftner, Blasco 2008). TERRA transcription usually originates from within the subtelomeric region and produces TERRA transcripts that range in length from approximately 0.1 kb to 9 kb (Azzalin et al. 2007, Schoeftner, Blasco 2008).

The function of TERRA is poorly understood, however it does seem to be important for telomere maintenance. When TERRA expression was inhibited using an siRNA approach it resulted in telomere free ends (TFE), telomere doublets (TD), and

telomere double minute (TDM) chromosomes, which are recapitulations of phenotypes commonly seen by the disruption of the shelterin complex (Deng et al. 2009). TERRA specifically localizes to telomeres and interacts directly with the Myb-like domain of TRF1 and TRF2, as well as the basic domain of TRF2 (Deng et al. 2009). These interactions are likely important for the recruitment of TERRA to telomeres since the overexpression of TRF2 lacking its basic domain prevents the accumulation of TERRA at telomeres (Deng et al. 2009). TERRA transcription is largely inhibited by the treatment of cells with α-amanitin, an inhibitor specific to RNA polymerase II (RNAPII) (Schoeftner, Blasco 2008). RNAPII and TRF1 interact *in vivo* which may provide communication between shelterin and the transcription complex (Schoeftner, Blasco 2008). Shelterin likely does regulate TERRA transcription, since when TRF1 is knocked down it results in a decrease in TERRA levels whereas the loss of TRF2 causes an increase in TERRA levels (Schoeftner, Blasco 2008, Caslini et al. 2009). TERRA enhances the interaction between TRF2 and ORC1, which also interacts with the basic domain of TRF2 (Deng et al. 2009, Deng et al. 2007). This interaction is important for H3K9me3 and heterochromatin protein 1 (HP1) association with telomeres, which creates a stable heterochromatic state (Deng et al. 2009).

Studies done *in vitro* and *in vivo* have hinted at TERRA being an inhibitor of telomerase. *In vitro* studies indicate that TERRA can directly inhibit telomerase by competing with the chromosome end for binding to the telomerase RNA template, whereas *in vivo* studies in yeast suggest that a stable RNA/DNA hybrid formed between

TERRA and telomeric DNA inhibits the access of telomerase to the chromosome end (Schoeftner, Blasco 2008, Luke et al. 2008). The removal of TERRA from the chromosome end by the 5'-3' exonuclease RAT1p is required for proper telomere length regulation (Luke et al. 2008). In the absence of RAT1p the TERRA levels are significantly increased and telomerase is inhibited, which results in telomere shortening (Luke et al. 2008). However, in humans the overexpression of TERRA does not prevent telomerase mediated telomere extension (Farnung et al. 2012). The explanation for this involves an elegant model of heterogeneous ribonucleoprotein A1 (hnRNPA1) switching between binding to TERRA and the telomere end. hnRNPA1 can bind both telomeric single strand DNA (ssDNA) and TERRA (Ding et al. 1999, Flynn et al. 2011). hnRNPA1 orchestrates a switch in the ssDNA binding of replication protein A (RPA) during replication to the ssDNA binding telomere complex POT1/TPP1, which is required for telomere protection. This switch requires the binding of hnRNPA1 to TERRA, which allows POT1/TPP1 to outcompete RPA for telomeric DNA while having the added bonus of preventing TERRA from being able to inhibit telomerase (Redon, Zemp & Lingner 2013).

A number of cancer cell lines have misregulated TERRA levels (Ng et al. 2009). Further insight into the relationship between TERRA and telomerase is of interest for its potential in the development of anti-cancer therapeutics. TERRA levels are particularly high in telomerase negative cancer cells that use a recombination mechanism to maintain

telomere length (Ng et al. 2009). However, whether high TERRA levels is required for the alternative lengthening of telomeres (ALT) mechanism has yet to be established.

1.3 TRF2 and its interacting proteins

TRF2 interacts with numerous proteins involved in the DNA damage response and DNA repair. These TRF2 interacting proteins are collectively known as telomere associated factors and they also play an important role in telomere maintenance. The majority of TRF2 interacting proteins are enzymes that can be either stimulated or inhibited by TRF2. How some TRF2 interacting proteins contribute to telomere maintenance is discussed below.

TRF2 associates with numerous proteins important for telomere maintenance. Many of these proteins contain a conserved Y(X)L(X)P motif that interacts with F120 of the TRFH domain (Chen et al. 2008, Kim et al. 2009). Some verified examples are Apollo, MCPH1 and PNUTS (Palm, de Lange 2008, Kim et al. 2009). Apollo was identified as a TRF2 interacting protein by mass spectrometry, and independently by another group through a yeast two-hybrid assay (van Overbeek, de Lange 2006, Lenain et al. 2006). Apollo is a 5'-3' exonuclease that also functions in interstrand crosslink repair (Bae et al. 2008). Its recruitment to telomeres occurs specifically during the S phase of the cell cycle, at which point it helps to protect telomeres against being recognized as DNA damage by promoting the reformation of a 3' overhang on the leading strand of DNA synthesis (van Overbeek, de Lange 2006, Lam et al. 2010, Wu et al. 2010). It also

plays an essential role in relieving topological stress that builds up during replication (Ye et al. 2010). The recruitment of Apollo to telomeres is entirely dependent on TRF2 (Chen et al. 2008). In the absence of Apollo telomeres resemble fragile sites due to the stalling and collapse of the replication fork (Ye et al. 2010). This can be visualized by fluorescence *in situ* hybridization as a telomere doublet phenotype, the occurrence of more than one telomere signal at a single chromosome end (Sfeir et al. 2009, van Overbeek, de Lange 2006, Ye et al. 2010).

Both MCPH1 and PNUTS function in the DNA damage response (Landsverk et al. 2010, Liang et al. 2010). The function of MCPH1 and PNUTS at telomeres is poorly understood; however the disruption of an interaction between TRF2 and MCPH1 or TRF2 and PNUTS results in a defect in the DNA damage response to dysfunctional telomeres, or a defect in telomere length regulation, respectively (Kim et al. 2009). When MCPH1 is knocked down it prevents a proper DNA damage response at telomeres to the overexpression of a TPP1 allele that is deficient in telomere maintenance (Kim et al. 2009). The authors showed that the exogenous expression of MCPH1 could rescue this defect, however an MCPH1 allele with its leucine and proline from its Y(X)L(X)P motif mutated to alanines was unable to rescue the defect in the DNA damage response at telomeres. When a non-functional form of PNUTS lacking its C-terminal region was overexpressed it resulted in telomere elongation in telomerase positive cancer cells, but there was no defect in telomere protection (Kim et al. 2009).

An important function of TRF2 is its role in chromosome end protection. A single unrepaired DNA double strand break (DSB) can be detrimental to cell survival (Bohgaki, Bohgaki & Hakem 2010). For this reason, the cell has evolved mechanisms to recognize and repair breaks in an efficient and timely manner. The two main responses to DSBs are non-homologous end joining (NHEJ) and homologous recombination (HR). In mammals NHEJ is the predominant pathway. Repair is initiated by Ku70/80 binding to each end of a break, which is then ligated in a ligase IV dependent and often deleterious manner (Brandsma, Gent 2012). On the other hand, HR is expected to be error free, however it requires a perfect template and therefore can only occur during S phase or G2/M when the chromosomes have duplicated but cell division has yet to occur (Brandsma, Gent 2012). TRF2 has been proposed to protect against Ku dependent NHEJ by directly interacting with helix 5 of Ku70. This region of Ku70 is important for its role in NHEJ and when bound to the dimerization domain of TRF2 it is left non-functional (Ribes-Zamora et al. 2013). Although TRF2 prevents Ku from performing its regular role in DSB repair, Ku proteins are also important for proper telomere maintenance. The loss of Ku80 is lethal in humans due to massive telomere loss (Fink et al. 2010, Hu et al. 2013). Ku70 functions in a pathway parallel to TRF2 to prevent T-SCE, an HR dependent process (Celli, Denchi & de Lange 2006).

When a DNA break occurs it is recognized by the sensing complex MRE11-RAD50-NBS1 (MRN) (Williams, Williams & Tainer 2007). The sensing of a DNA break activates the phosphatidylinositol 3-kinase related protein ataxia telangiectasia-

mutated (ATM) (Lisby et al. 2004, Falck, Coates & Jackson 2005). ATM then phosphorylates a complex network of proteins that halts cell cycle progression, initiates DNA repair and potentially promotes cellular senescence or apoptosis (Shiloh, Ziv 2013). Numerous proteins can be visualized by immunofluorescence at sites of DNA damage in an ATM dependent manner. Some examples are phosphorylated serine 139 H2AX (γH2AX), MDC1, 53BP1 and MRN (Shiloh, Ziv 2013). The main role of TRF2 is to protect the natural chromosome ends against being recognized as aberrant DNA DSBs (van Steensel, Smogorzewska & de Lange 1998). When functionally lost by the expression of a dominant negative allele lacking its Myb-like domain and basic domain, TRF2 is stripped from telomeric DNA, leading to the activation of an ATM dependent DNA damage response (Figure 1.3) (van Steensel, Smogorzewska & de Lange 1998, Karlseder et al. 1999). The ensuing colocalization of repair factors and telomeres are known as telomere dysfunction induced foci (TIF) (Takai, Smogorzewska & de Lange 2003, d'Adda di Fagagna et al. 2003). In the absence of TRF2 the exposed 3' overhang is lost due to nucleolytic attack, resulting in the formation of telomere-telomere fusions in a manner largely dependent on the endonuclease complex XPF/ERCC1, KU70/80, and DNA ligase IV (Celli, Denchi & de Lange 2006, Smogorzewska et al. 2002, Zhu et al. 2003). This indicates that the end protection provided by TRF2 is through the inhibition of classical NHEJ pathway (Denchi, de Lange 2007, Celli, Denchi & de Lange 2006). TRF2 prevents this from happening by promoting the t-loop formation which hides the chromosome end away from nucleases (Griffith et al. 1999, Stansel, de Lange & Griffith 2001). TRF2 also inhibits the DNA damage response in a two step mechanism by first

inhibiting ATM directly, and then by preventing the spread of a DNA damage response by severing the ubiquitylation signalling events that are essential to a properly functioning repair pathway (Okamoto et al. 2013, Denchi, de Lange 2007). The severing of the ubiquitylation signalling is dependent on a small motif located in the linker region called the inhibitor of DNA damage (IDD) (Okamoto et al. 2013).

Deletion of the basic domain alone results in the rapid loss of telomeric DNA due to t-loop sized deletions via the HR repair pathway (Wang, Smogorzewska & de Lange 2004). The ability of TRF2 to aid in t-loop formation, as well as its basic domain dependent affinity for DNA junctions are likely important characteristics in its ability to prevent HR at telomeres (Griffith et al. 1999, Stansel, de Lange & Griffith 2001, Amiard et al. 2007). The discovery that the DNA DSB response is inhibited by several mechanisms at telomeres reiterates the importance of preventing the chromosome ends from being recognized as DNA damage and highlights the central role TRF2 plays.

Several roles for TRF2 outside of telomere biology have also been reported. Using a reporter assay that quantifies repair efficiency by the NHEJ and HR pathways, it was uncovered that TRF2 overexpression suppresses NHEJ while increasing efficiency of HR (Mao et al. 2007). Knockdown of TRF2 had no effect on NHEJ, but inhibited HR, suggesting that TRF2 may play an active role in HR, while the effect of overexpression of TRF2 on NHEJ is a result of its intrinsic ability to prevent the activation of ATM (Mao et al. 2007). TRF2 has been visualized at sites of laser induced DNA DSBs (Bradshaw,

Stavropoulos & Meyn 2005). These results indicate that TRF2 may play a role in general DNA repair, however what that role may be is still uncertain.

TRF2 has been demonstrated to have non-telomere roles. TRF2 interacts with and stabilizes repressor element 1-silencing transcription factor (REST) in undifferentiated neuronal cells. REST binds to repressor element 1 (RE1) to negatively regulate genes involved in neuronal differentiation (Ballas et al. 2005). The authors show that TRF2 is required to prevent proteolytic degradation of REST and to maintain an undifferentiated state (Ballas et al. 2005). The authors later identified a splicing variant of TRF2 that lacked telomere binding capability and shuttled REST to the cytoplasm upon establishment of a differentiated state (Zhang et al. 2011).

1.4 Post-translational regulation of TRF2

1.4.1 A summary of TRF2 modifications

TRF2 function is regulated by a broad array of post-translational modifications including phosphorylation, ubiquitylation, SUMOylation, PARsylation and acetylation (Walker, Zhu 2012). TRF2 is phosphorylated by the DNA damage signalling kinases ATM and CHK2 (Buscemi et al. 2009, Zhou et al. 2010, Tanaka et al. 2005, Huda et al. 2009). Following ionizing radiation, T188 of TRF2 is transiently phosphorylated in an ATM dependent manner (Tanaka et al. 2005). TRF2 phosphorylated at this site does not bind telomere DNA (Tanaka et al. 2005). An active role for TRF2 in the DNA damage

response is supported by the finding that cells overexpressing a TRF2 mutant containing a T188A mutation showed decreased survival following IR and were defective in the fast pathway of DNA double strand break repair (Huda et al. 2009). However, T188 phosphorylation is dispensable for TRF2 recruitment to sites of DNA breaks (Huda et al. 2012). On the other hand, analysis of the recruitment of ectopically expressed TRF2 to laser induced DNA breaks suggests that it is dependent on the basic domain (Bradshaw, Stavropoulos & Meyn 2005).

TRF2 is also found in a complex with the checkpoint kinase CHK2, which is activated downstream of ATM in response to DNA double strand breaks (Perona et al. 2008). TRF2 interacts with the N-terminus of CHK2, which blocks the phosphorylation of T68 by ATM (Buscemi et al. 2009). Phosphorylation of T68 of CHK2 normally occurs in response to DNA damage and is required for checkpoint activation and the spread of the DNA damage response (Perona et al. 2008). The ability of TRF2 to directly inhibit the activation of CHK2 is another example of how TRF2 prevents the chromosome ends from initiating a DNA damage response. Interestingly, the induction of DNA DSBs elsewhere in the genome disrupts the CHK2/TRF2 interaction, and promotes phosphorylation of TRF2 at S20 (Buscemi et al. 2009). The phosphorylation of S20 decreases TRF2 binding to telomere DNA, which is in concurrence with the notion that TRF2 plays a role in double strand break repair away from telomeres (Mao et al. 2007, Buscemi et al. 2009).

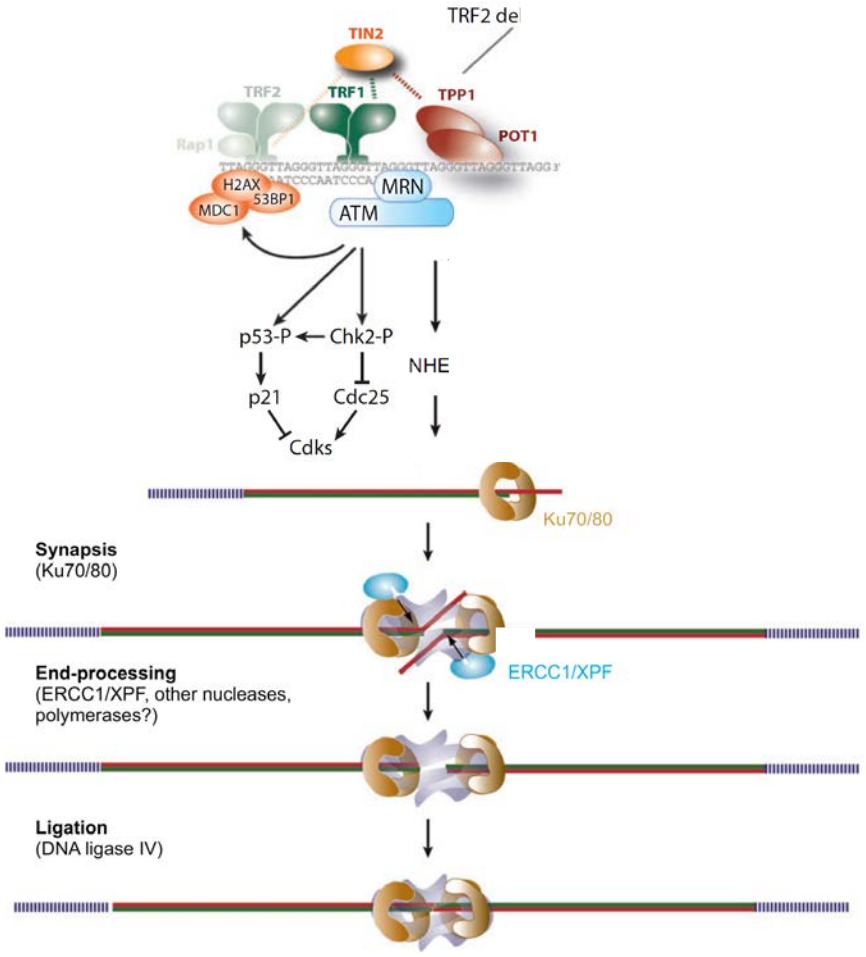

Figure 1.3. TRF2 prevents the activation of ATM. In the absence of TRF2 the MRN complex senses the chromosome ends as a DNA break. This activates the DNA damage kinase ATM which propagates signalling pathways involved in DNA repair cell cycle arrest. Downstream events from ATM include inhibiting cyclin kinases and activating p53, which in turn can trigger senescence through the transcription of p21. Simultaneously, ATM initiates the recruitment of the NHEJ repair proteins MDC1, γH2AX, 53bp1 which can be observed by immunofluorescence as telomere dysfunction induced foci (TIF). The fusion of telomere ends eventually occurs through Ku70/80 dependent and ERCC1/XPF dependent ligation of two chromosome ends. Modified from (Palm, de Lange 2008).

TRF2 was also identified as a substrate of Aurora C kinase through a yeast two-hybrid assay (Spengler 2007). Aurora C is the least well understood member of the Aurora kinases family, which also includes Aurora A and Aurora B. Aurora C has been implicated in regulating cell morphology and growth (Spengler 2007). TRF2 has a conserved Aurora B/C consensus sequence that can be phosphorylated *in vitro* on T358 by Aurora C. The physiological relevance of this phosphorylation has yet to be determined.

The level of TRF2 is regulated by a ubiquitin dependent proteasome degradation pathway in response to natural telomere shortening (Fujita et al. 2010). Cells that are reaching the end of their replicative capacity have shortened telomeres that are no longer able to prevent their recognition as DNA damage (d'Adda di Fagagna et al. 2003). This results in the phosphorylation of p53 by ATM, which promotes transcriptional activation of p53 regulated genes (d'Adda di Fagagna et al. 2003). One such gene is SIAH1, an E3 ligase that ubiquitylates TRF2 on K173, K180 and K184 (Fujita et al. 2010). The ubiquitylation and degradation of TRF2 further promotes telomere instability by uncapping the chromosome ends, which ensures that these cells will continue into, and maintain, a senescent state. SIAH1 dependent degradation of TRF2 explains the decreased level of TRF2 in senescent cells in the absence of a change in the level of TRF2 mRNA (Fujita et al. 2010). When SIAH1 was knocked down, the TRF2 level was stabilized and senescence was delayed (Fujita et al. 2010). This feedback loop between

TRF2 and p53 minimizes the risk that a cell carrying DNA damage and dysfunctional telomeres will escape senescence and lead to cancer (Fujita et al. 2010).

SUMOylation is a post-translational modification related to ubiquitylation. Unlike ubiquitylation which commonly targets its substrates for degradation, SUMOylation usually affects protein function through regulating cellular localization, protein-protein interaction or protein-DNA interaction (Gronholm et al. 2012, Dou et al. 2011, Muller et al. 2001). TRF2 is SUMOylated, along with TRF1, TIN2 and RAP1, by MMS21 (Potts, Yu 2007). SUMOylation of TRF1 and TRF2 is important for the alternative lengthening of telomeres (ALT) mechanism, which is a homologous recombination-based mechanism used by some cancer cells to regulate their telomere length (Bryan et al. 1995, Dunham et al. 2000). SUMOylated TRF2 is important for the formation of ALT associated PML bodies (APB), which is where HR dependent telomere lengthening is thought to occur (Potts, Yu 2007, Yeager et al. 1999).

TRF2 is acetylated on K293 by the histone acetyltransferase p300 (Her, Chung 2013). p300 is a transcriptional co-activator that interacts with basal transcription machinery and numerous transcription factors (Kalkhoven 2004). Histone acetylation by p300 has been shown to be associated with transcription activation (Kalkhoven 2004). p300 interacts with the basic domain of TRF2 (Her, Chung 2013). A band the size of TRF2 is detectable by immunoblot with an acetyl-lysine antibody in a TRF2 immunoprecipitation from cells overexpressing p300; however no band can be seen in cells overexpressing a catalytically dead p300 mutant (Her, Chung 2013). Acetylation of

TRF2 leads to an increase in TRF2 levels by blocking its ubiquitin dependent proteolysis (Her, Chung 2013). Mutating K293 to an arginine resulted in telomere deprotection and a cell proliferation defect (Her, Chung 2013).

Additionally, the Myb-like DNA binding domain of TRF2 is a target of poly(ADP-ribose)polymerase 1 (PARP1) and PARP 2; two proteins well characterized for their roles in base excision repair. PARsylation of TRF2 negatively regulates its binding to telomeres in response to DNA damage (Gomez et al. 2006, Dantzer et al. 2004). It has been proposed that the PARsylation of TRF2 relaxes the damaged telomere chromatin to facilitate DNA repair (Gomez et al. 2006).

Aside from the aforementioned modifications, I have shown that TRF2 undergoes arginine methylation. The role of arginine methylation in the cell is summarized below in prelude to our findings.

1.4.2 Arginine methylation

Arginine is a commonly found amino acid in protein, DNA and RNA interaction motifs (Thandapani et al. 2013). Arginine has a guanidino group that is protonated under physiological conditions, which also endows arginine with several unique characteristics. The guanidino group contains five hydrogen donor sites that can be used to make stable interactions with other biological molecules. The positive charge of the guanidino group also promotes stabilizing amino-aromatic interactions that are highly favourable for

protein-DNA and protein-RNA interactions, and the guanidino group can be modified by the addition of methyl groups (Thandapani et al. 2013, Yang, Bedford 2013, Boffa et al. 1977).

The human genome contains nine protein arginine methyltransferases (PRMTs), which are classified into three subtypes depending on the methylation pattern they produce. The methyl-arginine product can be ω-*NG*,*NG*-asymmetric dimethylarginine (ADMA), produced by type I enzymes (PRMT1 1-4, PRMT6, PRMT8), or ω-*NG*,*N'G*-symmetric dimethylarginine (SDMA) which are produced by the type II enzyme, PRMT5 and PRMT9 (Figure 1.4) (Yang, Bedford 2013). Both type I and type II enzymes can produce ω-*NG*-monomethylarginine (MMA), however the type III enzyme, PRMT7, solely monomethylates its substrates (Yang, Bedford 2013, Zurita-Lopez et al. 2012). All of these enzymes use S-adenosylmethionine (SAM) as their methyl donor (Di Lorenzo, Bedford 2011). The addition of a methyl group to arginine removes a potential hydrogen bond donor and changes its orientation. This adds bulkiness and hydrophobicity without neutralizing the charge or completely destroying the function of a domain (Yang, Bedford 2013). For example, a single motif in SAM68 can interact with both SH3 and WW domains. Arginine methylation of the motif inhibits its binding to SH3 domains, while having no effect on its interaction with WW domains (Bedford et al. 2000a, Bedford et al. 2000b).

Arginine methylation has been implicated in DNA repair, transcription regulation, RNA processing, signal transduction, protein trafficking and protein compartmentalization (Pahlich et al. 2009, Dery et al. 2008, Passos, Quaresma & Kobarg

2006, Wysocka, Allis & Coonrod 2006, Boisvert et al. 2005b). Most PRMTs prefer to methylate arginines in RGG/RG clusters referred to as glycine/arginine rich (GAR) domains (Thandapani et al. 2013, Boisvert, Chenard & Richard 2005). Gene ontology annotation indicates that the most enriched function for GAR motifs is in RNA binding (Thandapani et al. 2013). Methylation of GAR domains can increase RNA-protein binding by increasing hydrophobicity to facilitate stacking with RNA bases or alternatively it can decrease an interaction by removing hydrogen bond donor sites and creating steric hindrance (Blackwell, Ceman 2012). Arginine methylation has also been shown to regulate protein-protein and protein-DNA interactions both positively and negatively (Thandapani et al. 2013).

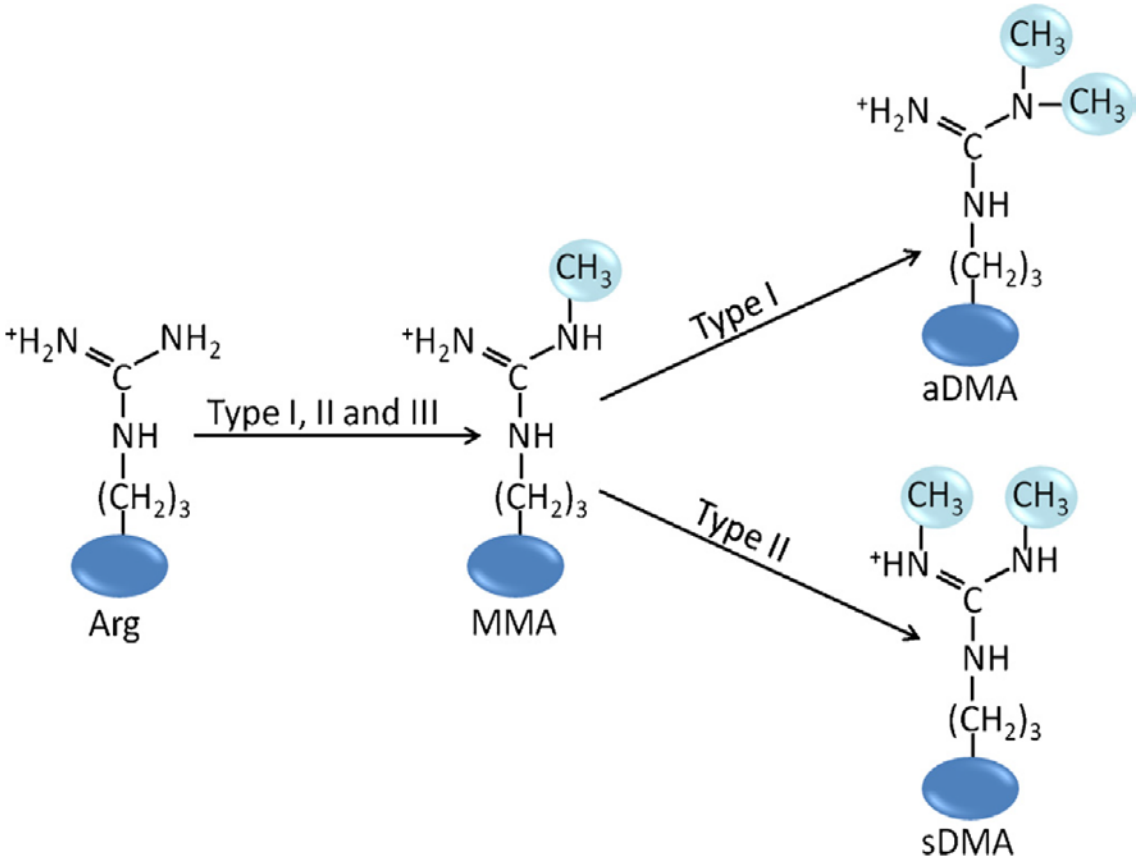

Fig. 1.4. Types of protein arginine methylation. Arginine can be monomethylated on the guanidino nitrogen by all three types of PRMTs. Type I enzymes (PRMT1-4, PRMT6 and PRMT8) can then asymmetrically dimethylate the arginine by catalyzing the addition of a second methyl group to the same guanidino nitrogen, whereas type II enzymes (PRMT5, PRMT9) catalyze symmetrical dimethylation by the addition of a methyl group to the opposite nitrogen. Type III enzymes (PRMT7) only produce monomethylated arginines. Reproduced from (Auclair, Richard 2013) with permission from Elsevier (license number: 3225340322939).

1.4.3 Protein Arginine Methyltransferase 1 (PRMT1)

PRMT1 was first identified in a two hybrid assay as a binding partner of TIS21 and BTG1, and was characterized as an arginine methyltransferase based on its homology to methyltransferases in lower eukaryotes (Lin et al. 1996, Scott et al. 1998). PRMT1 is a highly abundant and ubiquitously expressed protein found in both the cytoplasm and the nucleus (Lin et al. 1996, Tang, Kao & Herschman 2000). At least seven PRMT1 variants exist which differ in their N-terminal hydrophobic regions (Scorilas et al. 2000). Variants 4-7 are expressed in a tissue specific manner in the heart, pancreas and muscle cells, whereas variants 1-3 are found in all tissues (Goulet et al. 2007). The variant type also determines its cellular localization with variants 1 and 7 being predominantly nuclear, variant 2 being cytosolic and variants 3-6 being both cytosolic and nuclear (Goulet et al. 2007). The relative prevalence of the transcript variants are changed between cancer and normal cells. An increase in PRMT1v2 has been shown to be associated with increased invasiveness and breast cancer cell survival (Scorilas et al. 2000, Baldwin et al. 2012). Structural studies show that homodimerization of PRMT1 is required for binding to SAM and hence enzymatic activity, however a recent study has also indicated that the heterodimerization of PRMT1 with PRMT2 can also increase its enzymatic activity (Pak et al. 2011, Zhang, Cheng 2003).

Of all the arginine methylation that occurs in the cell, 85% is done by PRMT1 (Tang, Kao & Herschman 2000). As such, over 45 substrates of PRMT1 have been characterized in a variety of cellular functions. PRMT1 is the predominant arginine

methyltransferase in embryonic stem cells and PRMT1 null mice die at E6.5 (Pawlak et al. 2000, Yu et al. 2009). A few characteristics of PRMT1 null cells are spontaneous DNA damage, loss of checkpoint function, chromosome instability, aneuploidy and DNA repair deficiency (Yu et al. 2009). The importance of PRMT1 to genome stability is not surprising given that the list of substrates of PRMT1 includes the DSB repair factors MRE11, 53BP1 and BRCA1 (Boisvert et al. 2005a, Boisvert et al. 2005c, Guendel et al. 2010, Adams et al. 2005).

One particularly important target of PRMT1 is histone 4 arginine 3 (H4R3) which is associated with transcriptional activation by priming H4 for acetylation by p300 (Strahl et al. 2001, Wang et al. 2001). PRMT1 has been studied with expectations that it may be a useful therapeutic target in cancer. PRMT1 is commonly upregulated or alternatively spliced in a number of different types of cancer including prostate cancer, lung cancer, breast cancer, colon cancer, bladder cancer, gliomas and leukemia (Goulet et al. 2007, Baldwin et al. 2012, Wang et al. 2012, Yoshimatsu et al. 2011, Papadokostopoulou et al. 2009, Mathioudaki et al. 2008, Zou et al. 2012, Mathioudaki et al. 2011). An increase in H4R3 dimethylation is an indicator of poor prognosis and cancer reoccurrence (Seligson et al. 2005).

1.4.4 PRMT1 and the DNA damage response

MRE11 forms a complex with RAD50 and NBS1 called the MRN complex, which functions in DNA damage repair and telomere maintenance (Zhu et al. 2000,

Mirzoeva, Petrini 2003). MRE11 was the first DNA repair protein shown to contain methylated arginines (Boisvert et al. 2005a). MRE11 is methylated by PRMT1 on multiple arginines within a GAR motif, which is important for its exonuclease activity (Boisvert et al. 2005a). The compartmentalization of methylated MRE11 is unique compared to total MRE11 (Boisvert et al. 2005b). Methylated MRE11 is more tightly associated with the nuclear matrix and methylase inhibitors prevented MRE11 shuttling from PML bodies to sites of DNA damage (Boisvert et al. 2005b). The importance of methylation of the GAR motif was further dissected using a mouse knock-in allele of MRE11 ($MRE11^{RK/RK}$) that switched the known arginine methylation sites for lysines. These mice were hypersensitive to ionizing radiation (IR), had checkpoint defects, and chromosome abnormalities (Yu et al. 2012). Interestingly, the cells had no defect in ATM activation; however they were defective in ATR signalling (Auclair, Richard 2013). The defect in ATR activation was likely due to the exonuclease defect of $MRE11^{RK/RK}$ which would have prevented the formation of single strand DNA that is required for ATR activation (Auclair, Richard 2013, Shiotani, Zou 2009).

Downstream of ATM in DSB repair are the two tumor suppressor proteins, 53BP1 and BRCA1, which are involved in the choice between repair by NHEJ or HR (Chapman et al. 2013, Noon, Goodarzi 2011, Escribano-Diaz et al. 2013). 53BP1 promotes NHEJ through an effector molecule RIF1 by blocking HR promoting end resection by the BRCA1/CtIP complex (Noon, Goodarzi 2011, Escribano-Diaz et al. 2013, Zimmermann et al. 2013). Interestingly, both 53BP1 and BRCA1 are methylated on multiple arginines

by PRMT1 (Boisvert et al. 2005c, Guendel et al. 2010, Adams et al. 2005). The importance of arginine methylation of 53BP1 is poorly understood. However, methylation of BRCA1 alters its recruitment to a number of promoters where it acts as a transcriptional regulator (Guendel et al. 2010). It is unclear whether the effect of BRCA1 methylation on recruitment is due to changes in DNA binding efficiency or protein-protein interaction, however BRCA1 interacting protein STAT1 was shown to preferentially interact with hypermethylated BRCA1 (Guendel et al. 2010).

These findings suggest an important role for arginine methylation in DSB repair. PRMT1 was also identified in a large scale proteomics study for proteins associated with telomeric DNA (Dejardin, Kingston 2009). Given the close relationship between DNA DSB repair and telomere end protection it would be of interest to know whether arginine methylation plays a role in telomere maintenance.

1.4.5 Methyl-arginine binding domains

Arginine methylation has been shown to regulate numerous protein-protein interactions both positively and negatively (Thandapani et al. 2013, Bedford et al. 2000a, Friesen et al. 2001, Cote et al. 2003). Tudor domains are currently the only methylarginine binding domain that has been well characterized (Liu et al. 2010, Tripsianes et al. 2011). Tudor domains are divided into two groups; methylarginine binding domains and methyllysine binding domains (Chen et al. 2011). The methyl groups provide increased hydrophobicity within an aromatic cage of the Tudor domain

(Liu et al. 2010, Tripsianes et al. 2011). Each Tudor domain interacts with a single methylarginine, however many proteins have multiple tudor domains which could each interact with a methylarginine present in a GAR domain (Liu et al. 2010, Tripsianes et al. 2011, Hosokawa et al. 2007). The BRCT domain of BRCA1 was shown to specifically interact with methylated transcriptional coactivator p300 in response to DNA damage, which is required for its recruitment to the p21 promoter (Lee, Bedford & Stallcup 2011). Whether this is unique to this particular interaction or whether BRCT domains represent a novel methyl-binding domain has yet to be determined. Similarly, the PHD fingers of V(D)J recombination activating protein 2 (RAG2) and DNA methyltransferase 3A (DNMT3A) have been shown to possess methylarginine binding activity suggesting more methylarginine binding domains are likely to be discovered (Matthews et al. 2007, Zhao et al. 2009).

1.5 Telomeres and disease

1.5.1 A summary of diseases with a telomere defect

Telomere maintenance is essential for genome stability and therefore overall organismal health. Shorter telomeres correlate with an increased risk of age related diseases such as arteriosclerosis, cirrhosis, immunosenescence and bone marrow failure (Gunes, Rudolph 2013, Blasco et al. 1997, Armanios 2013). Whether telomere shortening is simply correlated with or causative of age related disease has been a long standing question. Recently, reactivation of telomerase in telomerase deficient mice was

shown to reverse the degenerative signs of aging seen in multiple tissues, including the brain, spleen, testes and intestine (Jaskelioff et al. 2011). This finding suggests that telomere dysfunction can be a driving force for age related disease, and highlights the potential for telomerase dependent regenerative medicine in the prevention of age related diseases (Jaskelioff et al. 2011).

Premature aging disorders have long been used as a model for studying the natural aging process. It should be noted that no premature aging disorder completely recapitulates all the phenotypes that can be associated with normal aging. For this reason these syndromes are more correctly referred to as segmental premature aging disorders, meaning that only a subset of tissues age at an accelerated rate (Hasty, Vijg 2004). It has been argued that these syndromes do not mimic the aging process and that extrapolating data about natural aging should be done with caution. However, the counterargument to this is that no single individual demonstrates all the phenotypes of natural aging, and therefore aging itself is a segmental process, likely depending on genetic variation within a population (Hasty, Vijg 2004). Similar to the variability in age related diseases displayed within the elders of a family, so too are the phenotypes varied between individuals of a family with a genetically inherited premature aging disorder (Hasty, Vijg 2004). However, an increased rate of telomere shortening and defects in telomere maintenance have been reported in numerous premature aging disorders, and have been suggested to be the root cause of the manifestation of some of the phenotypes of these disorders.

The first premature aging disorder shown to be primarily caused by a telomere defect was dyskeratosis congenita (DC) (Mitchell, Wood & Collins 1999). The first DC gene to be identified was DKC1, which codes for dyskerin, a protein important for proper telomerase assembly (Mitchell, Wood & Collins 1999, Knight et al. 1999). Since then, numerous mutations in both the telomerase reverse transcriptase (TERT) and the telomerase RNA component (TERC), as well as proteins involved in telomerase regulation (NOP10, NHP2, TCAB1, TIN2) have been shown to lead to DC (Savage et al. 2008, Walne et al. 2008, Dokal 2011, Vulliamy et al. 2004, Vulliamy et al. 2005, Walne et al. 2007, Vulliamy et al. 2008, Zhong et al. 2011).

Werner syndrome (WS) is one of the most well studied premature aging disorders. It is an autosomal recessive disorder caused by a mutation in the *WRN* gene, which codes for a DNA helicase involved in DNA repair, replication and transcription (Yu et al. 1996, Li et al. 2009, Huang et al. 2000, Rossi, Ghosh & Bohr 2010). WS manifests with the early onset of many phenotypes typically seen in the elderly, such as osteoporosis, atherosclerosis, cataracts, type II diabetes and a predisposition to cancer (Kong, Lee & Wang 2013, Li et al. 2009, Goto et al. 1996). WRN interacts with POT1, TRF1 and TRF2, and these interactions in turn regulate the ability of WRN to unwind telomeric D-loops and G4 structures (Opresko et al. 2002, Opresko et al. 2005b, Opresko et al. 2004, Li et al. 2001). The loss of WRN causes an increase in replication stress, which results in the deletion of large segments of telomeric DNA in WS cells (Crabbe et al. 2004, Damerla et al. 2012). Interestingly, the Wrn-null mouse model does not phenocopy the

human syndrome (Lombard et al. 2000). Mice have very long telomeres in excess of 50 Kb and it is only in late generation mice lacking both WRN and telomerase RNA that the telomere shortening and typical characteristics of WS are recapitulated (Chang et al. 2004, Du et al. 2004). These findings suggest that telomere defects play a central role in the manifestation of WS phenotypes.

The integrity of the nucleus is dependent on a group of proteins called lamins. Lamins are nuclear intermediate filaments which affect virtually all aspects of DNA function, including its organization, replication, transcription and repair (Hozak et al. 1993, Prokocimer, Barkan & Gruenbaum 2013, Misteli 2007, Gonzalez-Suarez et al. 2009). There are four main lamin proteins expressed in humans: lamin B1, lamin B2, lamin A and lamin C. Lamin B1 and B2 are expressed from the *LMNB1* and *LMNB2* genes, respectively, whereas lamin A and C are splice variants of the *LMNA* gene (Lin, Worman 1995, Lin, Worman 1993, Butin-Israeli et al. 2012). The lamins collectively make a meshwork of proteins, called the lamina, and are located on the nuclear side of the inner nuclear membrane (Dwyer, Blobel 1976). The lamins are also found throughout the nuclear volume, creating a fibrous network analogous to the cytoskeleton (Simon, Wilson 2011). Disease causing mutations in lamin B1 and B2 are extremely rare, however approximately 300 disease causing mutations have been mapped in the *LMNA* gene (Butin-Israeli et al. 2012). These diseases are collectively called laminopathies, with one of the most severe being Hutchinson-Gilford progeria syndrome (HGPS). More than 90% of HGPS patients have a *de novo* C1842T autosomal dominant mutation in the

LMNA gene. This is a silent Gly608 mutation, however it creates a cryptic splice site resulting in the expression of a 50 amino acid truncated lamin A protein termed progerin (Scaffidi, Misteli 2006). Progerin becomes stably farnesylated, unlike wildtype lamin A which during its maturation has its farnesyl group cleaved (Butin-Israeli et al. 2012). The expression of progerin disrupts the previously mentioned aspects of DNA metabolism. HGPS is a severe segmental premature aging disorder that is characterized by osteoporosis, amyotrophy, lipodystrophy, skin atrophy and results in death at the mean age 13.5, most commonly from myocardial infarction (Navarro, Cau & Levy 2006, Hennekam 2006). At the cellular level, the nuclei of HGPS cells are characteristically blebbed/lobulated, aneuploid, and the cells undergo premature senescence (Butin-Israeli et al. 2012, Taimen et al. 2009).

Exogenous telomerase expression has been shown to rescue the premature senescence phenotype of HGPS cells, suggesting that a defect in telomere maintenance may contribute to the disease (Benson, Lee & Aaronson 2010, Kudlow et al. 2008). Indeed, an accelerated rate of telomere shortening has been observed in HGPS (Decker et al. 2009). Additionally, $LMNA^{-/-}$ mice have accelerated telomere shortening and decreased TERRA expression. The decreased TERRA expression has been attributed to an increase in the heterochromatin mark H3K9me3 at telomeres (Gonzalez-Suarez et al. 2009). In normal nuclei the telomeres are evenly distributed throughout the nuclear volume, whereas the telomeres of HGPS cells become clustered and are mislocalized to the center of the nucleus (Uhlirova et al. 2010). The mislocalization of telomeres can be

reproduced by the exogenous expression of progerin, which in addition causes TIF formation (Cao et al. 2011, Benson, Lee & Aaronson 2010, Taimen et al. 2009). Similarly, the telomeres of normal senescent cells become centrally localized in the nucleus and preferentially associate with intranuclear lamina structures (Raz et al. 2008). Analysis of live cell imaging with citrine-TRF1, GFP-TRF1 and citrine-TRF2 has revealed that telomere localization of the shelterin proteins is affected by changes in the nuclear matrix in HGPS cells, which is in agreement with previous reports that telomeres are tethered to the nuclear matrix (Raz et al. 2008, Uhlirova et al. 2010, Luderus et al. 1996, de Lange 1992). Progerin expression has also been implicated in normal aging (Scaffidi, Misteli 2006). These findings are supportive of the idea that HGPS can be used as a model for natural human aging.

How telomeres are tethered to the nuclear matrix is poorly understood, however a link between several shelterin components and the nuclear lamina or nuclear matrix has been observed. TIN2 may ensure the proper localization of telomeres in the nucleus. A long isoform of TIN2 was found to localize to telomeres, but associated preferentially with the nuclear matrix (Kaminker et al. 2009). During postmitotic nuclear assembly, the TRF2 interacting factor RAP1 was shown to bind the nuclear envelope protein SUN1 (Crabbe et al. 2012). However, depletion of RAP1 did not prevent the relocalization of telomeres to the nuclear periphery following mitosis, suggesting that either this interaction is not important for this process or that other redundant pathways exist (Crabbe et al. 2012). TRF2 was reported to elute as part of the nuclear matrix fraction

from mouse cells (Voronin et al. 2003). Recently, it was observed that TRF2 expression is decreased in atypical Werner syndrome which is caused by a mutation in the *LMNA* gene rather than *WRN* (Saha et al. 2013, Bonne, Levy 2003, Vigouroux et al. 2003, Chen et al. 2003). The decreased level of TRF2 in atypical Werner syndrome is partially rescued by treatment with the proteasome inhibitor MG132 (Crabbe et al. 2012). Further studies elucidating the role of TRF2 in HGPS would be important and may provide further insight into its role in normal aging.

