THE REGULATION OF TRF1 FUNCTION BY PHOSPHORYLATION

FUNCTIONAL ANALYSIS OF TRF1 PHOSPHORYLATION IN TELOMERE MAINTENANCE, CELL CYCLE REGULATION, AND THE DNA DAMAGE RESPONSE

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Abstract

Telomeres are protein-DNA complexes found at the ends of human chromosomes. The function of telomeres is to protect chromosome ends from being recognized as damaged DNA. This protection is essential in preventing the erosion of telomeres, which has been shown to lead to genomic instability, a hallmark of cancer and aged cells. Precise regulation of telomere length and function is crucial to cell survival, and defects in this regulation are related to tumorigenesis and aging related disorders. The proteins that bind telomere DNA play an indispensable role in telomere maintenance. TRF1, *t*elomere *r*epeat binding *f*actor 1, is a protein that directly binds to mammalian telomeric DNA and participates in regulating telomere length. Post-translational modifications, such as phosphorylation, have been shown to modulate TRF1 function. The results presented here demonstrate that two phosphorylation sites on TRF1, S367 and T371, are involved in regulating the function and localization of TRF1. TRF1 S367 is phosphorylated by ATM, and this phosphorylation removes TRF1 from telomere DNA and directs TRF1 to sites of proteasome degradation. On the other hand, the phosphorylation of TRF1 at T371 prevents the association of TRF1 with telomere DNA but also protects TRF1 from degradation. We have demonstrated that the phosphorylation of T371 by CDK1 is important for the resolution of sister chromatids in mitosis. In interphase cells, in response to the induction of DNA damage, TRF1 phosphorylated at T371 is recruited to sites of damage and is involved in promoting efficient homologous recombination and in conferring checkpoint activation and cell survival. The work presented within this thesis sheds light on the regulation of TRF1 function by phosphorylation events and reveals novel functions of TRF1.

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List of Abbreviations

53BP1	p53 binding protein 1
6-4 PP	6-4 photoproduct
A	Adenine
ADP	Adenine diphosphate
ALT	Alternative lengthening of telomeres
AML	Acute myogenous leukemia
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
CDK	Cyclin dependent kinase
ChIP	Chromatin immunoprecipitation
Chk	Checkpoint kinase
CK	casein kinase
CPD	cyclobutane pyrimidine dimers
CPT	Camptothecin
CS	Crude serum
Cs	Cesium
CtIP	C-Terminal binding protein-interaction protein
Da	Dalton
DAPI	4'.6-diamidino-2-phenylindole
DKC	Dyskeratisos congenita
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DNA-PK	DNA-dependent protein kinase
DSB	Double strand break
dsDNA	double stranded DNA
EB1	End binding protein 1
ERCC1	Excision repair cross-complementation group 1
Est	Ever shorter telomeres
FACS	Fluorescence activated cell sorting

Fbx	F-box
FHA	Forkhead associated
FISH	Flourescence in site hybridization
FITC	Fluorescein isothiocyanate
G	Guanine
G1	Gap 1
G2	Gap 2
GFP	Green fluorescent protein
GGR	Global genome repair
GNL3L	Guanine nucleotide binding protein-like 3
Gy	Gray
HH	Hoyeraal-Hreidarsson
HP1	Heterochromatin protein 1
HR	Homologous recombination
hTERT	human telomerase reverse transcriptase
hTR	human telomerase RNA
HU	Hydroxyurea
IB	immunoblot
IF	Immunofluoresence
IP	Immunoprecipitation
IR	ionizing radiation
J	Joules
M	Mitosis
MDC1	Mediator of DNA damage checkpoint protein 1
Mre11	Meiotic recombination 11
MRN	MRE11-RAD50-NBS1
NA	nicotinamide
NBS1	Nijmegen Breakage syndrome 1
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NIMA	Never in mitosis A
NLS	Nuclear localization signal
Noc	Nocodazole
NS	Nucleostemin
OB	Oligosaccharide/oligonucleotide binding
P	Phospho
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly ADP-ribose polymerase
PD	Population doubling

Pin2	Protein interacting with NIMA-2
PINX1	PIN2-interacting protein 1
PIP1	POT1 interacting protein 1
PLK	Polo-like kinase
PML	Promyelocytic leukemia
POT1	Protection of telomeres 1
PTOP	POT1 and TIN2 organizing protein
Rap1	Repressor/ Activator protein
Rif1	Rap1 interacting factor 1
RLIM	Ring finger LIM domain-binding protein
RNA	Ribonucleic Acid
RNF	Ring finger
Ros	Roscovitine
RPA	Replication protein A
S	Synthesis
SA1	Stromal antigen
SDS	Sodium dodecyl sulfate
sh	Short hairpin
Siah	Seven in absentia homolog
SSB	Single stand break
ssDNA	single stranded DNA
SV40	Simian virus 40
T	Thymine
TCAB1	Telomerase cajal body protein 1
TCR	transcription coupled repair
TERRA	Telomeric repeat containing RNA
TIN2	TRF1 interacting nuclear protein 2
TINT1	TIN2 interacting protein
T-loop	Telomeric loop
TPE	Telomere position effect
TPP1	TINT1/ PTOP/PIP1
TRF1	Telomere repeat binding factor 1
TRF2	Telomere repeat binding factor 2
TRITC	Tetramethyl rhodamine isothiocyanate
UV	ultraviolet
WCE	Whole cell extract
WRN	Werner
WT	Wild type
XP	Xeroderma pigmentosum

XP (A/C/F)	Xeroderma pigmentosum group (A/C/F)
XRCC	X-ray repair complementing defective repair in Chinese hamster

Preface

This thesis presents a coherent exploration of the various functions associated with the phosphorylation of TRF1 at S367 and T371. There are three data chapters, each of which contributes information to our understanding of TRF1 function.

Chapters 2 and 3 have been published in peer reviewed journals, and Chapter 4 has been submitted for publication. Each of the published chapters contains a materials and methods section that describes the protocols utilized within that chapter. Similarly, each chapter that has been prepared as a manuscript is presented with an introduction reviewing literature relevant that that chapter, however a more comprehensive literature overview is provided as Chapter 1. Each chapter that has been prepared for publication or has been published has been presented with its own reference section, and so for consistency each chapter has been prepared with a discrete list of references.

I have performed all of the experiments included within this thesis, and the contributions made by other individuals to each publication have been described within the preface to each chapter. Each chapter discussed within this thesis contributes to the overall understanding of how post translational modifications on TRF1 regulate its function and localization.