Xeroderma pigmentosum (XP) is an autosomal recessive skin disorder characterized by hypersensitivity to UV light, premature aging, hyperpigmentation of the skin and an increased risk of skin cancer (Kraemer, Lee & Scotto 1987, Cleaver, Lam & Revet 2009). Individuals with XP begin to display symptoms early in life coinciding with their first exposure to sunlight, which results in freckling, severe burning and blistering (Cleaver, Lam & Revet 2009). The underlying cause is due to mutations in genes involved in the nucleotide excision repair (NER) pathway. NER is the major mechanism responsible for the removal of cyclobutane pyrimidine dimers and 6-4-photoproducts caused by exposure to UV light. NER can be divided into two major repair pathways; global genome repair (GGR), which is responsible for removing damage in non-transcribing DNA, and transcription-coupled NER (TCR), which removes damage from actively transcribing genes (Freitas, de Magalhaes 2011).

XP has garnered interest in the telomere field after the discovery that the NER protein complex XPF/ERCC1 is also important for telomere maintenance. XPF/ERCC1

is required for telomere fusions and telomere shortening caused by the disruption of, or the overexpression of TRF2, respectively (Zhu et al. 2003, Wu et al. 2007, Wu, Mitchell & Zhu 2008, Munoz et al. 2005). ERCC1$^{-/-}$ mice show increased telomere dysfunction in the absence of external stress, suggesting that ERCC1 is important for normal telomere function (Zhu et al. 2003). Transgenic mice overexpressing TRF2 in keratinocytes under the Keratin 5 promoter (K5-TRF2) have increased chromosome instability, skin hyperpigmentation, skin dryness and increased rates of spontaneous skin cancer, which are all hallmarks of XP (Martinez, Blasco 2010, Munoz et al. 2005). In addition, K5-TRF2 mice have accelerated telomere shortening (Munoz et al. 2005). Excessive telomere shortening in the K5-TRF2 mice is genetically linked to XPF since a K5-TRF2/XPF$^{-/-}$ double mutant was found to block the increased chromosome instability and accelerated telomere shortening phenotypes (Munoz et al. 2005). Additionally, TRF2 is upregulated in a number of cancers, including skin cancer, implicating it as a potential oncogene (Munoz et al. 2005, Matsutani et al. 2001, Oh et al. 2005). These studies identify a link between TRF2, NER and XP syndrome, and highlight the importance of proper telomere maintenance for genome stability.

1.5.2 Cockayne syndrome

The two NER sub-pathways use two distinct modes of recognizing damaged bases, which can allow for defects in one sub-pathway, but not the other. In GGR the repair process is initiated by XPC, whereas in TCR it is initiated by CSB and CSA.

Cockayne syndrome (CS) is an autosomal recessive premature aging syndrome caused by defects in the TCR pathway, and due most commonly to mutations in CSB or CSA. Like XP syndrome, CS patients are hypersensitive to UV light; however there is no increased risk of skin cancer, which is thought to be due to the retention of a fully functional GGR pathway and an increased rate of apoptosis in damaged cells (Diderich, Alanazi & Hoeijmakers 2011). CS patients are also characterized by neurodegeneration, cachetic dwarfism, delayed motor development, and retinal degeneration (Diderich, Alanazi & Hoeijmakers 2011). Approximately 80% of CS cases are due to compound mutations in the *CSB* gene (also referred to as *ERCC6*). CSB is a 1493 amino acid protein that belongs to the SNF2/SWI2 family of ATPases (Matson, Bean & George 1994, Flaus et al. 2006). The SNF2/SWI2 classification is based on its internal seven helicase-like motif ATPase domain stretching from amino acid 510-960, however CSB has not been shown to have any helicase activity (Troelstra et al. 1992, Selby, Sancar 1997). The ATPase domain is flanked by an NLS on each side and roughly divides CSB into three regions; the ATPase domain, the C-terminal region and the N-terminal region (Figure 1.5). Within the N-terminal region there is a short acidic domain and a glycine rich domain of unknown function (Troelstra et al. 1992). The C-terminal domain contains a ubiquitin binding domain (UBD) required for TCR and a nucleotide binding domain that has yet to be assigned a function (Anindya et al. 2010, Kristensen et al. 2013). A CSB mutant lacking the UBD is still capable of recruiting downstream NER factors; however excision of the lesion fails to occur (Anindya et al. 2010). The ubiquitylated protein(s) that CSB binds has yet to be elucidated (Anindya et al. 2010).

Figure 1.5 Domain structure of the CSB protein.

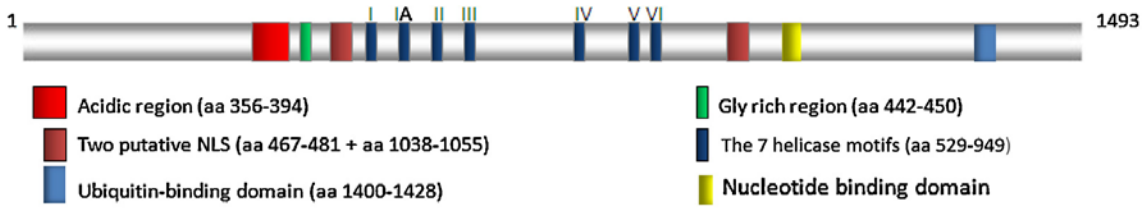

Figure 1.5 Domain structure of the CSB protein. CSB contains two putative nuclear localization signals (NLS) that flank a central seven helicase-like motif ATPase domain. CSB also contains an acidic region, glycine rich region, nucleotide binding domain and an ubiquitin binding domain. Reproduced from (Aamann et al. 2013) with permission from Elsevier (license: 3290470391159).

Numerous mutations in CSB have been uncovered in CS patients (Figure 1.6). The majority of missense mutations leading to CS are in the ATPase domain and have been shown to abolish DNA-stimulated ATP hydrolysis (Lake, Fan 2013, Lake et al. 2010). It is yet to be determined how the two missense mutations occurring in the C-terminal domain, P1042L and P1095R, disrupt CSB function. As to date, no disease causing missense mutations have been uncovered in the N-terminal domain; however numerous frameshift and nonsense mutations occur in both the N-terminal and C-terminal regions demonstrating that these regions are not less prone to mutagenesis (Lake, Fan 2013).

CS patients carrying mutations in *CSB/ERCC6* are also defective in base excision repair (BER) (Maynard et al. 2009). BER is the main pathway that repairs DNA bases damaged by reactive oxygen species (ROS) (Maynard et al. 2009). It has been demonstrated that 8-hydroxyguanine (8-oxoG), a common base lesion, is not repaired

efficiently in CS fibroblasts (Dianov et al. 1999). As such, CS fibroblasts have been shown to accumulate higher basal levels of oxidative lesions (Tuo et al. 2003). The role of CSB in BER is not yet understood, however it has been shown to be in an ATPase independent manner (Selzer et al. 2002). CSB has also been shown to directly or genetically interact with several important BER proteins, such as OGG1, NEIL1, APE1 and PARP1 (Muftuoglu et al. 2009, Tuo et al. 2002, Wong et al. 2007, Thorslund et al. 2005). Mammalian telomeres are innately sensitive to oxidative stress due to the abundance of guanines residue (Wang et al. 2010, Ames, Gold 1991). Oxidative lesions

Missense mutations			R670W N680D W686C S687L	W851R L871P V957G L987P	P1042L P1095R
Deletion mutations			ΔL860 ΔF665-Q723 ΔV724-Q792 ΔM752-Q762		Δ1240-1260
Stop codon or frame shift mutations	R68fs R77fs R77X Q156X E182fs Q184X E218fs W236X	E325fs K337X K345fs E379X V417fs S429fs P453X	P500fs P506fs W517X R637fs R652X T659fs R683X G715X Q723X	R735X D749fs W834fs Q854X R857X M867fs A944fs R947X Q956fs	R1087X K1172X Y1179fs K1198fs K1203fs K1239fs H1263fs R1288X D1355fs

Figure 1.6. A summary of mutations in CSB that are associated with Cockayne syndrome. Reproduced from (Lake, Fan 2013) with permission from Elsevier (License: 3290470144206).

in telomeric DNA inhibit the binding of TRF1 and TRF2 (Opresko et al. 2005a). OGG1 is the major DNA glycosylase that removes 8-oxoG from the genome. An increase in oxidative damage in OGG1$^{-/-}$ mice causes an increase in single and double strand breaks at telomeres, and results in a loss of the G-strand overhang (Wang et al. 2010). The loss of APE1 or PARP1 also causes telomere length defects (Gomez et al. 2006, Madlener et al. 2013, Beneke et al. 2008).

Telomeres are also hypersensitive to chronic UV radiation, which causes the accumulation of pyrimidine dimers in telomeric DNA at a faster rate than in other regions of the genome (Rochette, Brash 2010). Surprisingly, the accumulation of damage is due to the suppression of NER, suggesting that the cell prefers to tolerate the damage rather than to allow the access of repair proteins (Rochette, Brash 2010). These studies suggest that regulating both BER and NER pathways at telomeres is important for genome stability. Whether telomere dysfunction is an important contributing factor to CS, a disease characterized by defects in BER and NER, has not yet been studied.

1.6 Rationale and objectives

Telomeres shorten as we age. This shortening is viewed as a contributing factor to aging and cellular senescence; a theory which is supported by the observation that there is accelerated telomere shortening in many premature aging disorders. As reviewed in this chapter, TRF2 is a central component required for proper telomere maintenance.

TRF2 works in consortship with numerous telomere associated proteins to provide telomere length regulation and telomere protection. Therefore, TRF2 interacting proteins play essential roles in telomere maintenance. This general hypothesis formed the basis for my approach to understanding the roles of two novel TRF2 interacting proteins I have identified.

TRF2 has been shown to be regulated by numerous types of post-translational modifications. Sequence analysis of TRF2 suggests that the N-terminal basic domain is similar to the GAR motif which is commonly methylated by PRMT1. In Chapter 2, data presented demonstrates that TRF2 and PRMT1 interact *in vivo* and that the arginines of the basic domain are substrates of PRMT1. To determine the physiological relevance of TRF2 methylation, telomere stability was studied under conditions that would prevent *in vivo* arginine methylation of the basic domain. The main approach taken was to deplete PRMT1 through short hairpin RNA interference and then analyze the resulting telomere phenotypes.

Chapter 2 also introduces an antibody raised to specifically recognize dimethylated arginine 17 of TRF2. This antibody serves to validate our finding that TRF2 is a substrate of PRMT1 *in vivo*. In Chapter 3 this antibody is used to identify unique characteristics of methylated TRF2 that provide novel links between TRF2 and cellular aging.

Chapter 4 presents data on the novel interaction between TRF2 and CSB. Based on our hypothesis that TRF2 interacting proteins are important for telomere maintenance, we chose to investigate whether CSB is also important for telomere maintenance. Our

approach was to study telomere stability in CS cells that are defective in the known functions of CSB, as well as to study cells that were artificially knocked down for endogenous CSB.

Telomere health plays an important role in determining the replicative capacity of a cell, and changes in telomere dynamics are often associated with cellular aging and senescence. TRF2 and many of its interacting proteins are essential to telomere protection. Gaining further knowledge about TRF2 and its interacting proteins will provide valuable information that has real translational potential for improving how we age, as well as providing novel therapeutic targets in diseases with telomere defects. This thesis sheds further light on the mechanism of TRF2 dependent telomere maintenance.

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Chapter 2

Arginine Methylation Regulates Telomere Length and Stability

2.1 Preface

Shelterin requires the help of many additional cellular proteins in order to properly maintain telomere homeostasis (Palm, de Lange 2008). TRF2 is viewed as a protein hub that recruits many of these additional factors (Kim et al. 2009, Chen et al. 2008). TRF2 is subject to a number of different types of post-translational modification, such as phosphorylation, SUMOylation, ubiquitylation and PARsylation (Walker, Zhu 2012). When the ability of TRF2 to be modified is disrupted, either through mutation or the loss of the modifying enzyme, the result is often telomere dysfunction which can be seen through telomere length defects or the loss of telomere protection (Undarmaa et al. 2004, Walker, Zhu 2012).

The results presented here identify PRMT1 as a novel TRF2 interacting protein. We show that PRMT1 methylates arginines in the basic/GAR domain of TRF2 and that this methylation is important for proper telomere maintenance. This is the first time that arginine methylation has been reported to play a role in telomere maintenance.

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2.2 Publication

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Arginine Methylation Regulates Telomere Length and Stability[▽]

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2.2.1 Abstract

TRF2, a component of the shelterin complex, functions to protect telomeres. TRF2 contains an N-terminal basic domain rich in glycines and arginines, similar to the GAR motif that is methylated by protein arginine methyltransferases. However, whether arginine methylation regulates TRF2 function has not been determined. Here we report that amino acid substitutions of arginines with lysines in the basic domain of TRF2 induce telomere dysfunction-induced focus formation, leading to induction of cellular senescence. We have demonstrated that cells overexpressing TRF2 lysine mutants accumulate telomere doublets, indicative of telomere instability. We uncovered that TRF2 interacts with PRMT1, and its arginines in the basic domain undergo PRMT1-mediated methylation both *in vitro* and *in vivo*. We have shown that loss of PRMT1 induces growth arrest in normal human cells but has no effect on cell proliferation in cancer cells, suggesting that PRMT1 may control cell proliferation in a cell type-specific manner. We found that depletion of PRMT1 in normal human cells results in accumulation of telomere doublets, indistinguishable from overexpression of TRF2 lysine mutants. PRMT1 knockdown in cancer cells upregulates TRF2 association with telomeres, promoting telomere shortening. Taken together, these results suggest that PRMT1 may control telomere length and stability in part through TRF2 methylation.

2.2.2 Introduction

The integrity of telomeres is vital to cell survival and proliferation. Mammalian telomeric DNA is coated with a telomere specific complex, referred to as shelterin (18, 41). Shelterin, consisting of TRF1, TRF2, TIN2, RAP1, TPP1, and POT1, functions to control telomere length and stability (18, 41). Disruption or depletion of the shelterin complex and its interacting proteins has been shown to induce a variety of telomere abnormalities, such as telomere loss, telomere end-to-end fusions, telomere-containing double-minute chromosomes, and telomere doublets (more than one telomeric signal at a single chromatid end) (14, 45, 55, 56, 58, 66). Telomeres containing these abnormalities have been shown to be associated with DNA damage response factors, such as 53BP1, forming nuclear structures that are referred to as telomere dysfunction-induced foci (TIFs) (15, 30, 50, 55, 58).

TRF2, a component of the shelterin complex, binds to telomeric DNA as a dimer and has been shown to play a crucial role in telomere length maintenance and telomere protection. TRF2 contains an N-terminal basic domain rich in glycines and arginines, a central TRFH dimerization domain, and a C-terminal Myb DNA-binding domain (56). Overexpression of TRF2 has been shown to induce telomere shortening (2, 26, 37, 48). Loss of TRF2 from telomeres either through overexpression of a TRF2 dominant-negative allele ($TRF2^{\Delta B\Delta M}$) or depletion of TRF2 leads to an accumulation of telomere end-to-end fusions, resulting in ATM- and p53-dependent growth arrest or apoptosis depending upon the cell type (10, 25, 50, 56). Inhibition of TRF2 function at telomeres through overexpression of TRF2 lacking the basic domain has been shown to promote

DNA recombination at telomeres, leading to the induction of telomere loss (58). TRF2 has been shown to interact with many proteins involved in DNA repair, recombination, and replication, including Apollo (20, 30, 55), WRN (39), FEN1 (36), and ORC (3). While loss of WRN or inhibition of FEN1 results in telomere loss (14, 45), depletion of Apollo induces telomere doublets (55). Although DNA recombination is thought to play an important role in the formation of telomere loss, the mechanism underlying the formation of telomere doublets remains elusive. Furthermore, it has not been determined whether TRF2 inhibition may induce telomere doublets.

Protein arginine methyltransferases (PRMTs) represent a family of enzymes that utilize *S*-adenosyl methionine as a methyl donor and catalyze the direct transfer of the methyl group to one or two of the guanidino nitrogen atoms of arginine (5, 34). In mammalian cells, 11 PRMTs have been identified, and the majority of them are able to catalyze not only the formation of a monomethylated arginine intermediate but also the production of a dimethylated arginine (4, 40). Based on their substrate specificity, mammalian PRMTs can be classified into type I or type II enzymes. Type I enzymes, including PRMT1 (32), PRMT3 (53), CARM1 (11), PRMT6 (19), and PRMT8 (29), catalyze arginine dimethylation asymmetrically. On the other hand, type II enzymes, including PRMT5 (44), PRMT7 (21, 35), and PRMT9 (12), catalyze arginine dimethylation symmetrically.

PRMT1, the predominant mammalian type I enzyme (52, 54), accounts for more than 85% of all arginine methylation reactions in human cells (54). PRMT1 methylates a diverse range of proteins involved in transcription (1, 57), RNA processing (13, 24, 47),

and DNA damage repair (8, 9). Recently PRMT1 has also been shown to be associated with human telomeres (17). Most of its substrates contain a characteristic motif rich in glycines and arginines, referred to as the GAR motif (6, 7, 38). TRF2 contains an N-terminal basic domain rich in glycines and arginines, similar to the GAR motif. However, whether TRF2 is a substrate of PRMT1 has not been determined.

In this report, we show that the basic domain of TRF2 undergoes arginine methylation both *in vitro* and *in vivo*. We show that PRMT1 interacts with TRF2 and is the main enzyme responsible for methylating the basic domain of TRF2 both *in vivo* and *in vitro*. Overexpression of TRF2 carrying amino acid changes of arginines to lysines in the basic domain results in the formation of telomere doublets in hTERT-BJ cells, suggesting that arginines in the basic domain of TRF2 are essential for maintaining telomere stability. Knockdown of PRMT1 induces growth arrest in normal human cells but has no effect on cell proliferation in cancer cells, suggesting that PRMT1 may control cell growth in a cell type-dependent manner. We find that depletion of PRMT1 affects both telomere length and stability. While a loss of PRMT1 in cancer cells promotes telomere shortening, PRMT1 knockdown in normal human cells leads to an accumulation of telomere doublets, resembling the phenotype observed with overexpression of TRF2 mutants carrying amino acid changes of arginines to lysines. In addition, we show that depletion of PRMT1 mitigates TRF2 methylation but upregulates TRF2 association with telomeric DNA, suggesting that TRF2 methylation by PRMT1 may control its association with telomeric DNA. We propose that PRMT1 regulates telomere length and stability in part through TRF2 methylation.

2.2.3 Materials and Methods

DNA constructs. The constructs expressing wild-type TRF2 (pLPC-TRF2) and TRF2 lacking the basic domain (pLPC-TRF2$^{\Delta B}$) were generously provided by Titia de Lange, Rockefeller University. All TRF2 mutants except for TRF2- RK5-8 were first made in the pKS vector (Stratagene), followed by subcloning into the retroviral pLPC vector. Annealed oligonucleotides encoding the first 18 amino acids of TRF2 (TRF2$^{1–18}$), which either is wild type or contains amino acid substitutions of arginines to lysines at positions 13, 17, and 18, were ligated to BamHI- and HindIII-linearized pKS vector, generating an intermediate construct of pKS-TRF2$^{1–18}$ or pKS-TRF2$^{1–18}$-RK1-3, respectively. TRF2$^{19–500}$-RK4, which lacks the first 18 amino acids and contains an amino acid change of arginine to lysine at position 21, was obtained by PCR using a forward primer containing the mutation. Ligation of TRF219–500-RK4 into HindIII- and XhoI linearized pKS-TRF2$^{1–18}$-RK1-3 gave rise to pKS-TRF2-RK1-4, which contains substitutions of arginines with lysines at positions 13, 17, 18, and 21. TRF2$^{19–500}$- RK4-8 was made by PCR using a forward primer encoding TRF2 carrying amino acid changes of arginines to lysines at positions 21, 25, 27, 28, and 30. Subsequent ligation of TRF2$^{19–500}$-RK4-8 to HindIII- and XhoI-linearized pKS-TRF2$^{1–18}$- RK1-3 gave rise to pKS-TRF2-RK, which contains substitutions of arginines with lysines at positions 13, 17, 18, 21, 25, 27, 28, and 30. pKS-TRF2-RA was cloned in a similar manner except that oligonucleotides encoding amino acid changes of arginines to alanines were used. pLPC-TRF2-RK5-8 was made by PCR from the pLPC-TRF2 expression construct using a forward primer encoding TRF2 carrying amino acid changes of arginines to lysines at

positions 25, 27, 28, and 30. The presence of all TRF2 mutations was verified by DNA sequencing.

Wild-type TRF2 and various TRF2 mutants were also subcloned into the bacterial expression vector pGST-Parallel-2 (46) (a gift from Murray Junop, McMaster University).

The oligonucleotides encoding small interfering RNAs directed against TRF2, PRMT1, PRMT5, and PRMT6 have been described previously (49, 51, 62, 63). The annealed oligonucleotides were ligated into the pRetroSuper vector (kindly provided by Titia de Lange, Rockefeller University), giving rise to pRetroSuper-shPRMT1, pRetroSuper-shPRMT5, and pRetroSuper-shPRMT6.

Cell culture and retroviral infection. Cells were grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum for HT1080, Phoenix, 293T, and SV40-transformed skin fibroblast GM637 (Coriell) cells supplemented with nonessential amino acids, L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Supplemented Dulbecco's modified Eagle medium plus 15% fetal bovine serum was used to culture normal primary fibroblasts (IMR90, MRC5, and GM08399) (Coriell), Nbs1-deficient primary fibroblasts (GM07166) (Coriell), and hTERT-immortalized BJ (hTERT-BJ) cells (a kind gift from Titia de Lange, Rockefeller University). Retroviral gene delivery was carried out essentially as described previously (26). Phoenix amphotropic retroviral packaging cells were transfected with the desired DNA constructs. At 36, 48, 60, 72, and 84 h post-transfection, the virus-containing medium was collected and used to infect cells

in the presence of polybrene (4 µg/ml). Twelve hours after the last infection, puromycin (2 µg/ml) was added to the medium, and the cells were maintained in the selection medium for the entirety of the experiments.

Production of PRMT1 and TRF2 proteins. PRMT1, wild-type TRF2, and TRF2 mutants were expressed as glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli* strain BL21(DE3)pLysS. The expression construct for the GST-PRMT1 fusion protein was generously provided by Stéphane Richard, McGill University (Montreal, Canada). Expression of GST-PRMT1 was induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C, whereas expression of GST-TRF2 fusion proteins was induced with 0.3 mM isopropyl-1-thio-β-D-galactopyranoside overnight at room temperature. Following a wash with phosphate-buffered saline (PBS), the cell pellet for GST-PRMT1 was resuspended in PBS containing 0.5% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride and lysed by sonication. For GST-TRF2 proteins, the cell pellet was resuspended in buffer containing 50 mM potassium phosphate buffer (pH 7.0), 5 mM EDTA, 100 mM NaCl, and 1% Triton X-100 and then lysed by sonication. The lysate was subjected to centrifugation, and the supernatant was incubated with a 50% slurry of glutathione-Sepharose 4B (GE Life Sciences) for 4 h at 4 °C. For GST-PRMT1, bound proteins were eluted with 40 mM reduced glutathione in buffer containing 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 10 mM dithiothreitol and stored in aliquots at -80°C. For GST-TRF2 proteins, TEV protease was added to the mixture to release TRF2

proteins from GST. While GST remained bound to the beads, free TRF2 proteins were recovered in the supernatant.

***In vitro* methylation assays.** Five to seven micrograms of the recombinant TRF2 protein was incubated with 5 µg purified GST-PRMT1 in a 30-µl reaction mixture containing 1 µCi of *S*-adenosyl-L-[*methyl*-3H]methionine (Perkin Elmer) and 25 mM Tris-HCl (pH 7.5) for 2 h at 37°C. Following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, the gel was stained with Coomassie blue, destained with 20% (vol/vol) methanol and 8% (vol/vol) acetic acid, and treated with EN3HANCE solution (Perkin Elmer) according to the manufacturer. The gel was then dried and exposed to Kodak BioMax X-ray film at -80°C.

Identification of methylation sites in the basic domain of TRF2. Endogenous TRF2 was immunoprecipitated from whole-cell extracts of approximately 1.7 X 10^9 HeLaI.2.11 cells as described previously (65). The beads were washed five times in cold buffer D (20 mM HEPES-KOH, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). Mass spectrometry analysis of TRF2 was done through service provided by WEMB Biochem. Inc., Toronto, Canada, as described previously (64). Briefly, TRF2 bound to beads was digested with both trypsin and chymotrypsin overnight at room temperature. The sample was then acidified to pH 2, and the solution was treated with C_{18} ZipTip pipette tips prior to liquid chromatography tandem mass spectrometry analysis with an LCQ Deca XP

spectrometer (Thermo Finnegan). The data were analyzed using the Xcalibur software program, and peptide sequence data were rechecked manually.

Mass spectrometry analysis of *in vitro*-methylated TRF2 was done through the MALDI Mass Spectrometry Facility, University of Western Ontario, London, Ontario, Canada. Following *in vitro* methylation assays, recombinant wild-type TRF2 was separated by SDS-polyacrylamide gel electrophoresis, visualized with Coomassie blue, and excised. In-gel digestion was performed with trypsin using a MassPREP automated digester station (Perkin Elmer). Mass spectrometry data were obtained using a 4700 proteomics analyzer matrix-assisted laser desorption ionization time-of-flight TOF/TOF system (Applied Biosystems). Data acquisition and data processing, respectively, were done using the 4000 Series Explorer (Applied Biosystems) and Data Explorer (Applied Biosystems) software packages.

Immunoprecipitation and immunoblotting. Immunoprecipitation was performed with 750 µg of HeLa nuclear extract and non-immune serum, anti-TRF2 antibody, or anti-PRMT1 antibody as described previously (65). Immunoblotting was carried out with whole-cell extracts as described previously (65). Extracts were fractionated by 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose that was then immunoblotted. Rabbit anti-2meR17 antibody (no. 5510) was developed by Biosynthesis Inc. against a TRF2 peptide containing asymmetrically dimethylated arginine 17 (AAG-RMe2-RASRSSGRARRGRH). Antibodies to TRF1, TRF2, and TIN2 were kind gifts from Titia de Lange, Rockefeller University. Antibodies

to PRMT1 and Sam68 were generously provided by Stéphane Richard, McGill University. Antibodies to PRMT1 and PRMT5 were from Upstate. Anti-PRMT6 was from Bethyl Laboratory, and anti-γ-tubulin (GTU88) was from Sigma.

ChIP. Chromatin immunoprecipitation (ChIP) assays were carried out essentially as described previously (33, 59, 60). Cells were fixed with 1% (wt/vol) formaldehyde in PBS, followed by sonication (10 cycles of 20 s each, 50% duty, and output of 5). Cell lysate of 200 µl (equivalent to 2 X 10^6 cells) was used in each ChIP. Fifty microliters of cell lysate (equivalent to one-quarter of the amount used for immunoprecipitation) was used for quantifying the total telomeric DNA and processed along with the immunoprecipitation samples at the step of reversing the cross-links. One-fifth of the immunoprecipitated DNA was loaded on the dot blots, whereas 5% of total DNA was loaded in the input lane. The signals on the dot blots were quantified by ImageQuant analysis (GE Healthcare). The ratio of the signal from each ChIP to the signal from the input lane was multiplied by 5% (representing 5% of total DNA) and a factor of 5 (since only one-fifth of the precipitated DNA was loaded for each ChIP) to give the percentage of total telomeric DNA recovered from each ChIP.

Immunofluorescence. Immunofluorescence was performed essentially as described previously (50, 65). Cells were fixed at room temperature (RT) for 10 min in 3% paraformaldehyde and 2% sucrose, followed by permeablization at RT for 10 min in Triton X-100 buffer (0.5% Triton X-100, 20 mM HEPES-KOH, pH 7.9, 50 mM NaCl, 3

mM $MgCl_2$, 300 mM sucrose). Fixed cells were blocked with 0.5% bovine serum albumin (Sigma) and 0.2% gelatin (Sigma) in PBS and then incubated at RT for 2 h with both rabbit anti-TRF1 (a kind gift from Titia de Lange) and mouse anti-53BP1 (BD Biosciences). Following a wash in PBS, cells were incubated with both fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit and tetramethylrhodamine isocyanate-conjugated donkey anti-mouse antibodies (1:100 dilution; Jackson Laboratories) at RT for 1 h. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) (0.2 µg/ml). Cell images were then recorded on a Zeiss Axioplan 2 microscope with a Hammamatsu C4742-95 camera and processed using the Openlab software package.

Metaphase chromosome spreads and fluorescence *in situ* hybridization (FISH). Metaphase chromosome spreads were essentially prepared as described previously (56, 66). hTERT-BJ cells (≈80%) were arrested in colcemid (0.1 µg/ml) for 120 to 180 min. Following arrest, cells were harvested by trypsinization, incubated for 7 min at 37 °C in 75 mM KCl, and fixed in freshly made methanol-glacial acidic acid (3:1). Cells were stored overnight at 4 °C, dropped onto wet slides, and air dried overnight in a chemical hood.

FISH was carried out essentially as described previously (28, 66). Slides with chromosome spreads were incubated with 0.5 µg/ml FITC-conjugated- $(CCCTAA)_3$ PNA probe (Biosynthesis Inc.) for 2 h at room temperature. Following incubation, slides were washed, counterstained with 0.2 µg/ml DAPI, and embedded in 90% glycerol–10% PBS containing 1 mg/ml *p*-phenylene diamine (Sigma).

In-gel G-overhang assay and telomere blots. In-gel G-overhang assay was performed essentially as described previously (23, 66). Genomic DNA isolated from cells was digested with RsaI and HinfI and loaded onto a 0.7% agarose gel in 0.5 X Tris-borate-EDTA (TBE). Following electrophoresis, gels were dried down at 50 °C and prehybridized at 50 °C for 1 h in Church mix (0.5 M Na_2PO_4 [pH 7.2], 1 mM EDTA, 7% SDS, and 1% bovine serum albumin). Hybridization was done overnight at 50 °C with an end-labeled $(CCCTTA)_4$ oligonucleotide. After hybridization, gels were washed and exposed overnight to PhosphorImager screens. For telomere blots, gels were alkali denatured (0.5 M NaOH and 1.5 M NaCl), neutralized (3 M NaCl and 0.5 M Tris-HCl [pH 7.0]), rinsed with dH_2O, and hybridized with the end-labeled $(CCCTTA)_4$ oligonucleotide as described previously (23, 66). The average length of telomeric restriction fragments was determined by PhosphorImager analysis using the ImageQuant and Microsoft Excel software programs as described previously (31, 61).

For pulsed-field gel electrophoresis, genomic DNA isolated from hTERT-BJ cells expressing various TRF2 proteins was digested with RsaI and HinfI. Plugs containing the digested DNA were equilibrated with 0.5 X TBE and loaded on a 1% agarose gel in 0.5 X TBE. Gels were run for 20 h at 5.4 V/cm at a constant pulse time of 5 s using a CHEF DR-II pulsed-field apparatus (Bio-Rad). Following electrophoresis, the gel was processed as described above.

Growth curve assay and other assays. The growth curve assay and other assays were conducted essentially as described previously (59). For HT1080 cells expressing the

vector, wild-type TRF2, or TRF2 mutants, 2.5 X 10^5 cells per 10-cm plate were seeded in duplicate on the third day of selection in puromycin. Cells were counted every 2 days using a Coulter counter (Beckman). Cells expressing wild-type TRF2 or the vector alone were split 1:8 and 1:16 on the fifth day and the seventh day after seeding, respectively, whereas cells expressing TRF2 mutants were split 1:4 once on the fifth day after seeding. For hTERT-BJ cells expressing the vector, wild-type TRF2, or TRF2 mutants, 2 X 10^4 cells per well were seeded in duplicate on 12-well plates on day three of selection. Cells were counted every 2 days, and medium was changed every 4 days. For hTERT-BJ and MRC5 cells expressing short-hairpin RNA constructs shPRMT1, shPRMT5, or shPRMT6, 4.8 X 10^4 cells per well were seeded in triplicate on 12-well plates on the day before infection. Cells were first counted 2 days after selection in puromycin, followed by counting every 2 days.

For the senescence-associated β-galactosidase (β-Gal) assays, hTERT-BJ cells expressing TRF2 mutants were fixed on the 13th day of selection whereas IMR90 cells expressing knockdown constructs were fixed on the 7th day. The senescence- associated β-Gal assays were performed on the fixed cells using the senescence- associated β-Gal senescence kit (Cell Signaling).

The activity of telomerase in cells was determined using a Trapeze telomerase detection kit (Chemicon) according to the manufacturer's protocol. PCR amplification was performed for 31 cycles. The products were separated on a 12.5% nondenaturing polyacrylamide gel in 0.5 X TBE buffer and visualized using SYBR green (Invitrogen).

2.2.4 Results

Amino acid substitutions of arginines with lysines or alanines in the basic domain of TRF2 induce TIF formation and cellular senescence. Overexpression of TRF2 lacking the basic domain ($TRF2^{\Delta B}$) has been shown to promote recombination dependent telomere loss (58), leading to induction of cellular senescence (56, 58). It has been suggested that TRF2 may inhibit DNA recombination at telomeres through its basic domain (58). The basic domain of TRF2 contains nine arginines that are conserved between mouse and human (Fig. 1A) (56); however, whether these arginines are important for TRF2 function has not been determined. To address this question, we generated four TRF2 mutants (TRF2-RK, TRF2-RA, TRF2- RK1-4, and TRF2-RK5-8). In the TRF2-RK or TRF2-RA mutant, the first eight arginines were simultaneously changed to either lysines (TRF2-RK) or alanines (TRF2-RA). In the TRF2-RK1-4 mutant, arginines at positions 13, 17, 18, and 21 were replaced with lysines, whereas arginines at positions 25, 27, 28, and 30 were replaced with lysines in the TRF2-RK5-8 mutant. Expression of various TRF2 mutants was comparable to that of wild-type TRF2 (Fig. 1B). We found that overexpression of TRF2-RK, TRF2-RA, TRF2-RK1-4, or TRF2-RK5-8 led to induction of slow growth and cellular senescence in both human fibrosarcoma HT1080 cells and hTERT-immortalized normal human fibroblast hTERT-BJ cells (Fig. 1C to F and data not shown), similar to that of $TRF2^{\Delta B}$ (Fig. 1C to F). These results suggest that maintaining the charge alone in the basic domain is insufficient for TRF2 function. These results further imply that more than one arginine is required for TRF2 function.

Disruption of TRF2 function is known to result in induction of TIFs (50, 58). TIFs are sites where telomeres colocalize with DNA damage response factors, such as 53BP1 (50). To investigate whether arginines in the basic domain of TRF2 are essential for maintaining telomere integrity, we performed coimmunostaining in TRF2-RK-expressing cells using anti-53BP1 and anti- TRF1 antibodies. Compared to the vector alone, we observed a sharp increase in TIF formation in TRF2-RK-expressing cells (Fig. 2A and B). About 20% of TRF2-RK-expressing cells displayed TIFs, whereas less than 1% of the vector-expressing cells showed TIF formation (Fig. 2B). In addition, we found that overexpression of TRF2-RK led to a reduction in telomeric association of TIN2 (Fig. 2C and D), a component of shelterin (27). Overexpression of TRF2-RK had no impact on the level of TIN2 expression (Fig. 2E). Together, these results reveal that arginines in the basic domain of TRF2 are important for telomere integrity as well as shelterin association with telomeres.

Overexpression of TRF2 carrying amino acid changes of arginines to lysines promotes the formation of telomere doublets. We have shown that overexpression of TRF2-RK led to induction of TIFs (Fig. 2A and B). To gain further insight into the role of arginines in the basic domain of TRF2, we decided to examine whether overexpression of TRF2-RK, TRF2-RK1-4, or TRF2-RK5-8 may affect telomere length or stability. While no substantial change in the average telomere length was detected in hTERT-BJ or HT1080 cells overexpressing TRF2- RK, TRF2-RK1-4, or TRF2-RK5-8 (Fig. 2F and G and data not shown), we found that overexpression of TRF2-RK, TRF2- RK1-4, or

TRF2-RK5-8 promotes the formation of telomere doublets (Fig. 3A and B). FISH analysis revealed a significant increase in telomere doublets in cells overexpressing TRF2-RK (about fourfold; $P = 0.005$), TRF2-RK1-4 (more than threefold; $P = 0.003$), and TRF2-RK-5-8 (more than threefold; $P = 0.002$) compared to results with the vector alone (Fig. 3B). Accumulation of telomere doublets was not observed in cells overexpressing TRF2$^{\Delta B}$, whereas there was only a slight increase in telomere doublets in cells overexpressing TRF2 compared to results with the vector alone (Fig. 3B). These results suggest that TRF2-RK, TRF2-RK1-4, and TRF2-RK5-8 may affect telomere stability in a manner that is distinctive from that of TRF2$^{\Delta B}$.

Overexpression of TRF2$^{\Delta B}$ led to a drastic increase in telomere loss (more than 16-fold; $P << 0.0001$) compared to results with the vector alone or wild-type TRF2 (Fig. 3A and C), consistent with previous findings (58). However, only a slight increase in telomere loss was observed in cells overexpressing TRF2-RK, TRF2-RK1-4, and TRF2-RK5-8 compared to results with the vector alone (Fig. 3C). This increase in telomere loss was indistinguishable from that in cells overexpressing wild-type TRF2 (Fig. 3C). Taken together, these results suggest that arginines in the basic domain of TRF2 are essential for blocking the formation of telomere doublets.

TRF2$^{\Delta B}$-induced telomere loss has been shown to result from homologous recombination at telomeres (58). Loss of Nbs1, a component of the conserved Mre11/Rad50/Nbs1 complex essential for DNA recombination (22), has been shown to abrogate TRF2$^{\Delta B}$-induced telomere loss (58). To address whether TRF2-RK-induced telomere doublets require Nbs1- dependent DNA recombination, we performed FISH

analysis with Nbs1-deficient GM07166 cells expressing either TRF2-RK or the vector alone. We found that overexpression of TRF2-RK resulted in about a threefold increase in telomere doublets in Nbs1-deficient GM07166 cells (Fig. 3D), similar to that seen in Nbs1-proficient hTERT-BJ cells (Fig. 3B). These results suggest that Nbs1-dependent DNA recombination may not be required for the formation of telomere doublets. These results further imply that the basic domain of TRF2 may modulate telomere stability through multiple mechanisms.

PRMT1 interacts with TRF2 both *in vivo* and *in vitro*. Our findings that amino acid substitutions of arginines with lysines in the N terminus of TRF2 abrogate its function suggest that posttranslational modification, such as methylation on arginines, may be important for TRF2 function. To investigate whether TRF2 is methylated *in vivo*, we immunoprecipitated endogenous TRF2 from HeLa cells and subjected it to mass spectrometric analysis. Mass spectrometry analysis revealed that arginine at position 17 and arginine at position 18 of TRF2 were dimethylated and monomethylated, respectively (Fig. 4), demonstrating that the basic domain of TRF2 is methylated *in vivo*.

PRMT1, the major protein arginine methyltransferase in mammalian cells (54), has been shown to be associated with telomeres (17). We decided to examine whether PRMT1 is responsible for methylating arginines in the N terminus of TRF2 *in vivo*. Coimmunoprecipitation using anti-PRMT1 or anti-immunoglobulin G (IgG) antibody showed that TRF2 was associated with PRMT1 but not IgG (Fig. 5A). Anti-PRMT1 immunoprecipitation brought down Sam68, a known substrate of PRMT1 (13) (Fig. 5A).

A very weak interaction between PRMT1 and TRF1 was also observed (Fig. 5A). PRMT1 interaction with TRF2 appears to be specific, since anti-PRMT1 immunoprecipitation did not bring down γ-tubulin (Fig. 5A), an abundant protein in the cells. The interaction of PRMT1 with TRF2 was also detected in reverse immunoprecipitation using anti-TRF2 antibody (Fig. 5B). Addition of ethidium bromide to protein extracts prior to coimmunoprecipitation failed to disrupt TRF2 interaction with PRMT1 (data not shown), indicating that its interaction with PRMT1 is unlikely to be mediated through DNA. Furthermore, PRMT5, a type II PRMT, was not found to be associated with TRF2 (Fig. 5B). These results suggest that PRMT1 specifically interacts with TRF2 *in vivo*.

To investigate whether PRMT1 methylates arginines in the basic domain of TRF2, we performed *in vitro* methylation assays using [3H]*S*-adenosylmethionine, recombinant GST-tagged PRMT1, and various TRF2 proteins. As shown in Fig. 5C, we found that recombinant wild-type TRF2 derived from either bacteria or baculovirus was methylated by PRMT1. However, PRMT1 failed to methylate full-length recombinant TRF2-RK, TRF2-RK1-4, and TRF2-RK5-8 (Fig. 5C), suggesting that lysine substitutions of arginines in the basic domain abrogate methylation by PRMT1. Some methylation was observed on proteins migrating at or below a 37-kDa marker in the lanes containing TRF2-RK1-4 or TRF2-RK5-8 (Fig. 5C), which is likely nonspecific since anti-TRF2 antibody failed to recognize these proteins (data not shown). In addition, mass spectrometry analysis of *in vitro*-methylated wild-type TRF2 showed that PRMT1 was able to methylate essentially every arginine in the basic domain of TRF2 (Fig. 5D). Taken

together, these results demonstrate that PRMT1 methylates multiple arginines in the basic domain of TRF2 *in vitro*.

To gain further evidence that PRMT1 is responsible for methylating arginines in the basic domain of TRF2, we raised an antibody (5510) against a TRF2 peptide containing asymmetrically dimethylated arginine 17. Anti-2meR17 antibody 5510 showed a much higher affinity for the methylated TRF2 peptide than for the unmethylated peptide (Fig. 6A). To address whether 5510 specifically recognizes the methylated TRF2 peptide, we performed peptide competition assays. Although preincubation with unmethylated peptide completely abrogated binding of 5510 to unmethylated peptide (Fig. 6B), such preincubation failed to abolish binding of 5510 to methylated peptide (Fig. 6B and C). In contrast, preincubation with methylated peptide completely diminished 5510 binding to methylated peptide (Fig. 6C). These results together demonstrate that 5510 specifically recognizes the methylated TRF2 peptide.

When incubated with whole-cell extracts from cells, 5510 predominantly recognized a single protein band with an apparent molecular weight indistinguishable from that of endogenous TRF2 (Fig. 6D). Depletion of TRF2 led to a loss of TRF2 recognized by 5510 antibody, indicating that 5510 recognizes TRF2 *in vivo* (Fig. 6E). To investigate whether 5510 recognizes methylated TRF2 *in vivo*, we incubated 5510 with unmethylated peptide prior to Western analysis. As shown in Fig. 6F, preincubation with unmethylated peptide did not abrogate the ability of 5510 to recognize endogenous TRF2 from a number of human cell lines that are either primary, immortalized, or transformed, suggesting 5510 recognizes methylated TRF2 *in vivo*. The 5510 antibody also recognized

immunoprecipitated TRF2 in the presence of unmethylated peptide (Fig. 6G). We estimate that dimethylation of arginine 17 is present in about 1 to 5% of endogenous TRF2 in HeLa cells (Fig. 6G).

We have shown that PRMT1 methylates TRF2 *in vitro* (Fig. 5C). To examine whether PRMT1 methylates TRF2 *in vivo*, we carried out Western analysis on PRMT1-depleted cells using 5510 antibody that had been preincubated with unmethylated peptide. We found that depletion of PRMT1 mitigated R17 methylation in hTERT-BJ, HT1080, 293T, and HeLa cells (Fig. 6H and data not shown). Knockdown of PRMT1 had no effect on the level of TRF2 expression (Fig. 6H). Methylation of R17 was not affected by depletion of PRMT6 (19), also a type I enzyme (Fig. 6I). These results suggest that PRMT1 is the main enzyme responsible for methylating TRF2 R17 *in vivo*.

Depletion of PRMT1 promotes the formation of telomere doublets, leading to induction of growth arrest in normal human cells. We have shown that PRMT1 interacts with TRF2 and is responsible for methylating TRF2 R17 *in vivo*, suggesting that PRMT1 may play a role in telomere maintenance. To address this question, we stably knocked down PRMT1 in a number of normal human fibroblast cell lines (hTERT-BJ, IMR90, MRC5, and GM08399) (Fig. 7A). As a control, we also generated hTERT-BJ cell lines stably expressing shPRMT5, shPRMT6, or the vector alone (Fig. 7B and C). Compared to results with cells expressing the vector alone, knockdown of PRMT5 or PRMT6 had no impact on cell proliferation (Fig. 7D). In contrast, we found that depletion of PRMT1 resulted in growth arrest within 3 to 5 days after infection in hTERT-BJ,

IMR90, MRC5, and GM08399 cells (Fig. 7D to F). These results suggest that PRMT1 is essential for cell proliferation in normal human cells.

No substantial change in telomere length was detected in hTERT-BJ, IMR90, and MRC5 cells expressing shPRMT1 (Fig. 7G). However, FISH analysis revealed that loss of PRMT1 induced the formation of telomere doublets (Fig. 8). Compared to hTERT-BJ cells expressing the vector alone, we observed about a fourfold increase (P = 0.004) in telomere doublets in hTERT-BJ cells expressing shPRMT1 (Fig. 8A and B). This increase was not observed in hTERT-BJ cells expressing shPRMT5 or shPRMT6 (Fig. 8A and B). Induction of telomere doublets resulting from depletion of PRMT1 was also detected in MRC5 and GM08399 cells (Fig. 8D). Compared to MRC5 cells expressing the vector alone, MRC5 cells expressing shPRMT1 exhibited a fourfold increase (P = 0.023) in telomere doublets (Fig. 8D). Similarly, GM08399 cells expressing shPRMT1 showed a more than threefold increase (P = 0.002) in telomere doublets compared to results for the vector expressing cells (Fig. 8D). A 1.8-fold increase (P = 0.034) in telomere loss was also observed in hTERT-BJ cells expressing shPRMT1 compared to results with the vector alone (Fig. 8C). Taken together, these results suggest that PRMT1 is required for maintaining telomere stability in normal human cells.

Depletion of PRMT1 alters telomeric association of TRF2, promoting telomere shortening in cancer cells. To examine the effect of depletion of PRMT1 in telomere maintenance in transformed cells, several transformed cell lines (HT1080, 293T, and GM637) were infected with retrovirus expressing either shPRMT1 or the vector

alone, generating six stable cell lines (HT1080-pRS, HT1080-shPRMT1, 293T-pRS, 293T shPRMT1, GM637-pRS, and GM637-shPRMT1) (Fig. 9A). Knockdown of PRMT1 had no effect on cell proliferation in the transformed cells (Fig. 9B to D), suggesting that PRMT1 may not be essential for cell proliferation in cancer cells.

To assess telomere length dynamics, cells stably expressing either shPRMT1 or the vector alone were subjected to longterm culturing. While HT1080 cells expressing the vector alone experienced little change in their telomere length for 84 population doublings (PDs) (Fig. 10A and B), we detected a decline in telomere length at a rate of about 18 bp/PD in HT1080 cells expressing shPRMT1 (Fig. 10A and B). This decrease in telomere length resulting from PRMT1 knockdown appears to be specific. since we did not observe telomere shortening in HT1080 cells expressing shPRMT6 (data not shown). Furthermore, PRMT1 knockdown in HT1080 cells had no impact on telomerase activity (Fig. 10C) and 3' G-strand overhang (data not shown). The negative impact of PRMT1 depletion on telomere length maintenance was also observed in GM637 and 293T cells (Fig. 10D and E and data not shown). While the vector-expressing GM637 and 293T cells experienced growth in their telomere length during the long-term culturing (Fig. 10D and E), PRMT1 knockdown suppressed their telomere lengthening (Fig. 10D and E). The rebound in telomere length in shPRMT1-expressing GM637 cells after 30 PDs was likely due to the loss of PRMT1 knockdown observed in these cells at later passages (Fig. 9A). Taken together, these results suggest that PRMT1 is a positive regulator of telomere length maintenance.

Overexpression of TRF2 has been shown to promote telomere shortening (2, 26, 37, 48). We decided to investigate whether PRMT1 may modulate TRF2 association with telomeric DNA. ChIP using anti-TRF2 antibody was performed with HT1080 cells expressing shPRMT1 or the vector alone. ChIP analysis showed that cells expressing shPRMT1 exhibited a 65% increase (P = 0.0125) in TRF2 association with telomeric DNA compared to cells expressing the vector alone (Fig. 11A and B). Depletion of PRMT1 had no impact on telomeric association of TRF1 and TIN2, two other shelterin proteins (Fig. 11C to E). Furthermore, PRMT1 knockdown had no effect on TRF2, TRF1, and TIN2 expression in cells (Fig. 6H and data not shown). Altogether, these results suggest that PRMT1 negatively regulates TRF2 binding to telomeric DNA.

2.2.5 Discussion

In this report, we have shown that arginines in the basic domain of TRF2 undergo methylation both *in vitro* and *in vivo*. Amino acid changes of arginines to lysines in the basic domain of TRF2 alter telomeric association of the shelterin protein TIN2, promoting the formation of TIFs and telomere doublets (more than one telomeric signal at a single chromatid end), indicative of dysfunctional telomeres. We have further revealed that PRMT1 interacts with TRF2 and is the main enzyme responsible for methylating TRF2.

We have shown that depletion of PRMT1 in transformed cells leads to telomere shortening whereas removal of PRMT1 in normal human cells induces the formation of

telomere doublets. Our results suggest that arginine methylation by PRMT1 plays a crucial role in regulating telomere length and stability, perhaps in part through TRF2 methylation. We have shown that depletion of PRMT1 or overexpression of TRF2 carrying amino acid changes of arginines to lysines in the basic domain (TRF2-RK, TRF2-RK1-4, or TRF2-RK5-8) promotes the formation of telomere doublets. Telomere doublets have been seen at a very low frequency in human cells (43). An increase (≈3-fold) in the formation of telomere doublets has been shown to be associated with induction of growth arrest and cellular senescence (55). Consistent with this previous finding, we also observed an induction of growth arrest and cellular senescence in normal human cells depleted for PRMT1 or overexpressing TRF2-RK, TRF2-RK1-4, or TRF2-RK5-8, and these cells display about a three- to fourfold increase in telomere doublets.

Telomere doublets have been suggested to result from a DNA replication defect (55) (T. de Lange, personal communication). Consistent with this view, we find that Nbs1, a protein known to be involved in DNA recombination and repair (16, 22), is not required for the formation of TRF2-RK-induced telomere doublets. Overexpression of TRF2 lacking the basic domain ($TRF2^{\Delta B}$) has been shown to induce drastic telomere loss (58), which is dependent upon Nbs1-mediated DNA recombination at telomeres (58). Taken together, our results suggest that arginine methylation may play an important role in telomere replication and that the basic domain of TRF2 may be involved in multiple mechanisms to ensure telomere integrity.

PRMT1 is known to methylate multiple arginines in the GAR motif (8, 9, 13). Through mass spectrometry analysis, we have shown that arginines 17 and 18 are

methylated *in vivo*. We have shown that lysine substitutions of four arginines at positions 13 to 21 or at positions 25 to 30 abrogate TRF2 function *in vivo*. Furthermore, we have shown that lysine substitutions of either eight or four arginines abolish methylation of full-length TRF2 by PRMT1 *in vitro*. Our data suggest that PRMT1 may methylate multiple arginines in the basic domain of TRF2, including arginines at positions 25 to 30 *in vivo*. Using specific anti-2meR17 antibody, we have estimated that about 1 to 5% of endogenous TRF2 in HeLa cells contains dimethylated R17, suggesting a low abundance of methylated TRF2 in cells. However, whether all arginines in the basic domain are methylated equally *in vivo* requires further investigation. The low abundance of methylated TRF2 may in part account for our failure to detect arginine methylation at positions 25 to 30 *in vivo*.

PRMT1 is involved in a wide range of cellular processes (4, 5). Null mutations in PRMT1 leads to early embryonic lethality in mice shortly after implantation, whereas mouse embryonic stem cells lacking PRMT1 are viable (42). We have shown that knockdown of PRMT1 does not affect cell proliferation in multiple cancer cell lines (HT1080, 293T, GM637, and HeLa). However, loss of PRMT1 results in growth arrest and cellular senescence in four normal human fibroblasts tested (hTERT-BJ, IMR90, MRC5, and GM08399). These results suggest that PRMT1 may be dispensable for cell proliferation in transformed cells but essential in normal human cells. However, we cannot rule out the possibility that PRMT1 depletion is less severe in transformed cells than that in normal cells. The residual level of PRMT1 may also account for the observed discrepancy in growth phenotype between TRF2 lysine mutants and PRMT1 knockdown

in transformed cells. Alternatively, arginines in the basic domain of TRF2 might also have a function that is independent of PRMT1-mediated methylation. Our finding that depletion of PRMT1 results in growth arrest in telomerase-immortalized BJ cells implies that exogenous expression of telomerase in normal human cells is insufficient to bypass the requirement of PRMT1 for cell proliferation.

We have shown that loss of PRMT1 affects both telomere length and stability. Depletion of PRMT1 in normal human cells promotes telomere doublets in a manner indistinguishable from overexpression of TRF2 carrying lysine substitutions of arginines in the basic domain, suggesting that PRMT1 may control telomere stability in part through TRF2 methylation. In human fibrosarcoma HT1080 cells, we find that knockdown of PRMT1 leads to telomere shortening. Depletion of PRMT1 also suppresses telomere lengthening associated with long-term culturing of 293T and GM637 cells. Although loss of PRMT1 does not appear to affect telomerase activity in HT1080 cells, we find that telomeric association of TRF2 is upregulated in PRMT1-depleted cells. Excess TRF2 has been shown to lead to telomere shortening (2, 26, 37, 48). Our results suggest that PRMT1-mediated methylation may control TRF2 association with telomeres, which in turn modulates telomere length maintenance.

We have shown that about 1 to 5% of endogenous TRF2 is methylated. How such a small fraction of methylated TRF2 contributes to regulation of telomere function is unknown. TRF2 has been shown to interact with many non-shelterin proteins important for maintaining telomere length and stability, including Mre11/Rad50/Nbs1 (65), XPF/ERCC1 (66), Apollo (20, 30, 55), WRN (39), and FEN1 (36). A small fraction

(about 1 to 5%) of TRF2 has been shown to interact with Mre11/Rad50/Nbs1 (65) and XPF/ERCC1 (66), raising the possibility that TRF2 methylation by PRMT1 might be important for its interaction with non-shelterin proteins.

PRMT1 has been shown to be associated with human telomeres (17). We have identified that TRF2, a shelterin protein, is a substrate of PRMT1. Our finding that PRMT1 is required for telomere length maintenance and stability is consistent with the fact that TRF2 plays a multiple role in regulating telomere length maintenance and telomere protection (18). Our results suggest that PRMT1 may control telomere length and stability, perhaps in part through TRF2 methylation. Identification of additional PRMT1 substrates at telomeres may further our understanding of its role in telomere maintenance.

Acknowledgments

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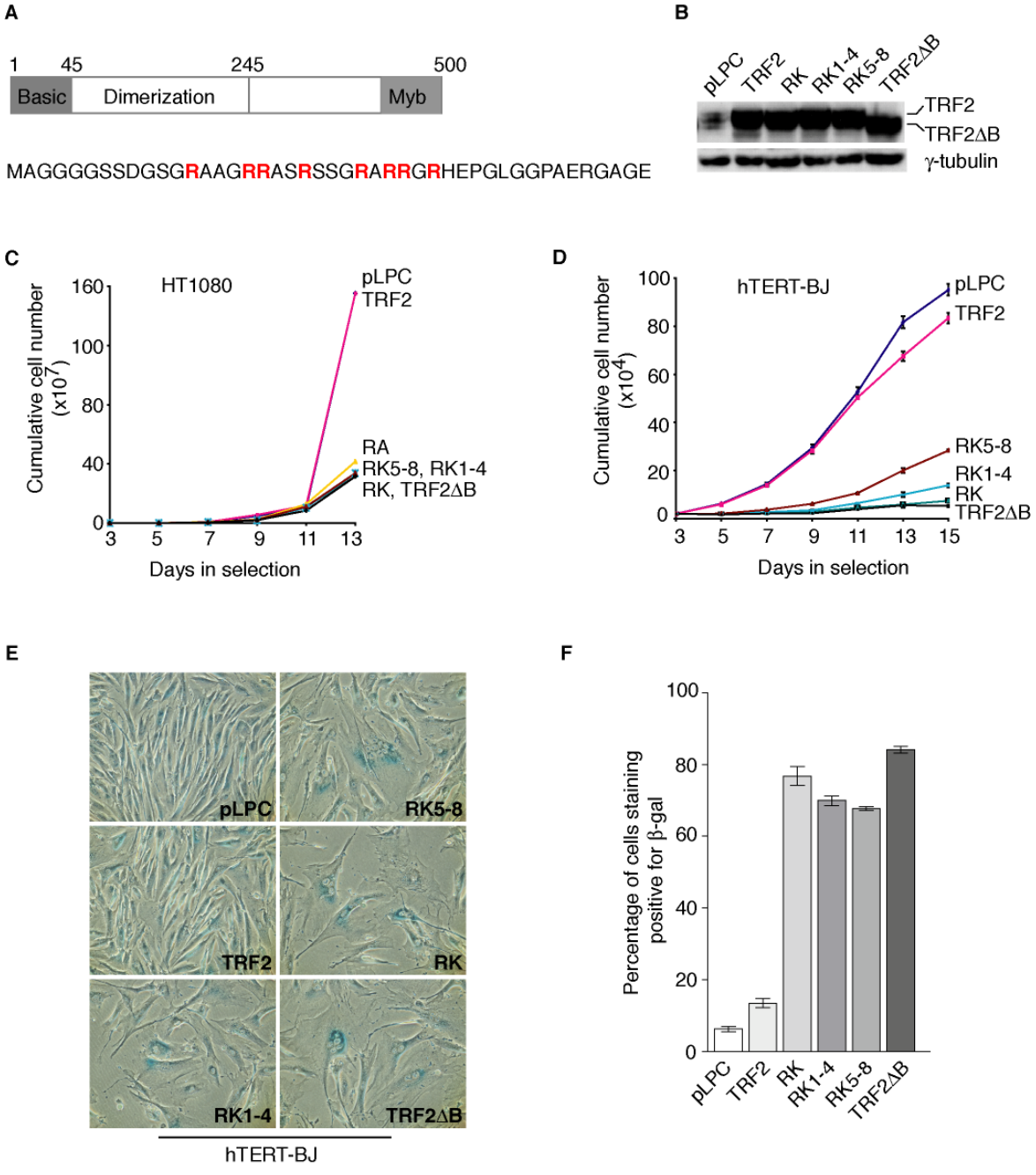
2.2.6 Figures and Figure Legends

Figure. 1. Arginines in the basic domain of TRF2 are crucial for its function. (A) Schematic diagram of human TRF2. Arginines in the basic domain are highlighted in red and they are conserved between human and mouse. (B) Western blot analysis of wild type and various mutant TRF2 expression. Whole cell extracts made from 200,000 cells were used and immunoblotting was performed with anti-TRF2 antibody. The γ-tubulin blot was used as a loading control. (C) Growth curve of HT1080 cells expressing TRF2 carrying amino acid changes of arginines to lysines or alanines. Cells were infected with retrovirus expressing either wild type TRF2, various TRF2 mutants or the vector alone. Following the last infection, cells were selected with puromycin (2 μg/ml) and maintained in the selection media for 13 days. (D) Growth curve of hTERT-BJ cells expressing TRF2 carrying amino acid changes of arginines to lysines. Cells were infected with indicated retrovirus. Following the last infection, cells were selected with puromycin (2 μg/ml) and maintained in the selection media for 15 days. Standard deviations derived from three independent experiments are indicated. (E) Overexpression of TRF2 carrying amino acid changes of arginines to lysines induces senescence. hTERT-BJ cells infected with indicated viruses were stained for senescence-associated β-galactosidase on day 13. (F) Quantification of percentage of cells staining positive for senescence-associated β-galactosidase. A total of more than 1500 cells from three independent experiments were scored. Standard deviations derived from three independent experiments are indicated.

Figure 1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45
245
500
Basic
Dimerization
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TRF2
RK
RK1-4
RK5-8
TRF2ΔB
TRF2
TRF2ΔB
γ-tubulin
C
HT1080
Cumulative cell number (x10^7)
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100
80
40
0
3
5
7
9
11
13
Days in selection
pLPC
TRF2
RA
RK5-8, RK1-4
RK, TRF2ΔB
D
hTERT-BJ
Cumulative cell number (x10^4)
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80
60
40
20
0
3
5
7
9
11
13
15
Days in selection
pLPC
TRF2
RK5-8
RK1-4
RK
TRF2ΔB
E
pLPC
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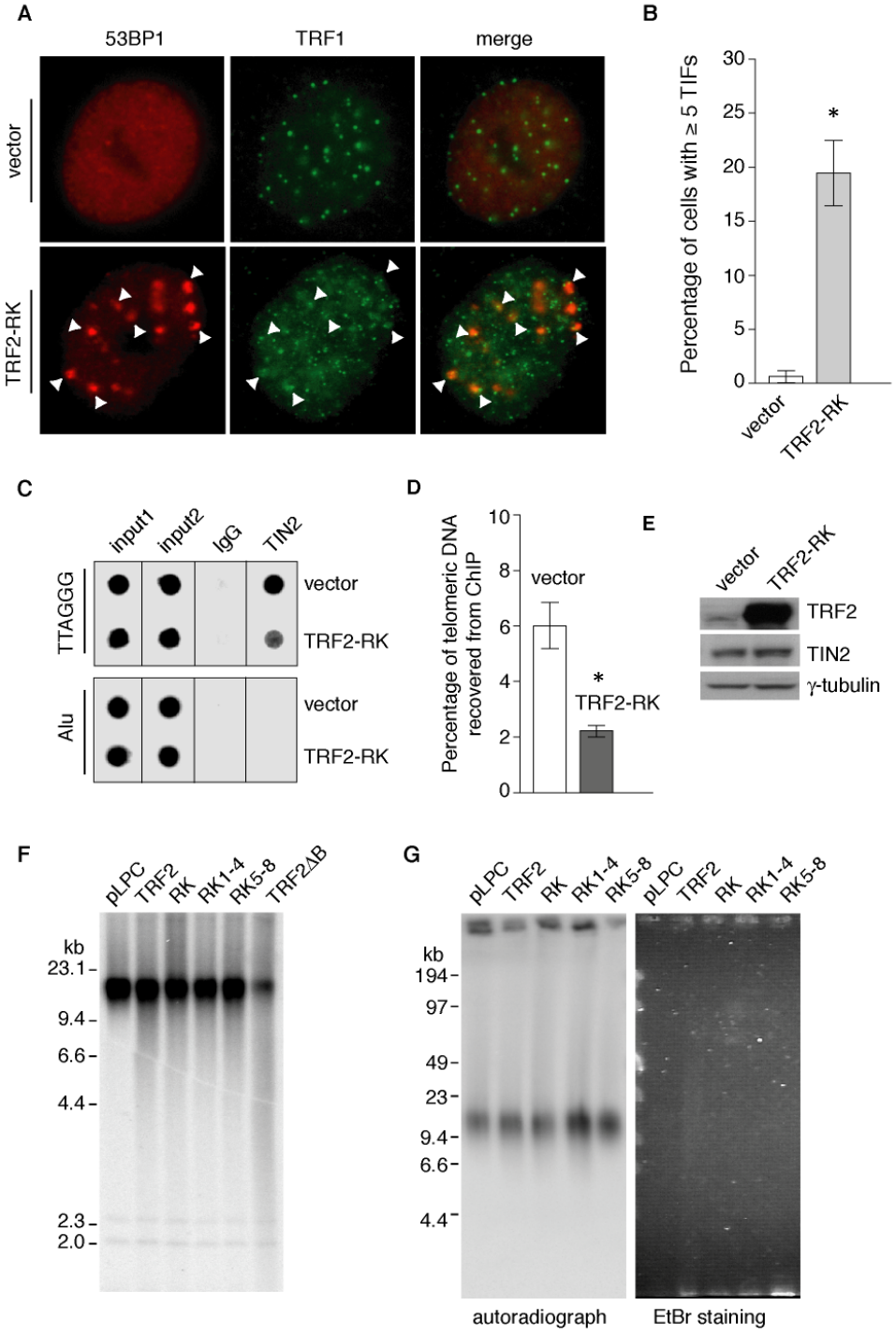
TRF2
RK
RK1-4
TRF2ΔB
hTERT-BJ
F
Percentage of cells staining positive for β-gal
100
80
60
40
20
0
pLPC
TRF2
RK
RK1-4
RK5-8
TRF2ΔB

Figure. 2. (A) Overexpresssion of TRF2-RK induces TIF formation. Indirect immunofluorescence using anti-TRF1 in conjunction with anti-53BP1 was performed with fixed hTERT-BJ cells expressing either TRF2-RK or the vector alone. Arrowheads indicate sites of colocalization of 53BP1 and TRF1. (B) Quantification of percentage of cells with more than five TIFs. For each cell line, a total of more than 800 cells from three independent experiments were scored. Standard deviations derived from three independent experiments are indicated. (C) Dot blots of ChIPs. ChIPs were performed with either anti-TIN2 or anti-IgG antibody in cell extracts from HT1080 cells overexpressing TRF2-RK or the vector alone. Precipitated DNA was analyzed for the presence of TTAGGG repeats and Alu repeats by Southern blotting. (D) Quantification of anti-TIN2 ChIPs. The signals were quantified by ImageQuant analysis. The percentage of precipitated DNA was calculated relative to the input signals. Standard deviations derived from three independent experiments are indicated. (E) Western blot analysis of TRF2 protein expression. Whole-cell extracts made from 200,000 cells were used, and immunoblotting was performed with anti-TRF2 and anti-TIN2 antibodies. The γ-tubulin blot was used as a loading control. (F) Genomic blot of telomeric restriction fragments of hTERT-BJ cells expressing various proteins as indicated above the blot. About 3 μg of RsaI/HinfI-digested genomic DNA from each sample was used for gel electrophoresis. The DNA molecular size markers are shown on the left of the blot. The bottom panel, taken from an ethidium bromide-stained agarose gel, is used as a loading control. (G) Genomic blot of telomeric restriction fragments of hTERT-BJ cells expressing various proteins as indicated above the blot. About 3 μg of RsaI/HinfI digested genomic DNA from each sample was separated on a CHEF gel. The picture of an ethidium bromide (EtBr)-staining gel is shown at the right of the blot, whereas the DNA molecular size markers are shown at the left of the blot.

Figure 2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53BP1
TRF1
merge
vector
TRF2-RK
B
Percentage of cells with ≥ 5 TIFs
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25
20
15
10
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vector
TRF2-RK
C
input1
input2
IgG
TIN2
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vector
TRF2-RK
Alu
vector
TRF2-RK
D
Percentage of telomeric DNA recovered from ChIP
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8
6
4
2
0
vector
*
TRF2-RK
E
vector
TRF2-RK
TRF2
TIN2
γ-tubulin
F
pLPC
TRF2
RK
RK1-4
RK5-8
TRF2ΔB
kb
23.1
9.4
6.6
4.4
2.3
2.0
G
pLPC
TRF2
RK
RK1-4
RK5-8
pLPC
TRF2
RK
RK1-4
RK5-8
kb
194
97
49
23
9.4
6.6
4.4
autoradiograph
EtBr staining

Figure. 3. Overexpression of TRF2 carrying amino acid changes of arginines to lysines promotes the formation of telomere doublets in both hTERT-BJ and Nbs1-deficient GM07166 cells. (A) Metaphase spreads from hTERT-BJ cells expressing TRF2-RK, $TRF2^{\Delta B}$, or the vector alone. Chromosomes were stained with DAPI and false colored in red. Telomeric DNA was detected by FISH using a FITC-conjugated $(CCCTAA)_3$-containing PNA probe (green). Arrows indicate telomere doublets. Enlarged images of chromosomes with telomere doublets are shown. (B) Quantification of telomere doublets in hTERT-BJ cells stably infected with retrovirus as indicated. For each cell line, a total of 1,600 to 1,900 chromosomes from 42 to 45 metaphase cells were scored. Standard deviations derived from at least three independent experiments are indicated. (C) Quantification of telomere loss in hTERT-BJ cells expressing TRF2 alleles as indicated. For each cell line, a total of 1,600 to 1,900 chromosomes from 42 to 45 metaphase cells were scored. Standard deviations derived from at least three independent experiments are indicated. (D) Quantification of telomere doublets in Nbs1-deficient GM07166 cells stably infected with retrovirus as indicated. For each cell line, a total of 1,372 to 1,650 chromosomes from 38 to 40 metaphase cells were scored. Standard deviations derived from three independent experiments are indicated. Asterisks indicate $P < 0.05$ using a two tailed student's t test.

Figure 3

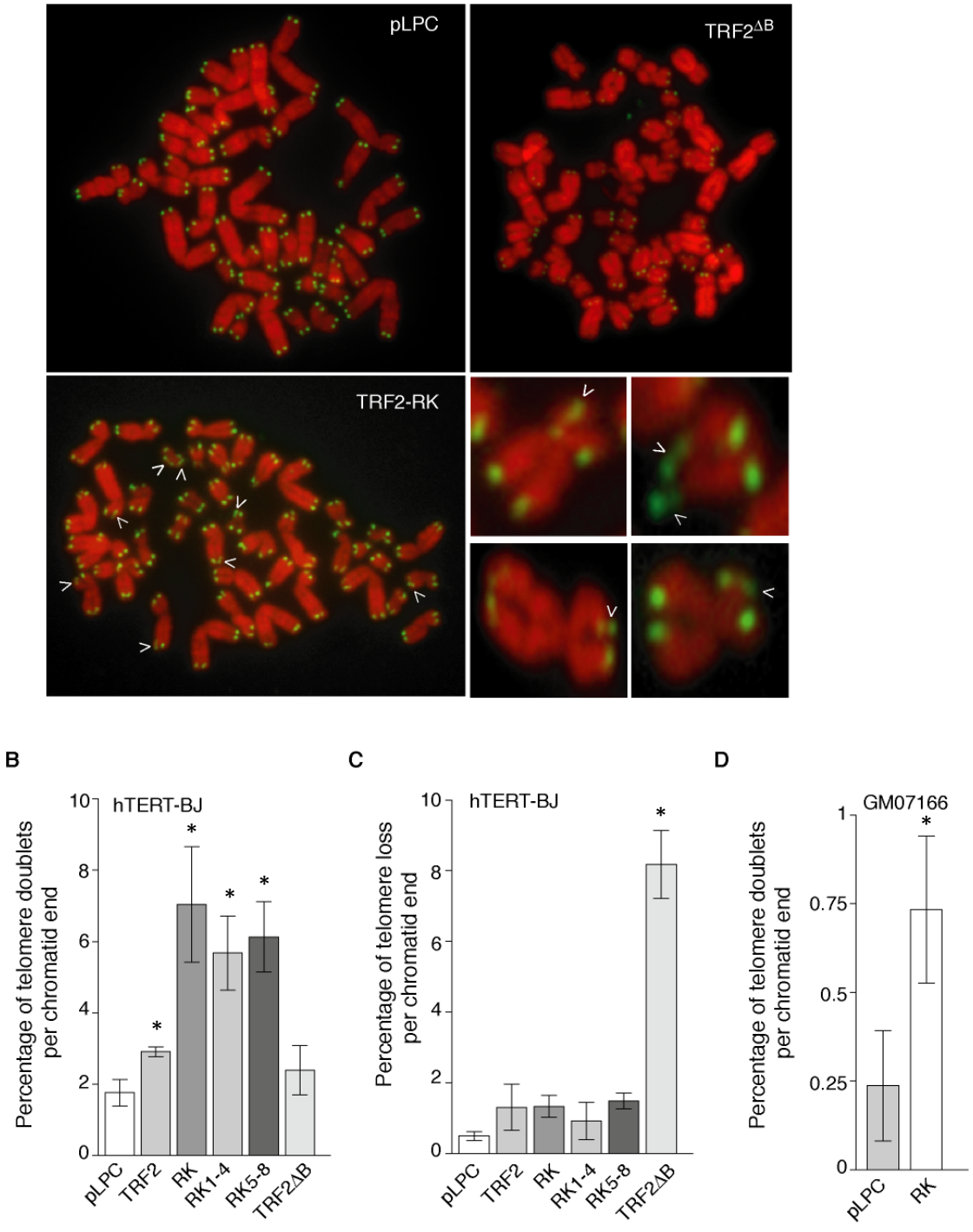
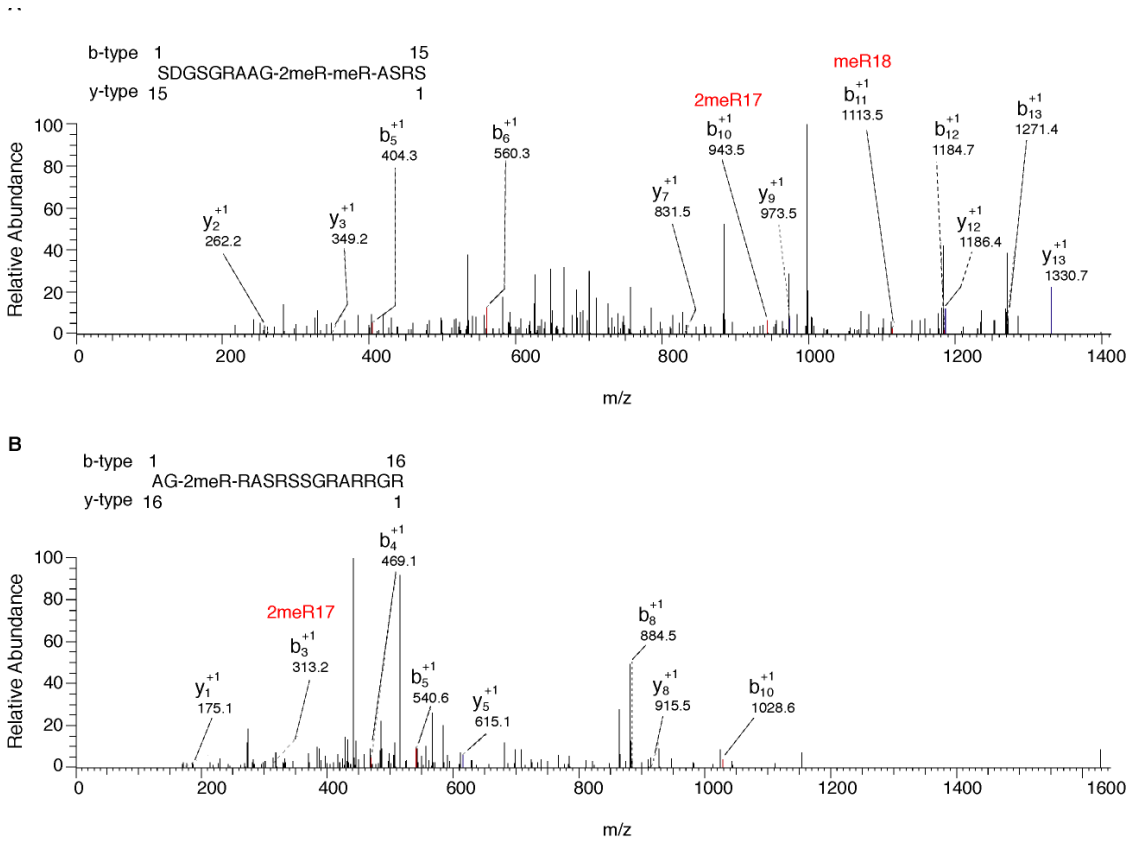

pLPC
TRF2ΔB
TRF2-RK
B
hTERT-BJ
Percentage of telomere doublets per chromatid end
pLPC
TRF2
RK
RK1-4
RK5-8
TRF2ΔB
C
hTERT-BJ
Percentage of telomere loss per chromatid end
D
GM07166
Percentage of telomere doublets per chromatid end

Figure. 4. Arginines of TRF2 at positions 17 and 18 are methylated *in vivo*. Liquid chromatography-tandem mass spectrometry analysis was performed with endogenous TRF2 immunoprecipitated from HeLa cells. The spectra of two peptides identified to contain methylated arginines in the basic domain are shown, with the relative abundance plotted against the monoisotopic mass (*m*/*z*). The *m*/*z* peaks from both b-type and y-type ions are indicated. The peptide presented in panel A contained dimethylated arginine at position 17 and monomethylated arginine at position 18, whereas the peptide presented in panel B contained dimethylated arginine at position 17. Asterisks indicate $P < 0.05$ using a two-tailed student's t test.

Figure 4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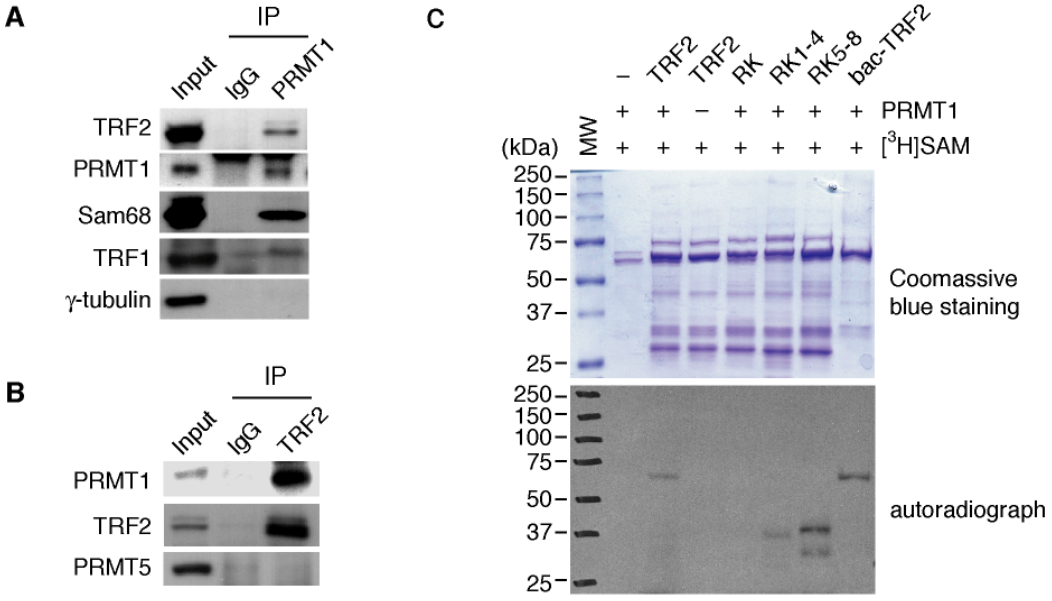
y-type 15 1
meR18
2meR17
Relative Abundance
m/z
B
b-type 1 16
AG-2meR-RASRSSGRARRGR
y-type 16 1
2meR17
Relative Abundance
m/z

Figure. 5. Interaction of PRMT1 with TRF2. (A) Association of PRMT1 with TRF2 *in vivo*. Coimmunoprecipitations with HeLa nuclear extracts were conducted with either anti-IgG or anti-PRMT1 antibody. Western analysis was done with anti-PRMT1, anti-TRF2, anti-TRF1, anti-Sam68, and anti-γ-tubulin antibodies. (B) Association of TRF2 with PRMT1 *in vivo*. Coimmunoprecipitations with HeLa nuclear extracts were conducted with either anti-IgG or anti-TRF2 antibody. Western analysis was done with anti-PRMT1, anti-TRF2, and anti-PRMT5 antibodies. (C) PRMT1 methylates arginines in the basic domain of TRF2. *In vitro* methylation assays were conducted using [3H]SAM, GST-PRMT1, and recombinant TRF2 as indicated. Both the Coomassie blue-staining gel (top panel) and autoradiograph (bottom panel) are shown. The protein molecular mass markers are shown on the left of the gel and blot. All proteins except for bac-TRF2 were expressed in bacteria. Bac-TRF2 (a gift from Titia de Lange) refers to wild-type TRF2 derived from baculovirus. (D) PRMT1 methylates multiple arginines of the basic domain *in vitro*. Bacterium expressed wild-type TRF2 was methylated *in vitro* and then subjected to matrix-assisted laser desorption ionization mass spectrometry analysis. Peptides containing methylated arginines are shown. Arginines in the basic domain are highlighted in bold.

Figure 5



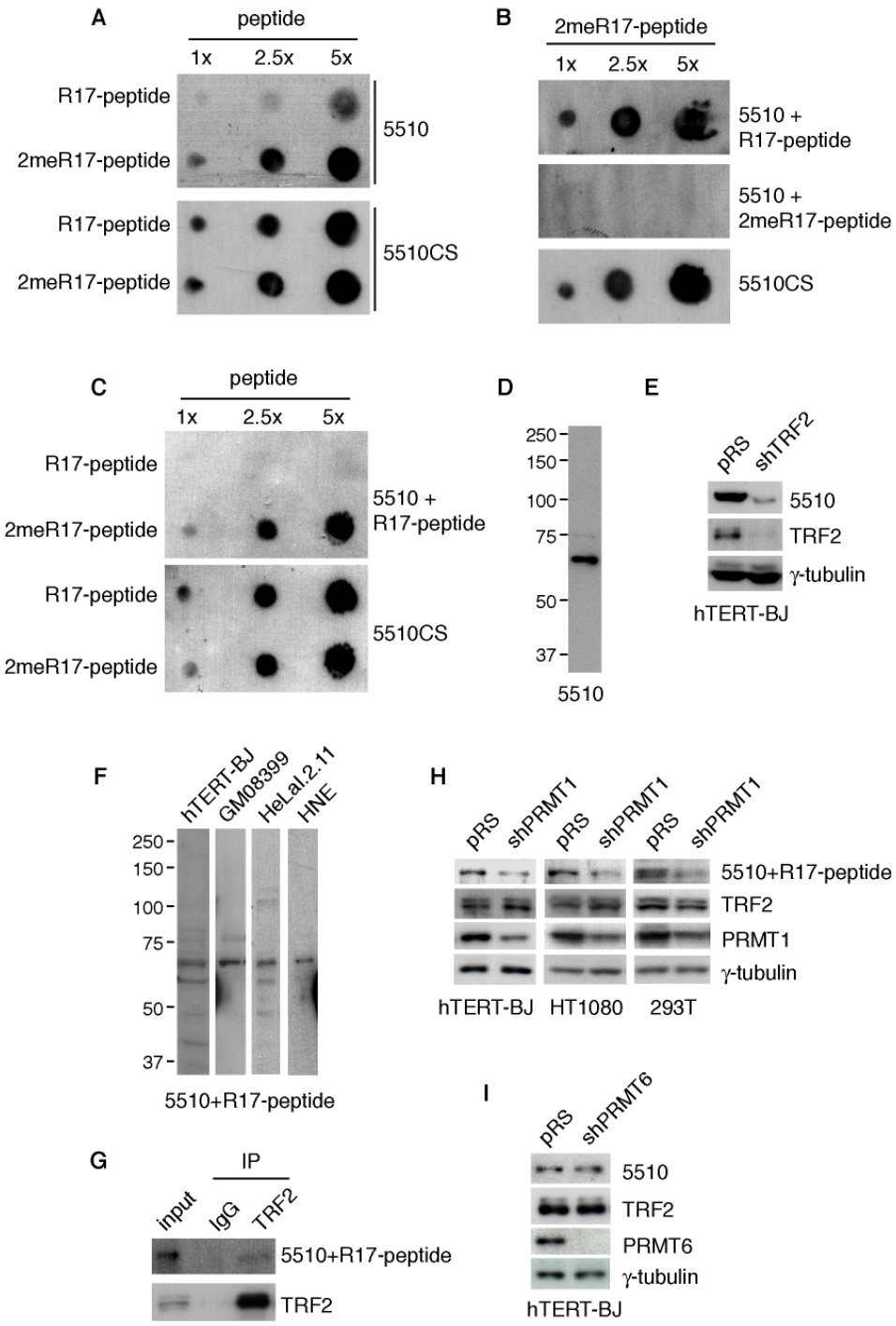
D

Sequence	position	monoisotopic mass	modifications
(R)*GS*AGGGGSSDGSG**R**(A)	2-13	1122.4770	1 methylation
(R)*DLVPRGS*AGGGGSSDGSG**R**AAG**RR**(A)	2-18	2284.1856	6 methylation
(R)*GS*AGGGGSSDGSG**R**AAG**RR**AS**R**SSG**R**(A)	2-25	2391.1944	5 methylation
(R)AAG**RR**AS**R**SSG**R**A**R**(R)	14-27	1514.8735	4 methylation
(R)AS**R**SSG**R**A**RR**G**R**(H)	19-30	1372.7992	4 methylation
(R)SSG**R**A**R**(R)	22-27	661.3740	2 methylation
(R)SSG**R**A**RR**G**R**(H)	22-30	1030.5977	2 methylation
(R)SSG**R**A**RR**G**R**(H)	22-30	1058.6290	4 methylation
(R)A**RR**G**R**(H)	26-30	671.4424	4 methylation

Figure. 6. PRMT1 is the main enzyme responsible for methylating TRF2 *in vivo*. (A) Affinity-purified 5510 antibody specifically recognizes TRF2 peptide containing asymmetrically dimethylated arginine 17 (R17). Increasing amounts of peptide carrying either unmodified R17 or asymmetrically dimethylated R17 were spotted onto a nitrocellulose membrane, followed by immunoblotting with affinity-purified 5510 or crude serum (5510CS). The amounts of peptide spotted, from left to right, are 0.5 µg, 1.3 µg, and 2.7 µg. (B) Peptide competition assays. Affinity-purified 5510 antibody was incubated with 5.5 µg of unmodified peptide prior to immunoblotting. Crude serum 5510 was used to show the presence of the TRF2 peptide on the nitrocellulose membrane. Amounts of peptide spotted, from left to right, for both modified and unmodified, are 0.5 µg, 1.3 µg, and 2.7 µg. (C) Peptide competition assays. Prior to immunoblotting TRF2 peptide carrying asymmetrically dimethylated R17, affinity-purified 5510 antibody was incubated with 5.5 µg of either unmodified or modified peptide as indicated. Crude serum 5510 was used to show the presence of the methylated TRF2 peptide. (D) Western analysis of methylated TRF2. Twenty micrograms of the whole-cell extract from hTERT-BJ cells was immunoblotted with affinity-purified 5510 antibody. (E) Depletion of endogenous TRF2 leads to loss of methylated TRF2 bound by 5510 antibody. hTERT-BJ cells were infected with retrovirus expressing either shTRF2 or the vector pRS alone. Western analysis was performed with 5510 and anti-TRF2. The γ-tubulin blot was used as a loading control. (F) Western analysis of methylated TRF2. Affinity-purified 5510 antibody was incubated with 5.5 µg of unmodified peptide prior to immunoblotting. Twenty micrograms of the whole-cell extract from several cell lines, as indicated, was

used for immunoblotting. (G) 5510 recognizes immunoprecipitated TRF2. Endogenous TRF2 was immunoprecipitated from HeLa cells using anti-TRF2 antibody. Immunoblotting was performed with anti-TRF2 antibody or affinity-purified 5510 antibody that had been preincubated with 5.5 µg of unmodified peptide. (H) Depletion of endogenous PRMT1 decreases asymmetrical dimethylation of R17. hTERT-BJ, HT1080, and 293T cells were infected with retrovirus expressing either shPRMT1 or the vector pRS alone. For detection of methylated TRF2, Western analysis was done with 5510 that had been preincubated with 5.5 µg of unmodified peptide. Western analysis was also done with anti-TRF2 and anti-PRMT1. The γ-tubulin blot was used as a loading control. (I) Depletion of endogenous PRMT6 has no effect on dimethylation of R17. hTERT-BJ cells were infected with retrovirus expressing either shPRMT6 or the vector pRS alone. Western analysis was done with 5510, anti-TRF2, and anti-PRMT6. The γ-tubulin blot was used as a loading control.