Chapter 1

Introduction

1.1 Telomere Maintenance

1.1.1 Telomere Composition

Telomeres are nucleoprotein complexes found at the ends of linear eukaryotic chromosomes. Telomeres consist of a short sequence of repetitive double stranded DNA, TTAGGG repeats in humans (and all mammals), and a complex of 6 proteins, termed the shelterin complex (de Lange, 2005; Palm and de Lange, 2008). Due to the nature of the repeat sequence, there is a nucleotide bias between the stands of DNA in the duplex, and the TTAGGG repeat containing strand has come to be known as the "G strand", and the complementary CCCTAA containing strand as the "C strand". These repeats span approximately 10 to 15 kilobase pairs in humans at birth, and this length decreases with age (de Lange et al., 1990; Harley et al., 1990; Hastie et al., 1990). The telomere terminates in a single stranded 3' overhang of the G-rich strand (de Lange, 2005; Makarov et al., 1997; McElligott and Wellinger, 1997; Wright et al., 1997). Telomere ends generated by lagging strand synthesis would naturally have an overhang product, as DNA polymerase is unable to replicate the end of a linear template after the removal of the last primer, however leading strand synthesis would result in a blunt end product at the other end of the chromosome (Olovnikov, 1973; Watson, 1972). Telomeres exhibit a 3'overhang at each end of the chromosome, which has been determined to be the result of Apollo nuclease activity at the leading strand end, which is monitored by POT1 to avoid excessive resection (Makarov et al., 1997; Sfeir et al., 2005; Wu et al., 2012). Both stands are further processed by the resection activity of EXO1, and each 5' end terminates precisely with an ATC sequence (Sfeir et al., 2005; Wu et al., 2012). The final overhang product is 50-500 nucleotides in length and is important for the formation of higher order structures, such as t-loops (de Lange, 2005; Griffith et al., 1999; Palm and de Lange, 2008). T-loops are the structures formed when the 3' overhang invades the double stranded telomere repeats (Griffith et al., 1999). During

this process, it is postulated that the overhang forms complementary base pairs with the C strand of the duplex DNA and displaces the G strand in that region, which bubbles out to form the D-loop (displacement loop). The size of the t-loop varies based upon where the overhang invades the double strand telomere DNA, but regardless of the size, t-loops are thought to "hide" telomere ends from DNA damage response machinery, by tucking the exposed ends away (Griffith et al., 1999; Stansel et al., 2001; Wei and Price, 2003). The structure of chromosome ends is evolutionarily conserved, and therefore likely plays a fundamental role in telomere maintenance (Griffith et al., 1999; Munoz-Jordan et al., 2001; Murti and Prescott, 1999).

For the purpose of this thesis, human telomeres will be reviewed and discussed.

1.1.2 Telomere Function

Linear chromosomes pose challenges, such as the end replication problem, which telomeres help to alleviate (Olovnikov, 1971; Watson, 1972). Lagging strand synthesis is unable to replicate the chromosomes to the very terminus. DNA polymerase can synthesize DNA from template strands only in the 5'-3' direction, so the lagging strand DNA must be synthesized in small fragments (Okazaki fragments). Each of these fragments is generated individually in the 5'-3' direction with the help of a RNA primer (as a start site), and ligated together after the primer is removed and the gap is filled in. The removal of the last RNA primer leaves a gap at the end of the chromosome that cannot be replicated, however, which results in the gradual shortening of chromosomes and the loss of DNA with each cell division, in the absence of telomere extension. It has recently been elucidated that the last RNA primer is not at the very terminus of the chromosome, but 70-100 nucleotides from the end, and therefore chromosomes shorten by an amount larger than the size of the RNA primer with each cell division (Chow et al., 2012). The presence of telomere DNA at chromosome ends acts as a buffer to ensure that no vital coding DNA is lost with this shortening, but, as a result, telomeres

are shortened with each cell division in the absence of telomerase. If telomeres reach a critically short point, and are less than 400-1000 bases long, cells enter replicative senescence; an irreversible state in which cells are no longer replicating or dividing (Baird, 2008; Baird and Kipling, 2004; Damm et al., 2001). Telomere shortening serves a second function; to impose a limit on the number of times that a cell can divide, referred to as the Hayflick limit, and determines the lifespan of a cell (Hayflick, 1965). The barrier to uncontrolled proliferation imposed by telomere shortening is thought to function as a tumor suppressor mechanism.

Telomeres also serve an important function in preventing the recognition of the chromosome end as a site of DNA damage requiring repair. The t-loop structure at telomeres is thought to hide the end of the chromosome from this response, and the proteins bound to telomeres act in concert to maintain telomere ends and prevent a DNA damage response (Griffith et al., 1999). The proteins that are predominantly associated with telomere ends, collectively referred to as the shelterin complex, help to accomplish this function in conjunction with additional accessory factors (de Lange, 2005). Shelterin is a complex of 6 proteins which were originally characterized as having telomere specific functions and localization, and an abundant presence at telomere ends throughout the cell cycle, as shown in Figure 1.1 (de Lange, 2005; Palm and de Lange, 2008). Removal of each component of the shelterin complex has significant effects on telomere function, and removing the entire complex from telomeres demonstrates that in the absence of shelterin, chromosome ends are recognized and processed by the ATM and ATR signalling pathways, non-homologous end joining, homologous recombination, and resection (Hockemeyer et al., 2006; Hockemeyer et al., 2005; Kim et al., 2004; Martinez et al., 2010; Martinez et al., 2009; Sfeir and de Lange, 2012; Sfeir et al., 2010; Sfeir et al., 2009; Tejera et al., 2010; Wu et al., 2006; Ye et al., 2004a). The severity of this defect demonstrates a crucial role for the shelterin complex in preventing the

recognition of telomeres as damaged DNA. The six members of the shelterin complex will be discussed in the next section.

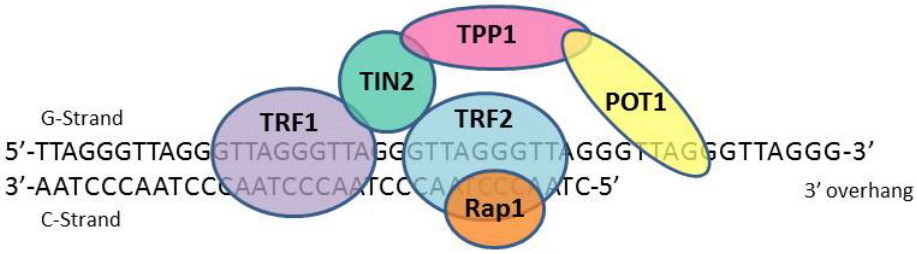

Figure 1.1 The Shelterin Complex
Schematic of the human shelterin complex bound to telomere DNA. Picture is a representation and is not drawn to scale.