Figure 6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peptide
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2.5x
5x
R17-peptide
2meR17-peptide
5510
R17-peptide
2meR17-peptide
5510CS
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1x
2.5x
5x
5510 +
R17-peptide
5510 +
2meR17-peptide
5510CS
C
peptide
1x
2.5x
5x
R17-peptide
2meR17-peptide
5510 +
R17-peptide
R17-peptide
2meR17-peptide
5510CS
D
250
150
100
75
50
37
5510
E
pRS
shTRF2
5510
TRF2
γ-tubulin
hTERT-BJ
F
hTERT-BJ
GM08399
HeLa1.2.11
HNE
250
150
100
75
50
37
5510+R17-peptide
H
pRS
shPRMT1
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shPRMT1
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γ-tubulin
hTERT-BJ
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IgG
TRF2
5510+R17-peptide
TRF2
I
pRS
shPRMT6
5510
TRF2
PRMT6
γ-tubulin
hTERT-BJ

Figure. 7. Depletion of PRMT1 results in induction of growth arrest in normal human fibroblast cells. (A) Western analysis of PRMT1 expression. Whole-cell extracts made from 200,000 cells were used, and immunoblotting was performed with anti-PRMT1 antibody. The γ-tubulin blot was used as a loading control. (B) Western analysis of PRMT5 expression. Immunoblotting was conducted with anti-PRMT5 antibody, and the γ-tubulin blot was used as a loading control. (C) Western analysis of PRMT6 expression. Immunoblotting was conducted with anti-PRMT6 and anti-γ-tubulin antibodies, the latter serving as a loading control. (D) Growth curve of hTERT-BJ cells infected with indicated virus. hTERT-BJ cells expressing shPRMT1 undergo growth arrest within 3 days of infection, and seeding after infection was associated with massive cell loss. To overcome this problem, 4.8 X 10^4 cells were seeded in triplicate on day -4. Cells were infected five times with indicated virus over 12-h intervals between day -3 and day -1. Selection with puromycin (2 μg/ml) started on day 0, and cells were maintained in the selection medium for 9 days. Standard deviations derived from three independent experiments are indicated. (E) Growth curve of normal primary fibroblast MRC5 cells infected with indicated virus. MRC5 cells expressing shPRMT1 undergo growth arrest. Seeding, retroviral infection, and selection of MRC5 were done as described for panel D. Standard deviations derived from three independent experiments are indicated. (F) Loss of PRMT1 results in growth arrest in normal human cells. Live cell images show hTERT-BJ, IMR90, or GM08399 cells infected with retrovirus expressing shPRMT1 or the vector pRS alone. Images were taken 5 days after infection. (G) Genomic blots of telomeric restriction fragments from hTERT-BJ, IMR90, and MRC5 cells expressing either shPRMT1 or pRS. About 3 μg of RsaI/HinfI-digested genomic DNA from each sample was used for gel electrophoresis. The DNA molecular size markers are shown to the left of the blots.

Figure 7

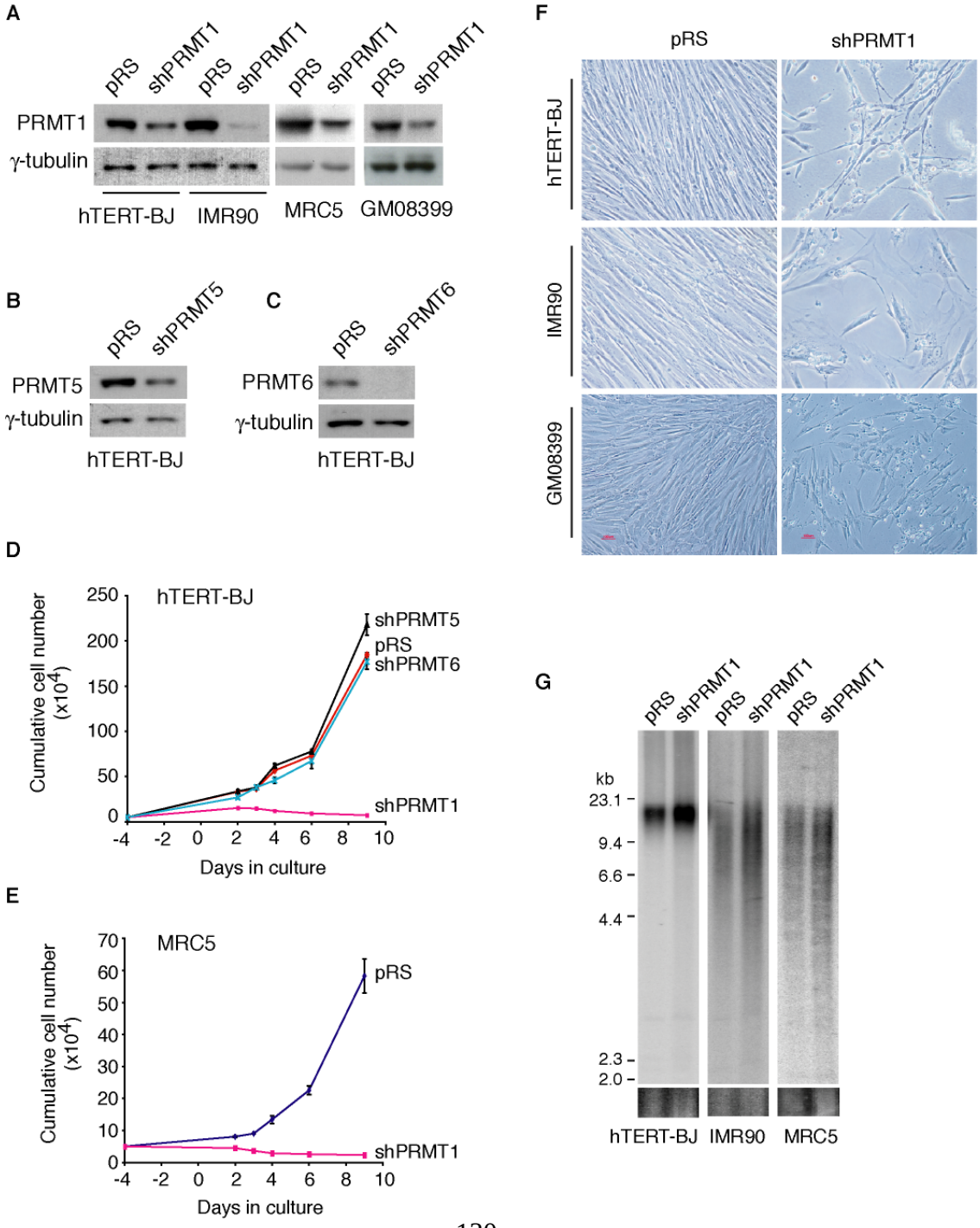

A
pRS
shPRMT1
pRS
shPRMT1
pRS
shPRMT1
pRS
shPRMT1
PRMT1
γ-tubulin
hTERT-BJ
IMR90
MRC5
GM08399
B
pRS
shPRMT5
PRMT5
γ-tubulin
hTERT-BJ
C
pRS
shPRMT6
PRMT6
γ-tubulin
hTERT-BJ
D
hTERT-BJ
Cumulative cell number (x10^4)
250
200
150
100
50
0
-4
-2
0
2
4
6
8
10
Days in culture
shPRMT5
pRS
shPRMT6
shPRMT1
E
MRC5
Cumulative cell number (x10^4)
70
60
50
40
30
20
10
0
-4
-2
0
2
4
6
8
10
Days in culture
pRS
shPRMT1
F
pRS
shPRMT1
hTERT-BJ
IMR90
GM08399
G
pRS
shPRMT1
pRS
shPRMT1
pRS
shPRMT1
kb
23.1
9.4
6.6
4.4
2.3
2.0
hTERT-BJ
IMR90
MRC5

Figure. 8. Depletion of PRMT1 in human normal cells results in telomere instability, promoting the formation of telomere doublets. (A) Analysis of metaphase chromosomes from hTERT-BJ cells infected with indicated virus. Chromosomes were stained with DAPI and false colored in red. Telomeric DNA was detected by FISH using a FITC-conjugated $(CCCTAA)_3$-containing PNA probe (green). Arrows indicate telomere doublets, and the asterisk represents telomere loss. Enlarged images of chromosomes with telomere doublets are shown at the bottom. (B and C) Quantification of telomere doublets and telomere loss in hTERT-BJ cells stably infected with the indicated virus. For each cell line, a total of more than 2,700 chromosomes from at least 60 metaphase cells were scored. Standard deviations derived from at least three independent experiments are indicated. (D) Quantification of telomere doublets in MRC5 and GM08399 cells stably infected with the indicated virus. For each cell line, a total of more than 1,600 chromosomes from 40 to 44 metaphase cells were scored. Standard deviations derived from at least three independent experiments are indicated. Asterisks indicate $P < 0.05$ using a two-tailed student's t test.

Figure 8

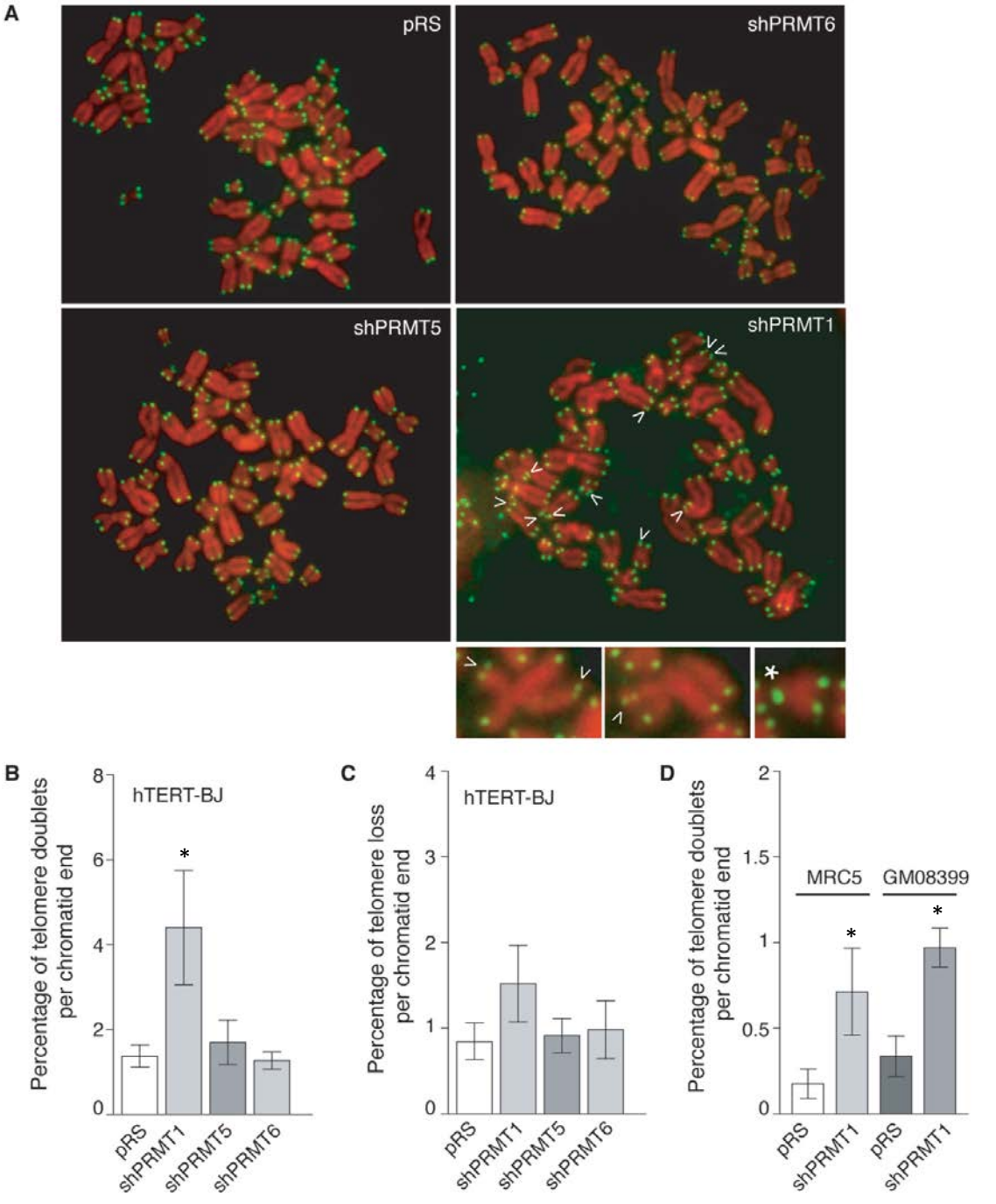

A
pRS
shPRMT6
shPRMT5
shPRMT1
B
hTERT-BJ
Percentage of telomere doublets per chromatid end
pRS
shPRMT1
shPRMT5
shPRMT6
C
hTERT-BJ
Percentage of telomere loss per chromatid end
D
MRC5
GM08399
Percentage of telomere doublets per chromatid end

Figure. 9. Depletion of PRMT1 does not affect cell proliferation in human cancer cells. (A) Western analysis of PRMT1 expression in HT1080, 293T, and GM637 cells expressing shPRMT1 or pRS. The γ-tubulin blot was used as a loading control. (B) Growth curve of 293T cells expressing shPRMT1 or pRS. The number of PDs was plotted against days in culture. (C) Growth curve of HT1080 cells expressing shPRMT1 or pRS. The number of PDs was plotted against days in culture. (D) Growth curve of GM637 cells expressing shPRMT1 or pRS. The number of PDs was plotted against days in culture.

Figure 9

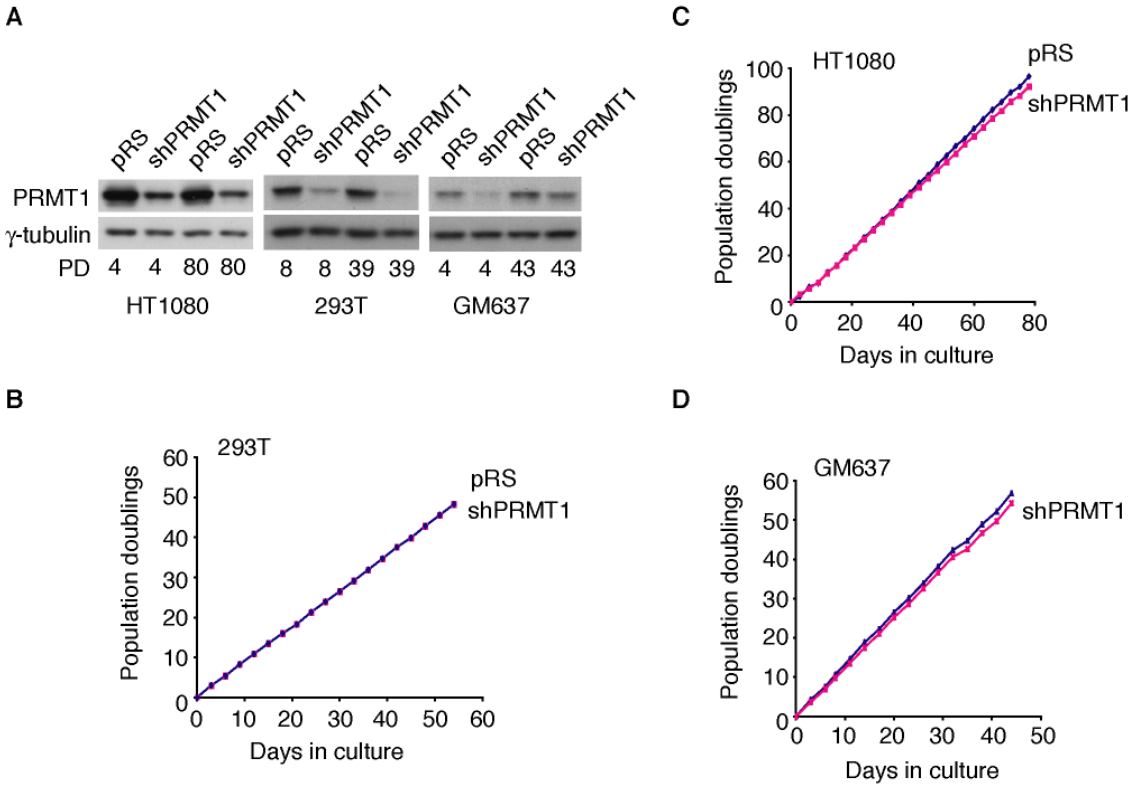

A
pRS
shPRMT1
PRMT1
γ-tubulin
PD 4 4 80 80 8 8 39 39 4 4 43 43
HT1080
293T
GM637
B
293T
pRS
shPRMT1
Population doublings
Days in culture
C
HT1080
pRS
shPRMT1
Population doublings
Days in culture
D
GM637
shPRMT1
Population doublings
Days in culture

Figure. 10. PRMT1 regulates telomere length maintenance in human cancer cells. (A) Genomic blot of telomere restriction fragments from HT1080 cells expressing shPRMT1 or the vector pRS alone after indicated numbers of PDs. About 5 µg of RsaI/HinfI-digested genomic DNA from each sample was used for gel electrophoresis. The DNA molecular size markers are shown to the left of the blot. The bottom panel, taken from an ethidium bromide-stained agarose gel, is used as a loading control. (B) Average telomere length of HT1080 expressing shPRMT1 or pRS was plotted against PDs. (C) Depletion of endogenous PRMT1 has no impact on telomerase activity. Ten thousand HT1080 cells expressing either shPRMT1 or pRS were used to measure telomerase activity. TSR8 was used as a positive control, whereas 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate buffer was used as a negative control. (D) Average telomere length of GM637 cells expressing shPRMT1 or pRS was plotted against PDs. (E) Average telomere length of 293T cells expressing shPRMT1 or pRS was plotted against PDs.

Figure 10

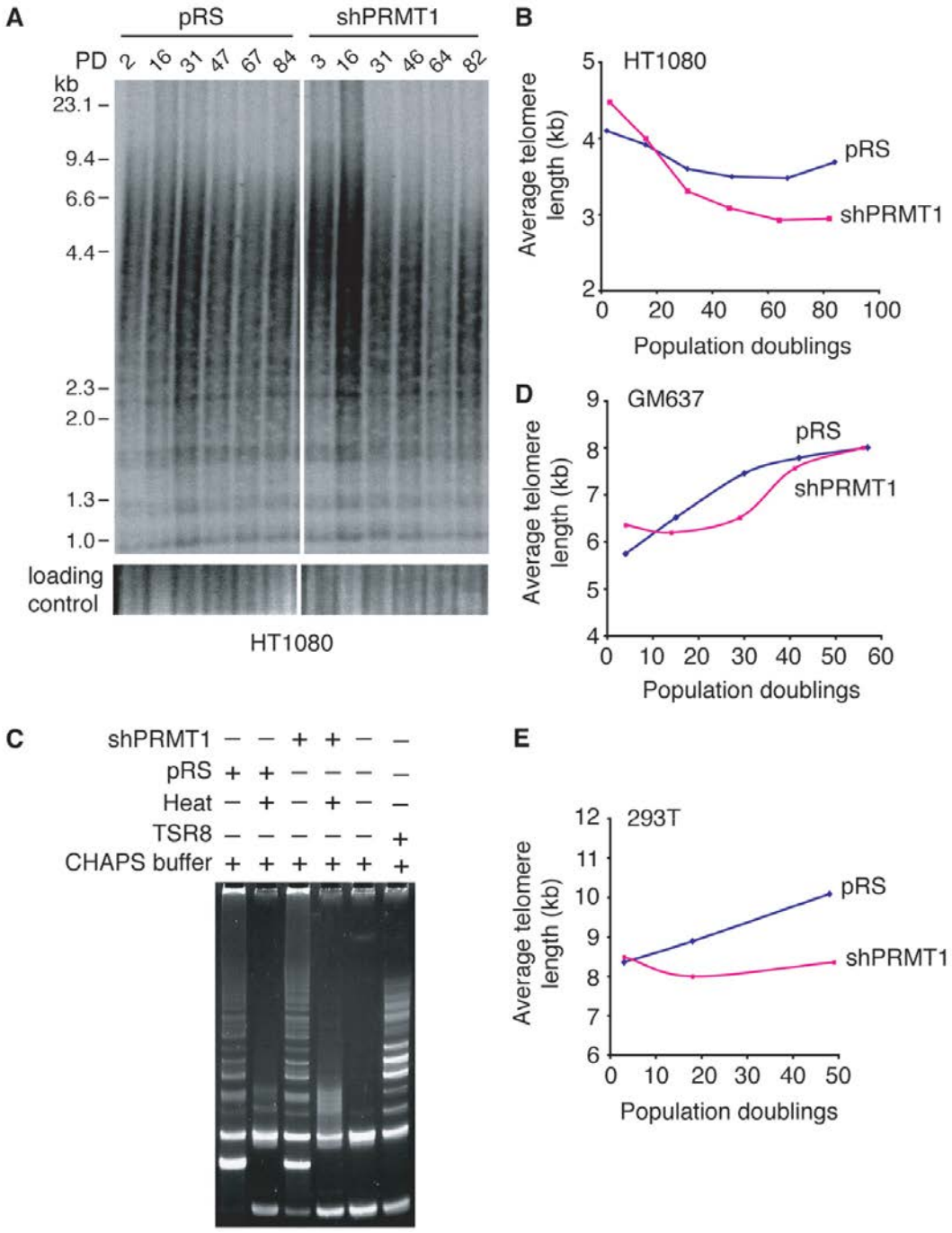
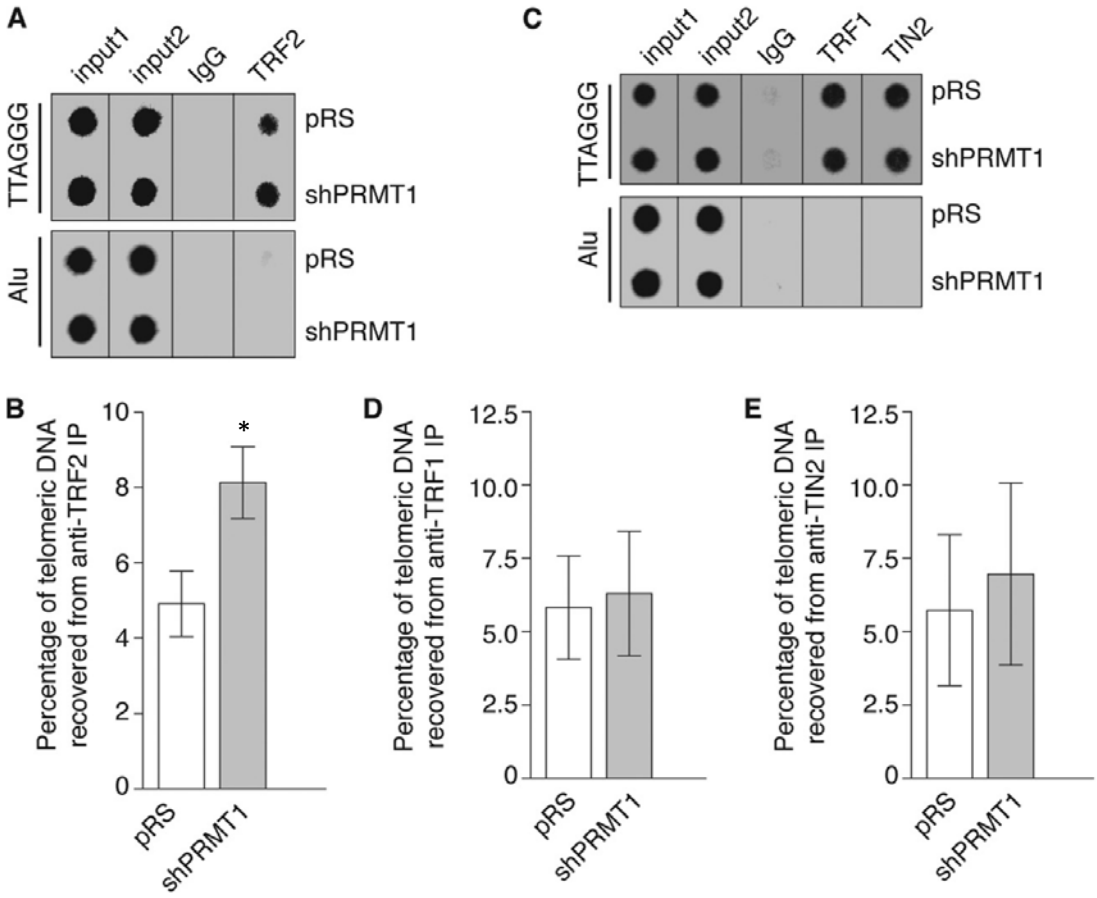

A
pRS
shPRMT1
PD 2 16 31 47 67 84 3 16 31 46 64 82
kb
23.1
9.4
6.6
4.4
2.3
2.0
1.3
1.0
loading control
HT1080
B
HT1080
Average telomere length (kb)
pRS
shPRMT1
Population doublings
C
shPRMT1 – – + + – –
pRS + + – – – –
Heat – + – + – –
TSR8 – – – – – +
CHAPS buffer + + + + + +
D
GM637
pRS
shPRMT1
Average telomere length (kb)
Population doublings
E
293T
pRS
shPRMT1
Average telomere length (kb)
Population doublings

Figure. 11. (A) Dot blots of ChIPs. ChIPs were performed with either anti-TRF2 or anti-IgG antibody in cell extracts from HT1080 cells expressing shPRMT1 or the vector pRS alone. Precipitated DNA was analyzed for the presence of TTAGGG repeats and Alu repeats by Southern blotting. (B) Quantification of anti-TRF2 ChIPs. The signals were quantified by ImageQuant analysis. The percentage of precipitated DNA was calculated relative to the input signals. Standard deviations derived from three independent experiments are indicated. (C) Dot blots of anti-TRF1 and anti-TIN2 ChIPs from HT1080 cells expressing shPRMT1 or the vector pRS alone. Precipitated DNA was analyzed for the presence of TTAGGG repeats and Alu repeats by Southern blotting. (D) Quantification of anti-TRF1 ChIPs from dot blots in panel C. Standard deviations derived from three independent experiments are indicated. (E) Quantification of anti-TIN2 ChIPs from dot blots in panel C. Standard deviations derived from three independent experiments are indicated. The asterisk indicates a P value < 0.05 using a two-tailed student's t test.

Figure 11


A
input1
input2
IgG
TRF2
TTAGGG
pRS
shPRMT1
Alu
pRS
shPRMT1
C
input1
input2
IgG
TRF1
TIN2
TTAGGG
pRS
shPRMT1
Alu
pRS
shPRMT1
B
Percentage of telomeric DNA recovered from anti-TRF2 IP
10
8
6
4
2
0
*
pRS
shPRMT1
D
Percentage of telomeric DNA recovered from anti-TRF1 IP
12.5
10.0
7.5
5.0
2.5
0
pRS
shPRMT1
E
Percentage of telomeric DNA recovered from anti-TIN2 IP
12.5
10.0
7.5
5.0
2.5
0
pRS
shPRMT1

2.2.7 References

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2.3 Complementary Data

Methods and Materials

***In vitro* gel shift assays.** *In vitro* methylation of recombinant TRF2 was done as described (Mitchell et al. 2009). TRF2 from the *in vitro* methylation assay was then used for the gel shift assays. *In vitro* gel shift assays were done as described (Li, Oestreich & de Lange 2000, Zhong et al. 1992). Each reaction contained binding buffer (4% glycerol, 0.5 µg *E. coli* DNA, 20 mM Glycine-KOH [ph 9], 10 mM DTT and 50 ng β-casein), end-labeled 188-bp BglII-XhoI fragment from pTH12 and recombinant TRF2. The samples were incubated for 30 minutes at 4 ^{o}C and then loaded on a 0.6% agarose gel in 0.1 X TBE. The gels were run at 200 V for 30 minutes at 4 ^{o}C and then dried and analyzed by autoradiography using PhosphorImager screens.

RNA preparation and Northern analysis. Total RNA was isolated by TRIzol (Invitrogen) extraction according to the manufacturer's protocol. The purified RNA was resuspended in DEPC treated water. Northern analysis was performed essentially as described (Azzalin et al. 2007, Batenburg et al. 2012). A total of 10 µg of RNA was loaded onto a 1.3% formaldehyde agarose gel and run at 70V for 5 h. The gel was stained with ethidium bromide to visualize the 28S and 18S ribosomal RNA. The RNA was transferred to a Nylon membrane (Hybond-N, GE) and after blocking was incubated in church mix (0.5 M NaPi, 1 mM EDTA pH 8.0, 7% SDS, 1% BSA) overnight at 65 ^{o}C with a radioactively labeled 800 bp TTAGGG fragment (Loayza, De Lange 2003). The

membranes were stripped in 10 mM Tris-HCl, 0.2% SDS at 75 ^{o}C for 2 h and incubated with a radiolabeled fragment of the GAPDH gene. The washed membranes were exposed to a phosphorimager screen and quantified using ImageQuant analysis.

RNA immunoprecipitation (RNA-IP). RNA-IP was done essentially as described (Deng et al. 2009). Cells were lysed (~1x10^{7}/mL) in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% Na deoxycholate, 0.05% SDS, 1 mM EDTA, 150 mM NaCl, 2 mM vanadyl ribonucleoside complex, 1.2 mM PMSF, 1mM DTT, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 10 µg/ml pepstatin) for 20 mins on ice. The extracts were briefly sonicated and clarified by centrifugation at 12,000 rpm for 10 mins at 4 ^{o}C. Immunoprecipitation was carried out with TRF2 antibody or an IgG control overnight at 4 ^{o}C, incubated the following day for 1 h with pre blocked G beads and washed 5 times with 1 ml RIPA buffer at 4 ^{o}C. One fifth of the G beads was boiled in 2X Laemmli sample buffer, and analyzed by western blot. The remaining beads were TRIzol extracted and analyzed by dot blot. Where indicated the RNA was treated with RNase A for 1 hr at 37 ^{o}C prior to loading. For inputs 10% of starting lysate was collected and processed appropriately.

Results

2.3.1 An amino acid substitution of arginine 17 to lysine is not sufficient to induce telomere dysfunction

In vitro methylation of TRF2 by PRMT1 followed by mass spectrometry indicated that all eight arginines of the basic domain are methylated. Mass spectrometry on endogenously isolated TRF2 indicated that R13 and R17 are dimethylated and R18 is monomethylated. We have reported that the substitution of all eight arginines to lysines, as well as the substitution of the first four or the last four arginines of the basic domain to lysines resulted in a similar fold increase in telomere doublets compared to cells overexpressing an empty vector or wildtype TRF2. In our studies we verified that R17 is dimethylated *in vivo* using an antibody that specifically recognizes this modification. We found that a substitution of R17 to a lysine did not cause a significant difference in telomere doublets compared to the overexpression of wildtype TRF2 (Figure 2.3.1). PRMT1 is known to methylate clusters of arginines present in GAR motifs (Boisvert et al. 2003). Our finding presented in figure 2.3.1 is consistent with the notion that PRMT1 methylates a cluster of arginines present in the GAR motif of TRF2, and that the methylation of multiple arginines in the GAR motif is likely important for telomere maintenance.

Figure 2.3.1

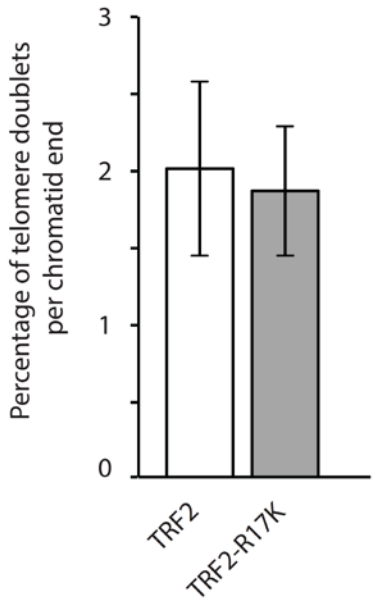


Figure 2.3.1 A Mutation of arginine 17 alone is not sufficient to induce telomere dysfunction

Quantification of telomere doublets in hTERT-BJ cells stably infected with retrovirus expressing TRF2 or TRF2-R17K. A total of 2,120 chromosomes for TRF2 and 1,728 chromosomes for TRF2-R17K were scored. Standard deviations are derived from at least three independent experiments.

2.3.2 Methylation of TRF2 does not change the affinity of TRF2 for telomeric DNA

We observed that in PRMT1 knockdown cells there is an increased enrichment of TRF2 at telomeres, suggesting that the methylation of TRF2 may be a negative regulator of its binding to telomeric DNA (chapter 2.2 Figure 11). We wished to investigate whether methylation of TRF2 had a direct effect on its binding to telomeric DNA sequence. Recombinant His-TRF2 was incubated with GST-PRMT1 in an *in vitro* methylation assay and then used for gel shift assays. TRF2 is only methylated when PRMT1 is present in the methylation reaction (Chapter 2.2.6 Figure 5C) We found that there was no distinguishable difference in the gel shift binding of TRF2 from the methylation assays in which PRMT1 was either present or absent (Figure 2.3.2). These results suggest that methylation of TRF2 does not directly affect its binding to telomeric DNA.

Figure 2.3.2

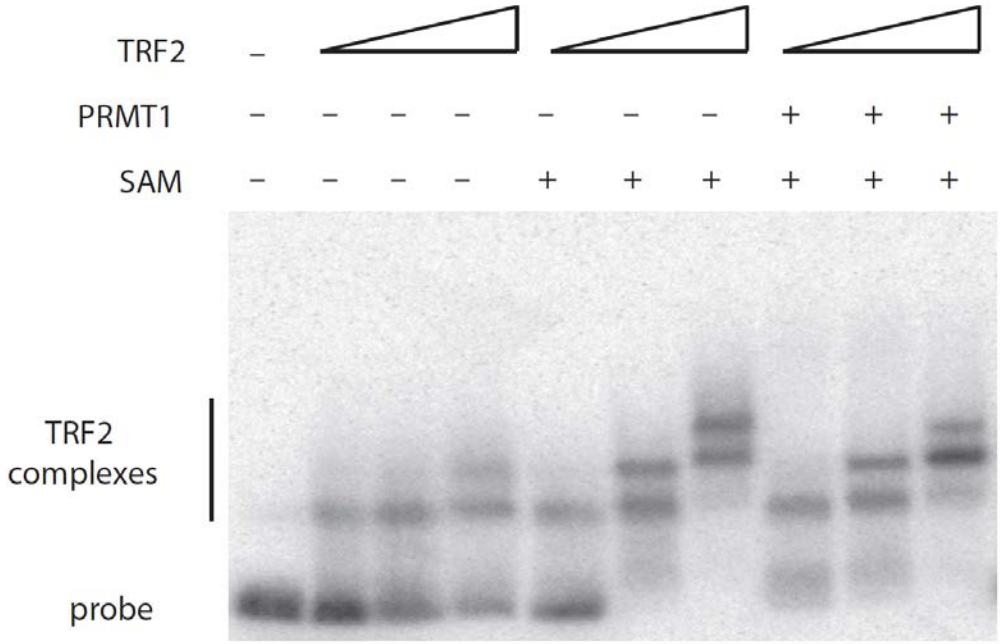


Figure 2.3.2 Methylation of TRF2 by PRMT1 does not change its affinity for telomeric DNA

The Gel shift assay using TRF2 from *in vitro* methylation assays. Recombinant His-TRF2 was subject to an *in vitro* methylation assay containing the components as indicated above each lane. TRF2 (50 ng, 100 ng or 200 ng) from the methylation assay was then used for a gel shift assay. The three distinct complexes represent whether 1, 2 or 3 TRF2 dimers have bound to the telomeric DNA substrate.

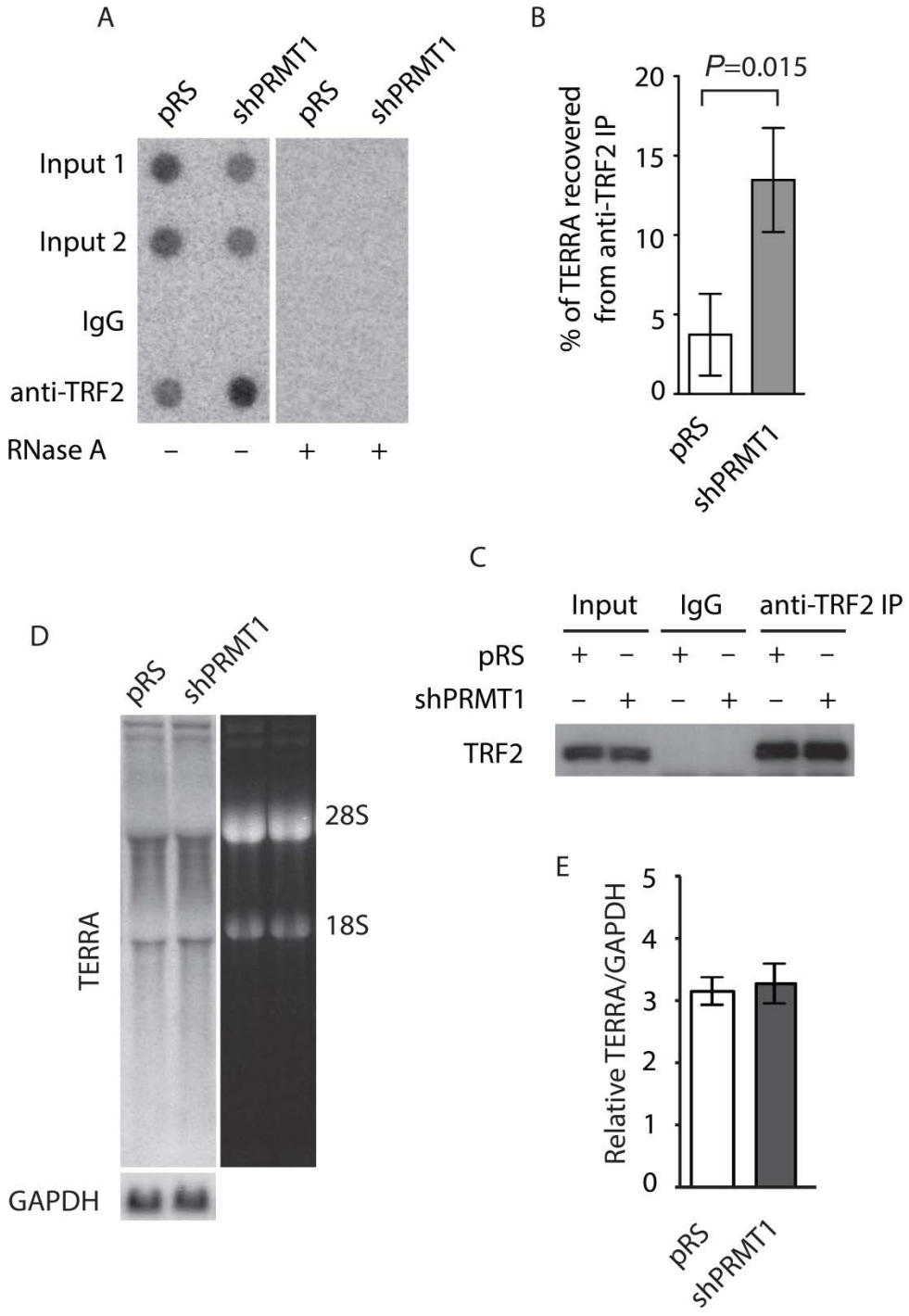
2.3.3 Methylation of the basic domain decreases binding to TERRA

RNA has a strong preference for interacting with arginine or lysine residues (Blackwell, Ceman 2012). It is therefore not surprising that the arginine rich basic domain of TRF2 binds TERRA (Deng et al. 2009). Arginine methylation has been shown to affect the ability of proteins to bind RNA, both positively and negatively (Blackwell, Ceman 2012). TRF2 forms a complex at telomeres with TERRA (Deng et al. 2009). To assess whether methylation might affect TRF2 binding to TERRA we performed RNA-IP on PRMT1 knockdown and vector control cells. Immunoprecipitation with TRF2 antibody, but not IgG immunoglobulin brought down TERRA, which was increased three fold in PRMT1 knockdown cells compared to the control cell line (Figure 2.3.3A, B). The difference in TERRA enrichment was not due to a change in the level of or immunoprecipitation efficiency of TRF2 in these cells (Figure 2.3.3C). Likewise, there was no significant change in TERRA levels in PRMT1 knockdown cells compared to the vector expressing cells (Figure 2.3.3D, E). These results suggest that methylation of the basic domain is a negative regulator of the TRF2/TERRA complex. These results are in agreement with our previous finding that the knockdown of PRMT1 increases the enrichment of TRF2 at telomeres. These results further support the notion that methylated TRF2 is not predominantly localized to telomeres.

Figure 2.3.3 Methylation of the basic domain decreases TRF2 binding to TERRA

A) RNA immunoprecipitation of TRF2 and TERRA. 293T cells expressing empty vector (pRS) or knocked down for PRMT1 (shPRMT1) were harvested in RIPA buffer plus the RNase inhibitor vanadyl ribonucleoside. Immunoprecipitations were carried out with anti-TRF2 or IgG control. Half of the IP was treated with RNase A prior to loading and the blots were probed with a radiolabeled C-rich telomere sequence. B) Quantification of TERRA recovered in TRF2-IP. Analysis was carried out using ImageQuant. The amount in the TRF2-IP was normalized to the amount in the inputs (2.5%) for each cell line. The experiment was performed in triplicates. C) Immunoblotting was used to determine the input levels and IP efficiency of TRF2. Immunoblotting was done using anti-TRF2. D) Northern blot of cells expressing empty vector (pRS) or PRMT1 knock-down. Northern blotting was done using a radiolabeled C-rich telomere fragment (top left). The blot was stripped and probed for GAPDH, which was used as a loading control (bottom left). The ethidium bromide stained gel showing the position of the 18S and 28S ribosomal RNA is shown on the right. (E) Quantification of relative TERRA levels from D). The northern blot was quantified using ImageQuant analysis. The TERRA signal from each lane was normalized against GAPDH to obtain the relative TERRA/GAPDH ratio. The standard deviations come from three independent experiments.