1.1.3 The Shelterin Complex

Telomere Repeat Binding Factor 1 (TRF1)

The first mammalian telomere protein identified, TRF1, binds as a homodimer to duplex telomere DNA (Bianchi et al., 1997; Chong et al., 1995). TRF1 was identified *in vitro* based on its ability to bind to telomere repeat tracts (Zhong et al., 1992). It was characterized shortly after as a protein which bound telomeres *in vivo* (Chong et al., 1995). TRF1 was also identified in a genetic screen for proteins that interacted with NIMA protein kinase, and termed Pin2 (Shen et al., 1997). The Pin2 isomer differs from TRF1 in that it is missing TRF1 residues 296-316, and therefore is 20 amino acids shorter. Pin2 and TRF1 are able to heterodimerize with one another (Shen et al., 1997). TRF1 is 56 kDa in size, and predominantly localized in the nucleus. It is transcribed from chromosome 8, band q13 (Broccoli et al., 1997a). TRF1 is ubiquitously expressed and is present at each stage of the cell cycle (de Lange, 2005; Palm and de Lange, 2008). Structurally, TRF1 consists of a C-terminal DNA binding domain, a TRF homodimerization (or homology) domain, a flexible linker (or hinge) region, and an N terminal acidic

domain (de Lange, 2005), as illustrated in Figure 1.2. The crystal structure of the DNA binding domain of TRF1 revealed 3 alpha helices which contact TAGGGTT sequences (Court et al., 2005). It has been demonstrated that each TRF1 molecule preferentially binds an YTAGGGTTR motif, and that this binding may result in looping of the intervening DNA (Bianchi et al., 1999). The binding of TRF1 to telomeres bends telomere DNA by 120˚, which may play a role in modulating the formation of higher order structures at chromosome ends (Bianchi et al., 1997). The hinge domain of TRF1 confers enough flexibility that the two DNA binding domains in one homodimer can bind separate telomeres, bind in different orientations, or bind at a distance apart, which can bend or tether two regions of telomere DNA (Bianchi et al., 1997; Bianchi et al., 1999). The function of TRF1 is in part mediated by its interaction with other proteins. Position 142 of TRF1, phenylalanine, mediates the interaction between TRF1 and proteins containing an FxLxP motif, including TIN2 and PINX1 (Chen et al., 2008; Yoo et al., 2009; Zhou and Lu, 2001).

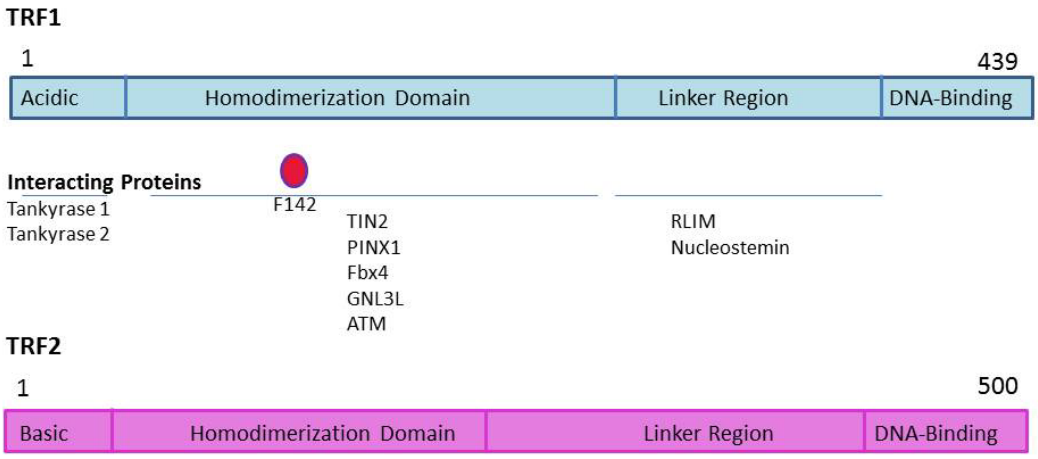

Figure 1.2 TRF1 and TRF2 Domain Structure
A representation of the domain structure of TRF1 and TRF2, illustrating the similarities. Diagram is not to scale. Proteins known to interact with specific domains of TRF1 are indicated under the appropriate region. F142, a residue of TRF1 described to mediate certain protein-protein interactions, is shown in pink.

A comparison between human and mouse TRF1 reveals a high level of conservation of the N terminal acidic domain, the homodimerization domain, and the C-terminal DNA binding domain (Broccoli et al., 1997a). The flexible linker region of the proteins, however, is very poorly conserved and the overall identity of the two proteins is only 67% (Broccoli et al., 1997a). Mouse TRF1 has been demonstrated to play a similar function as human TRF1; it binds as a dimer to telomere DNA and negatively regulates telomere length (Broccoli et al., 1997a).

Telomere Repeat Binding Factor 2 (TRF2)

TRF2, like TRF1, binds double stranded telomere DNA. It was identified based on its sequence similarity to TRF1 in a database screen (Bilaud et al., 1997; Broccoli et al., 1997b). It binds to telomere DNA as a homodimer, and forms homotypic interactions through its TRF homology domain (TRFH), like TRF1 (Broccoli et al., 1997b). TRF2 also has a C terminal DNA binding domain, a TRFH domain, and a linker region (de Lange, 2005; Palm and de Lange, 2008). Its N-terminal region, however, is a glycine-arginine rich basic domain, unlike the acidic domain of TRF1 (Palm and de Lange, 2008). A comparison between the domain structure of TRF1 and TRF2 is shown in Figure 1.2. The basic domain has an affinity for DNA junctions, including Holliday junctions (Fouche et al., 2006; Nora et al., 2010; Poulet et al., 2009). TRF2 is important for the formation of t-loops, and has been suggested to promote these structures *in vitro* (Stansel et al., 2001). TRF2, like TRF1, is abundant, ubiquitously expressed, and present at all stages of the cell cycle. There is twice as much TRF2 in the cell as TRF1; subcomplexes of shelterin containing different combinations of protein components have been observed (Liu et al., 2004a; Takai et al., 2010). There is a high degree of homology between TRF1 and TRF2, specifically in their TRFH and C-terminal domains. Despite the similarity in TRFH domains, TRF1 and TRF2 do not interact with one another (Broccoli et al., 1997b; Fairall et al., 2001). TIN2 and Rap1 form direct connections with TRF2; linking it to the rest of

the shelterin complex (Kim et al., 2004; Li et al., 2000; Ye et al., 2004a). TRF2 plays an essential role in telomere protection, which is dependent upon the ATM and p53 mediated DNA damage response (Ancelin et al., 2002; Karlseder et al., 1999; Zhu et al., 2000). The removal of TRF2 from telomeres leads to the formation of telomere end-to-end fusions and an induction of dysfunctional telomere-induced foci (TIF) formation (Celli and de Lange, 2005; Denchi and de Lange, 2007; Karlseder et al., 1999; Smogorzewska et al., 2002; van Steensel et al., 1998). In addition, TRF2 is implicated in telomere length regulation (Karlseder et al., 2002; Munoz et al., 2006; Smogorzewska et al., 2000; van Steensel et al., 1998). The levels of TRF2 are tightly controlled to ensure its function in the protection of telomeres. Siah1, an E3 ubiquitin ligase, ubiquitinates TRF2 and targets it for proteasome mediated degradation (Fujita et al., 2010). Siah1 is a transcriptional target of p53, and therefore this regulation links the damage response to the role of TRF2 in protecting chromosome ends. The activation of p53 induces Siah1, resulting in the degradation of TRF2 (Fujita et al., 2010). The levels of TRF2 are also regulated at the transcriptional stage, in part by the WNT/ β-catenin signalling pathway, which promotes TRF2 expression (Diala et al., 2013). In telomerase positive cancer cells, with reduced β-catenin, reduced TRF2 expression results in telomere uncapping and dysfunction (Diala et al., 2013). In a telomerase negative background, conversely, increasing TRF2 levels promotes recombination, both at telomeres and elsewhere in the genome, suggesting that TRF2 may be involved in initiating the ALT pathway of telomere lengthening (Blanco et al., 2007).