Figure 2.3.3


A
pRS
shPRMT1
pRS
shPRMT1
Input 1
Input 2
IgG
anti-TRF2
RNase A
– – + +
B
P=0.015
% of TERRA recovered from anti-TRF2 IP
20
15
10
5
0
pRS
shPRMT1
C
Input
IgG
anti-TRF2 IP
pRS
shPRMT1
TRF2
D
pRS
shPRMT1
28S
18S
TERRA
GAPDH
E
Relative TERRA/GAPDH
5
4
3
2
1
0
pRS
shPRMT1

2.3.4 TRF2$^{\Delta M}$ induced fusions are exacerbated by the loss of PRMT1

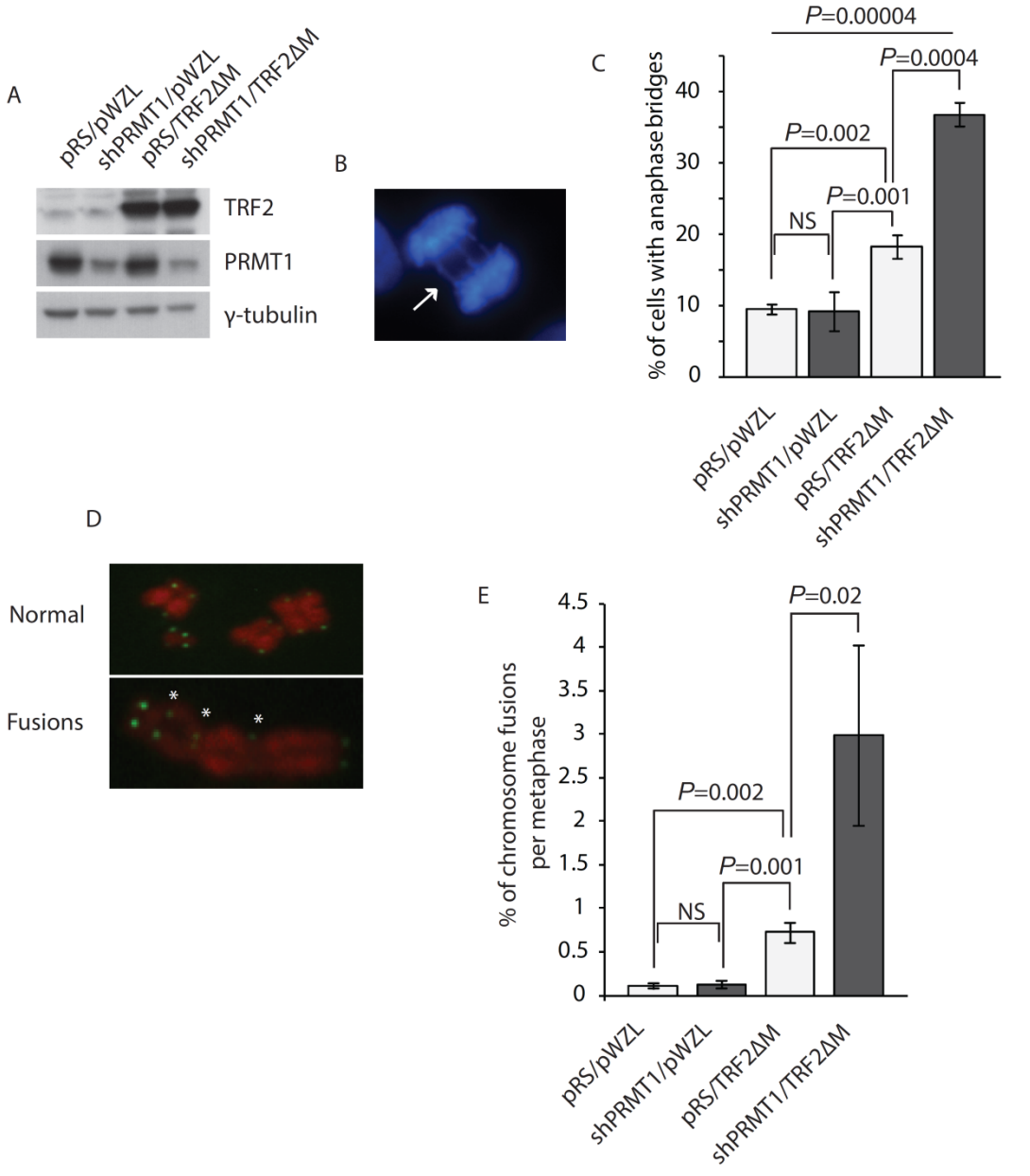
TRF2 requires both its basic domain and Myb-like DNA binding domain in order to properly suppress a DNA damage response at telomere. Deletion of the Myb-like domain alone is sufficient to induce telomere fusions, however TRF2$^{\Delta B\Delta M}$ or TRF2-RK$^{\Delta M}$ have been shown to produce a higher rate of telomere fusions than TRF2$^{\Delta M}$ (Glenfield, 2008). If the loss of arginine methylation in the TRF2-RK$^{\Delta M}$ allele is responsible for the increase in telomere fusions in comparison to TRF2$^{\Delta M}$, then we reasoned that the expression of TRF2$^{\Delta M}$ concurrent with the knockdown of PRMT1 should mimic the TRF2-RK$^{\Delta M}$ phenotype.

We have observed an increase in telomere instability in cells coexpressing the dominant negative allele TRF2$^{\Delta M}$ and a short hairpin RNA against PRMT1 (shPRMT1). Cells expressing TRF2$^{\Delta M}$/shPRMT1 had a two fold increase in anaphase bridges compared to TRF2$^{\Delta M}$/vector cells, whereas cells expressing empty vectors or only knocked down for PRMT1 had only background levels of anaphase bridges (Figure 2.3.4A-C). Anaphase bridges are an indication of fused chromosomes that are being pulled to opposite poles during mitosis (van Steensel, Smogorzewska & de Lange 1998), however they are difficult to differentiate from proteinaceous non-covalently linked chromosomes. Telomere-telomere fusions can be unmistakably distinguished from proteinaceously linked fusion events by metaphase FISH. Analysis of metaphase FISH demonstrated there was a three-fold increase in telomere-telomere fusions in the TRF2$^{\Delta M}$/shPRMT1cells compared to those expressing TRF2$^{\Delta M}$/vector (Figure 2.3.4D and E). These data support our earlier finding that PRMT1 is important for proper telomere maintenance.

Figure 2.3.4 TRF2$^{\Delta M}$ induced fusions are exacerbated by the loss of PRMT1

A) Immunoblot for protein expression. Cell lysates were harvested from HT1080 cells expressing the indicated constructs. Immunoblotting was carried out with anti-TRF2, anti-PRMT1 and anti-γ-tubulin. B) An example of an anaphase bridge. Cells were fixed and stained with DAPI using standard methods. The cells were then scored for the presence of anaphase bridges (arrow). C) Quantification of the percentage of anaphase bridges in each of the indicated cell lines. At least 100 cells were counted in triplicate. Significance was calculated using a two tailed student's t-test. D) An example of telomere-telomere fusions. Metaphase spreads were stained with DAPI and false coloured in red. The telomere ends were detected by hybridization to a PNA probe containing FITC-conjugated $(CCCTAA)_3$. An example of a normal metaphase chromosome (top panel) is shown in comparison to chromosomes that have undergone telomere-telomere fusions (bottom panel). The asterisks indicate telomere fusions. E) Quantification of telomere fusions in HT1080 cells expressing the indicated constructs. pWZL is the vector containing TRF2$^{\Delta M}$ and pRS contains the PRMT1 knockdown sequence. A total of 37 to 47 metaphase spreads were scored for each cell line. Statistical analysis was done using a two tailed student's t-test.

Figure 2.3.4


A
pRS/pWZL
shPRMT1/pWZL
pRS/TRF2ΔM
shPRMT1/TRF2ΔM
TRF2
PRMT1
γ-tubulin
B
C
P=0.00004
P=0.0004
P=0.002
P=0.001
NS
% of cells with anaphase bridges
0
10
20
30
40
pRS/pWZL
shPRMT1/pWZL
pRS/TRF2ΔM
shPRMT1/TRF2ΔM
D
Normal
Fusions
E
P=0.02
P=0.002
P=0.001
NS
% of chromosome fusions per metaphase
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
pRS/pWZL
shPRMT1/pWZL
pRS/TRF2ΔM
shPRMT1/TRF2ΔM

2.3.6 Complementary Data References

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Chapter 3

Methylated TRF2 associates with the nuclear matrix and may serve as a potential biomarker for cellular senescence

3.1 Preface

The results presented in this chapter are the follow up studies to the work performed for chapter 2. The following studies analyze the localization of methylated TRF2 in the nucleus and make observations about changes in its organization with the onset of cellular senescence.

These studies strengthen the link between aging and telomeres and hint that mechanisms exist, that have yet to be fully elucidated, which further connect these two processes.

All of the experiments were performed by me, and the writing and experimental design were a collaborative effort between me and Dr. Zhu.

3.2 Submitted Manuscript

Methylated TRF2 associates with the nuclear matrix and may serve as a potential biomarker for cellular senescence

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Key words: TRF2, arginine methylation, nuclear matrix, cellular senescence.

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3.2.1 Abstract

Methylation of N-terminal arginines of the shelterin component TRF2 is important for cellular proliferation. While TRF2 is found at telomeres, where it plays an essential role in maintaining telomere integrity, little is known about the cellular localization of methylated TRF2. Here we report that the majority of methylated TRF2 is resistant to extraction by high salt buffer and DNase I treatment, indicating that methylated TRF2 is tightly associated with the nuclear matrix. We show that methylated TRF2 drastically alters its nuclear staining as normal human primary fibroblast cells approach and enter replicative senescence. This altered nuclear staining can be suppressed by hTERT and is barely detectable in transformed and cancer cell lines. In addition, we find that dysfunctional telomeres and DNA damage, both of which are potent inducers of cellular senescence, promote the altered nuclear staining of methylated TRF2, which is found to be dependent upon the ATM-mediated DNA damage response. Taken together, these results suggest that the altered nuclear staining of methylated TRF2 may represent ATM-mediated nuclear structural alteration associated with cellular senescence. Our data further imply that methylated TRF2 may serve as a potential biomarker for cellular senescence.

3.2.2 Introduction

Cellular senescence refers to a state of permanent arrest of cell proliferation and it is generally thought to be a response to potentially oncogenic stimuli [1, 2], which include telomere shortening, DNA damage either at telomeres or elsewhere in the genome, strong mitogenic stimuli and epigenomic perturbations. Since it was first described approximately fifty years ago [3], cellular senescence has emerged as an important mechanism linked to both tumor suppression and aging. In most human somatic cells, telomeres shorten with each round of DNA replication, in part because DNA polymerases are unable to fill in the gap generated from removal of the last RNA primer [4]. When telomeres become critically short, they activate a DNA damage response and trigger the induction of cellular senescence [5]. Telomerase, a reverse transcriptase that can replenish the repetitive telomeric DNA *de novo*, is able to circumvent telomere shortening and allows cells to gain unlimited growth potential, a feature associated with cancer. Therefore cellular senescence is widely considered as a tumor suppressive mechanism. In addition, cellular senescence is also implicated in tissue repair and inflammation associated with aging and cancer progression [1, 2].

The nuclear matrix is a filamentous network of protein, DNA and RNA that is refractory to high salt extraction [6-8]. This structure serves as an architectural skeleton to the nucleus and provides support for chromatin organization and various nuclear functions including DNA replication, transcription and DNA repair [8]. Changes in the composition of the nuclear matrix have been observed in senescent cells [9]. Alterations in the nuclear matrix have been implicated in restricting cellular proliferation in mortal

human fibroblasts [10]. Furthermore, nuclei with abnormal nuclear structure are found to accumulate in aged and prematurely senescent cells [11, 12].

Telomeres, heterochromatic structures found at the ends of linear eukaryotic chromosomes, function to protect natural chromosome ends from being recognized as damaged DNA. Mammalian telomeric DNA consists of tandem repeats of TTAGGG and is bound by a six-subunit protein complex [13, 14], referred to as shelterin or telosome, which is composed of TRF1, TRF2, TIN2, POT1, TPP1 and hRap1. Telomeres are attached to the nuclear matrix [15, 16] and the components of the shelterin complex have been reported to be associated with the nuclear envelope as well as the nuclear matrix [10, 17-19]. TIN2L, an isoform of TIN2, has been suggested to mediate the interaction between telomeres and the nuclear matrix [17] whereas hRap1 has been implicated in tethering telomeres to the nuclear envelope [18]. TRF2, a shelterin protein that interacts directly with both TIN2 and hRap1 [20-23], has also been implicated in mediating the interaction between telomeres and the nuclear matrix [19]. However, little is known about the role of post-translational modification in mediating the interaction between shelterin proteins and the nuclear matrix.

TRF2, a subunit of the shelterin complex, binds to duplex telomeric DNA [24, 25] and plays a crucial role in telomere protection [26]. TRF2 contains an N-terminal basic domain rich in glycines and arginines (the GAR domain), a central TRFH dimerization domain, a flexible linker and a C-terminal Myb-like DNA binding domain [24, 25]. It has been shown that loss of TRF2 from telomeres through either TRF2 knockout or

overexpression of a dominant-negative allele of TRF2 promotes the formation of telomere end-to-end fusions [26, 27]. On the other hand, overexpression of TRF2 lacking the basic/GAR domain induces telomere rapid deletion [28] whereas overexpression of TRF2 carrying amino acid substitutions of arginines to lysines in the basic/GAR domain promotes the formation of fragile telomeres [29]. In all aforementioned cases, the formation of dysfunctional telomeres resulting from disruption of TRF2 function results in the induction of cellular senescence [26-29].

TRF2 undergoes extensive post-translational modification [30], which in turn regulates its stability, DNA binding and cellular localization. Ubiquitylation of TRF2 by Siah1, an E3 ligase, promotes TRF2 degradation and replicative senescence of human primary fibroblasts [31]. Acetylation, SUMOlyation and poly(ADP-ribosyl)ation have been implicated in modulating TRF2 binding to telomeric DNA [32-35]. TRF2 is phosphorylated in response to DNA damage and this phosphorylation has been implicated in DNA double strand break repair [36, 37]. Arginines in the N-terminal basic/GAR domain of TRF2 are methylated by protein arginine methyltransferase 1 (PRMT1) [29]. Loss of arginine methylation in TRF2 induces DNA-damage response foci at telomeres and triggers cellular senescence [29]. Arginine methylation is also implicated in negatively regulating the amount of telomere-bound TRF2 [29], raising a question as to whether methylated TRF2 is associated with telomeres *in vivo*.

Here we report that the majority of methylated TRF2 is not released by the treatment with high salt buffer and DNase I digestion and that it co-fractionates with

lamin A, a component of the nuclear matrix [38, 39], suggesting that methylated TRF2 interacts with the nuclear matrix. We find that methylated TRF2 is largely not localized at telomeres, indicating that association of TRF2 with the nuclear matrix is likely independent of telomeres. We demonstrate that methylated TRF2 dramatically changes its nuclear staining as normal human primary fibroblast cells approach and enter replicative senescence. Introduction of hTERT into human primary fibroblast cells suppresses the altered nuclear staining of methylated TRF2, suggesting that progressive telomere shortening may contribute to the altered staining of methylated TRF2 in normal primary fibroblasts. In addition to telomere shortening, dysfunctional telomeres and ionizing radiation-induced DNA damage, both of which are potent inducers of cellular senescence, also promote the altered nuclear staining of methylated TRF2. Furthermore, we show that the lack or inhibition of ATM blocks the formation of IR-induced altered nuclear staining of methylated TRF2, indicating that the formation of the altered nuclear staining of methylated TRF2 is mediated by the ATM-dependent DNA damage response. Taken together, our results reveal that methylated TRF2-associated nuclear matrix undergoes an ATM-mediated structural alteration during the process of cellular senescence. Our data further imply that methylated TRF2 may serve as a potential biomarker for cellular senescence.

3.2.3 Results

Methylated TRF2 is associated with nuclear matrix. We have previously reported that PRMT1 methylates arginines in the N-terminal GAR domain of TRF2 and

that this arginine methylation negatively regulates TRF2 association with telomere chromatin [29], suggesting that methylated TRF2 is not associated with telomeres *in vivo*. To investigate the nuclear compartmentalization of methylated TRF2, we first subjected hTERT-immortalized BJ (hTERT-BJ) cells to analysis of sequential extraction of the nuclear matrix, which began with the treatment of cells with CSK buffer to remove the majority of soluble proteins. The treatment with RSB-magik buffer further removed the cytoskeleton, leaving behind the nuclei and their attached filaments. Digestion of the nuclei with DNase I released chromatin bound proteins, and the DNase I-resistant pellet was then further fractionated to release the outer nuclear matrix components by 2M NaCl. Treatment with RNase A disassembled ribonucleoproteins, and the final insoluble pellet contained the core nucleofilament proteins. The DNaseI-resistant fractions including the pellet are referred to as the nuclear matrix-associated fractions.

Examination of cell fractionations of hTERT-BJ with anti-Lamin A antibody revealed that Lamin A, a nuclear matrix-associated protein [38, 39], was predominantly found in the final pellet as well as fractions treated with 2M NaCl and RNase A (Fig. 1A), in agreement with these DNase I-resistant fractions being nuclear matrix. On the other hand, we found that the majority of chromatin-bound histone H2AX protein was released by DNase I digestion (Fig. 1A), suggesting that the sequential cell fractionation protocol was working as expected. A very small amount of PRMT1 was detected in fractions associated with the nuclear matrix, consistent with previous findings [40]. In addition, a small amount of shelterin proteins including TRF1, TRF2 and hRap1 was also found in

fractions associated with nuclear matrix (Fig. 1A), indicative of the nuclear matrix association of the shelterin proteins.

We have previously raised an antibody specifically against TRF2 methylated at R17 (anti-2meR17) [29], which specifically recognized methylated TRF2 (Fig. 1B) but not TRF2 carrying amino acid substitutions of arginines to lysines in its N-terminal domain (TRF2-RK) or lacking its N-terminal domain (TRF2-ΔB) (Fig. 1C). Using anti-2meR17 antibody, we found that although some methylated TRF2 was released by DNase I digestion, the majority of methylated TRF2 was recovered in nuclear matrix-associated fractions (Fig. 1A). The association of methylated TRF2 with the nuclear matrix was also observed in two other primary fibroblasts IMR90 and GM9503 cells as well as cancer cell line HeLaI.2.11 (Fig. 1D-1F). Taken together, these results suggest that methylated TRF2 preferentially associates with the nuclear matrix.

Methylated TRF2 exhibits nuclear staining that is predominantly not associated with human telomeres. To further investigate the nuclear localization of methylated TRF2, we performed indirect immunofluorescence with anti-2meR17. We found that methylated TRF2 exhibited nuclear staining in both human primary (IMR90, GM9503) and cancer (HeLaI.2.11) cells (Fig. 2A). Analysis of dual indirect immunofluorescence with anti-2meR17 in conjunction with antibody against TRF1 [41], a marker for interphase telomeres, revealed that although there appeared to be some overlap between anti-2meR17 staining and anti-TRF1 staining (Fig. 2B), the majority of

methylated TRF2 was not found to localize at telomeres (Fig. 2B). Similarly, there was only occasional overlap of foci seen by immuno-FISH using a FITC-conjugated $(CCCTAA)_3$ PNA probe to visualize telomeric DNA along with anti-me2R17 (Fig. 2C). To investigate whether the observed anti-2meR17 staining might be due to any non-specific binding, we performed indirect immunofluorescence with anti-2meR17 antibody in the presence of TRF2 peptide containing either unmodified R17 or dimethylated R17. We found that while TRF2 peptide containing unmodified R17 had little effect on the nuclear staining of methylated TRF2 in these cell lines (Fig. 2D and data not shown), TRF2 peptide containing dimethylated R17 abrogated the nuclear staining of methylated TRF2 (Fig. 2D and data not shown). These results suggest that the observed anti-2meR17 staining is unlikely due to a non-specific binding. Taken together, these results suggest that methylated TRF2 localizes in nuclear domains largely free of human telomeres. These results are in agreement with our previous report that arginine methylation negatively regulates TRF2 association with telomere chromatin [29].

Methylated TRF2 is associated with the altered nuclear structure induced by replicative senescence. We have shown that methylated TRF2 is associated with the nuclear matrix, which is known to undergo alterations in the process of replicative senescence [42]. To investigate whether the association of methylated TRF2 with the nuclear matrix might be affected by cellular senescence, we performed the sequential extraction of nuclear matrix in both early and late passages of primary skin fibroblast

GM9503 cells. GM9503 cells typically entered replicative senescence at passage 45 (Fig. 3A). Analysis of cell fractionations with anti-2meR17 antibody revealed that the association of methylated TRF2 with the nuclear matrix in senescent GM9503 (p45) cells was indistinguishable from that in young GM9503 (p21) cells (Fig. 1E), suggesting that methylated TRF2 does not dissociate from the nuclear matrix during the process of senescence.

We also examined the nuclear staining of methylated TRF2 in senescent GM9503 cells. Analysis of indirect immunofluorescence with anti-2meR17 antibody revealed that although in young GM9503 (p15) cells methylated TRF2 showed a rather homogeneous nuclear staining (Fig. 3B), its staining in senescent GM9503 cells (p45) was drastically altered (Fig. 3B), resembling abnormal nuclear structures previously described to be associated with cellular senescence [11, 12, 42]. We detected an over 10-fold induction in the formation of altered TRF2 staining in senescent GM9503 (p45) cells compared to that in young GM9503 (p15) cells (Fig. 3C). To investigate whether this altered nuclear staining might be due to any non-specific binding of anti-2meR17 antibody in senescent cells, we performed indirect immunofluorescence with anti-2meR17 antibody in the presence of TRF2 peptide containing either unmodified R17 or dimethylated R17. We found that TRF2 peptide containing unmodified R17 had little effect on the altered nuclear staining of methylated TRF2 whereas TRF2 peptide containing dimethylated R17 abolished TRF2 staining in senescent cells (Fig. 3D), arguing against the possibility that

the observed altered nuclear staining of methylated TRF2 is due to a non-specific binding of the anti-2meR17 antibody.

To investigate whether the altered nuclear staining of methylated TRF2 might be a general feature associated with replicative senescence, we examined methylated TRF2 staining in several other human normal primary fibroblast cells. The primary fibroblast AG02261 cells entered replicative senescence after 45 passages (Fig. 3E). We found that senescent AG02261 cells exhibited a 3-fold induction in the altered nuclear staining of methylated TRF2 compared to AG02261 cells at p17 (Fig. 3C and 3F). The induction of this altered staining of methylated TRF2 was also observed in late passage IMR90 cells and GM1706 cells (Fig. 3G and 3H). On the other hand, we did not detect any significant accumulation of the altered staining of methylated TRF2 in cancer and transformed cell lines including HeLa and WI38VA13/2RA (Fig. 3C). These results suggest that the altered nuclear staining of methylated TRF2 is a characteristic associated with replicative senescence.

It has been reported that senescent and aged cells are associated with distorted nuclear defects [11, 12, 42]. To investigate whether the altered staining of methylated TRF2 might represent a distorted nuclear structure, we performed dual indirect immunofluorescence with anti-2meR17 antibody in conjunction with an antibody against Lamin A, a marker for distorted nuclear defects in aged cells [11, 12]. We observed an overlap between altered TRF2 staining and anti-Lamin A staining in senescent GM9503

(p45) cells (Fig. 3I). These results further support the notion that methylated TRF2 is tightly bound to the nuclear matrix.

Overexpression of hTERT prevents the formation of senescence-induced altered nuclear staining of methylated TRF2. Overexpression of hTERT can prevent replicative senescence, resulting in immortalization of normal primary fibroblasts [43, 44]. To investigate whether hTERT may repress the formation of the altered nuclear staining of methylated TRF2, we introduced hTERT into GM9503 cells of passage 38 (p38) and cultured hTERT-GM9503 cells in parallel with parental GM9503 cells (p38) until GM9503 cells entered replicative senescence at p47 (Fig. 4A). Exogenous expression of hTERT was sufficient to immortalize GM9503 cells and prevented telomere shortening associated with replicative senescence (Fig. 4B). Analysis of indirect immunofluorescence with anti-2meR17 antibody revealed an induction of an altered nuclear staining of methylated TRF2 in GM9503 cells at passage 47 compared to those at passage 39 (Fig. 4C and 4D), consistent with our earlier finding. On the other hand, no increase in the altered nuclear staining of methylated TRF2 was observed in hTERT-immortalized GM9503 cells. Instead, we detected a 30% decrease (P=0.005) in the number of hTERT-GM9503 cells with an altered nuclear staining of methylated TRF2 when compared to GM9503 cells of passage 39 (Fig. 4D). These results suggest that overexpression of hTERT suppresses the formation of replicative senescence-induced altered nuclear staining of methylated TRF2.

Dysfunctional telomeres induce the formation of the altered nuclear staining of methylated TRF2. In addition to programmed telomere shortening, dysfunctional telomeres resulting from disruption of TRF2 function can also induce cellular senescence [26, 28, 29]. It has been well documented that overexpression of TRF2 lacking the N-terminal basic/GAR domain (TRF2-ΔB) promotes telomere rapid deletion whereas overexpression of TRF2 lacking both the N-terminal basic/GAR and C-terminal Myb-like DNA binding domains (TRF2-ΔBΔM) induces the formation of telomere fusions, both of which result in the induction of cellular senescence [26, 28]. Overexpression of TRF2 carrying amino acid substitutions of arginines to lysines in its N-terminal domain (TRF2-RK) has been shown to induce fragile telomeres, triggering cellular senescence [29]. To investigate whether the altered nuclear staining of methylated TRF2 might be associated with cellular senescence induced by dysfunctional telomeres, we infected GM9503 (p23) cells with retrovirus expressing TRF2-RK, TRF2-ΔB, TRF2-ΔBΔM or the vector alone. Fourteen days post infection (Fig. 5A), we observed an induction of cellular senescence in GM9503 cells overexpressing either TRF2-RK, TRF2-ΔB or TRF2-ΔBΔM but not in GM9503 cells expressing the vector alone (Fig. 5B), consistent with previous reports [26, 28, 29]. Analysis of indirect immunofluorescence revealed a drastic accumulation of the altered nuclear staining of methylated TRF2 in GM9503 cells overexpressing either TRF2-RK, TRF2-ΔB or TRF2-ΔBΔM when compared to GM9503 cells expressing the vector alone (Fig. 5C and 5D). Overexpression of TRF2-RK, TRF2-ΔB or TRF2-ΔBΔM

had little effect on the level of endogenous methylated TRF2 (Fig. 5E). These results suggest that the altered nuclear staining of methylated TRF2 is associated with cellular senescence induced by dysfunctional telomeres.

DNA damage induces the formation of the altered nuclear staining of methylated TRF2 in an ATM-dependent manner. Oncogene-induced cellular senescence is known to be a DNA damage response [45] and therefore we asked whether the altered nuclear staining of methylated TRF2 might be associated with DNA damage-induced senescence. To address this question, GM9503 cells (p23) were either mock-treated or treated with 12 Gy IR. We found that 48 h post IR, all GM9503 cells entered cellular senescence as evidenced by analysis of senescence-associated β-galactosidase assays (Fig. 6A). Analysis of indirect immunofluorescence with anti-2meR17 revealed an over 3-fold induction in the number of GM9503 cells with the altered staining of methylated TRF2 48 h post 12 Gy IR (Fig. 6B and 6C). We did not detect any significant change in the level of methylated TRF2 (Fig. 6D). Collectively, these results suggest that the altered nuclear staining of methylated TRF2 is associated with DNA damage-induced cellular senescence.

ATM is a master regulator of DNA damage response following ionizing radiation [46, 47] and therefore we also examined whether IR-induced altered nuclear staining of methylated TRF2 might be dependent upon the ATM-mediated DNA damage response. GM9503 cells (p23) were treated with DMSO or KU55933, a potent and specific ATM

inhibitor [48], prior to 12 Gy IR. Analysis of indirect immunofluorescence with anti-2meR17 antibody revealed that treatment with KU55933 impaired the induction of IR-induced altered nuclear staining of methylated TRF2 (Fig. 6E). The impairment of IR-induced altered nuclear staining of methylated TRF2 was also observed in primary ataxia telangiectasia (AT) fibroblast cells (AT2RO) lacking functional ATM (Fig. 6F). Treatment with KU55933 or loss of ATM did not affect the association of methylated TRF2 with the nuclear matrix (Fig. 6G and 6H). Taken together, these results suggest that ATM is important for the formation of IR-induced altered nuclear staining of methylated TRF2.

3.2.4 Discussion

In this report, we have shown that methylated TRF2 is associated with the nuclear matrix and that this localization is largely free of human telomeres. In addition we have uncovered that the nuclear staining of methylated TRF2 is drastically altered upon induction of cellular senescence, suggesting that methylated TRF2 may serve as a potential biomarker for cellular senescence.

Through sequential extraction of the nuclear matrix, we have shown that the nuclear matrix contains a small amount of endogenous TRF1, TRF2 and hRap1, suggesting that components of the shelterin complex are associated with the nuclear matrix, in agreement with previous reports [10, 17]. We have previously estimated that about 1-5% of endogenous TRF2 is methylated by PRMT1 and that PRMT1 negatively

regulates TRF2 interaction with telomere chromatin [29]. Our finding that the majority of methylated TRF2 is associated with the nuclear matrix suggests that arginine methylation plays an important role in regulating the crosstalk between telomeres and the nuclear matrix. In addition, we have demonstrated that overexpression of TRF2-RK carrying amino acid substitutions of arginines to lysines (TRF2-RK), which cannot undergo PRMT1-dependent arginine methylation [29], induces cellular senescence, consistent with a previous report [29]. Overexpression of TRF2-RK has been shown to promote the formation of telomeres with multiple telomere signals [29], also known as fragile telomeres [49, 50], which are thought to arise from a defect in telomere replication. Taken together, these findings suggest that the arginine methylation-dependent TRF2 interaction with the nuclear matrix may be important for supporting efficient telomere replication.

Although we have shown that methylated TRF2 co-fractionates with Lamin A, we have not been able to detect a direct interaction between TRF2 and Lamin A (T. Mitchell and X.-D. Zhu, unpublished data), suggesting that the association of methylated TRF2 with the nuclear matrix may not be mediated by Lamin A. While human telomeres are attached to the nuclear matrix [16], we have shown through indirect immunofluorescence that the nuclear staining of methylated TRF2 is largely free of telomere signals, suggesting that methylated TRF2 interaction with the nuclear matrix is unlikely mediated through telomeric DNA. How methylated TRF2 interacts with the nuclear matrix remains unknown, but our finding nevertheless suggests that post-translational modification plays a role in regulating TRF2 interaction with the nuclear matrix.

We have shown that methylated TRF2 exhibits an altered nuclear staining upon induction of replicative senescence in normal human primary fibroblasts and that this altered staining can be suppressed by overexpression of hTERT, suggesting that telomere erosion may be a trigger for the altered nuclear staining of methylated TRF2. It has been suggested that IR- and oncogene-induced cellular senescence is caused by irreparable DNA damage at telomeres [51, 52]. Our observation that the altered nuclear staining of methylated TRF2 is associated with IR- and dysfunctional telomeres-induced cellular senescence further supports the notion that telomere damage resulting from either genotoxic insults or programmed telomere erosion is a major inducer of the altered nuclear staining of methylated TRF2.

It has been well established that the ATM-dependent DNA damage response is needed for initiating and maintaining cellular senescence [53-56]. Consistent with previous reports [53-56], we have observed that primary AT2RO fibroblasts lacking functional ATM fail to undergo cell cycle arrest and start to die within days after ionizing radiation (T.R.H Mitchell and X.-D. Zhu, unpublished data). We have demonstrated that loss or inhibition of ATM abrogates IR-induced altered nuclear staining of methylated TRF2, indicating that the ATM-dependent DNA damage response is needed for the formation of the altered nuclear staining of methylated TRF2. ATM has been implicated in regulating the nuclear matrix attachment of human telomeres [57, 58], raising a possibility that the altered telomere attachment to the nuclear matrix might be a underlying cause for the altered nuclear staining of methylated TRF2. Furthermore, we

have shown that the altered nuclear staining of methylated TRF2 is associated with distorted nuclear structures in senescent cells. Taken together, these findings suggest that methylated TRF2 may serve as a potential biomarker for aging- and oncogene-induced cellular senescence.

3.2.5 Methods

DNA constructs and antibodies. The retroviral construct expressing shRNA against PRMT1 (pRS-shPRMT1) has been previously described [29]. The retroviral construct expressing hTERT (pBabe-hTERT) was generously provided by Robert Weinberg, Whitehead Institute for Biomedical Research.

Antibody specifically raised against TRF2 methylated at R17 (2meR17) has been previously described [29]. Antibodies against TRF1, TRF2 and hRap1 were kind gifts from Titia de Lange, Rockefeller University. Other antibodies used include Lamin A (Millipore), PRMT1 (Millipore) and H2A.X (Upstate).

Cell culture and retroviral infection. Cells were grown in DMEM medium with 10% fetal bovine serum (FBS) for HeLaI.2.11, 293T, WI38VA13/2RA, hTERT-BJ and Phoenix cells supplemented with non-essential amino acids, L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Supplementary DMEM medium plus 15% FBS was used to culture normal primary human fibroblasts (IMR90, GM9503, AG02261 and

GM1706) (Coriell) and ATM-deficient primary fibroblast AT2RO (a kind gift from Jan Hoeijmakers). For inhibition of ATM, cells were treated with KU55933 (20 μM, Sigma) for 3 h before 12 Gy IR treatment. Ionizing radiation was delivered from a Cs-137 source at McMaster University (Gammacell 1000).

Retroviral gene delivery was carried out essentially as described [29, 59]. Phoenix amphotropic retroviral packaging cells were transfected with the desired DNA constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer. At 36, 48, 60, 72, and 84 h post-transfection, the virus-containing medium was collected and used to infect cells in the presence of polybrene (4 μg/ml). Twelve hours after the last infection, puromycin (2 μg/ml) was added to the medium, and the cells were maintained in the selection media for the entirety of the experiments.

Sequential extraction of nuclear matrix. Extraction of nuclear matrix components was conducted essentially as described [60, 61]. Briefly, PBS-washed cells were resuspended in 5X pellet volume cytoskeleton (CSK) buffer (10 mM Pipes pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 2 mM vanadyl ribonucleoside complex, 1.2 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 μg/ml pepstatin and 0.5% Triton X-100). Following centrifugation at 1000g for 5 min, the cytoskeleton framework was further extracted by incubating the pellet in RSB-magik buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 2 mM vanadyl ribonucleoside complex, 1.2 mM phenylmethylsulfonyl fluoride, 1

mM DTT, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 10 µg/ml pepstatin, 1% Tween 20 and 0.5% sodium deoxycholate) for 5 min. Upon centrifugation, the pellet was treated with 30-50 U of RNase-free DNaseI (Fermentas) per 10^6 cells in digestion buffer (10 mM Pipes pH6.8, 50 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 2 mM vanadyl ribonucleoside complex, 1.2 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 10 µg/ml pepstatin and 0.5% Triton X-100) for 1 h at room temperature. Chromatin was then removed by elution with 0.25 M ammonium sulfate, leaving a complete nuclear matrix-intermediate filament scaffold containing nuclear ribonuclear-protein complexes [60]. The complete nuclear matrix was further extracted with 2M NaCl to release the outer nuclear matrix proteins, and in some cases followed by digestion with DNase-free RNase A to remove the core filaments of the matrix. All incubations and centrifugations were performed at 4 ^oC except where indicated.

Immunofluorescence. Immunofluorescence was performed as described [29, 62, 63]. All cell images were recorded on a Zeiss Axioplan 2 microscope with a Hammamatsu C4742-95 camera and processed using the Openlab software package.

Telomere length analysis and senescence-associated β-galactosidase assays. For telomere length analysis, genomic DNA isolated from cells was digested with *Rsa*I

and *Hinf*I and loaded onto a 0.7% agarose gel in 0.5 X TBE. Blotting for telomeric fragments was carried out as described [64, 65].

Senescence-associated (SA) β-galactosidase assays were carried out using the SA-β-gal senescence kit (Cell Signaling) according to the manufacturer's instructions. The cells were seeded two to four days prior to processing.

Statistical Analysis. A student's two-tailed t test was used to derive all *P* values.

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3.2.7 Figures and Figure Legends

Figure 1. Methylated TRF2 is associated with the nuclear matrix. (**A**) Sequential extraction of the nuclear matrix from hTERT-BJ cells. Immunoblotting was performed with anti-TRF2-2meR17, anti-TRF2, anti-hRap1, anti-TRF1, anti-Lamin A, anti-H2AX, or anti-PRMT1 antibody. (**B**) Western analysis of 293T cells expressing shPRMT1 or the vector alone. Immunoblotting was performed with anti-PRMT1, anti-TRF2-2meR17 or anti-TRF2 antibody. The γ-tubulin blot was used a loading control. (**C**) Western analysis of 293T cells overexpressing Myc-tagged wild type TRF2, TRF2 carrying amino acid substitutions of arginines to lysines (TRF2-RK) or TRF2 lacking the N-terminal GAR/basic domain (TRF2-ΔB). Immunoblotting was carried out with anti-TRF2-2meR17 or anti-Myc antibody. (**D**) Sequential extraction of the nuclear matrix from IMR90 cells. Immunoblotting was performed with anti-TRF2-2meR17, anti-TRF2, anti-Lamin A or anti-H2AX antibody. (**E**) Sequential extraction of the nuclear matrix from GM9503 cells. Immunoblotting was performed with anti-TRF2-2meR17, anti-TRF2 or anti-Lamin A antibody. (**F**) Sequential extraction of the nuclear matrix from HeLaI.2.11 cells. Immunoblotting was performed with anti-TRF2-2meR17, anti-TRF2, anti-Lamin A or anti-H2AX antibody.

Figure 1

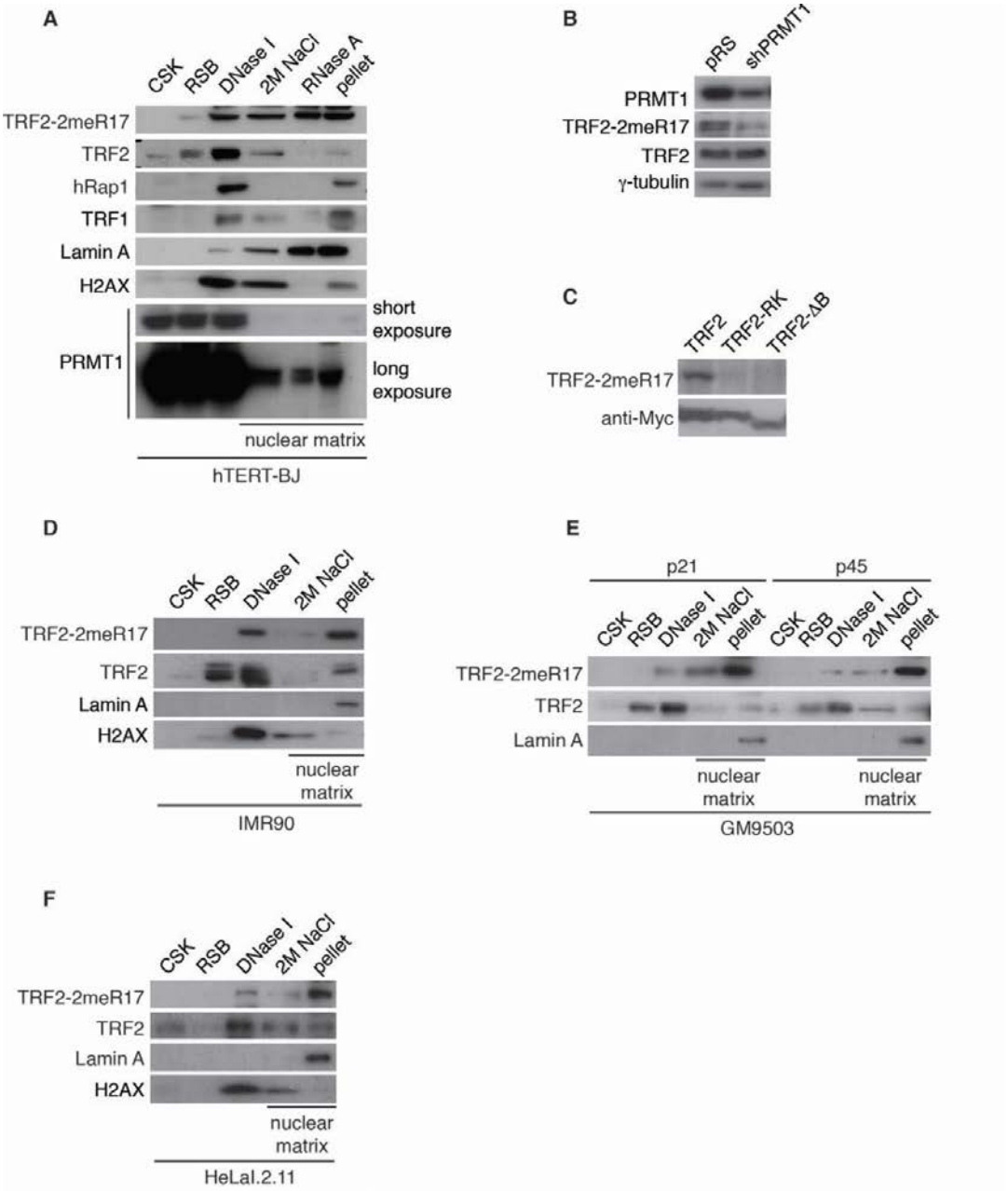

A
CSK
RSB
DNase I
2M NaCl
RNase A
pellet
TRF2-2meR17
TRF2
hRap1
TRF1
Lamin A
H2AX
PRMT1
short exposure
long exposure
nuclear matrix
hTERT-BJ
B
pRS
shPRMT1
PRMT1
TRF2-2meR17
TRF2
γ-tubulin
C
TRF2
TRF2-RK
TRF2-ΔB
TRF2-2meR17
anti-Myc
D
CSK
RSB
DNase I
2M NaCl
pellet
TRF2-2meR17
TRF2
Lamin A
H2AX
nuclear matrix
IMR90
E
p21
p45
CSK
RSB
DNase I
2M NaCl
pellet
TRF2-2meR17
TRF2
Lamin A
nuclear matrix
nuclear matrix
GM9503
F
CSK
RSB
DNase I
2M NaCl
pellet
TRF2-2meR17
TRF2
Lamin A
H2AX
nuclear matrix
HeLaI.2.11

Figure 2. Methylated TRF2 exhibits nuclear staining largely free of human telomeres. (**A**) Analysis of indirect immunofluorescence of three different cell lines with anti-TRF2-2meR17 antibody. Cell nuclei were stained with DAPI in blue. (**B**) Analysis of dual indirect immunofluorescence with anti-TRF2-2meR17 (green) in conjunction with anti-TRF1 antibody (red). HeLaI.2.11 cell nuclei were stained in blue. (**C**) Immuno-FISH on HeLa and BJ cells using anti-2meR17 (red) and FITC-conjugated PNA probe containing telomere sequence (green). The DNA was stained with DAPI in blue. (**D**) Analysis of indirect immunofluorescence with anti-TRF2-2meR17 in conjunction with 100 ng of TRF2 peptide containing either modified or unmodified arginine 17. HeLaI.2.11 cell nuclei were stained with DAPI in blue.