TRF2 has many interacting partners that aid in modulating its function. The homodimerization domain of TRF2, specifically phenylalanine 120, recognizes a short peptide motif, YxLxP, in its interacting partners (Kim et al., 2009). One of these partners is Apollo, which has 5’ exonuclease activity, and, together with TRF2, helps to relieve topological stress at telomeres during replication (Ye et al., 2010). Cells depleted for Apollo display TIF formation, which is a DNA damage signal elicited at telomere ends,

specifically in cells progressing through S phase, suggesting that these damage signals are replication dependent (Lam et al., 2010; van Overbeek and de Lange, 2006; Wu et al., 2010; Ye et al., 2010). Apollo is also involved in processing telomere ends to create the 5'overhang (Wu et al., 2012; Wu et al., 2010). Apollo depletion results in a DNA damage signal at telomere ends, and deleting Apollo leads to fragile telomeres and the appearance of multiple telomere signals at chromosome ends (Lenain et al., 2006; van Overbeek and de Lange, 2006; Wu et al., 2010). The same region of TRF2 also mediates interactions with the proteins PNUTS and MCPH1, the exact function of which remains to be elucidated (Kim et al., 2009). Mutating F120 of TRF2 to alanine resulted in TIF formation and telomere deprotection, suggesting for an importance of these interactions, perhaps in the recognition of telomeres as sites of damage (Kim et al., 2009).

TRF2 directly binds WRN, a RecQ helicase with 3'-5' exonuclease activity, and stimulates its helicase activity (Crabbe et al., 2004; Machwe et al., 2004; Opresko et al., 2002). WRN interaction with TRF2 is important for WRN recruitment to telomere DNA, which happens primarily in S phase (Machwe et al., 2004). Cells depleted for WRN display chromosome instability, telomere dysfunction, and senescence (Crabbe et al., 2004). WRN may be important at telomeres to prevent recombination events from happening either between or within telomere ends. It may also be important in alleviating G-quartet structures during replication, allowing for the passage of replication forks (Bhattacharyya et al., 2010).

TRF2 is subject to numerous post translational modifications which affect its activity. For instance, TRF2 is methylated by PRMT1, which is important for genomic stability and telomere length maintenance (Mitchell et al., 2009). TRF2 is acetylated by the histone acetyltransferase p300, which increases the stability of TRF2 and promotes its binding to telomere DNA (Her and Chung, 2013).

Apart from telomeres, TRF2 has been described to localize at sites of DNA double strand breaks that are not telomere associated (Huda et al., 2012; Mao et al., 2007). TRF2 has been reported to facilitate homologous recombination at non-telomeric DNA DSBs, and is important for the formation of Rad51 foci (Bradshaw et al., 2005; Huda et al., 2012; Huda et al., 2009; Mao et al., 2007; Tanaka et al., 2005). The full significance of the involvement of TRF2 in DNA repair is not yet understood, although its role in preventing a DNA damage response at telomeres is clear.

TRF1- Interacting Nuclear Protein 2 (TIN2)

TIN2 plays a central role in connecting all of the other members of the shelterin complex; forming direct interactions with TRF1, TRF2, and TPP1, all of which may happening simultaneously (de Lange, 2005; Palm and de Lange, 2008). TIN2 was originally identified in a yeast two-hybrid screen for proteins interacting with TRF1 (Kim et al., 1999). Depleting TIN2 destabilizes the entire shelterin complex (Kim et al., 2004; Ye and de Lange, 2004). TRF2 and Rap1 are lost from telomere ends in this setting, and TRF1 levels are decreased (Ye et al., 2004a). Interestingly, the association of TRF2 with telomeres is reduced when TRF1 is knocked down, suggesting that the connection between TRF1 and TRF2, bridged by TIN2, may stabilize the shelterin complex (Ye et al., 2004a). The expression of TIN2 mutants which maintain an interaction with TRF1 at telomeres results in telomere lengthening in telomerase positive cells, suggesting that TIN2 may mediate the function of TRF1 in telomere length maintenance (Kim et al., 1999). TIN2, being the central scaffolding protein in shelterin, may be responsible for the formation of shelterin sub complexes by interacting with shelterin components in different combinations, and aids in controlling the spatial arrangement of shelterin proteins (Kim et al., 2008b; Takai et al., 2010). TPP1-POT1 complexes are shuttled out of the nucleus and into the cytoplasm by a nuclear export signal on TPP1, and an interaction between TPP1 and TIN2 is important for targeting these proteins to the

nucleus from the cytoplasm (Chen et al., 2007). In this way, TIN2 is important for mediating the functions of TPP1, which includes recruiting telomerase to telomere ends (Abreu et al., 2010). The levels of TIN2 are of importance in moderating the other shelterin components, and the stability of TIN2 is regulated by its ubiquitination by Siah2, which is an E3 ligase that targets it for proteasome-mediated protein degradation (Bhanot and Smith, 2012). In addition to its role at telomeres, TIN2 has been suggested to play a function in cellular metabolism in the mitochondria (Chen et al., 2012). It is able to localize at the mitochondria, which is negatively regulated by its interaction with TPP1. The N terminus of TIN2 mediates both its interaction with TPP1 and its mitochondrial localization; when TIN2 is bound to TPP1 it does not go to mitochondria (Chen et al., 2012). Depleting TIN2 with siRNA results in a decrease in glycolysis and reactive oxygen species production and an increase in oxygen consumption in cancer cells, indicating that TIN2 plays an important functional role at mitochondria (Chen et al., 2012). Different isoforms of TIN2 have been shown to have differential subnuclear localizations. TIN2 may play a role in tethering telomeres to the nuclear matrix; in certain splice variants of TIN2 a C-terminal region appears to regulate this interaction (Kaminker et al., 2009; Smith, 2009).

Precise regulation of TIN2 is crucial to maintaining genomic integrity; mutations in TIN2 are directly linked to the development of the disease dyskertatosis congenita (DKC) (Vulliamy et al., 2012; Walne et al., 2008). Cases of DKC resulting from TIN2 mutations are extremely severe (Dokal, 2011; Sasa et al., 2012; Vulliamy et al., 2011). The region of TIN2 that is associated with these mutations is also responsible for an association between TIN2 and HP1, which is required to establish and maintain cohesion at telomeres (Canudas et al., 2011). TIN2 binds the cohesin protein SA1 and is involved in regulating the cohesion of sister telomeres, alongside TRF1 (Canudas et al., 2007; Dynek and Smith, 2004). Patients with TIN2-associated DKC are defective in their cohesion (Houghtaling et al., 2012). TIN2 is also responsible for mediating the

recruitment of telomerase to telomeres, and mutations in the same region of TIN2 that facilitates its interaction with HP1 reduce the association of telomerase with telomeres (Yang et al., 2011a).

Repressor/Activator Protein 1 (Rap1)

Rap1 was identified through a yeast-two hybrid screen for proteins interacting with TRF2 (Li et al., 2000). There is a 1:1 relationship between the presence of TRF2 and Rap1 at telomeres (Takai et al., 2010; Zhu et al., 2000). TRF2 depletion abrogates the localization of Rap1 to telomeres and also affects Rap1 stability (Celli and de Lange, 2005). Rap1 is an important component of shelterin in preventing homology directed repair at chromosome ends, which is evidenced by an increase in sister-chromatid exchange at telomeres upon Rap1 deletion (Martinez et al., 2010; Sfeir et al., 2010). The presence of Rap1 at telomeres is involved in preventing non-homologous end joining (NHEJ) of chromosome ends (Bae and Baumann, 2007; Sarthy et al., 2009). In cells with an activated DNA damage response at telomeres, targeting Rap1 to these ends prevents repair by NHEJ, and therefore Rap1 aids in maintaining genomic stability (Sarthy et al., 2009).

Rap1 has been demonstrated to have functions apart from telomere maintenance, at non telomere sites. One of these functions is in transcriptional regulation, as Rap1 deficient cells exhibit an upregulation of a number of genes, some of which are subtelomeric (Martinez et al., 2010). Supporting this suggestion is the observation the Rap1 binds DNA at sites distinct from chromosome ends, but which may have 1-2 telomere repeats, which may be the sites at which Rap1 is regulating transcription (Martinez et al., 2010; Yang et al., 2011b). Another role for Rap1 is as a positive regulator of the NFκB signalling pathway (Teo et al., 2010). Rap1 associates with proteins that are responsible for phosphorylating and degrading inhibitors of NFκB, thereby stabilizing it (Teo et al., 2010).

TPP1 (known formerly as TINT1/ PTOP/ and PIP1)

TPP1 was identified by three separate groups and given three distinct names, TINT1, PTOP, and PIP1, which have since been consolidated into TPP1 (Houghtaling et al., 2004; Liu et al., 2004b; Ye et al., 2004b). TPP1 is responsible for bridging the interaction between TIN2 and POT1 (Abreu et al., 2010; Takai et al., 2010; Takai et al., 2011). TPP1 contains a C-terminal TIN2 interaction domain, a serine-rich region of unknown function, a central POT1 interaction domain, and an N-terminal OB-fold, which mediates the association of telomerase with telomeres (Ye et al., 2004a; Zhong et al., 2012). TPP1 is important for the recruitment of POT1 to telomeres, increasing the affinity of POT1 to telomeres by 5-10 times (Hockemeyer et al., 2007). Phenotypes associated with TPP1 depletion are similar to the telomere deprotection effects observed with POT1 loss (Denchi and de Lange, 2007; Hockemeyer et al., 2007). TPP1 null mice exhibit phenotypes associated with telomere dysfunction, including telomere fusions and telomere doublets, and undergo a p53-dependent cell cycle arrest (Tejera et al., 2010). TPP1 deleted cells show a decreased association of telomerase at telomeres and an increase in telomere shortening (Tejera et al., 2010). TPP1 has been shown to be important for telomerase recruitment to telomeres and for telomerase processivity; the processivity of telomerase being enhanced by a "TEL" patch on TPP1 (Abreu et al., 2010; Latrick and Cech, 2010; Nandakumar et al., 2012; Zhong et al., 2012). TPP1 promotes this recruitment in a cell cycle dependent manner, through a phosphorylation event in its OB fold which is restricted to S and G2 phases of the cell cycle (Zhang et al., 2013). These observations suggest that TPP1 is important at chromosome ends for regulating telomere elongation by telomerase.

Protection of Telomeres 1 (POT1)

POT1 binds the 3' single stranded telomere DNA overhang, and also the single stranded DNA present in the D-loop (Palm and de Lange, 2008). It was identified based

on its sequence homology with telomere binding proteins in the ciliates *Oxytrichia nova* and *Euplotes crassus* (Baumann and Cech, 2001). POT1 has multiple OB-folds in its N-terminus, which are characteristically involved in binding to single stranded oligonucleotides (Baumann and Cech, 2001; Lei et al., 2004; Loayza and De Lange, 2003; Loayza et al., 2004). POT1 has a binding preference for TAGGGTTAG, and, as mentioned previously, is present at telomeres in a manner promoted by TPP1 (Hockemeyer et al., 2007; Wang et al., 2007). POT1 coats the single stranded DNA at telomeres and helps to compact these strands and promote the formation of higher ordered structures (Taylor et al., 2011).

Depletion or conditional knockout of POT1 results in a DNA damage signal at telomeres (Hockemeyer et al., 2006; Hockemeyer et al., 2005; Veldman et al., 2004; Wu et al., 2006; Yang et al., 2005; Ye et al., 2004b). The result of this is chromosomal abnormalities, and a decrease in cellular proliferation. The deprotection of telomeres with POT1 depletion is accompanied by alterations in the processing of the C-strand telomere end, suggesting that POT1 is important in regulating this process (Hockemeyer et al., 2006; Hockemeyer et al., 2005; Sfeir et al., 2005; Wu et al., 2006). The interaction between POT1 and TPP1, as well as the binding of POT1 to single stranded telomere DNA, are necessary for the protection of telomere ends and the prevention of an ATR-dependent DNA damage signal (Barrientos et al., 2008; Churikov and Price, 2008; Denchi and de Lange, 2007; Guo et al., 2007). POT1 binding to single stranded telomere DNA is thought to prevent the binding of RPA, and the initiation of an ATR damage signalling cascade (Denchi and de Lange, 2007; Gong and de Lange, 2010). Knocking down POT1 also results in telomere lengthening, which suggests that POT1 may regulate the access of telomerase to telomere ends (Veldman et al., 2004; Yang et al., 2005; Ye et al., 2004b).

The components of the shelterin complex are differentially regulated and play diverse roles at chromosome ends, but they function cooperatively to maintain telomere length and prevent a DNA damage response at telomeres.

1.1.4 Telomere Lengthening

Telomerase Mediated Telomere Elongation

Telomere shortening with each cell division imposes a barrier to uncontrolled proliferation and suppresses tumorigenesis. As a prerequisite to tumorigenesis, then, cells must overcome telomere shortening. One mechanism by which this occurs is through telomerase-mediated telomere lengthening.