Figure 2

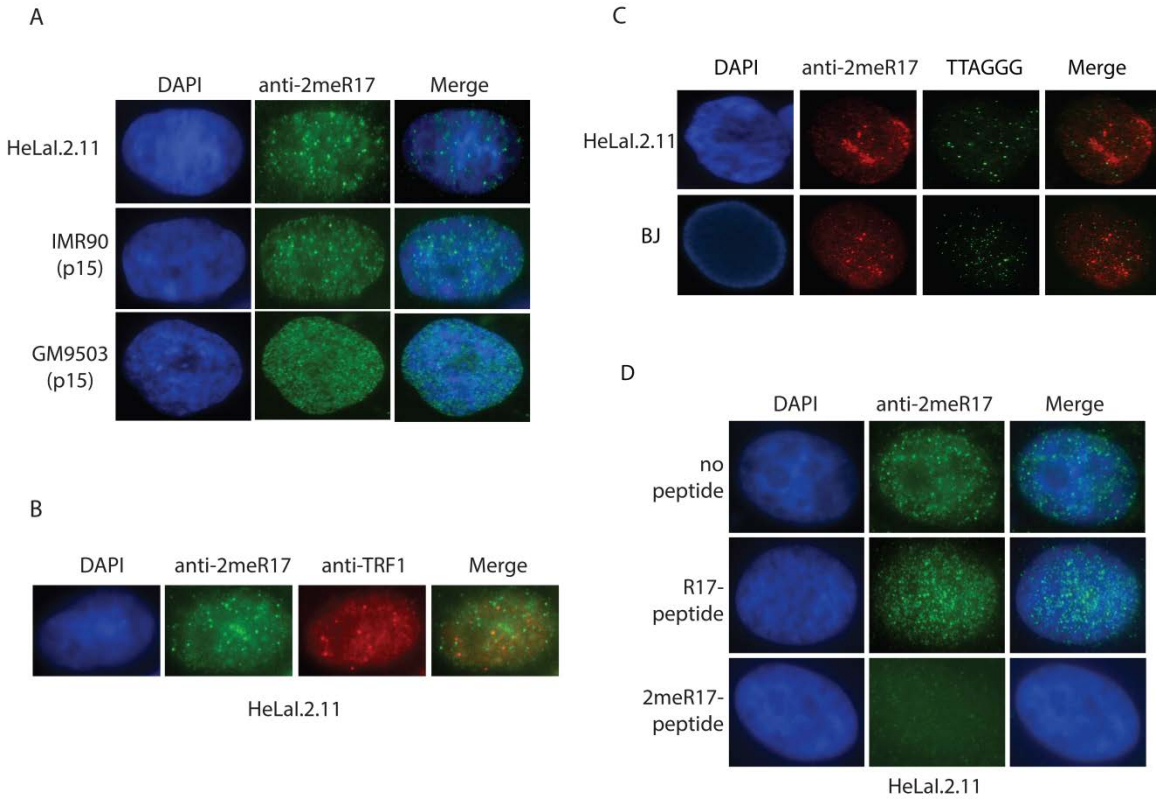

A
DAPI
anti-2meR17
Merge
HeLaI.2.11
IMR90 (p15)
GM9503 (p15)
B
DAPI
anti-2meR17
anti-TRF1
Merge
HeLaI.2.11
C
DAPI
anti-2meR17
TTAGGG
Merge
HeLaI.2.11
BJ
D
DAPI
anti-2meR17
Merge
no peptide
R17-peptide
2meR17-peptide
HeLaI.2.11

Figure 3. Methylated TRF2 exhibits an altered nuclear staining associated with induction of replicative senescence. (**A**) Senescence-associated β-galactosidase assays for GM9503 cells at either p15 or p45. (**B**) Analysis of indirect immunofluorescence with anti-TRF2-2meR17 antibody in GM9503 cells at either p15 or p45. Cell nuclei were stained with DAPI in blue. (**C**) Quantification of percentage of cells with altered nuclear staining of methylated TRF2. At least 900 cells in triplicate were scored in blind for each transformed cell line or each normal primary fibroblast cell line at a given passage as indicated. Both HeLa and WI38VA13 are transformed cell lines whereas GM9503 and AG02261 are normal primary fibroblasts. Standard deviations from three independent experiments are indicated. (**D**) Analysis of indirect immunofluorescence with anti-TRF2-2meR17 in conjunction with 100 ng of TRF2 peptide containing either modified or unmodified arginine 17. Cell nuclei were stained in DAPI in blue. (**E**) Senescence-associated β-galactosidase assays for AG02261 cells at either p15 or p45. (**F**) Analysis of indirect immunofluorescence with anti-TRF2-2meR17 antibody in AG02261 cells at either p15 or p45. Cell nuclei were stained with DAPI in blue. (**G**) Quantification of percentage of normal primary fibroblast GM1706 cells with altered nuclear staining of methylated TRF2. A total of at least 900 cells in triplicate were scored in blind for each passage as indicated. Standard deviations from three independent experiments are indicated. (**H**) Quantification of percentage of normal primary fibroblast IMR90 cells with altered nuclear staining of methylated TRF2. A total of at least 900 cells in triplicate were scored in blind for each passage as indicated. Standard deviations from three independent experiments are indicated. (**I**) Analysis of dual indirect immunofluorescence in GM9503 (p45) with anti-TRF2-2meR17 antibody in conjunction with anti-Lamin A antibody. Cell nuclei were stained with DAPI in blue.

Figure 3

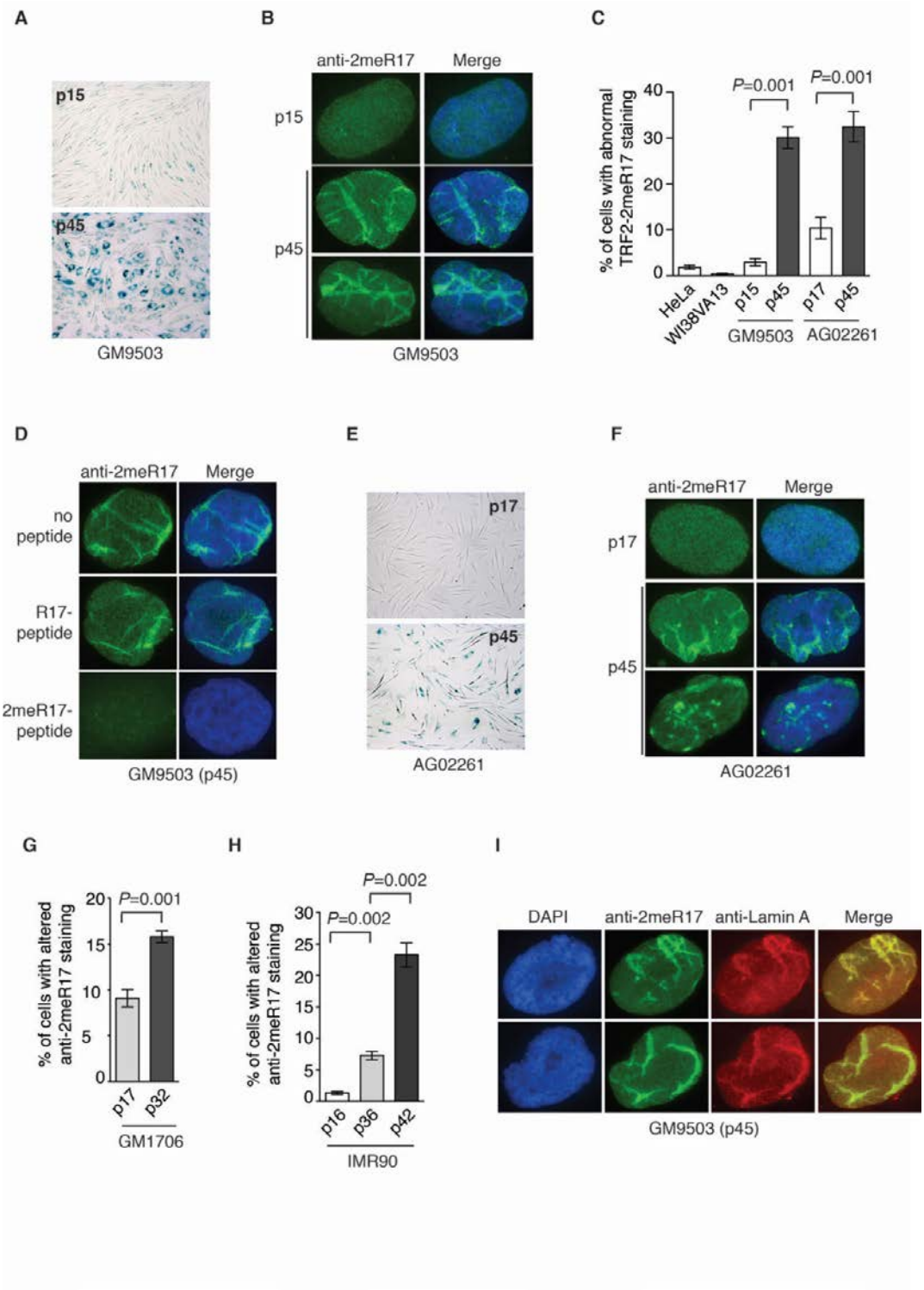

A
p15
p45
GM9503
B
anti-2meR17
Merge
p15
p45
GM9503
C
% of cells with abnormal TRF2-2meR17 staining
P=0.001
P=0.001
HeLa
WI38VA13
p15
p45
p17
p45
GM9503
AG02261
D
anti-2meR17
Merge
no peptide
R17-peptide
2meR17-peptide
GM9503 (p45)
E
p17
p45
AG02261
F
anti-2meR17
Merge
p17
p45
AG02261
G
% of cells with altered anti-2meR17 staining
P=0.001
p17
p32
GM1706
H
P=0.002
P=0.002
% of cells with altered anti-2meR17 staining
p16
p36
p42
IMR90
I
DAPI
anti-2meR17
anti-Lamin A
Merge
GM9503 (p45)

Figure 4. Introduction of hTERT into normal primary fibroblast cells suppresses the formation of senescence-associated altered nuclear staining of methylated TRF2. (**A**) Schematic diagram of the experimental setup. At day 0, GM9503 cells were infected with retrovirus expressing hTERT, generating hTERT-GM9503 cells. Both GM9503 and hTERT-GM9503 cells were cultured continuously for 67 days. (**B**) Genomic blots of telomeric restriction fragments from GM9503 (p38), GM9503 (p47) and hTERT-GM9503 at day 67. About 3 µg of RsaI/HinfI-digested genomic DNA from each sample was used for gel electrophoresis. The DNA molecular size markers are shown to the left of the blots. (**C**) Analysis of indirect immunofluorescence with anti-TRF2-2meR17 antibody. Cell nuclei of GM9503 and hTERT-GM9503 were stained with DAPI in blue. (**D**) Quantification of percentage of cells with altered nuclear staining of methylated TRF2. At least 900 cells in triplicate were scored in blind for each cell line as indicated. Standard deviations from three independent experiments are indicated.

Figure 4

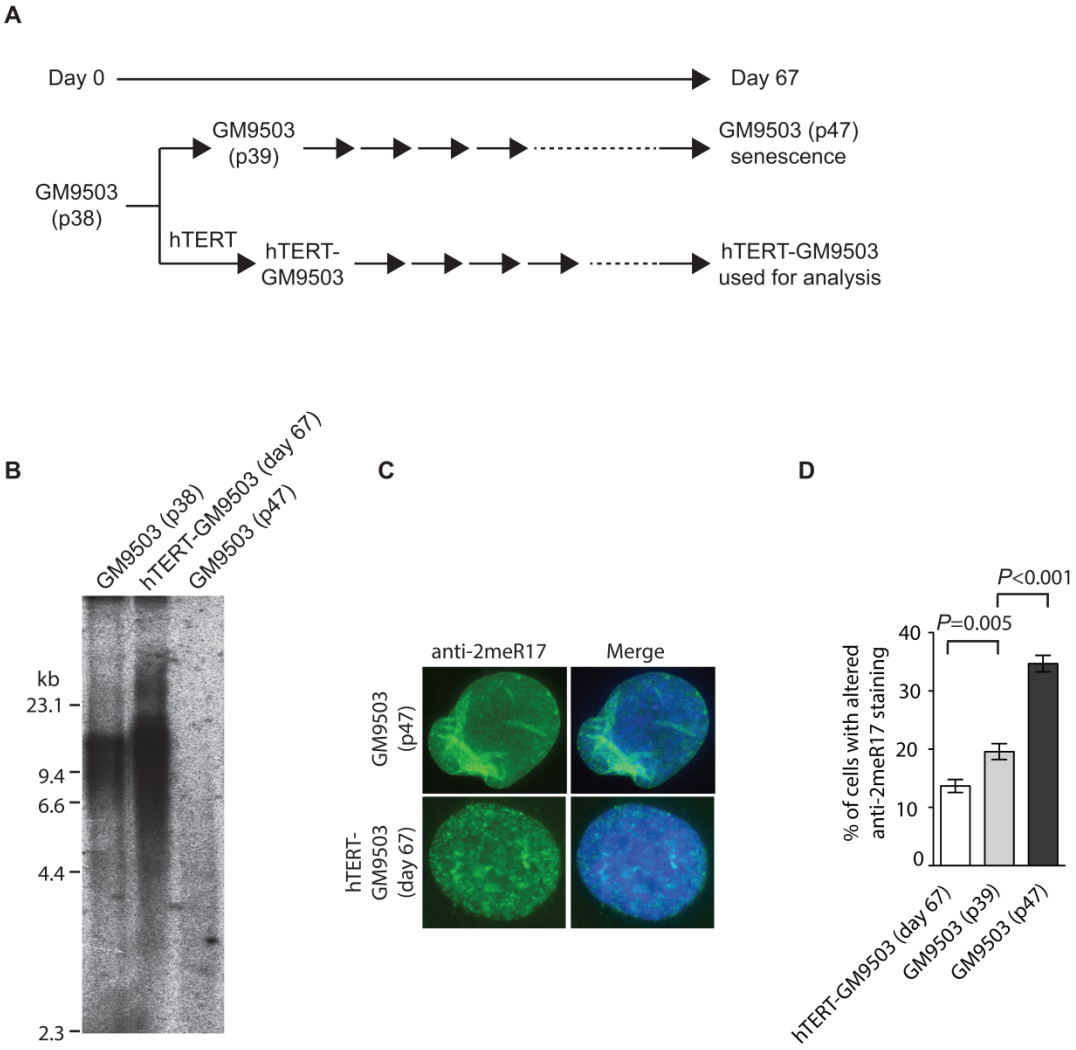

A
Day 0
Day 67
GM9503 (p38)
GM9503 (p39)
GM9503 (p47) senescence
hTERT
hTERT-GM9503
hTERT-GM9503 used for analysis
B
GM9503 (p38)
hTERT-GM9503 (day 67)
GM9503 (p47)
kb
23.1
9.4
6.6
4.4
2.3
C
anti-2meR17
Merge
GM9503 (p47)
hTERT-GM9503 (day 67)
D
P<0.001
P=0.005
% of cells with altered anti-2meR17 staining
40
30
20
10
0
hTERT-GM9503 (day 67)
GM9503 (p39)
GM9503 (p47)

Figure 5. Dysfunctional telomeres induce altered nuclear staining of methylated TRF2. (**A**) Schematic diagram of experimental setup. GM9503 cells (p23) were infected with retrovirus expressing various TRF2 mutant alleles at day -6. After the three-day selection ended on day 0, the cells were cultured for 14 days and then subjected to analysis of immunofluorescence (IF) and cellular senescence. (**B**) Senescence-associated β-galactosidase assays of GM9503 cells overexpressing the vector alone or various TRF2 mutant alleles as indicated. (**C**) Indirect immunofluorescence with anti-TRF2-2meR17 antibody in fixed GM9503 cells overexpressing the vector alone or various TRF2 mutant alleles as indicated. Cell nuclei were stained with DAPI in blue. (**D**) Quantification of percentage of cells with altered nuclear staining of methylated TRF2. A total of 1000 cells in triplicate were scored in blind for each cell line. Standard deviations from three independent experiments are indicated. The asterisks indicate a P value < 0.05 by student's t test compared to the vector cell line. (**E**) Western analysis of GM9503 cells overexpressing various TRF2 mutant alleles as indicated. Immunoblotting was carried out with anti-TRF2, anti-TRF2-2meR17 or anti-γ-tubulin antibody.

Figure 5

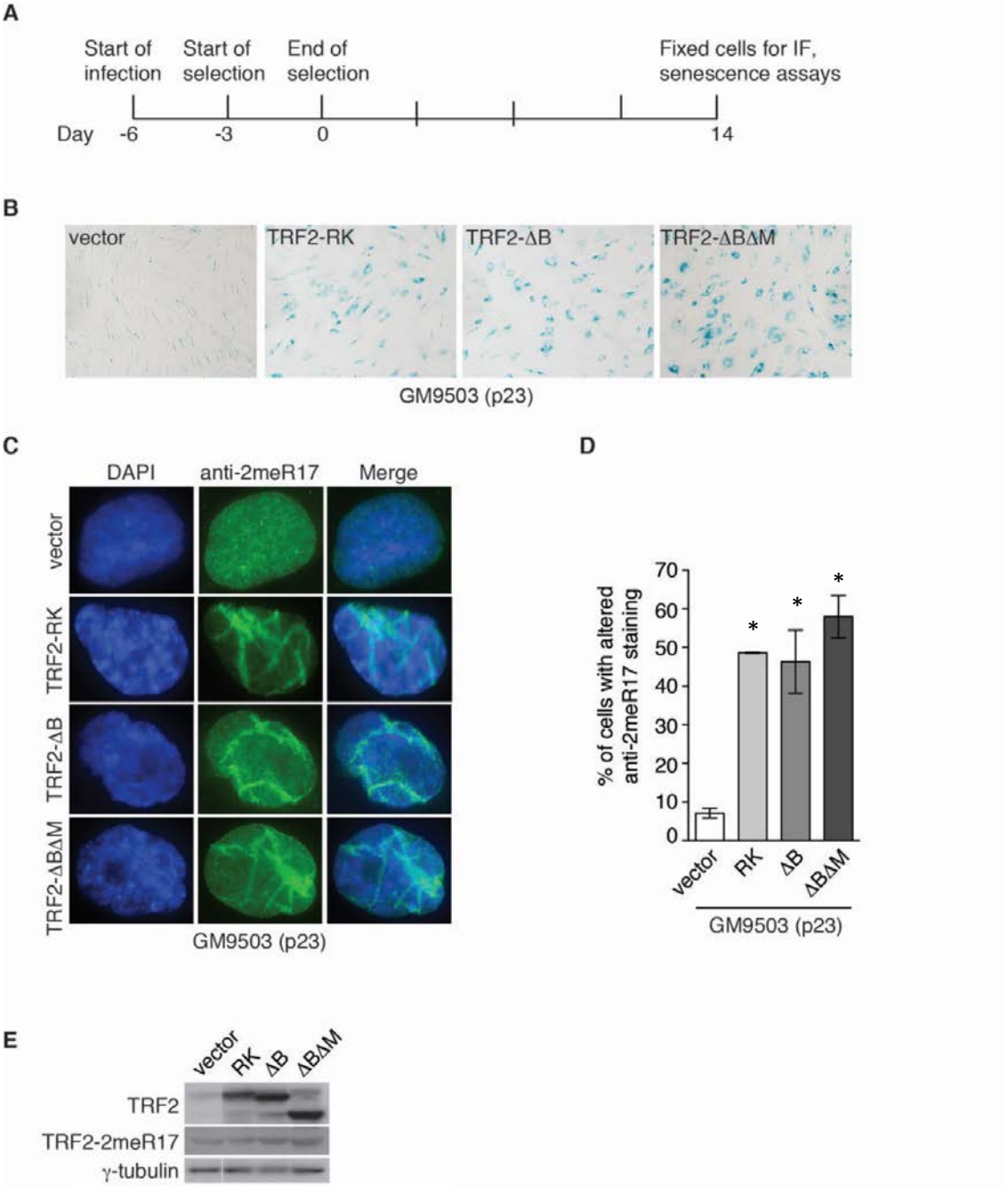
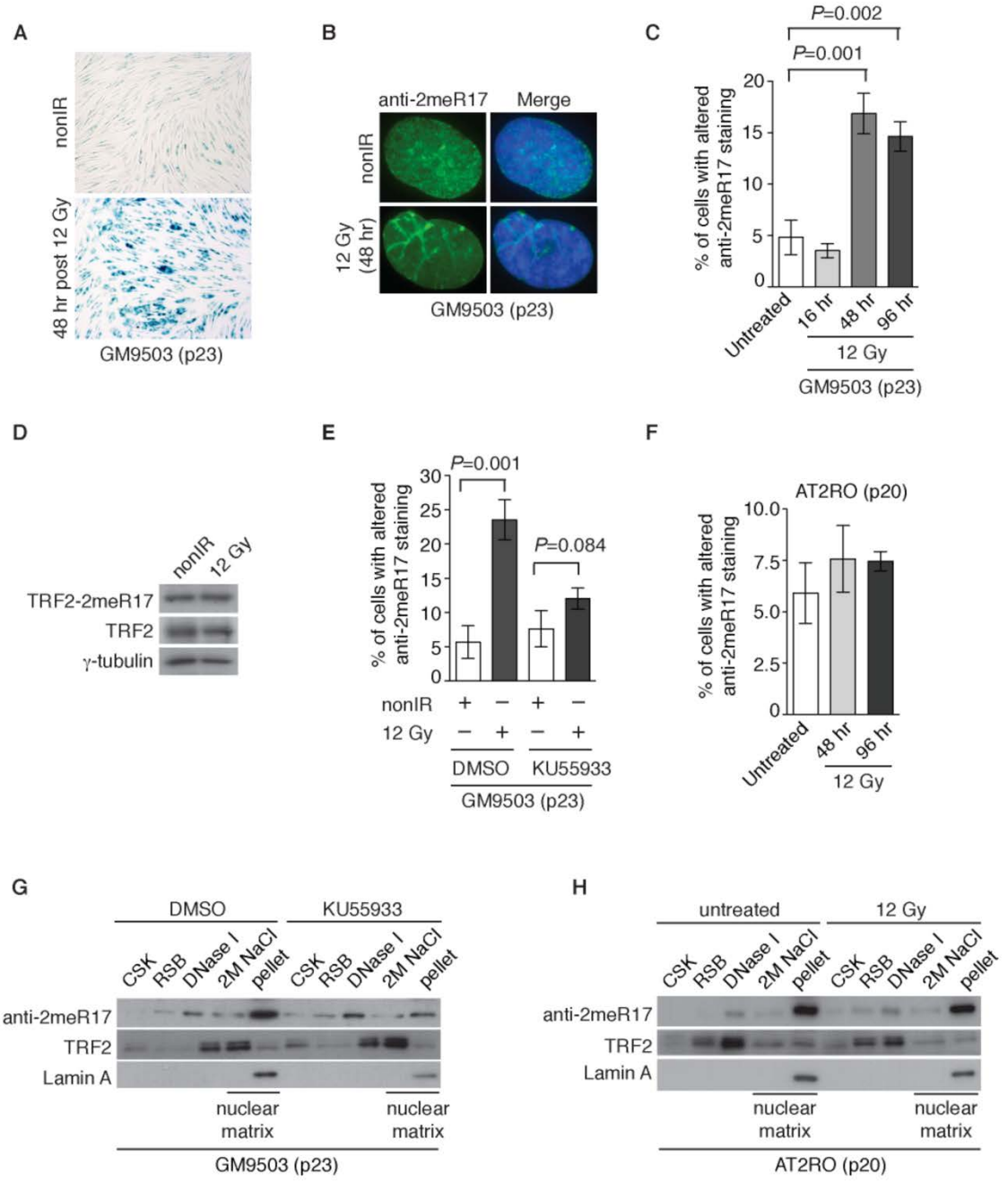

A
Start of infection
Start of selection
End of selection
Fixed cells for IF, senescence assays
Day -6 -3 0 14
B
vector
TRF2-RK
TRF2-ΔB
TRF2-ΔBΔM
GM9503 (p23)
C
DAPI
anti-2meR17
Merge
vector
TRF2-RK
TRF2-ΔB
TRF2-ΔBΔM
GM9503 (p23)
D
% of cells with altered anti-2meR17 staining
70
60
50
40
30
20
10
0
*
*
*
vector
RK
ΔB
ΔBΔM
GM9503 (p23)
E
vector
RK
ΔB
ΔBΔM
TRF2
TRF2-2meR17
γ-tubulin

Figure 6. Ionizing radiation induces altered nuclear staining of methylated TRF2 in a ATM-dependent manner. (**A**) Ionizing radiation induces cellular senescence in GM9503 cells. GM9503 (p23) cells were treated with 12 Gy IR. Senescence-associated β-galactosidase assays were performed 48 h post IR. (**B**) Indirect immunofluorescence with anti-TRF2-2meR17 antibody in mock- or IR-treated GM9503 cells (p23). Cell nuclei were stained with DAPI in blue. (**C**) Quantification of percentage of cells with altered nuclear staining of methylated TRF2. A total of 1000 cells in triplicate were scored in blind for untreated or IR-treated cells fixed at various time points post IR as indicated. Standard deviations from three independent experiments are indicated. (**D**) Western analysis of GM9503 cells (p23) that were either mock- or IR-treated. Immunoblotting was performed with anti-TRF2-2meR17 or anti-TRF2 antibody. The γ-tubulin blot was used as a loading control. (**E**) ATM inhibition abrogates IR-induced altered nuclear staining of methylated TRF2. GM9503 cells (p23) were treated with DMSO or KU55933 prior to 12 Gy IR treatment. Forty-eight hours post IR, cells were processed for indirect immunofluorescence with anti-TRF2-2meR17 antibody. Quantification of percentage of cells with altered nuclear staining of methylated TRF2. A total of 1000 cells in triplicate were scored in blind. Standard deviations from three independent experiments are indicated. (**F**) Little IR-induced altered nuclear staining of methylated TRF2 is observed in AT2RO cells lacking functional ATM. Quantification of percentage of cells with altered nuclear staining of methylated TRF2. A total of 1000 cells in triplicate were scored in blind. Standard deviations from three independent experiments are indicated. (**G**) Sequential extraction of the nuclear matrix from GM9503 cells treated with either

DMSO or KU55933. Immunoblotting was performed with anti-TRF2-2meR17, anti-TRF2 or anti-Lamin A antibody. (**H**) Sequential extraction of the nuclear matrix from AT2RO cells that were either untreated or treated with 12 Gy IR. Immunoblotting was performed with anti-TRF2-2meR17, anti-TRF2 or anti-Lamin A antibody.

Figure 6


A
nonIR
48 hr post 12 Gy
GM9503 (p23)
B
anti-2meR17
Merge
nonIR
12 Gy (48 hr)
GM9503 (p23)
C
P=0.002
P=0.001
% of cells with altered anti-2meR17 staining
Untreated
16 hr
48 hr
96 hr
12 Gy
GM9503 (p23)
D
nonIR
12 Gy
TRF2-2meR17
TRF2
γ-tubulin
E
P=0.001
P=0.084
% of cells with altered anti-2meR17 staining
nonIR
12 Gy
DMSO
KU55933
GM9503 (p23)
F
AT2RO (p20)
% of cells with altered anti-2meR17 staining
Untreated
48 hr
96 hr
12 Gy
G
DMSO
KU55933
CSK
RSB
DNase I
2M NaCl
pellet
anti-2meR17
TRF2
Lamin A
nuclear matrix
GM9503 (p23)
H
untreated
12 Gy
anti-2meR17
TRF2
Lamin A
nuclear matrix
AT2RO (p20)

3.3 Complementary Data

3.3.1 The staining of methylated TRF2 is altered in HGPS cells

Since methylated TRF2 is part of the nuclear matrix and is altered in aged cells, we hypothesized that methylated TRF2 may also be mislocalized in cells derived from Hutchinson-Gilford progeria syndrome (HGPS). HGPS patients age prematurely due to defects in the nucleoskeleton protein, Lamin A (Eriksson et al. 2003, Scaffidi, Misteli 2006). We looked at methylated TRF2 staining in two HGP cell lines, AG11498 and AG11513 that express a truncated form of lamin A called progerin (Eriksson et al. 2003). Both AG11498 p15 and AG11513 p14 had significantly more abnormal nuclei than similar passage normals (Figure 3.3.1A and B). Interestingly, cells co-stained with methylated TRF2 and Lamin A had little overlap (Figure 3.3.1C). This indicates that in HGPS cells, which have altered nuclear matrix, methylated TRF2 is also mislocalized, albeit uniquely from that of Lamin A. However, sequential cell fractionation of AG11498 demonstrated that methylated TRF2 is retained in the nuclear matrix fraction, indicating that progerin expression does not dislodge methylated TRF2 (Figure 3.3.1D). These findings support our work that demonstrates methylated TRF2 is part of the nuclear matrix and indicates that its proper localization is dependent on lamin A.

Figure 3.3.4

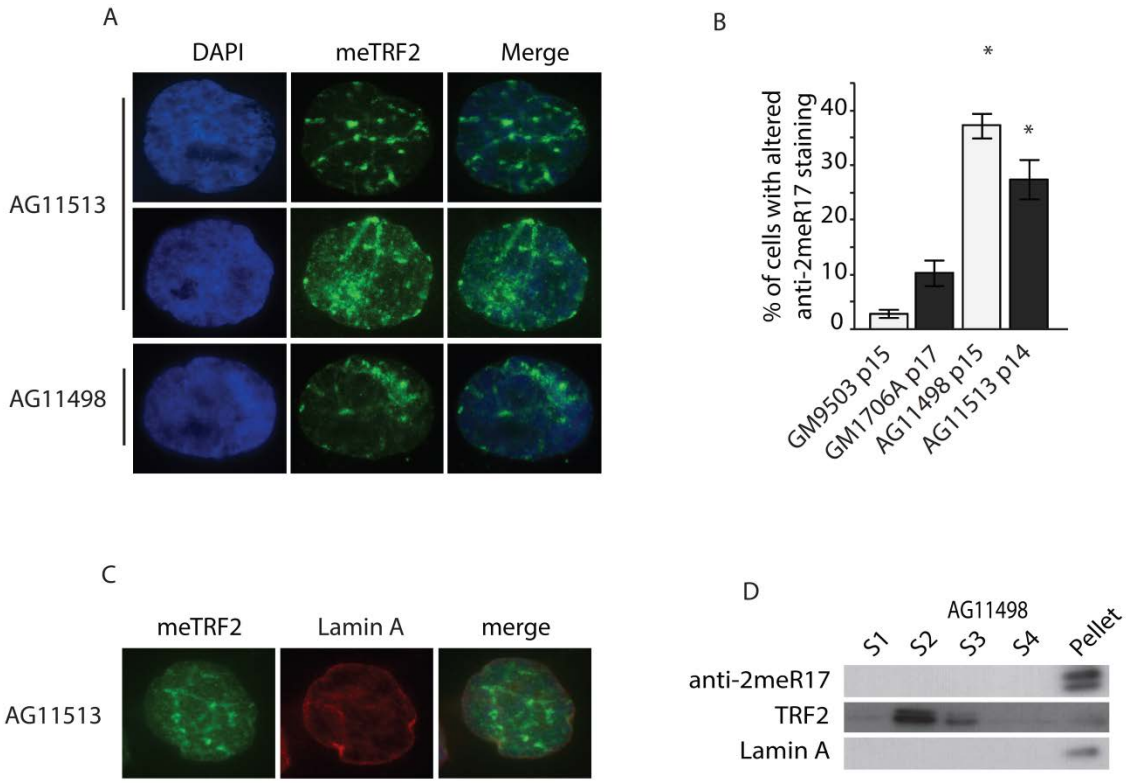

A
DAPI
meTRF2
Merge
AG11513
AG11498
B
*
*
% of cells with altered anti-2meR17 staining
40
30
20
10
0
GM9503 p15
GM1706A p17
AG11498 p15
AG11513 p14
C
meTRF2
Lamin A
merge
AG11513
D
AG11498
S1
S2
S3
S4
Pellet
anti-2meR17
TRF2
Lamin A

Figure 3.3.4 The staining of methylated TRF2 is altered in HGPS cells.

A) Indirect immunofluorescence of HGP cells. AG11498 (p15) and AG11513 (p14) were processed for immunofluorescence by standard methods. The cells were stained with anti-2meR17 (green) and DNA was counterstained with DAPI (blue). B) Quantification of the percentage of cells with altered nuclear staining. The asterisks indicate a $P < 0.05$ compared to the vector control. Statistical analysis was done by a two-tailed student's t test. C) Costaining of methylated TRF2 and Lamin A. AG11513 cells were fixed for immunofluorescence by standard methods. The cells were co-stained with anti-2meR17 (green) and anti-lamin A (red). The DNA was counterstained with DAPI (blue). D) Sequential fractionation of AG11498 (p12) cells. The fractionation was carried out as previously described. The extracts were immunoblotted with anti-2meR17, anti-TRF2 and anti-lamin A

3.3.5 Complementary data references

Eriksson, M., Brown, W.T., Gordon, L.B., Glynn, M.W., Singer, J., Scott, L., Erdos, M.R., Robbins, C.M., Moses, T.Y., Berglund, P., Dutra, A., Pak, E., Durkin, S., Csoka, A.B., Boehnke, M., Glover, T.W. & Collins, F.S. 2003, "Recurrent de novo point mutations in lamin A cause Hutchinson-Gilford progeria syndrome", *Nature*, vol. 423, no. 6937, pp. 293-298.

Gonzalez-Suarez, I., Redwood, A.B., Perkins, S.M., Vermolen, B., Lichtensztejin, D., Grotsky, D.A., Morgado-Palacin, L., Gapud, E.J., Sleckman, B.P., Sullivan, T., Sage, J., Stewart, C.L., Mai, S. & Gonzalo, S. 2009, "Novel roles for A-type lamins in telomere biology and the DNA damage response pathway", *The EMBO journal*, vol. 28, no. 16, pp. 2414-2427.

Scaffidi, P. & Misteli, T. 2006, "Lamin A-dependent nuclear defects in human aging", *Science (New York, N.Y.)*, vol. 312, no. 5776, pp. 1059-1063.

Chapter 4

Cockayne Syndrome group B protein interacts with TRF2 and regulates telomere length and stability

4.1 Preface

Telomere defects have been found in several disorders that manifest with a premature aging phenotype, such as Werner's syndrome, Xeroderma Pigmentosum and Hutchinson Gilford Progeria (Decker et al. 2009, Benson, Lee & Aaronson 2010, Crabbe et al. 2004, Zhu et al. 2003). Cockayne Syndrome (CS) is a segmental progeroid syndrome that has previously not been studied with respect to telomere maintenance. It has long been thought that CSB must have other unknown functions in the cell in order to account for the premature aging phenotypes associated with it. The work presented in this chapter supports a role for CSB in telomere maintenance. The identification of a novel TRF2 interacting factor increases our understanding of telomere maintenance mechanisms, and draws new links between DNA repair proteins and telomere proteins. This work also advances our understanding of CS and may be helpful in deciding future treatment options for CS patients.

The original idea to look whether cells from Cockayne patients had telomere defects was through a collaborative effort between myself and Derrik Leach. From this effort we determined TRF2 and CSB interact *in vivo.* Derrik Leach and Dr. Rainbow provided exceptional insight into the direction of the project, as well as, the patient cell lines and supporting work not presented here. All the figures in the paper are the work of either myself or Nicole Batenburg. I produced in its entirety figures 1 and 2, 4E-G, 5D, F-H and Supplementary Figures S2A and S4. The paper was written as a collaborative effort between myself, Nicole Batenburg and Dr. Zhu.

4.2 Publication

Nucleic Acids Research, 2012, **1–14**
doi:10.1093/nar/gks745

Cockayne Syndrome group B protein interacts with TRF2 and regulates telomere length and stability

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4.2.1 Abstract

The majority of Cockayne syndrome (CS) patients carry a mutation in Cockayne Syndrome group B (CSB), a large nuclear protein implicated in DNA repair, transcription and chromatin remodeling. However, whether CSB may play a role in telomere metabolism has not yet been characterized. Here, we report that CSB physically interacts with TRF2, a duplex telomeric DNA binding protein essential for telomere protection. We find that CSB localizes at a small subset of human telomeres and that it is required for preventing the formation of telomere dysfunction-induced foci (TIF) in CS cells. We find that CS cells or CSB knockdown cells accumulate telomere doublets, the suppression of which requires CSB. We find that overexpression of CSB in CS cells promotes telomerase-dependent telomere lengthening, a phenotype that is associated with a decrease in the amount of telomere-bound TRF1, a negative mediator of telomere length maintenance. Furthermore, we show that CS cells or CSB knockdown cells exhibit misregulation of TERRA, a large non-coding telomere repeat-containing RNA important for telomere maintenance. Taken together, these results suggest that CSB is required for maintaining the homeostatic level of TERRA, telomere length and integrity. These results further imply that CS patients carrying CSB mutations may be defective in telomere maintenance.

4.2.2 Introduction

Telomeres are heterochromatic structures found at the ends of linear eukaryotic chromosomes. Mammalian telomeric DNA consists of tandem repeats of TTAGGG that are bound by a telomere-specific complex known as shelterin/telosome (1–3). Shelterin, composed of six protein subunits, including TRF1, TRF2, TIN2, hRap1, TPP1 and POT1, functions not only to regulate telomere length maintenance but also to protect natural chromosome ends from being recognized as damaged DNA (1,2,4). Telomeric DNA has been shown to be transcribed into a large non-coding telomere repeat-containing RNA (5), referred to as TERRA, which is implicated in maintaining the integrity of telomere heterochromatin (5,6). Disruption of the shelterin complex or the telomere heterochromatic state can lead to induction of telomere abnormalities, including telomere end-to-end fusions, telomere loss and telomere doublets/fragile telomeres (1,2,6). These dysfunctional telomeres have been shown to be associated with DNA damage response factors, such as γH2AX and 53BP1, resulting in the formation of nuclear structures that are referred to as telomere dysfunction-induced foci (TIF) (7–10).

TRF2 is one of the two shelterin subunits that bind specifically to duplex telomeric DNA (11,12), the other being TRF1 (13). Overexpression of TRF1 leads to telomere shortening, whereas removal of TRF1 from telomeres promotes telomerase-dependent telomere lengthening (14–16), implying that TRF1 may restrict the access of telomerase to the ends of telomeres. While TRF1 has been implicated in telomere length maintenance, TRF2 is best known for its role in telomere protection. TRF2 contains a N-terminal basic domain, a central TRF homology (TRFH) domain and a C-terminal Myb-

like DNA binding domain (11,12). The N-terminal basic domain is rich in glycine and arginine residues, also referred to as a GAR domain. The TRFH domain of TRF2 not only mediates homo-dimerization but also acts as a protein interaction platform at telomeres to recruit additional shelterin subunits and other accessory proteins (17,18). Removal of TRF2 from telomeres either by conditional knockout or overexpression of a dominant-negative allele of TRF2 lacking both the N-terminal basic/GAR domain and the C-terminal Myb-like DNA binding domain promotes telomere end-to-end fusions (19,20). Overexpression of TRF2 lacking its N-terminal basic/GAR domain promotes telomere loss (8), whereas overexpression of TRF2 carrying amino acid substitutions in the same basic/ GAR domain induces the formation of telomere doublets (10).

Cockayne syndrome (CS) is a rare human hereditary disorder characterized by severe postnatal growth failure, progressive neurological degeneration and segmental premature aging, including sensorineural hearing loss, retinal degeneration and loss of subcutaneous fat (21,22). CS patients show hypersensitivity to UV light and the average life span of CS patients is ≈12 years (23–25). Although five genes have been identified to be responsible for the disease, including CSA, CSB, XPB, XPD and XPG, the majority of CS patients carry a defect in the CSB gene (21,22,25).

Cockayne Syndrome group B (CSB) protein, also known as ERCC6, is a nuclear protein of 1493 amino acids in length, containing several distinct domains, including an acidic domain, a glycine rich domain, a SWI/SNF-like ATPase domain, a nucleotide binding (NTB) domain and a ubiquitin binding domain (UBD) (Figure 1A) (21,26–28).

CSB has been shown to play a key role in transcription-coupled repair (21,29), a subpathway of nucleotide excision repair (NER) responsible for removing bulky lesions such as UV-induced DNA damage (cyclobutane pyrimidine dimers and 6-pyrimidine-4-pyrimidone photoproducts). In addition to NER, CSB has also been implicated in base excision repair (30,31), transcription (32–35), chromatin maintenance and remodeling (36). However, whether CSB may play a role in telomere maintenance relevant to cancer and aging has not yet been characterized.

Here, we report that CSB physically interacts with TRF2. While multiple domains of CSB are engaged in its interaction with TRF2, the TRFH domain of TRF2 is required and sufficient for binding CSB. We show that CS cells or CSB knockdown cells exhibit an accumulation of telomere doublets and an induction of TIF formation. Re-introduction of wild-type CSB into CS cells suppresses the formation of telomere doublets and TIFs, indicative of its role in telomere protection. In addition, we find that CS cells undergo telomere shortening, whereas overexpression of CSB into CS cells results in telomerase-dependent telomere lengthening. The latter is associated with a reduction in the amount of telomere-bound TRF1, a negative mediator of telomere length maintenance (14–16). Furthermore, we find that CS cells or CSB knockdown cells display misregulation of TERRA expression. Collectively, these results suggest that CSB is required for maintaining the homeostatic level of TERRA, telomere length and stability.

4.2.3 Materials and Methods

DNA constructs and antibodies. The complementary DNA (cDNA) for CSB purchased from mammalian gene collection contained three missense mutations (C666, P1041 and P1294). The QuickChange site-directed mutagenesis kit (Strategene) was used to revert these mutations to wildtype. The corrected CSB cDNA was then subcloned into the retroviral vector pLPC-puro (37) or pLPC-N-Myc-puro (37). The pLPC-N-Myc-CSB plasmid was used as a template for PCR to generate CSB truncation alleles CSB-N (amino acids 2–510), CSB-ATPase (amino acids 510–960) and CSB-C (amino acids 972–1493). The cDNA for TRF2 was a generous gift from Titia de Lange, Rockefeller University. The TRF2 truncation alleles $TRF2^{\Delta B \Delta M}$ (amino acids 45–453), $TRF2^{TRFH}$ (amino acids 45–245) and $TRF2^{linker}$ (amino acids 246–453) were generated by PCR and cloned into pLPC-FH2 (38) (a kind gift from Titia de Lange, Rockefeller University). pBabe-neo-hTERT was kindly provided by Robert Weinberg, MIT.

The oligonucleotides encoding siRNA directed against CSB have been previously described (39). The annealed oligonucleotides were ligated into pRetroSuper vector (kindly provided by Titia de Lange, Rockefeller University), giving rise to pRetroSuper-shCSB.

Antibodies to TRF1 (13), TRF2 (40) and hRap1 (41) were kind gifts from Titia de Lange, Rockefeller University. Commercial antibodies used were rabbit anti-CSB (Bethyl A301–345A), mouse anti-CSB (Abcam Ab66598), anti-Myc (9E10, Calbiochem), anti-γ-H2AX (Upstate) and anti-γ-tubulin (GTU88, Sigma).

Cell culture and retroviral infection. HeLaI.211 cells were a gift from Titia de Lange, Rockefeller University. Primary fibroblast cell lines GM38 (normal), GM9503 (normal), GM8399 (normal), GM10901 (heterozygote), GM10905 (CS), GM739 (CS), GM1428 (CS) and a transformed CS cell line (GM16095) were obtained from the NIGMS Human Genetic Cell Repository (Coriell Institute for Medical Research, Camden, NJ, USA). GM16095 is a SV40-transformed cell line derived from GM739 (27). Supplementary Table S1 lists the nature of CSB mutations and the age of the individuals from whom biopsies were taken to establish the primary cell lines. Cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) for transformed cell lines GM16095, HeLa and Phoenix cells, and 15% FBS for all primary fibroblasts, supplemented with non-essential amino acids, glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Retroviral gene delivery was carried out as described (42,43). Phoenix amphotropic retroviral packaging cells were transfected with the desired DNA constructs. For hTERT-mediated immortalization, 3 days after the last infection, neomycin (600 μg/ml) was added to the medium to select for hTERT-expressing cells. Otherwise, 12 h after the last infection, puromycin (2 μg/ml) was added to the medium and the cells were maintained in the selection medium for the entirety of the experiments.

Immunoblotting and immunoprecipitation. Immunoblotting was carried out as previously described (10,40). Immunoprecipitation (IP) of endogenous TRF2 was

performed essentially as described (10,40). For IP of endogenous CSB, HeLa cells were collected and resuspended in ice-cold NP-40 buffer (1% NP-40, 150 mM NaCl, 10 mM sodium phosphate, pH 7.2). Following incubation on ice for 20 min, the supernatant was recovered by micro-centrifugation at 13 000 rpm for 10 min. Protein extracts of 1.5 mg were mixed with 2 ml mouse anti-CSB antibody (Abcam) and the mixture was incubated overnight at 4 °C. Protein G-beads (30 µl) were added to the mixture on the next day and the IP pellet was washed five times each with 1 ml of ice-cold NP-40 buffer containing 1mM DTT, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 10 µg/ml pepstatin and 1mM PMSF.

Co-immunoprecipitation from 293T cells was carried out essentially as described (38) except for the method of transfection used. Human 293T cells grown on 6-cm plates with 95% confluency were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For each co-transfection, a total of 8 mg DNA mixture was used. The ratio of CSB constructs to TRF2 constructs in each DNA mixture was 3:1.

Chromatin Immunoprecipitations. Chromatin immunoprecipitations (ChIPs) were carried out essentially as described (44–46). Cells were directly fixed with 1% formaldehyde in phosphate-buffered saline (PBS) for 1 h, followed by sonication (10 cycles of 20 s each, 50% duty and 5 output). For each ChIP, 200 µl cell lysate (equivalent to 2 X 10^6 cells) was used. For the total telomeric DNA, 50 µl supernatant (corresponding to one-quarter of the amount of lysate used for IP) were processed along with the IP samples at the step of reversing the crosslinks. Four-fifths of the immunoprecipitated

DNA was loaded on the dot blots whereas two inputs each containing 5% of total DNA were included to assess the consistency of loading. The ratio of the signal from each ChIP relative to the signal from the input lane was multiplied by 5% (5% represents 5% of total DNA) and a factor of 1.25 (since four-fifths of the precipitated DNA was loaded for each ChIP), giving rise to the percentage of total telomeric DNA recovered from each ChIP.

Immunofluorescence and fluorescence *in situ* hybridization. Immunofluorescence was performed essentially as described (40,43). Briefly, cells grown on coverslips were rinsed with PBS, incubated in Triton X-100 buffer [0.5% Triton X-100, 20mM HEPES–KOH (pH 7.9), 50 mM NaCl, 3 mM $MgCl_2$ and 300 mM sucrose] at room temperature (RT) for 5 min and then fixed for 10 min in PBS buffered 3% paraformaldehyde and 2% sucrose. Following permeablization at RT for 10 min in Triton X-100 buffer, fixed cells were blocked with 0.5% bovine serum albumin (Sigma) and 0.2% gelatin (Sigma) in PBS and then incubated at RT for 2 h with both rabbit anti-hRap1 and mouse anti-γ-H2AX or mouse anti-CSB.

Immunofluorescence (IF)–fluorescence *in situ* hybridization (FISH) analyses were conducted as described (9). Briefly, cells grown on coverslips were fixed at RT for 10 min in PBS buffered 2% para-formaldehyde, washed in PBS twice for 5 min each, followed by incubation at RT for 30 min in blocking buffer containing 1 mg/ml bovine serum albumin (BSA), 3% goat serum, 0.1% Triton X-100 and 1mM ethylenediaminetetraacetic acid (EDTA) in PBS. Blocked coverslips were incubated with

anti-Myc antibody in blocking buffer at RT for 1 h. After three washes in PBS, coverslips were incubated with tetramethyl rhodamine isothiocyanate (TRITC)- conjugated donkey anti-mouse (1:100, Jackson Laboratories) at RT for 30 min. Subsequently, cells on coverslips were fixed again in PBS buffered 2% paraformaldehyde for 5 min and followed by dehydration in a series of 70, 85 and 100% ethanol. The air-dried coverslips were denatured at 80 °C for 10 min and hybridized with 0.5 µg/ml fluorescein isothiocyanate (FITC)- conjugated-$(CCCTAA)_3$ PNA probe (Biosynthesis Inc.) for 2 h in dark at RT. Following incubation, cover slips were washed with 70% formamide and 10mM Tris–HCl (pH 7.2) twice for 15 min. After three washes in PBS, DNA was counter-stained with 4', 6-diamidino-2-phenylindole (DAPI; 0.2 µg/ml) and embedded in 90% glycerol/10% PBS containing 1 mg/ml *p*-phenylene diamine (Sigma). All cell images were recorded on a Zeiss Axioplan 2 microscope with a Hammamatsu C4742-95 camera and processed in Open Lab.

Metaphase chromosome spreads. Metaphase chromosome spreads were essentially prepared as described (19,43). Cells were arrested in nocodazole (0.1 µg/ml) for 90–120 min. Following arrest, cells were harvested by trypsinization, incubated for 7 min at 37 °C in 75 mM KCl and fixed in freshly made methanol/glacial acetic acid (3:1). Cells were stored overnight at 4 °C, dropped onto slides and air-dried overnight in a chemical hood.

FISH analysis on metaphase chromosome spreads was carried out essentially as described (43,47). Slides with chromosome spreads were incubated with 0.5 µg/ml FITC-conjugated-$(CCCTAA)_3$ PNA probe (Biosynthesis Inc.) for 2 h at room temperature. Following incubation, slides were washed, counter-stained with 0.2 µg/ml DAPI and embedded in 90% glycerol/10% PBS containing 1 mg/ml *p*-phenylene diamine (Sigma). All cell images were recorded on a Zeiss Axioplan 2 microscope with a Hammamatsu C4742-95 camera and processed in Open Lab.

Northern analysis of TERRA. Total RNA was isolated from cells using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Northern analysis was performed essentially as described with minor modifications (5). Briefly, 20 µg of the RNA was loaded onto 1.3% formaldehyde agarose gels and run at 60V for 7 h. The gel was then stained with ethidium bromide to inspect the presence of the 28S and 18S ribosomal RNA, both of which were indicators of RNA quality. RNA was then transferred to a Nylon membrane (Hybond-N, GE) and was blocked in Church mix [0.5M Na_2PO_4 (pH 7.2), 1mM EDTA, 7% SDS and 1% BSA] for 1 h at 65 °C. The membrane was then incubated overnight at 65 °C with a radioactively labeled 800-bp TTAGGG repeat-containing fragment as previously described (44). For the GAPDH control, the membrane was incubated with a radioactively labeled DNA fragment containing the GAPDH gene. Following incubation, the membrane was washed once with 1 X SSC, 0.1% SDS at room temperature, three times in 0.5 X SSC at 65 °C and then exposed to a

PhosphorImager screen. The signals on the membrane were quantified by ImageQuant analysis.

Telomere length analysis and telomeric repeat amplification protocol (TRAP) assays. Genomic DNA isolated from cells was digested with RsaI and HinfI and loaded onto a 0.7% agarose gel in 0.5 X TBE. Blotting for telomeric fragments was carried out according to standard protocols (48,49). The average telomeric restriction fragment length was determined by PhosphorImager analysis using ImageQuant and MS Excel as described (50).

The activity of telomerase in cells was determined using a Trapeze telomerase detection kit (Chemicon) according to the protocol provided by the manufacturer. PCR amplification was performed for 31 cycles. The products were separated on a 12.5% non-denaturing polyacrylamide gel in 0.5 X TBE buffer and visualized using SYBR green (Invitrogen).

4.2.4 Results

Physical interaction between CSB and TRF2. To investigate the role of CSB in telomere biology, we decided to ask whether CSB might interact with components of the shelterin complex essential for telomere maintenance. Co-immunoprecipitation with anti-CSB antibody brought down endogenous TRF2 (Figure 1B). CSB association with TRF2

was also detected in a reverse IP using anti-TRF2 antibody and HeLa nuclear extracts (Figure 1C). The interaction of CSB with TRF2 was further confirmed when Flag-tagged TRF2 was co-expressed with Myc-CSB in 293T cells (Figure 1D). Taken together, these results reveal that CSB interacts with TRF2 *in vivo*.

To gain further understanding of CSB interaction with TRF2, we examined the interaction between various CSB domains and TRF2. Flag-TRF2 was coexpressed with Myc-tagged CSB-N carrying the first 510 amino acids, including the acidic and the glycine-rich domains, Myc-tagged CSB-ATPase containing the central 450 amino acids or Myc-tagged CSB-C carrying the last 521 amino acids including the NTB domain and UBD in 293T cells. Co-immunoprecipitation studies with anti-Myc antibody revealed that all three CSB truncation mutants were able to pull down Flag-TRF2 (Figure 1D), suggesting that multiple domains of CSB may be engaged in its interaction with TRF2.

TRF2 contains an N-terminal basic/GAR domain, a central TRFH domain, a linker region and a C-terminal Myb-like DNA binding domain (Figure 1E). To investigate the domain of TRF2 important for its interaction with CSB, we coexpressed Myc-CSB with Flag-tagged TRF2$^{\Delta B \Delta M}$ lacking both the basic domain and the Myb-like domain, Flag-tagged TRF2 carrying the TRFH dimerization domain alone (Flag-TRF2^{TRFH}) or Flag-tagged TRF2 carrying the linker region alone (Flag-TRF2^{linker}) in 293T cells. Co-immunoprecipitation with anti-Myc antibody showed that both Flag-tagged TRF2 $^{\Delta B \Delta M}$ and Flag-tagged TRF2^{TRFH} were able to interact with Myc-CSB (Figure 1F). In contrast, no interaction between Myc-CSB and Flag-TRF2^{linker} was

detected despite a high level of expression of Flag-TRF2^{linker} (Figure 1F). These results suggest that the TRFH domain is required and sufficient for TRF2 interaction with CSB.

CSB localizes at a fraction of human telomeres and is required to suppress the formation of TIFs in CS cells. To investigate whether CSB may be associated with human telomeres, we performed dual indirect immunofluorescence with anti-CSB antibody in conjunction with anti-hRap1 antibody, a marker for interphase telomeres (41). We observed an overlap between several anti-hRap1 staining (green) and anti-CSB staining (red) foci in HeLa cells (Figure 2A). The co-localization of CSB with several hRap1 foci was also detected in CSB-complemented immortalized CS cells hTERT-GM10905 (Figure 2A). In addition, we also performed IF–FISH analysis with anti-Myc antibody in conjunction with a FITC conjugated telomeric DNA-containing PNA probe in SV40-transformed CS cells GM16095 stably expressing Myc-tagged CSB. We again observed the presence of CSB (red) at several telomeres (green) (Figure 2B). Taken together, these results suggest that CSB may be associated with a small subset of human telomeres although we cannot rule out the possibility that observed costaining of CSB with telomeres may be coincidental.

Dysfunctional telomeres are known to attract DNA damage response factors including γ-H2AX (7–10). To investigate whether CS cells may accumulate dysfunctional telomeres, dual indirect immunofluorescence was performed on hTERT-GM10905 expressing either CSB or the vector alone with anti-hRap1 antibody in conjunction with

anti-γ-H2AX antibody. We observed an induction of TIFs in vector-expressing hTERT-GM10905 cells when compared to CSB-complemented hTERT-GM10905 cells (Figure 2C). While 18% of the vector-expressing hTERT-GM10905 cells exhibited five or more TIFs, such TIFs were detected in only 1% of CSB-complemented hTERT-GM10905 cells (Figure 2D). These results suggest that CSB is required for telomere protection.

Primary fibroblasts derived from CS patients carrying a CSB mutation show an accumulation of telomere doublets. To investigate whether CSB may be required for maintaining telomere structure, we performed FISH analysis of metaphase spreads on two cell lines (GM10901 and GM10905) at various passages to inspect for the presence of any telomere abnormalities, including telomere loss (chromatid ends without a detectable telomeric signal), telomere fusions, telomere-containing double minute chromosomes (TDM) and telomere doublets/fragile telomeres (more than one telomeric signal at a single chromatid end). GM10901 and GM10905 are two respective primary fibroblast cell lines derived from a mother heterozygote for a CSB mutation and her CS offspring. We did not observe any significant accumulation of TDM and telomere fusions in either GM10901 or GM10905 (Figure 3A). While telomere loss was detected in both GM10901 and GM10905 (Figure 3B), no significant difference in the formation of telomere loss was found when GM10901 and GM10905 cells of various passages were compared (Figure 3B). In contrast, we found that various passages of GM10905 cells

consistently exhibited an accumulation of telomere doublets when compared to the heterozygote GM10901 cells of similar passages (Figure 3C).

We also examined the presence of telomere loss and telomere doublets in two other CS cell lines GM1428 and GM739 in comparison to three fibroblast cell lines (GM38, GM9503, GM8399) derived from normal individuals. We found that when compared to the normal control cells, both GM1428 and GM739 displayed an increase in the formation of telomere loss and telomere doublets (Figure 3D and E), the latter consistent with our earlier finding. No full-length CSB was detected in any CS cells examined (Supplementary Figure S1). Taken together, these results suggest that CSB is required for maintaining the integrity of telomere structure.

Introduction of wild-type CSB into CS cells suppresses the formation of telomere doublets. Formally, it is possible that the increased formation of telomere doublets observed in CS primary fibroblasts might be due to the difference in the genetic background between CS cells and normal control cells. To address this question, we decided to examine telomere structures in several pairs of cell lines with isogenic background.

CS primary fibroblasts GM10905 were immortalized with exogenously expressed catalytic subunit of telomerase (hTERT) (Supplementary Figure S2A) to overcome poor growth and premature senescence associated with CS cells. Subsequently, retrovirus expressing either wildtype CSB or the vector alone was used to infect hTERT-GM10905 cells, generating two stable isogenic cell lines (hTERT-GM10905-vector and hTERT-

GM10905-CSB). FISH analysis revealed that overexpression of hTERT drastically reduced telomere loss (Figure 4A and Supplementary Figure S2B); however, it had little effect on the accumulation of telomere doublets in GM10905 cells (Figure 4B and Supplementary Figure S2B). On the other hand, we found that introduction of wild-type CSB into hTERT-GM10905 cells led to a reduction in the formation of telomere doublets (Figure 4C and Supplementary Figure S3). We observed a 40% decrease (P=0.009) in the formation of telomere doublets in CSB-complemented hTERT-GM10905 cells when compared to vector expressing hTERT-GM10905 cells (Figure 4C).

We also examined the formation of telomere doublets in a second pair of isogenic CS cell lines (GM16095) complemented with either the vector alone or wild-type CSB. Introduction of wild-type CSB also resulted in a reduction in the formation of telomere doublets in GM16095 (Figure 4D). To further investigate the role of CSB in the formation of telomere doublets, we knocked down CSB in HeLaI.2.11 cells (Figure 4E) and found that depletion of CSB led to an induction of telomere doublets (Figure 4F and Supplementary Figure S4). Taken together, these results suggest that CSB prevents the formation of telomere doublets.

Aphidicolin, an inhibitor of DNA replication, has been shown to induce telomere doublets (9,51). We found that treatment with aphidicolin resulted in a further increase in the formation of telomere doublets in CS cells (GM16095) (Figure 4G), consistent with previous findings that the effect of aphidicolin was additive (9,46,48). We also observed an increase in the formation of telomere doublets in CSB-complemented GM16095 cells

upon aphidicolin treatment, although such increase was less than that observed in GM16095 cells expressing the vector alone (Figure 4G). These results suggest that telomere doublets observed in CS cells may have arisen from a defect associated with telomere replication.

Introduction of wild-type CSB into CS cells promotes telomerase-dependent telomere lengthening. We observed that the median telomere length in hTERT-immortalized heterozygote mother GM10901 cells was longer than that in hTERT-immortalized CS offspring GM10905 cells (Figure 5A). Therefore, we decided to examine whether CSB might be involved in telomere length maintenance. To address this question, pools (not single cell clones) of hTERT-GM10905 cells stably expressing the vector alone or wild-type CSB were continuously cultured for over 60 population doublings (PDs) and their telomere length dynamics was examined. Analysis of telomere restriction fragments revealed that the median telomere length in hTERT-GM10905 cells expressing the vector alone declined at a rate of ≈11.6 bp/ PD, whereas the median telomere length increased at a rate of ≈21.5 bp/PD for the first 42 PDs and then plateaued in hTERT-GM10905 cells expressing wild-type CSB (Figure 5B and C). A decline in the level of CSB expression in hTERT-GM10905 CSB cells was noticed after PD60 (Figure 5D), suggesting that the loss of CSB expression may in part contribute to the plateau of the median telomere length seen between PD42 and PD61 in these cells. We did not observe any significant difference in the growth rate between hTERT-GM10905 vector

and hTERT-GM10905 CSB cells (Figure 5E). Taken together, these results suggest that CSB is required for telomerase-dependent telomere elongation.

We also performed ChIP analysis with an antibody against TRF1 or TRF2, both of which are mediators of telomere length maintenance (14–16,42). We found that introduction of wild-type CSB into hTERT-GM10905 cells had little effect on telomeric association of TRF2 (Figure 5F and G), but it led to a significant increase in TRF1 association with telomeric DNA (Figure 5F and G). When compared to CSB-complemented hTERT-GM10905 cells, we observed a 54% (P=0.006) increase in the amount of telomere-bound TRF1 in hTERT-GM10905 cells expressing the vector alone (Figure 5G). The level of TRF1 in the vector-expressing hTERT-GM10905 cells was indistinguishable from that in the CSB-complemented hTERT-GM10905 cells (Figure 5H). These results suggest that association of TRF1 with telomeric DNA may be deregulated in CS cells carrying a CSB mutation.

CSB is required for maintaining the homeostatic level of TERRA. CSB has been implicated in transcription (32–35) and therefore we decided to examine whether CSB may be involved in regulating the expression of TERRA, a large non-coding telomere repeat-containing RNA (5). Northern analysis on three pairs of isogenic cell lines revealed a misregulation of TERRA associated with CS cells or CSB knockdown cells. We observed a 35% increase (P=0.017) in the level of TERRA in hTERT-GM10905 expressing the vector alone when compared to hTERT-GM10905 cells

complemented with wild-type CSB (Figure 6A and B). On the other hand, the level of TERRA in GM16095 cells expressing the vector alone was ≈45% (P=0.016) less than that in GM16095 complemented with wild-type CSB (Figure 6C and D). Knockdown of CSB led to a 38% (P=0.038) reduction in the level of TERRA in HeLaI.2.11 cells (Figure 6E and F). These results suggest that CSB is required for the homeostatic level of TERRA and that the level of TERRA may increase or decrease in CS cells depending upon the nature of CSB mutations.

4.2.5 Discussion

CSB, a multifunctional protein, plays an important role in DNA repair, transcription and chromatin remodeling. In this report, we have uncovered a role for CSB in telomere maintenance and protection. We have shown that CSB interacts physically with TRF2, a key component of the shelterin complex essential for telomere maintenance. We have demonstrated that CS cells or CSB knockdown cells exhibit an accumulation of telomere doublets and an induction of TIF formation. We have shown that CS cells carrying a CSB mutation are defective in telomerase dependent telomere elongation whereas introduction of CSB into CS cells results in telomerase-dependent telomere elongation, suggesting that CSB is required for telomere length maintenance. Furthermore, we have shown that the level of TERRA is misregulated in CS cells or CSB knockdown cells. Taken together, these results reveal an important role of CSB in the maintenance of telomere length and integrity. These results further imply that CS patients

lacking functional CSB are defective in telomere maintenance, which is associated with cancer and aging.

Our co-immunoprecipitation studies suggest that a small percentage of endogenous TRF2 (estimated to be ≈1–5%) interacts with CSB and vice versa. This low level of interaction is similar to previously reported association between TRF2 and several other DNA repair proteins including XPF/ERCC1 and Mre11/Rad50/Nbs1 (40,43), indicating that CSB interaction with TRF2 may be dependent upon a specific functional requirement.

Analysis of domain mapping suggests that the TRFH domain of TRF2 is sufficient and required for its interaction with CSB. The TRFH domain of TRF2 has been shown to interact with proteins containing the Y/FxLxP motif (17,18). CSB contains one YxLxP motif corresponding to amino acids 402–406 but also seven degenerate Y/FxLxx motifs spread throughout the entire protein. Double mutations at positions L404 and P406 did not abrogate CSB interaction with TRF2 (T.R.H. Mitchell and X.D. Zhu, unpublished data). These results, in conjunction with our finding that multiple domains of CSB are engaged in its interaction with TRF2 raise the possibility that TRF2 might interact with degenerate Y/FxLxx motifs of CSB. Alternatively, TRF2 may interact with CSB through a mechanism independent of Y/FxLxP motifs. Future studies are required to investigate the mechanism underlying CSB interaction with TRF2.

The physical interaction between TRF2 and CSB raises the possibility that TRF2 may play a role in recruiting and/or modulating CSB function at telomeres. We have

observed localization of CSB at a small subset of human telomeres. Several shelterin accessory proteins have been reported to localize at one or a few human telomeres, including HP1, BLM, PNUTS and MCPH1 (53–55). Perhaps, like these shelterin accessory factors, CSB might be needed by only a few telomeres at a given time although we cannot rule out the possibility that the colocalization of CSB with a few telomeres may be coincidental.

We have shown that overexpression of wild-type CSB has little effect on the telomere association of TRF2 but results in a reduction in the amount of telomere-bound TRF1, a negative mediator of telomerase-dependent telomere elongation. Perhaps, the reduction in the level of telomere-bound TRF1 may in part contribute to the telomerase-dependent telomere elongation observed in CSB-expressing hTERT-GM10905 cells. We have not been able to detect any interaction between CSB and endogenous TRF1 (T.R.H Mitchell and X.D. Zhu, unpublished data), suggesting that the effect of CSB on TRF1 binding to telomeric DNA may be indirect.

While we have observed a greater accumulation of telomere loss in CS primary fibroblast GM739 (p19) and GM1428 cells (p15) than in the control cells GM38 (p19) and GM9503 (p18), no significant difference in the formation of telomere loss has been detected between the heterozygote mother GM10901 and her CS offspring GM10905. It is possible that the lack of difference in telomere loss between the heterozygote mother and her CS offspring may be due to CSB haploinsufficiency. Alternatively, the level of

accumulation of telomere loss observed in CS cells may vary depending upon their genetic background.

We have found that while knockdown of CSB leads to a reduction in the level of TERRA, overexpression of wildtype CSB can have an opposite effect on the level of TERRA in CS cells. Introduction of wild-type CSB into CS cells hTERT-GM10905 results in a decrease in the level of TERRA whereas introduction of wild-type CSB into CS cells GM16095 leads to an increase in the level of TERRA. Both CS cell lines carry a nonsense mutation (Supplementary Table S1), which converts R735 to a stop codon in GM10905 (22,56) and K337 to a stop codon in GM16095 (27). The level of overexpressed CSB in hTERT-GM10905 cells is comparable to that in GM16095 (N. Batenburg, T.R.H. Mitchell and X.D. Zhu, unpublished data), suggesting that it is unlikely that exogenously expressed CSB may account for its opposite effect on the level of TERRA in these two cell lines. Although both cell lines do not express full-length CSB, GM10905 cells express a CSB-PiggyBac fusion protein (Figure 5D) (52), which is not present in GM16095 (27). CSB-PiggyBac is a product of alternative splicing involving the first five exons of CSB and a conserved PiggyBac transposable element (PGBD3) located within the intron 5 of the CSB gene (52). How overexpression of CSB differentially affects the level of TERRA remains unknown. Our finding suggests that the nature of CSB mutations may play a role in influencing TERRA expression. Taken together, our data suggest that CSB is required for maintaining the homeostatic level of

TERRA, excess expression or depletion of which has been shown to impair the maintenance of telomere length and integrity (5,6,57,58).

We have shown that CSB mutations or CSB depletion promotes the formation of telomere doublets, also known as fragile telomeres (9,51). It has been shown that fragile telomeres can arise from a defect in telomere replication (9,51). Consistent with this notion, we have observed that treatment with aphidicolin further induces the formation of telomere doublets in CS cells, suggesting that telomere replication is compromised in CS cells. It is likely that the compromised telomere replication in CS cells may be in part caused by misregulation of TERRA, an integral component of telomere heterochromatin. Perhaps misregulation of TERRA associated with CS cells could lead to an altered telomere heterochromatin, which could impede the progression of the replication fork.

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Conflict of interest statement

None declared.

4.2.6 Figures and Figure Legends

Figure 1. CSB interacts physically with TRF2. (A) Schematic diagram of CSB. NLS: nuclear localization sequence; NTB: nucleotide binding domain; and UBD: ubiquitin binding domain. (B) Co-immunoprecipitation with HeLa cell extracts and anti-CSB antibody. Anti-IgG IP was used as a negative control. Immunoblotting was carried out with anti-CSB or anti-TRF2 antibody. (C) Co-IP with HeLa nuclear extracts and anti-TRF2 antibody. Anti-IgG IP was used as a negative control. Immunoblotting was carried out with anti-CSB or anti-TRF2 antibody. (D) IP with anti-Myc antibody was carried out with protein extracts from 293T cells coexpressing Flag-TRF2 in conjunction with either the vector alone, Myc-CSB, Myc-CSB-N, Myc-CSB-ATPase or Myc-CSB-C. Immunoblotting was performed with anti-Myc or anti-Flag antibody. (E) Schematic diagram of TRF2. B stands for basic domain. (F) IP with anti-Myc antibody was carried out with protein extracts from 293T cells coexpressing the vector or Myc-CSB in conjunction with Flag-TRF2^{linker}, Flag-TRF2^{TRFH} or Flag-TRF2$^{\Delta B \Delta M}$. Immunoblotting was performed with anti-Myc or anti-Flag antibody.

Figure 1

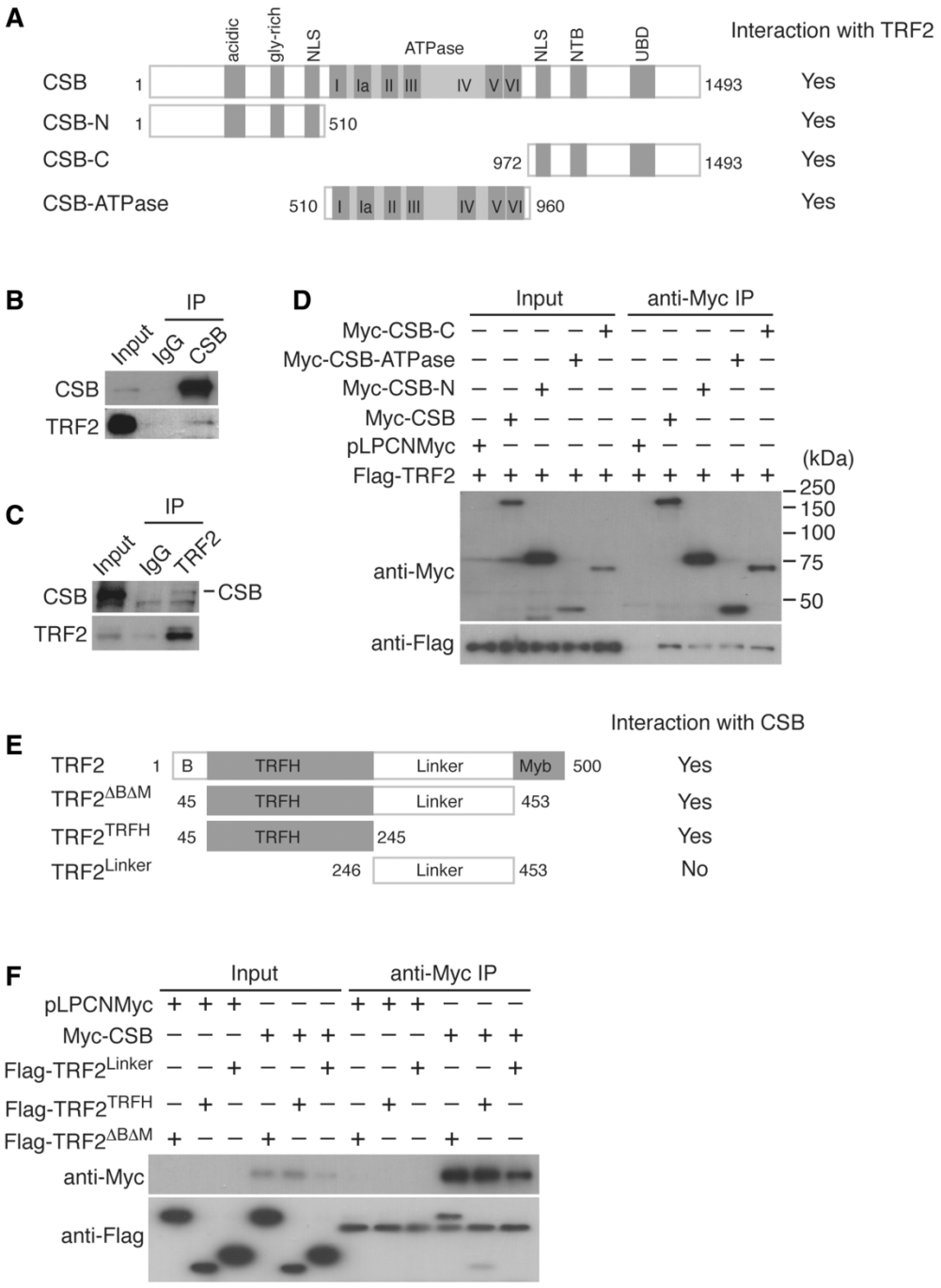

A
acidic
gly-rich
NLS
ATPase
NLS
NTB
UBD
Interaction with TRF2
CSB 1 I Ia II III IV V VI 1493 Yes
CSB-N 1 510 Yes
CSB-C 972 1493 Yes
CSB-ATPase 510 I Ia II III IV V VI 960 Yes
B
IP
Input
IgG
CSB
CSB
TRF2
C
IP
Input
IgG
TRF2
CSB
CSB
TRF2
D
Input
anti-Myc IP
Myc-CSB-C
Myc-CSB-ATPase
Myc-CSB-N
Myc-CSB
pLPCNMyc
Flag-TRF2
(kDa)
250
150
100
75
50
anti-Myc
anti-Flag
E
Interaction with CSB
TRF2 1 B TRFH Linker Myb 500 Yes
TRF2ΔBΔM 45 TRFH Linker 453 Yes
TRF2TRFH 45 TRFH 245 Yes
TRF2Linker 246 Linker 453 No
F
Input
anti-Myc IP
pLPCNMyc
Myc-CSB
Flag-TRF2Linker
Flag-TRF2TRFH
Flag-TRF2ΔBΔM
anti-Myc
anti-Flag

Figure 2. CSB localizes at a small subset of human telomeres and prevents the formation of TIFs in CS cells. (A) Analysis of indirect immunofluorescence (IF) on HeLaII and CSB-complemented hTERT-GM10905 cells. IF was performed with mouse anti-CSB (red) in conjunction with rabbit anti-hRap1 (green). Cells were extracted with detergent prior to fixation by paraformaldehyde to remove soluble proteins. Cell nuclei were stained with DAPI shown in blue. Arrowheads indicate the overlap between anti-CSB and anti-hRap1 staining. (B) Analysis of IF-FISH on GM16095 cells expressing Myc-CSB. IF–FISH analyses were performed with anti-Myc antibody (red) in conjunction with a FITC-conjugated $(CCCTAA)_3$-containing PNA probe (green). Cell nuclei were stained with DAPI shown in blue. Arrowheads indicate the colocalization of CSB with telomeric DNA. (C) Indirect immunofluorescence using anti-hRap1 in conjunction with anti-γ-H2AX was performed with fixed hTERT-GM10905 cells expressing either the vector alone or wild-type CSB. Arrowheads indicate sites of colocalization of γ-H2AX and hRap1. (D) Quantification of percentage of cells with five or more TIFs. For each cell line, a total of 300 cells from three independent experiments were scored. Standard deviations from three independent experiments are indicated.

Figure 2

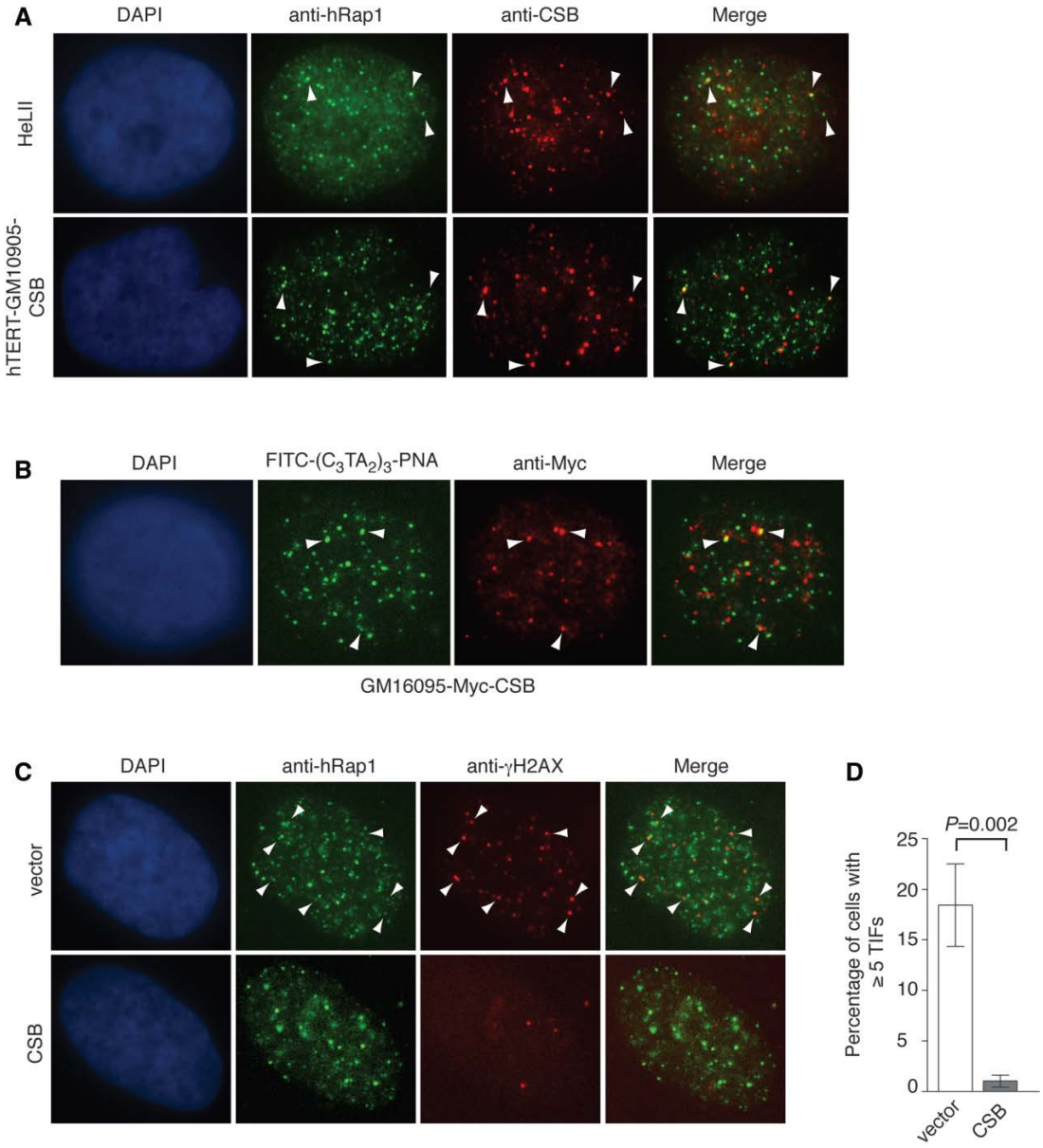

A
DAPI
anti-hRap1
anti-CSB
Merge
HeLII
hTERT-GM10905-CSB
B
DAPI
FITC-(C3TA2)3-PNA
anti-Myc
Merge
GM16095-Myc-CSB
C
DAPI
anti-hRap1
anti-γH2AX
Merge
vector
CSB
D
P=0.002
Percentage of cells with ≥ 5 TIFs
25
20
15
10
5
0
vector
CSB

Figure 3. CS primary fibroblasts carrying CSB mutations accumulate telomere doublets. (A) Analysis of metaphase chromosomes from GM10901 and GM10905. Chromosomes were stained with DAPI and false colored in red. Telomeric DNA was detected by FISH using a FITC-conjugated $(CCCTAA)_3$-containing PNA probe (green). Open arrows represent telomere doublets whereas asterisks indicate telomere loss. Enlarged images of chromosomes with telomere doublets or telomere loss are shown at the bottom. (B–E) Quantification of telomere loss or telomere doublets from indicated cell lines. For each cell line, a total of 2410–2699 chromosomes from 60 metaphase cells were scored in a blind manner for the presence of telomere loss (B and D) as well as telomere doublets in (C and E). Standard deviations derived from three independent experiments are indicated. Passage numbers of cell lines used are indicated above the bars.

Figure 3

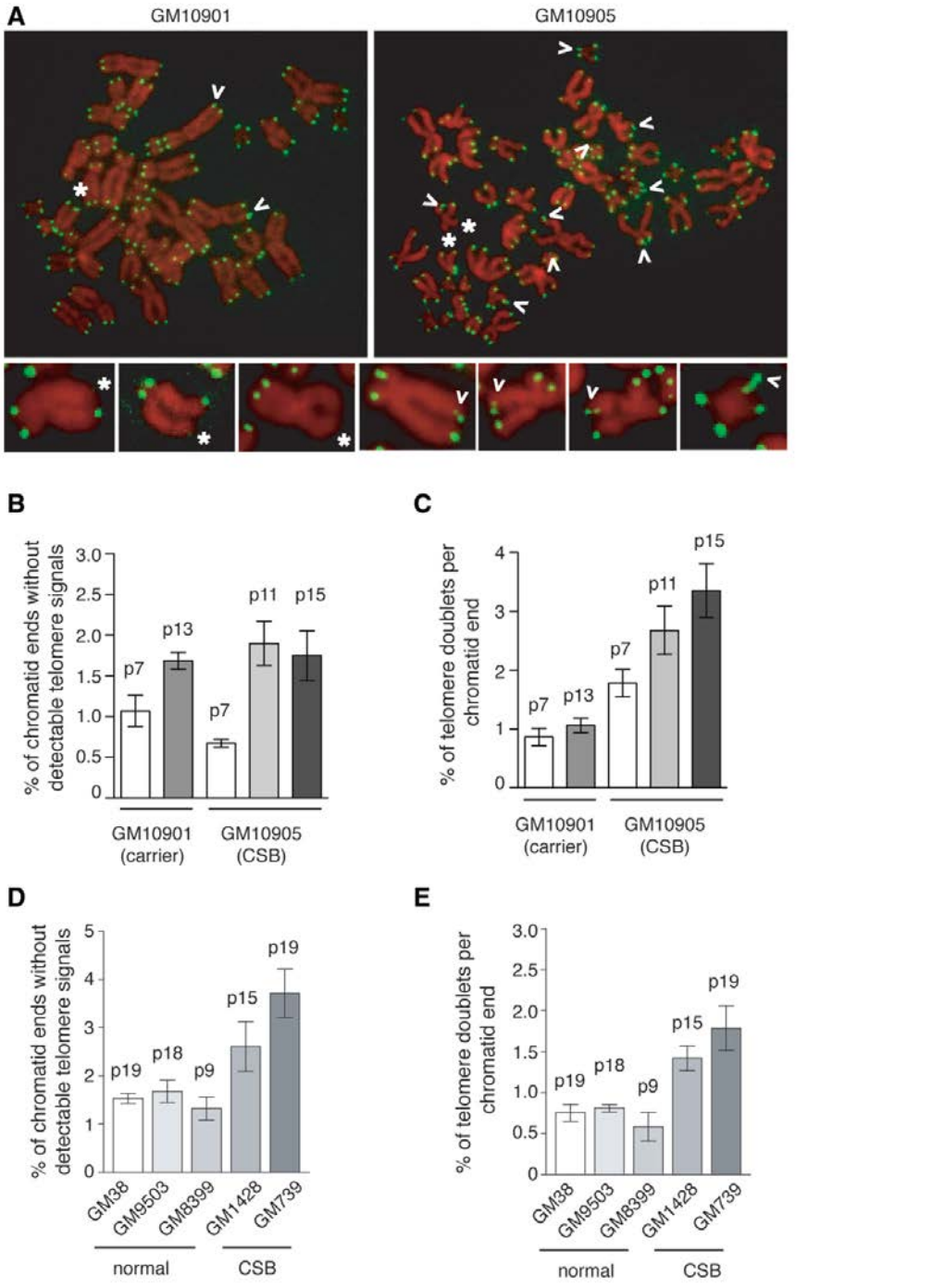

A
GM10901
GM10905
B
% of chromatid ends without detectable telomere signals
p7
p13
p7
p11
p15
GM10901 (carrier)
GM10905 (CSB)
C
% of telomere doublets per chromatid end
p7
p13
p7
p11
p15
GM10901 (carrier)
GM10905 (CSB)
D
% of chromatid ends without detectable telomere signals
p19
p18
p9
p15
p19
GM38
GM9503
GM8399
GM1428
GM739
normal
CSB
E
% of telomere doublets per chromatid end
p19
p18
p9
p15
p19
GM38
GM9503
GM8399
GM1428
GM739
normal
CSB

Figure 4. CSB is required to prevent the formation of telomere doublets. (A) Quantification of telomere loss from indicated cell lines. For each cell line, a total of at least 2649–2668 chromosomes from 60 metaphase cells were scored in a blind manner. Standard deviations derived from three independent experiments are indicated. (B) Quantification of telomere doublets from indicated cell lines. For each cell line, a total of 2649–2668 chromosomes from 60 metaphase cells were scored in a blind manner. Standard deviations derived from three independent experiments are indicated. (C) Quantification of telomere doublets from hTERT-GM10905 cells expressing indicated constructs. For each cell line, a total of 2707–2754 chromosomes from 60 metaphase cells were scored in a blind manner. Standard deviations derived from three independent experiments are indicated. (D) Quantification of telomere doublets from GM16095 cells expressing indicated constructs. For each cell line, a total of 4774–4923 chromosomes from 60 metaphase cells were scored in a blind manner. Standard deviations derived from three independent experiments are indicated. (E) Western analysis of CSB expression. CSB was stably knocked down in HeLaI.2.11 cells. Immunoblotting was performed with anti-CSB or anti-γ-tubulin antibody. The latter was used as a loading control. (F) Quantification of telomere doublets from HeLaI.2.11 cells expressing the vector alone or pRS-shCSB. For each cell line, a total of 2678–2961 chromosomes from at least 43 metaphase cells were scored in a blind manner. Standard deviations derived from three independent experiments are indicated. (G) Quantification of telomere doublets from GM16095 cells expressing indicated constructs. Cells were treated with DMSO or aphidicolin (0.2 μM) for 16 h. For each cell line, a total of 3879–4321 chromosomes from 51 to 53 metaphase cells were scored in a blind manner. Standard deviations derived from three independent experiments are indicated.