Telomerase is a ribonucleoprotein reverse transcriptase that adds telomere repeats *de novo* onto telomere ends with the use of RNA as a template (Greider and Blackburn, 1985). Telomerase is repressed in most somatic cells, and therefore these cells exhibit telomere shortening, at approximately 100 base pairs per cell division (Harley et al., 1990; Huffman et al., 2000). Cells with extended proliferative potential, such as germ cells or committed progenitor cells, have active telomerase and have overcome the replication barrier imposed by telomere loss (Cifuentes-Rojas and Shippen, 2012). Approximately 85-90% of human tumors have activated telomerase, and the rest utilize the alternative lengthening of telomeres (ALT) pathway to overcome telomere shortening (Reddel, 2003). Amplification of the loci encoding either hTERT or hTR is related to tumor growth (Soder et al., 1997). On the flip side, in highly proliferative cells, telomerase must be active to overcome telomere shortening, and insufficient telomerase could result in disorders including dyskertatosis congenita or aplastic anemia (Cifuentes-Rojas and Shippen, 2012; Nelson and Bertuch, 2012). These findings reinforce that telomerase expression must be maintained within a precise limit as defined by the cell type.

Human telomerase is composed of two subunits; the catalytic reverse transcriptase, or TERT, and the RNA subunit, or hTR (human telomerase RNA) (Podlevsky and Chen, 2012). Human TERT has a reverse transcriptase domain (RT) which is catalytically active, a "telomerase essential N-terminal" domain (TEN), a telomerase RNA binding domain (TRBD), as well as a C-terminal extension domain (CTE) (Blackburn, 2005; Bryan and Cech, 1999; Lingner et al., 1997). The TEN domain is important for the interaction of telomerase with single stranded DNA at telomere ends. Implicitly, the TRBD interacts with the telomerase RNA subunit, forming multiple connections with telomerase RNA (Podlevsky and Chen, 2012). The RT and C-terminal domains both contribute to the catalytic and extension activity of hTERT (Podlevsky and Chen, 2012).

The architecture of telomerase RNA (TR) has been thoroughly investigated and reveals evolutionarily conserved structural features which are essential for telomerase function (Podlevsky and Chen, 2012). In humans, the 451 nucleotide TR contains, from 5' to 3', the pseudoknot or core domain (containing the template region), conserved regions 4 and 5, or CR4/CR5 (the catalytic core), the H/ACA scaRNA motif, and conserved region 7 (Blasco et al., 1995; Chen et al., 2000; Chen and Greider, 2004, 2005). The core and CR4/CR5 domains mediate the interaction of hTR with hTERT (Mitchell and Collins, 2000). The 3'end H/ACA element is important for the association of hTR with cajal bodies, and is important for the localization and accumulation of telomerase and for the processing of hTR (Jady et al., 2004; Zhu et al., 2004). The 3' CR7 domain mediates the association between hTR and other proteins involved in the telomerase holoenzyme, such as dyskerin, TCAB1, and NOP10 (Egan and Collins, 2010). The H/ACA motif in vertebrate TRs mediates dyskerin binding, which is essential for catalytic telomerase activity (Mitchell et al., 1999).

The regulation of the localization of telomerase is an important step in modulating its activity. The telomerase protein subunit goes to cajal bodies with the

help of the TCAB1 protein, where it interacts with the RNA telomerase component, at which point cajal bodies facilitate the localization of the holoenzyme to telomere ends (Cristofari et al., 2007; Jady et al., 2006; Stern et al., 2012; Theimer et al., 2007; Tomlinson et al., 2008; Venteicher and Artandi, 2009). Proteins associated with chromosome ends aid in recruiting telomerase and regulating telomere extension. One of these proteins is the shelterin component TPP1, which interacts with the TEN domain of hTERT, and is important for telomerase association at telomere ends and also processivity (Abreu et al., 2010; Latrick and Cech, 2010; Nandakumar et al., 2012; Wang et al., 2007; Zaug et al., 2010). The double stranded telomere binding protein TRF1, in conjunction with TIN2, inhibits telomerase dependent telomere elongation (Ancelin et al., 2002; Broccoli et al., 1997b; de Lange, 2005; Loayza and De Lange, 2003; Okamoto et al., 2008; Smogorzewska et al., 2000; Takai et al., 2010; van Steensel and de Lange, 1997).

There exist mechanisms in the cell to prevent the excessive lengthening of telomeres by telomerase, and regulate a stable telomere length. This phenomenon has been referred to as “telomere trimming”, and is observed in germline cells and cells with upregulated telomerase activity, including cancer cells (Pickett et al., 2009; Pickett et al., 2011). Telomere trimming uses the HR protein XRCC3 to trim telomeres to form t-circles (double stranded telomere DNA loops) and shortened telomeres, through the resolution of t-loops by a homologous recombination based mechanism (Pickett et al., 2009; Pickett et al., 2011; Pickett and Reddel, 2012). Telomere trimming results in a shortening effect much more rapid than that observed as a result of incomplete replication, and aids in the maintenance of the telomere length equilibrium. Another mechanism preventing the excessive lengthening of telomeres is the inhibition of telomerase by PINX1. PINX1 interacts directly with TRF1, and also with both hTERT and hTR, and reduces telomerase activity (Banik and Counter, 2004; Soohoo et al., 2011; Zhou and Lu, 2001). Telomere length, and the number of shelterin complexes bound to telomeres,

may work as a feedback loop in preventing the lengthening of long telomeres. The "protein counting model" has been proposed to explain this, and suggests that the longer telomeres are, the more TRF1 and POT1 are bound, and the more strongly telomerase is inhibited from extending telomere ends (Loayza and De Lange, 2003; Marcand et al., 1997; van Steensel and de Lange, 1997). In contrast, short telomeres, with few telomerase-inhibiting shelterin components bound, would be more vulnerable to telomerase elongation (Ancelin et al., 2002; Smogorzewska and de Lange, 2004).

The function of telomerase is to add *de novo* telomere repeats onto chromosome ends, but telomerase may mistake DNA double strand breaks for chromosome ends and inappropriately add telomere repeats to break sites, a process referred to as chromosome healing (Flint et al., 1994; Myung et al., 2001; Schulz and Zakian, 1994). Chromosome healing may lead to terminal deletions and gross chromosome rearrangements (Flint et al., 1994; Lasko et al., 1991; Sprung et al., 1999). Telomerase is phosphorylated by c-Abl in response to DNA damage, which also functions to reduce its activity (Kharbanda et al., 2000). Lastly, the localization of telomerase is altered after DNA damage, and it is shuttled to the nucleolus, to limit the accessibility of telomerase to sites of DNA damage (Wong et al., 2002). The combined inhibitory mechanisms promote the accurate repair of DNA breaks and avoid chromosome healing.

Alternative Lengthening of Telomeres

Telomere lengthening is an essential step in tumorigenesis, and cells that do not accomplish this task by expressing telomerase do so through the alternative lengthening of telomeres, or ALT, pathway. Approximately 10-15% of cancers utilize the ALT pathway to overcome telomere shortening and replicative senescence (Reddel, 2003; Reddel et al., 2001; Shay and Bacchetti, 1997). For reasons not well understood, cancers which utilize the ALT pathway typically have a particularly poor prognosis (Cesare and Reddel, 2010; Henson and Reddel, 2010; Reddel, 2003).

ALT cells have a few defining characteristics that distinguish them from telomerase positive cells. ALT cells have double-stranded extrachromosomal telomere DNA in circles, t-circles, or partially single stranded telomere DNA in circles, referred to as C-circles or G-circles, based on which strand composes the majority of the circle (Cesare and Griffith, 2004; Henson et al., 2009). PML (promyelocytic leukemia) bodies containing telomere chromatin are specifically found in ALT cells (Yeager et al., 1999). A great heterogeneity in telomere length is observed in ALT cells as compared to telomerase positive cells, and telomere length may change very rapidly (Bryan et al., 1995).

T-circles may result as either a consequence of a recombination based mechanism at telomeres and the resolution of a t-loop as if it were a recombination intermediate, or they may be the result of telomere trimming to shorten extremely long telomeres generated as a result of the ALT pathway (Cesare and Reddel, 2010; Pickett et al., 2009; Reddel, 2003). While t-circles are prominent in ALT cells, they are present in other cell types as well. C-circles are characteristic of ALT cells, and as such are a more useful way to define ALT cells, although their exact origin is not fully elucidated (Henson et al., 2009; Lau et al., 2013).

The mechanism behind ALT extension is based on homologous recombination, which explains the rapid change in telomere length observed in ALT cells. The specific mode of recombination is debated and multiple theories exist to explain this process. Evidence for recombination at telomeres includes the observation that ALT cells undergo telomere sister chromatid exchanges at a high frequency (Londono-Vallejo et al., 2004). Another model suggests that recombination at telomeres happens when one telomere uses another telomere (its sister chromatid or a telomere on another chromosome) as a template for synthesis during replication (Dunham et al., 2000). In furthering this theory, it is possible that instead of using another telomere as a template, telomeres use a t-

circle, and synthesize telomere repeats using a rolling circle mechanism (Henson et al., 2002). Rolling circles would occur when the 3' end of the G-rich overhang forms complementary base pairs with a t-circle or single stranded C-circle, and the G-strand is synthesized using the circle as a template and the overhang as a primer (Hartig and Kool, 2004; Natarajan and McEachern, 2002; Nosek et al., 2005). The new strand is released as it is synthesized, and in this way the circle can "roll", or synthesis of the new strand can go around the circle continuously and result in extensive telomere elongation (Nosek et al., 2005). The process of rolling circles has been observed in yeast, and C-circles have been shown to work as a substrate for telomere amplification *in vitro* (Henson et al., 2009). As ALT-elongation requires recombination, it is reasonable that proteins required for homologous recombination, such as the MRN complex, are also important in the ALT pathway (Wang et al., 2004; Zhong et al., 2007).

PML bodies are present in many cell types and play roles in cellular senescence, DNA repair, and the stress response. PML bodies in ALT cells are unique in that they contain telomere chromatin; so unique that they have been labelled "ALT-associated PML bodies", or APBs (Chung et al., 2012; Yeager et al., 1999). Numerous DNA damage response factors, for instance 53BP1 and γH2AX, have been detected at APBs, lending support to the notion that ALT extension occurs by a recombination dependent mechanism (Cesare et al., 2009). The function of APBs has been proposed to be in facilitating HR between telomeres by clustering together telomere ends in close proximity, facilitating telomere extension (Draskovic et al., 2009). It has been suggested that TRF1 is important in regulation of the ALT pathway of telomere lengthening. TRF1 is important in the assembly of APBs, and is one of the proteins that is capable of initiating the formation of a *de novo* PML body (Chung et al., 2011; Yu et al., 2010). The sumoylation of TRF1 by MMS21 promotes APB formation in ALT cells (Potts and Yu, 2007). The MRN complex is required for APB formation, and depleting ALT cells for

components of the MRN complex results in telomere shortening and reduces the formation of t-circles (Compton et al., 2007; Jiang et al., 2007; Zhong et al., 2007).

Another link between TRF1 and the ALT pathway was uncovered with the discovery that TRF1 interacts with NBS1. In a yeast two-hybrid screen for proteins interacting with NBS1, TRF1 was identified, and this interaction was confirmed *in vivo* (Wu et al., 2000). The C-terminal region of NBS1 interacts with TRF1, and these proteins localize at PML bodies in ALT cells; and rarely go to PML bodies in telomerase positive cells (Wu et al., 2000). The colocalization between TRF1 and NBS1 happens specifically when there is active DNA synthesis in these PML bodies, in late S phase, which submits that NBS1 may be involved in telomere lengthening in ALT cells (Wu et al., 2000). These results are consistent with the model that proposes that telomeres in ALT cells are elongated by a recombination dependent system accomplished, in part, by proteins involved in HR DNA damage repair (Cesare and Reddel, 2010; Dunham et al., 2000; Henson et al., 2002).

1.1.5 Telomere Chromatin Structure

A general telomere chromatin state can typically be described as heterochromatic in nature; enriched for trimethylated H3K9, trimethylated H4K20, and HP1, and having low levels of H3 and H4 acetylation (Blasco, 2007; Canudas et al., 2011). The nucleosome structure at telomeres is also different than at other heterochromatin sites; being more mobile and having a smaller repeat length (Pisano et al., 2010). TRF1 has been demonstrated to induce the mobility of nucleosomes and also supports the compaction of telomere DNA in the presence of nucleosomes, perhaps by causing sliding of the telomere nucleosome (Galati et al., 2006; Pisano et al., 2010).The effect of TRF1 on telomere compaction is mediated by the TRFH domain of TRF1, which is very similar to the same region on TRF2, however TRF2 exerts a different effect on chromatin structure. TRF2 condenses telomeric chromatin and creates negative torsion which is partially responsible for its ability to promote t-loops and participate in Holliday junctions

(Fouche et al., 2006; Poulet et al., 2009; Poulet et al., 2012). The differing roles of TRF1 and TRF2 in chromatin remodelling are a result of the differences in their N-termini (Poulet et al., 2012).

It is interesting to note that as telomere length increases, the density of heterochromatin marks on telomeres also increases, providing a possible explanation for the differential regulation of long versus short telomeres (Arnoult et al., 2012). Along this line, short telomeres are typically associated with subtelomere regions that have less DNA methylation, but unaltered telomere binding of shelterin proteins TRF1 and TRF2 (Benetti et al., 2007). Chromatin changes associated with telomere length, which relax the chromatin structure when telomeres are shorter, may be involved in transmitting information regarding telomere length.

1.1.6 Telomere Transcription

Telomere repeats are transcribed by DNA-dependent RNA polymerase II, generating a telomere repeat containing RNA product which has been dubbed “TERRA” (Azzalin et al., 2007; Schoeftner and Blasco, 2008). TERRA molecules have been shown to contain predominantly UUAGGG repeats, and therefore are mostly transcribed from the C strand of telomeres, and are polyadenylated, which enhances their stability (Porro et al., 2010; Schoeftner and Blasco, 2008). Telomeres are transcribed from promoters which are subtelomere in origin, and produce products that vary widely in size, ranging from 100 to 9000 bases in mammals (Azzalin et al., 2007; Schoeftner and Blasco, 2008). TERRA is not translated, but rather functions as a non-coding RNA molecule (Porro et al., 2010). TERRA molecules remain associated with telomeres after transcription, and negatively regulate telomerase activity *in vitro*, perhaps by binding with the template region of hTR (Azzalin et al., 2007; Luke et al., 2008; Schoeftner and Blasco, 2008). TERRA levels are regulated throughout the cell cycle, being the least abundant during S phase, perhaps to allow for telomere replication, and increasing to peak levels in G1 phase (Porro et al., 2010). There appears to be a positive correlation between telomere length and telomere

transcript abundance, with more TERRA production in cells with longer telomeres, which may be a consequence of altered chromatin marks on longer telomeres (Arnoult et al., 2012; Schoeftner and Blasco, 2008). Altered TERRA production may provide a feedback mechanism which would allow for the precise regulation of telomere length.