Figure 4

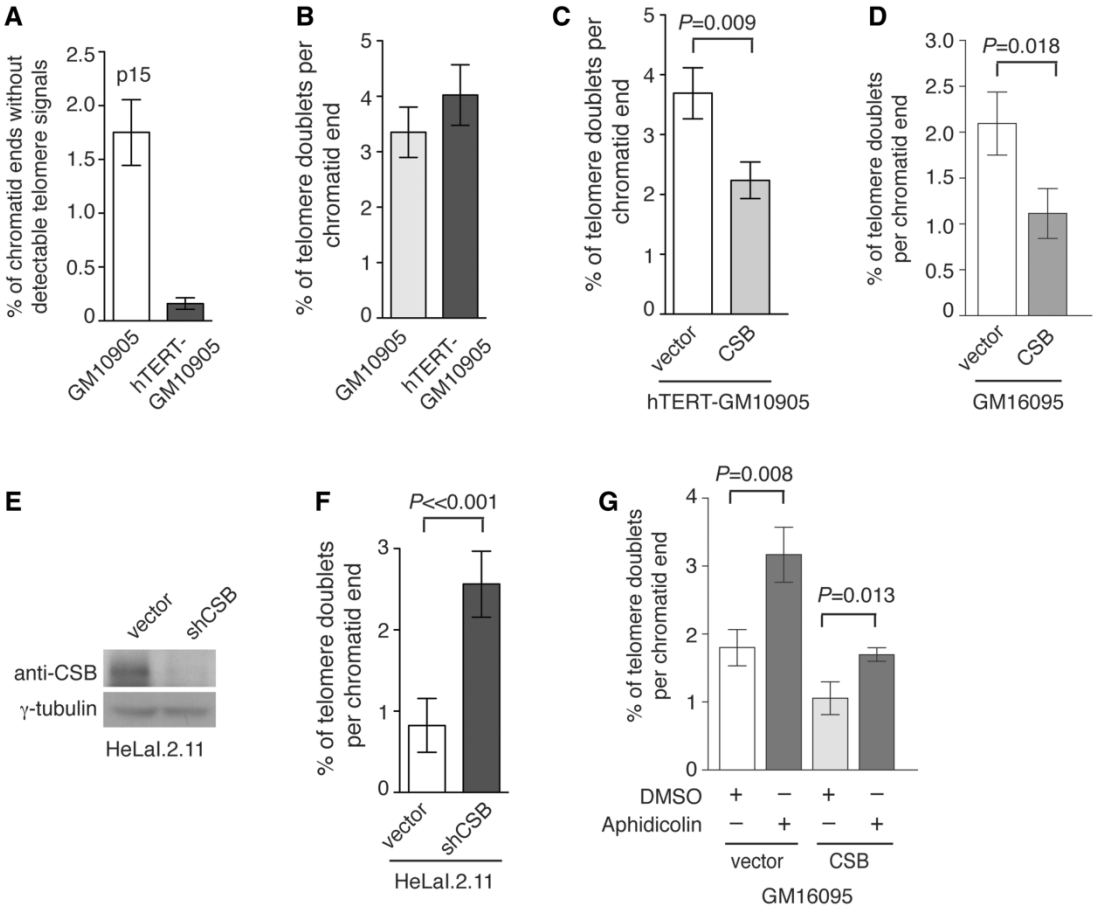

A
% of chromatid ends without detectable telomere signals
p15
GM10905
hTERT-GM10905
B
% of telomere doublets per chromatid end
GM10905
hTERT-GM10905
C
% of telomere doublets per chromatid end
P=0.009
vector
CSB
hTERT-GM10905
D
% of telomere doublets per chromatid end
P=0.018
vector
CSB
GM16095
E
vector
shCSB
anti-CSB
γ-tubulin
HeLaI.2.11
F
% of telomere doublets per chromatid end
P<<0.001
vector
shCSB
HeLaI.2.11
G
% of telomere doublets per chromatid end
P=0.008
P=0.013
DMSO
Aphidicolin
vector
CSB
GM16095

Figure 5. CSB is required for telomere length maintenance. (A) Genomic blot of telomeric restriction fragments from hTERT-immortalized GM10901 and GM10905 cells. About 3 μg of RsaI/HinfI-digested genomic DNA from each sample was used for gel electrophoresis. DNA molecular weight markers are shown on the left of the blot. Median telomere lengths of indicated cell lines are shown on the bottom of the blot. (B) Genomic blots of telomeric restriction fragments from hTERT-GM10905 cells expressing either the vector alone or wild-type CSB as indicated above the lanes. About 3 mg of RsaI/HinfI-digested genomic DNA from each sample was used for gel electrophoresis. PDs are indicated above the lanes whereas DNA molecular weight markers are shown on the left of the blots. The bottom panel, taken from an ethidium bromide-stained agarose gel, is used as a loading control. (C) Median telomere length of indicated cell lines was plotted against PDs. (D) Western analysis of CSB expression in hTERT-GM10905 cells. Immunoblotting was performed with anti-CSB or anti-γ-tubulin antibody. The indicated CSB-PiggyBac fusion protein is a product of alternative splicing involving the first five exons of CSB and a conserved PGBD3 located within the intron 5 of the CSB gene (52). (E) Growth curve of hTERT-GM10905 cells expressing various constructs as indicated. The number of PDs was plotted against days in culture. (F) Dot blots of ChIPs with anti-TRF1 or anti-TRF2 antibody. ChIPs were performed with lysates from hTERT-GM10905 cells expressing either the vector alone or wild-type CSB. Anti-IgG ChIP was used as a control. (G) Quantification of ChIPs from (E). The signals from dot blots were quantified by ImageQuant (IQ) analysis. Standard deviations from three independent experiments are shown. (H) Western analysis of protein expression. Immunoblotting was carried out with anti-TRF1, anti-TRF2 or anti-γ-tubulin antibody.

Figure 5

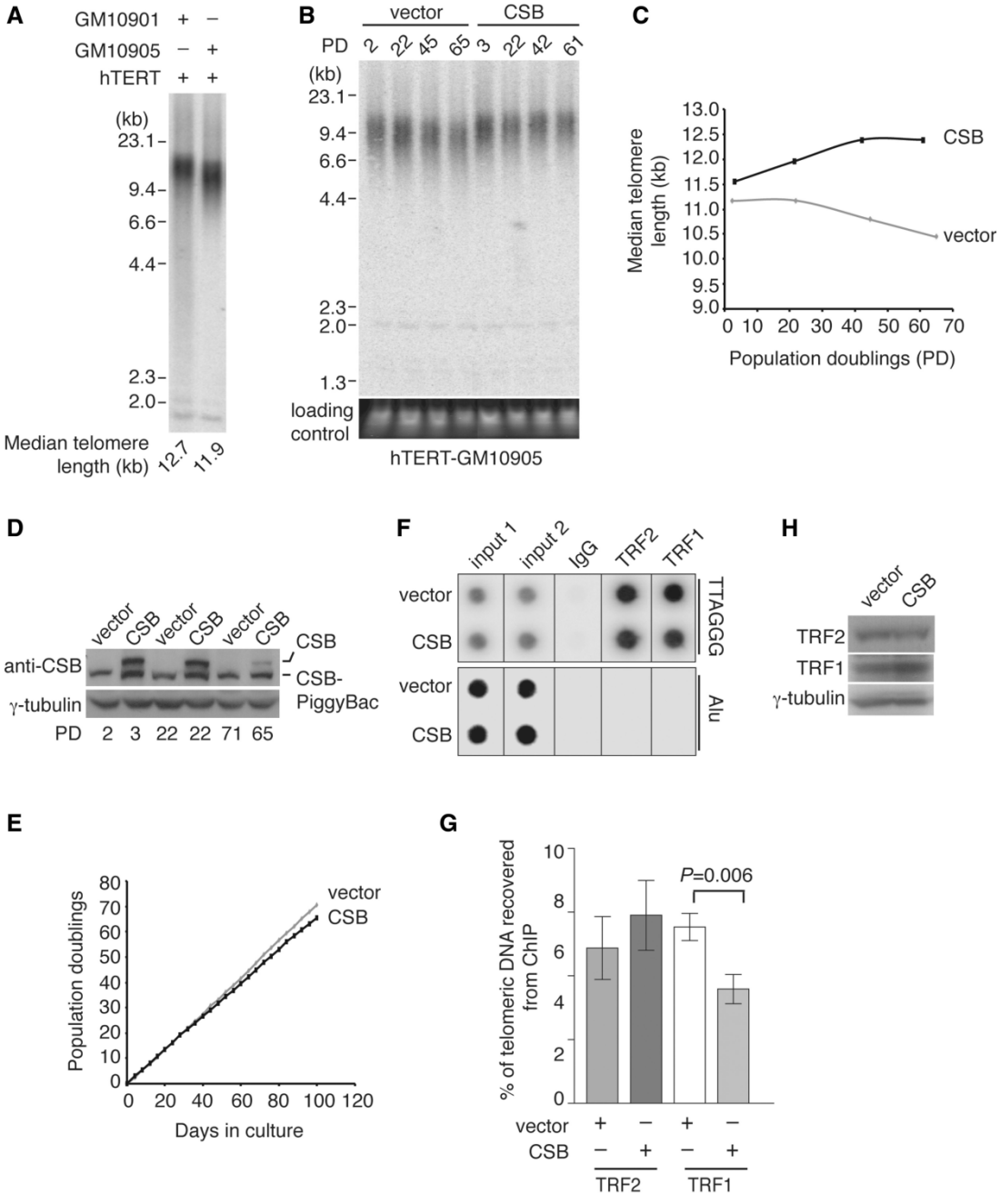
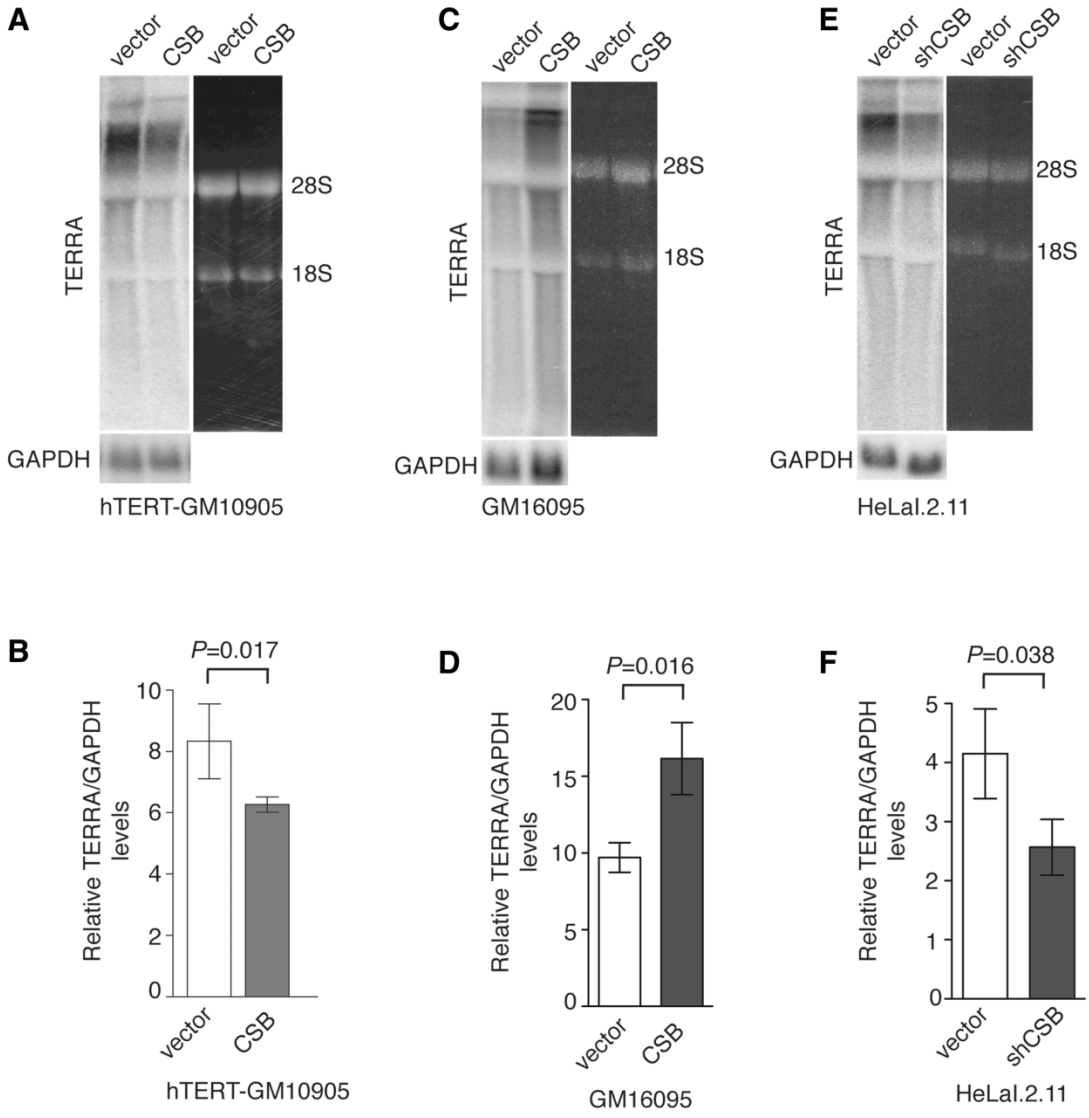

A
GM10901 + –
GM10905 – +
hTERT + +
(kb)
23.1
9.4
6.6
4.4
2.3
2.0
Median telomere length (kb) 12.7 11.9
B
vector
CSB
PD 2 22 45 65 3 22 42 61
(kb)
23.1
9.4
6.6
4.4
2.3
2.0
1.3
loading control
hTERT-GM10905
C
Median telomere length (kb)
13.0
12.5
12.0
11.5
11.0
10.5
10.0
9.5
9.0
CSB
vector
0 10 20 30 40 50 60 70
Population doublings (PD)
D
vector CSB vector CSB vector CSB
anti-CSB
CSB
CSB-PiggyBac
γ-tubulin
PD 2 3 22 22 71 65
F
input 1
input 2
IgG
TRF2
TRF1
vector
CSB
TTAGGG
vector
CSB
Alu
H
vector
CSB
TRF2
TRF1
γ-tubulin
E
Population doublings
80
70
60
50
40
30
20
10
0
vector
CSB
0 20 40 60 80 100 120
Days in culture
G
% of telomeric DNA recovered from ChIP
10
8
6
4
2
0
P=0.006
vector + – + –
CSB – + – +
TRF2
TRF1

Figure 6. CSB is required for maintaining the homeostatic level of TERRA. (A) Analysis of TERRA expression from hTERT-GM10905 cells expressing the vector alone or CSB. Northern blotting was performed with a 32P-labeled telomeric DNA-containing probe shown on the left top panel. The northern blot of GAPDH shown on the left bottom panel was used as a loading control. The right panel was taken from the ethidium bromide-stained agarose gel. The position of 28S or 18S ribosomal RNA is indicated. (B) Quantification of relative TERRA levels from (A). The signals from northern blots were quantified by ImageQuant analysis. The TERRA signal from each lane was normalized to the GAPDH signal in the corresponding lane, giving rise to the relative level of TERRA to GAPDH. (C) Northern analysis of TERRA expression from GM16095 cells expressing the vector alone or wild-type CSB. The northern blot of GAPDH shown on the left bottom panel was used as a loading control. The right panel was taken from the ethidium bromide-stained agarose gel. The position of 28S or 18S ribosomal RNA is indicated. (D) Quantification of relative TERRA levels from (C). Quantification was performed as described in (B). (E) Northern analysis of TERRA expression from HeLaI.2.11 cells stably expressing the vector alone or pRS-shCSB. The northern blot of GAPDH shown on the left bottom panel was used as a loading control. The right panel was taken from the ethidium bromide-stained agarose gel. The position of 28S or 18S ribosomal RNA is indicated. (F) Quantification of relative TERRA levels from (E). Quantification was performed as described in (B).

Figure 6


A
vector
CSB
vector
CSB
28S
18S
TERRA
GAPDH
hTERT-GM10905
C
vector
CSB
vector
CSB
28S
18S
TERRA
GAPDH
GM16095
E
vector
shCSB
vector
shCSB
28S
18S
TERRA
GAPDH
HeLaI.2.11
B
P=0.017
Relative TERRA/GAPDH levels
10
8
6
4
2
0
vector
CSB
hTERT-GM10905
D
P=0.016
Relative TERRA/GAPDH levels
20
15
10
5
0
vector
CSB
GM16095
F
P=0.038
Relative TERRA/GAPDH levels
5
4
3
2
1
0
vector
shCSB
HeLaI.2.11

4.2.7 Supplementary Figures

Figure S1.

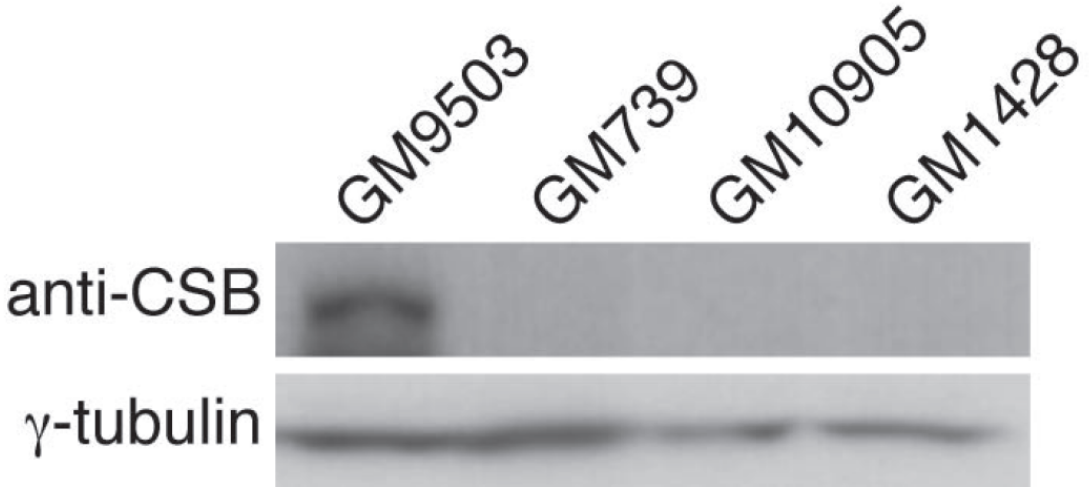


Figure S1. Western analysis of CSB expression in normal and CS primary fibroblasts. Immunoblotting was carried out with anti-CSB or anti-γ-tubulin antibody. The latter was used as a loading control.

Figure S2.

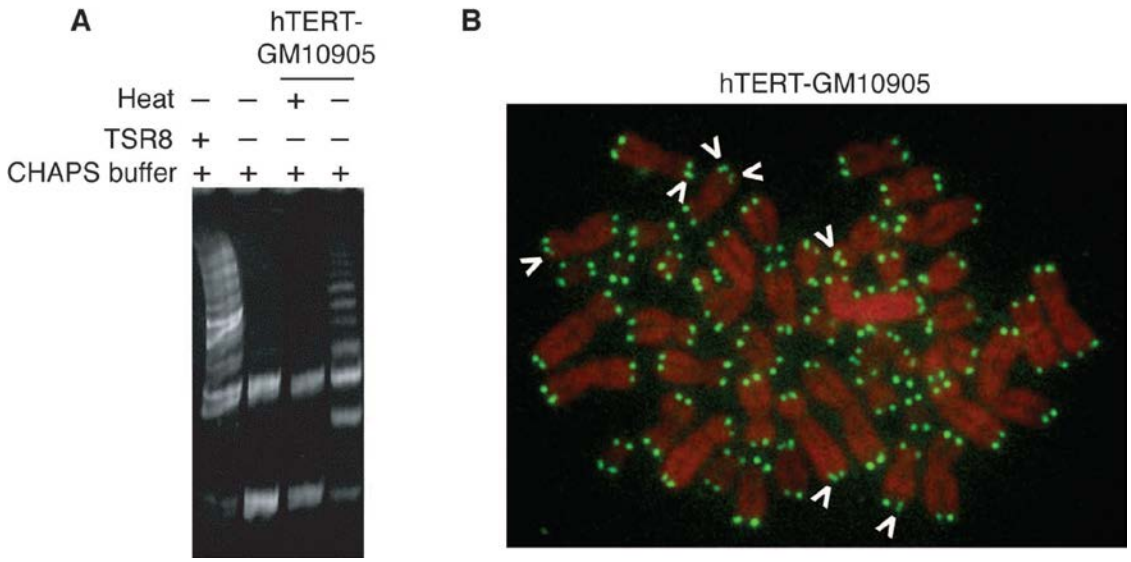


Figure S2. (**A**) Analysis of hTERT expression in GM10905 cells. One thousand cells of hTERT-immortalized GM10905 cells were used to measure telomerase activity. TSR8 was used as a positive control whereas 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate buffer (CHAPS) was used as a negative control. (**B**) Analysis of metaphase chromosomes from hTERT-GM10905. Chromosomes were stained with DAPI and false colored in red. Telomeric DNA was detected by FISH using a FITC-conjugated $(CCCTAA)_3$-containing PNA probe (green).

Figure S3.

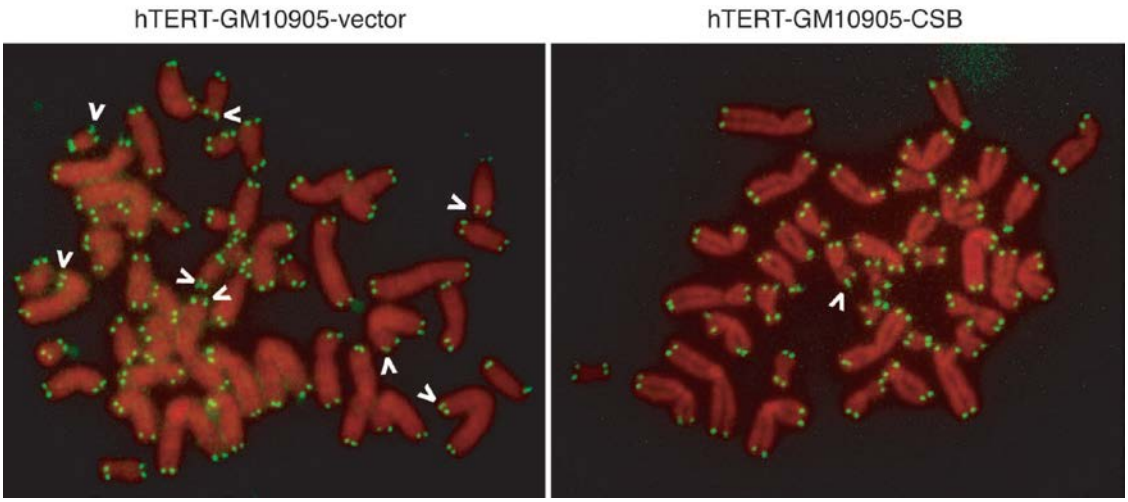


Figure S3. Analysis of metaphase chromosomes from hTERT-GM10905 stably expressing either the vector alone or wild type CSB. Chromosomes were stained with DAPI and false colored in red. Telomeric DNA was detected by FISH using a FITC-conjugated $(CCCTAA)_3$-containing PNA probe (green). Open arrows represent telomere doublets.

Figure S4.

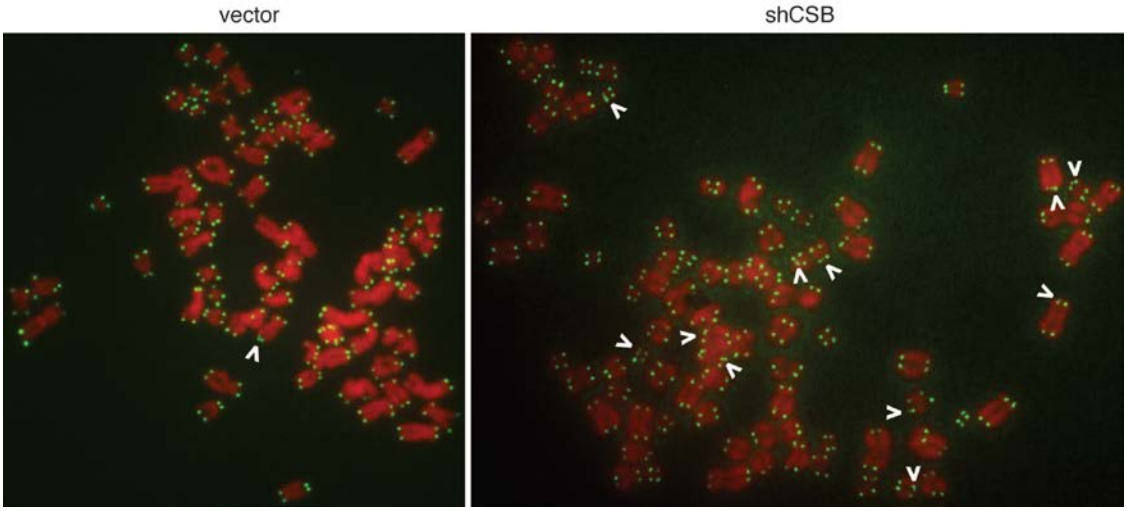


Figure S4. Analysis of metaphase chromosomes from HeLaI.2.11 stably expressing either the vector alone or pRS-shCSB. Chromosomes were stained with DAPI and false colored in red. Telomeric DNA was detected by FISH using a FITC-conjugated $(CCCTAA)_3$-containing PNA probe (green). Open arrows represent telomere doublets.

Supplementary Table S1. Summary of normal and CS primary fibroblast cell lines used.

Cell Line	Age at Biopsy	CSB Allele 1	CSB Allele 2	Source
GM38	9 YR	Normal	Normal	NIGMS
GM9503	10 YR	Normal	Normal	NIGMS
GM8399	19 YR	Normal	Normal	NIGMS
GM10901	42 YR	R735X	Normal	NIGMS
GM10905	10 YR	R735X	R735X	NIGMS
GM739	3 YR	K377X	R857X	NIGMS
GM1428	8 YR	N/A	N/A	NIGMS

4.2.8 References

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Chapter 5

Discussion

5.1 Summary of work

The work presented in this thesis has revealed novel TRF2 interacting factors important for telomere maintenance, which is relevant to cancer and aging. I have found that TRF2 is methylated on multiple arginines in its basic domain and that this arginine methylation plays an important role in telomere maintenance. In addition, I have identified that CSB interacts with TRF2 and that this interaction is important for telomere maintenance.

I have revealed a novel role for PRMT1 in telomere maintenance. I have identified that PRMT1 and TRF2 interact *in vivo*, and that PRMT1 is the methyltransferase responsible for TRF2 methylation. The overexpression of TRF2 carrying mutations of arginines to lysines resulted in growth inhibition, telomere dysfunction induced foci, and telomere doublets. The knockdown of PRMT1 recapitulated the telomere doublet phenotype and resulted in accelerated telomere shortening in telomerase positive cancer cell lines. I have also shown that TRF2 binding to telomeric DNA and TERRA is enriched in PRMT1 knockdown cells. These findings suggest that PRMT1 knockdown leads to a change in telomere organization, which may be a contributing factor to the telomere instability reported (Mitchell et al. 2009).

I have shown that methylated TRF2 is predominantly not localized to telomeres, and that methylated TRF2 fractionates with the core nuclear matrix component, lamin A. Immunofluorescence using a methylated TRF2 specific antibody revealed that it forms homogenous nuclear staining in early passage primary fibroblasts; however in late

passage senescent fibroblasts methylated TRF2 has an altered irregular staining pattern. The percentage of cells with an altered staining of methylated TRF2 was reliably increased upon replicative senescence, ionizing radiation and acute telomere dysfunction-induced cellular senescence. Additionally, I have found that the altered methylated TRF2 staining was not present in irradiated cells deficient in ATM. Our findings suggest that methylated TRF2 may serve as a potential biomarker for cellular aging.

I have identified an interaction between TRF2 and CSB. Mapping of this interaction suggests that multiple domains of CSB interact with TRF2, most likely through the TRFH domain of TRF2. Cockayne syndrome fibroblast cells have an increase in telomere doublets and telomere loss compared to normal fibroblasts. The telomere loss phenotype could be rescued by the introduction of hTERT; however the telomere doublet phenotype was only rescued once CSB was exogenously expressed. I have validated this phenotype by knocking down CSB in HeLa cells, which results in a three-fold increase in telomere doublets. I have shown that CSB is important for the regulation of TERRA levels, as well as TRF1 enrichment at telomeric DNA. Taken together, our findings suggest that CSB plays an important role in telomere maintenance.

5.2 TRF2 arginine methylation

There are over 300 different protein post translational modifications (PTM) that have been identified (Zhao, Jensen 2009). The characterization of PTM sites provides valuable information about the molecular pathways and cellular functions of a protein.

Mass spectrometry is a common and effective method used to map PTM sites, which can then be further analyzed through *in vitro* studies and mutational analyses to shed further light on the *in vivo* characteristics of a PTM protein. The use of a PTM specific antibody can provide information that would be difficult to ascertain through other methods, such as novel protein localization or compartmentalization. This is especially true if it is only a fraction of the total protein population that becomes modified. For example, when staining with a pan H2A.X antibody following ionizing radiation the staining pattern is indistinguishable from non-irradiated cells, whereas staining of irradiated cells with an antibody that specifically recognizes H2A.X phosphorylated at S139 (γ-H2A.X) allows for the visualization of foci at sites of DNA DSBs which are absent from non-irradiated cells (Rogakou et al. 1999). PTM specific antibodies have also been a useful method for uncovering the novel localization of shelterin components. A phosphorylation specific antibody against S367 of TRF1 demonstrated that TRF1 phosphorylated at this site is targeted to nuclear proteasome centers (McKerlie, Lin & Zhu 2012). A TRF1 phospho-specific T371 antibody showed that there is an increase in pT371-TRF1 during mitosis, which is unbound to telomeres (McKerlie, Zhu 2011). As well, a small population of pT371-TRF1 is recruited to sites of DNA double strand breaks (McKerlie et al. 2013), indicating that pT371-TRF1 is important for multiple roles of TRF1 in the cell. In both cases, the currently available pan TRF1 antibodies are not capable of elucidating the localization of endogenous TRF1 to proteasome centers or DNA DSBs. Therefore, without the aid of a phospho-specific antibody these novel roles would have been difficult to elucidate.

Our methylated TRF2 antibody is another example of how PTM antibodies can be used to uncover a sub-population of a protein that is unique compared to the total population. Since methylated TRF2 is only a small percentage of the total TRF2 in the cell its localization is not observed using a pan TRF2 antibody, which will predominantly recognize the more abundant non-methylated TRF2. However, because only a small amount of TRF2 is methylated, this does not mean that it does not have an important cellular function. Methylation events that use AdoMet as a methyl donor, such as arginine methylation, do so at a cost of 12 ATP, making methylation the most expensive form of post-translational modification in terms of energy/carbon (Lake, Bedford 2007). Therefore, from an evolutionary standpoint, arginine methylation events likely play important and essential roles; otherwise they would be selected against over time (Lake, Bedford 2007). This assumption is in agreement with our findings that methylated TRF2 is important for proper telomere maintenance and possibly senescent signalling.

There are a number of useful biomarkers that can be used to indicate senescence and *in vivo* aging; however each has its own limitations and no single marker is indisputably used to indicate senescence. Probably the most commonly used biomarker for senescent cells is β-galactosidase staining. Some limitations of β-galactosidase staining are that some cells that are not senesced, such as melanocytes, show strong β-galactosidase staining, as well, confluency of the cells at the time of the assay can alter β-galactosidase activity (Dimri et al. 1995, Debacq-Chainiaux et al. 2009). Another limitation is that senescence associated β-galactosidase staining can be inhibited by the

loss $p16^{INK4a}$ (Bertrand-Vallery et al. 2010, Lewis et al. 2008). Other biomarkers have similar limitations due to cell type specific gene expression, genetic variability, and the heterogeneous nature of the induction of cellular senescence (Kuilman et al. 2010). For this reason, it is important that new biomarkers for cellular senescence are found that can be used to identify with the highest validity senescent cells *in vitro* and *in vivo*.

Although we have yet to fully elucidate the mechanism(s) involving methylated TRF2, we have shown that it has a homogeneous nuclear staining pattern which becomes altered during cellular senescence. Altered methylated TRF2 staining is seen in replicative senescent cells, as well as cells forced into early senescence via DNA damage, implying that methylated TRF2 may be important for the dual roles of cellular senescence in both aging progression and cancer prevention. Our data places the altered methylated TRF2 staining downstream of ATM, since lack of functional ATM prevents the formation of the altered staining pattern of methylated TRF2. Replicative senescence has been shown to be predominantly p53, p21 and ATM dependent, whereas the role of the pRB-$p16^{INK4a}$ pathway is less clear and possibly cell type specific (Kuilman et al. 2010, Herbig et al. 2004). However, pRB-$p16^{INK4a}$ can be the prominent pathway activated by oncogene induced senescence (OIS), and it would be beneficial to know whether methylated TRF2 staining is altered in OIS as well. In conclusion, altered methylated TRF2 staining could be used in conjunction with other senescence markers to allow for better identification of senescent cells *in vivo.* More tools to help identify senescent cells

in vivo will help improve our understanding of senescence in both pathological and non-pathological settings.

Although shelterin has traditionally been implicated in telomere metabolism, components of the shelterin complex have recently been suggested to play non-telomeric functions. TRF1 has been shown to facilitate DNA double strand break repair (McKerlie et al. 2013), whereas TIN2 has been reported to regulate glycolysis in the mitochondria (Chen et al. 2012). RAP1 has been found to be involved in transcriptional control of genes that protect against obesity (Martinez et al. 2013, Yeung et al. 2013). Furthermore, TRF2 has also been observed to function away from telomeres. TRF2 interacts with REST in neural cells and sequesters it at PML bodies (Zhang et al. 2008). This interaction controls neural stem cell fate (Zhang et al. 2008). A number of reports have also indicated that TRF2 may function in DNA double strand break repair. TRF2 colocalizes with DNA double strand breaks and regulates their repair process in a basic domain dependent manner (Bradshaw, Stavropoulos & Meyn 2005, Tanaka et al. 2005, Huda et al. 2009, Mao et al. 2007). Our finding that methylated TRF2 is associated with the nuclear matrix further supports the notion that TRF2 is involved in non-telomeric functions.

TRF2 acts as a protein hub for the recruitment of numerous proteins involved in telomere maintenance. The regulation of these interactions can be done spatially, temporally, or dependent on a number of post-translational modifications that occur on TRF2. The importance of these interactions are highlighted by the number of studies

showing that the loss of TRF2 or its interacting proteins induces the formation of dysfunctional telomeres, leading to the induction of cellular senescence (Mitchell et al. 2009, Zhang et al. 2008, Walker, Zhu 2012, Buscemi et al. 2009, Wu et al. 2007, Zhu et al. 2000). The disruption of TRF2 can lead to telomere fusions, telomere loss or telomere doublets depending on which domain is manipulated. Each type of telomere dysfunction is associated with the loss of a different aspect of telomere regulation: loss of protection against NHEJ, loss of protection against HR and the inability to replicate properly, respectively (Mitchell et al. 2009, van Steensel, Smogorzewska & de Lange 1998, Wang, Smogorzewska & de Lange 2004). These findings highlight the important role of TRF2 in telomere metabolism.

We have shown that PRMT1 plays an important role in telomere maintenance. PRMT1 is often misregulated in cancer and is regarded as a potential therapeutic target. Drugs that affect telomere maintenance are also considered potential cancer therapeutics. Our finding that arginine methylation regulates telomere length homeostasis brings awareness to the complexity in cancer therapeutics involved in targeting these two pathways.

5.3 CSB functions in telomere maintenance

I have shown that CSB is a TRF2 interacting protein and that it is important for telomere maintenance. In our publication, Nicole Batenburg uncovered a telomere defect

in a panel of CS cells. I have shown that HeLa cells expressing shRNA against CSB exhibit an accumulation of telomere doublets. Telomere doublets are an indication of telomere fragility that can be brought on by increased replication stress (Martinez et al. 2009, Sfeir et al. 2009), indicating that CSB may be important for telomere replication.

The amount of TRF2 and CSB that exist in a complex is of low abundance, however this is similar to previous reports for other TRF2 interacting factors, including MRE11, XPF, BLM, MCPH1 and PNUTS (Zhu et al. 2000, Zhu et al. 2003, Barefield, Karlseder 2012, Kim et al. 2009, Canudas et al. 2011).

The TRFH domain of TRF2 was found to be sufficient and required for its interaction with CSB, whereas the N-terminal, the C-terminal and the internal ATPase domains of CSB were all able to bind TRF2. Phenylalanine 120 (F120) resides in the TRFH domain of TRF2, which binds to a Y/FXLXP motif found in several TRF2 interacting proteins (Kim et al. 2009, Chen et al. 2008). CSB contains one YXLXP motif corresponding to amino acids 402–406. However, when I mutated L404 and P406 to alanines, these mutations did not abrogate the interaction (T.R.H. Mitchell and X-D. Zhu, unpublished data). The TRFH domain of TRF2 binds the core sequence Y/FXL and CSB contains seven of these motifs spread throughout the entire protein (Kim et al. 2009). Further studies will be needed to determine whether multiple Y/FXL motifs in CSB may be engaged in its interaction with TRF2. Having multiple interacting regions is not a unique mode of regulation for TRF2 or CSB. The basic domain of TRF2 interacts with multiple regions of WRN (Opresko et al. 2002). CSB is also known to interact with p53

through multiple domains including the ATPase and C-terminal regions (Lake, Basheer & Fan 2011).

We have shown that the level of TERRA is regulated by CSB and that the lack of functional CSB suppresses telomerase dependent telomere elongation (Batenburg et al. 2012). These findings indicate that telomere dysfunction is likely a contributing factor to the CS phenotype. This is yet another example of a premature ageing disorder that has telomere dysfunction, and serves to further strengthen the link between telomere health and aging.

5.4 Future direction

I have shown that methylation of the basic domain is important for telomere maintenance. The knockdown of PRMT1 inhibited telomerase dependent telomere elongation in three different cancer cell lines, whereas the knockdown of PRMT1 in primary fibroblasts hTERT-BJ caused an increase in telomere doublets, which is an indication of a replication defect. Future studies will be needed to investigate the molecular mechanism by which methylated TRF2 contributes to efficient telomere replication. The disruption of several TRF2-interacting proteins (Apollo, BLM, FEN1, WRN and RTEL), have been shown to cause a similar phenotype to the loss of PRMT1 or the overexpression of TRF2-RK, and therefore it would be of interest to investigate whether arginine methylation may regulate any of these interactions (Sfeir et al. 2009, van Overbeek, de Lange 2006, Saharia et al. 2010, Liu, Barchowsky & Opresko 2010).

Apollo is a TRF2 interacting protein that when knocked down has a telomere doublet phenotype similar to the disruption of methylation of the basic domain. Apollo and TRF2 cooperate with TOP2α to relieve replication stress built up due to positive supercoiling of DNA (Ye et al. 2010). The 5'-3' exonuclease activity of Apollo is essential for its role in telomere maintenance, and this activity is stimulated by TRF2 (Ye et al. 2010, Lenain et al. 2006). It would be interesting to know whether methylation of TRF2 might have an effect on the enzymatic activity of Apollo or its recruitment to exposed 5' ends, especially since the basic domain of TRF2 has an affinity for DNA junctions and overhangs (Poulet et al. 2009, Amiard et al. 2007, Fouche et al. 2006). Knockdown of Apollo in conjunction with the overexpression of TRF2-RK could be used to determine whether they have an epistatic relationship.

The role of the basic domain of TRF2 in telomere maintenance is poorly defined. The overexpression of $TRF2^{\Delta B}$ causes HR dependent deletion of large segments of telomere DNA, while TRF2-RK results in telomere doublets (Mitchell et al. 2009, Wang, Smogorzewska & de Lange 2004). The formation of telomere doublets is attributed to telomere fragility as a result of replication stress (Sfeir et al. 2009). The DNA helicases, RTEL and BLM work in an epistatic pathway with TRF1 to prevent fork collapse (Sfeir et al. 2009, Lillard-Wetherell et al. 2004, Deng et al. 2013). Interestingly, RTEL and BLM are also interacting partners of TRF2 (Opresko et al. 2002). TRF2 can stimulate the enzymatic activity of BLM, which suggests TRF2 and BLM also have an important functional relationship (Opresko et al. 2002, Lillard-Wetherell et al. 2004). The

interaction between BLM and TRF2 has not been mapped, however WRN has been shown to interact with the basic domain of TRF2 through its RecQ C-terminal region (RQC), which is conserved between WRN and BLM (Opresko et al. 2002, Kim, Hakoshima & Kitano 2013, Larsen, Hickson 2013, Li et al. 2008). It would be of interest to know whether the telomere doublets arising from the overexpression of TRF2-RK are also epistatic to BLM. If they are, then one could investigate whether BLM also interacts with the basic domain of TRF2 and if so, whether methylation of the basic domain might regulate this interaction or the enzymatic activity of BLM. Therefore, along with Apollo, BLM also represents a potential TRF2 interacting protein that may work in a functional pathway with methylated TRF2. These studies would provide a novel understanding of telomere replication, which could have important implications for how we view both aging and cancer.

ATM is an important regulator of cellular senescence and is required for both its initiation and maintenance of senescence (Fumagalli et al. 2012, Shiloh, Ziv 2013, Bhatti et al. 2011). I have found that IR does not induce altered methylated TRF2 staining in ATM deficient cells or cells treated with an ATM inhibitor. This finding suggests that altered methylated TRF2 staining is dependent on the ATM-mediated signalling. ATM is also important for the nuclear matrix attachment of telomeres (Pandita, Dhar 2000, Hancock 2000). In AT cells, 90% of telomeric DNA is tightly associated with the nuclear matrix compared to 50% in normal cells (Hancock 2000). Furthermore, telomere localization is altered in senescent cells compared to their younger counterparts (Raz et al.

2008). It would be of interest to investigate whether a link might exist between these correlative observations, such as whether the status of ATM might affect the 3D organization of telomeres and whether methylated TRF2 might be required for the binding of telomeres to the nuclear matrix. TRF2 is a substrate of ATM (Tanaka et al. 2005), and it would be interesting to determine whether phosphorylation and methylation might be occurring together on a population of TRF2. This could be a mechanism by which TRF2 might link telomeres with global ATM dependent senescent signalling.

CSB functions in telomere maintenance to prevent telomere doublets, implicating it has a role in supporting proper telomere replication. We have demonstrated that CSB regulates TERRA expression. TERRA downregulation results in telomere doublets (Deng et al. 2009). It has been suggested that TERRA acts as a switch between RPA and POT1 to facilitate telomere replication (Flynn et al. 2011). Further studies would be needed to investigate the mechanism by which CSB regulates TERRA expression.

CSB has a DNA dependent ATPase activity and it belongs to the SWI2/SNF2 family of proteins (Velez-Cruz, Egly 2013). The ATPase activity of CSB is required for both its chromatin remodeling and TCR function (Lake, Fan 2013, Cho et al. 2013). It will be necessary to determine whether the enzymatic activity of CSB is required for its role in telomere maintenance. Telomeres are highly susceptible to thymidine dimers caused by UV radiation (Rochette, Brash 2010). Since CSB is essential for transcription coupled repair of thymidine dimers, it would be interesting to know whether chronic UV treatment may affect telomere maintenance in CS cells. Determining the contribution of

telomere defects to the pathology of CS would provide useful insight that could provide new methods of treatment for CS patients. Overall, the elucidation of the actions by which CSB protects telomeres will improve our understanding of telomere biology in general, as it applies to both natural aging and CS progression.

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