Both TRF1 and TRF2 are able to bind directly to TERRA, which helps to maintain heterochromatin structure at telomeres, as TERRA is involved in recruiting HP1 and maintaining an enrichment of trimethylated H3K9 (Deng et al., 2009; Schoeftner and Blasco, 2008). Depleting TERRA results in the induction of TIFs, chromosomal aberrations, and a loss of heterochromatin marks at telomeres (Deng et al., 2009). Depleting TRF2, which induces dysfunctional telomeres, increases the levels of TERRA in a manner dependent upon p53 (Caslini et al., 2009). The presence of TERRA at telomeres promotes efficient POT1 binding to single stranded telomere DNA (Flynn et al., 2011). For these reasons, TERRA plays an important role in modulating telomere stability, although the precise mechanism by which TERRA accomplishes this is not yet clear.

TRF1 interacts with RNA polymerase II, as demonstrated through coimmunoprecipitation experiments (Schoeftner and Blasco, 2008). Depleting TRF1 decreases the amount of TERRA production, but does not affect the ability of RNA polymerase II to associate with telomere ends (Schoeftner and Blasco, 2008). These data suggests that TRF1 may be involved in facilitating telomere transcription and TERRA production.

1.1.7 Diseases Associated with Telomere Dysfunction

The tight regulation of telomere maintenance is so crucial to genomic integrity that mutations in a number of the proteins associated with telomeres have been linked to diseases and disorders. After early embryogenesis, telomerase activity is repressed in most human cells, with the exception of cancerous cells and proliferative tissues such as bone marrow, skin, intestine, and lymphocytes; some of which may contain stem cell populations (Chiu et al., 1996; Harle-Bachor and Boukamp, 1996; Hiyama et al., 1995). It

is interesting to consider within the following discussion that these are the populations of cells which are most often affected in disorders of telomere dysfunction.

Dysketatosis congenita (DKC) is a rare genetic disease with a childhood onset that is directly related to improper telomere maintenance and telomerase function. DKC was the first disorder characterized with links to telomere dysfunction (Mitchell et al., 1999). DKC is a disease of telomere shortening which is predominantly caused by mutations in hTR, hTERT, dyskerin, or TCAB1 genes (Dokal, 2000; Dokal and Vulliamy, 2003; Zhong et al., 2011). The most devastating symptom of DKC is bone marrow failure, which is often the cause of death for these patients. Other symptoms include developmental delays, an increased incidence of cancer (including solid tumors and AML, acute myogenous leukemia), aging related features (such as graying hair), pulmonary disease, abnormal skin pigmentation, nail dystrophy, and mucosal leukoplakia (Dokal, 2000; Dokal and Vulliamy, 2003). The last three symptoms have been referred to as the DKC triad, and are typically the basis for diagnosis (Dokal, 2011). Those affected with DKC have less hTR than unaffected individuals, and also display shorter telomeres, chromosome end fusions, and overall telomere dysfunction (Dokal et al., 1992). A number of severe DKC cases have been linked to mutations in the shelterin protein TIN2 (Sasa et al., 2012; Walne et al., 2008). There is a cluster of TIN2 mutations that have been identified which are in the same region of TIN2 which binds HP1, and has been demonstrated to be important for the cohesion of sister telomeres (Canudas et al., 2011). Patients with DKC resulting from mutations in this region of TIN2 have defective sister chromatid cohesion (Savage et al., 2008; Walne et al., 2013; Walne et al., 2008). This information confirms the importance of precise telomerase regulation and telomere maintenance.

Hoyeraal-Hreidarsson (HH) syndrome and Revesz syndrome can be considered to be severe forms of DKC. Those with HH have the symptoms described for DKC, and may

also experience cerebellar hypoplasia, immunological abnormalities, microcephaly, and severe developmental delays (Sznajer et al., 2003). Patients with Revesz syndrome also suffer from many DKC symptoms, and also intracranial calcifications and bilateral exudative retinopathy (Revesz et al., 1992). Both of these diseases present with short telomeres and mutations in the gene coding for TIN2 (Savage et al., 2008; Sznajer et al., 2003). It has been suggested that HH syndrome may also be associated with mutations in Apollo, and that this defect is responsible for the more severe telomere dysfunction observed in a subset of patients, including telomere doublets, fusions, and shortened 3' overhangs (Lamm et al., 2009; Touzot et al., 2010).

Aplastic anemia is another disorder which can arise from mutations in the hTR or hTERT genes, in which case it is referred to as hereditary aplastic anemia (Yamaguchi et al., 2003). Aplastic anemia is a complex disorder of bone marrow failure which is mediated by the immune system. Damaged bone marrow stem cells fail to regenerate hematopoietic cells. Telomeres in patients with aplastic anemia are often shorter than average age matched comparisons. Mutations in hTERT have also been found to be correlated with the development of hematologic malignancies, including AML, chronic lymphocytic lymphoma, and diffuse large-B cell lymphoma (Hills and Lansdorp, 2009). Similarly, some people with idiopathic pulmonary fibrosis (IPF) have mutations in TERT or TR genes (Armanios et al., 2007). IPF is characterized by respiratory failure resulting from progressive lung scarring, and telomere shortening. Some of these patients have symptoms overlapping with DKC, and these disorders may be considered to be on the same spectrum. In a similar way, liver disease that presents without a causative link to alcohol may be rooted in a telomere dysfunction and patients should be examined for other symptoms of DKC or a genetic basis for telomere dysfunction. There have been reports which also suggest that telomerase deficiency may lead to liver cirrhosis (Calado et al., 2009). These disorders manifest later than DKC, and typically have an adult-onset.

AT, ataxia telangiectasia, is an early onset disorder caused by mutations in the ATM gene. This rare genetic disorder is characterized by telomere shortening, premature senescence, immunodeficiency, a predisposition to cancer, developmental problems, ataxia, and a sensitivity to ionizing radiation (Abraham, 2001; Savitsky et al., 1995b). ATM is a crucial mediator in the cellular response to DNA damage and without its functionality DNA damage would accumulate and propagate, leading to genomic instability and possibly tumorigenesis. ATM is associated with telomeres is S and G2 phases of the cell cycle (Buscemi et al., 2009; Kishi et al., 2001a; Verdun et al., 2005). AT, as well as most of the diseases discussed previously, have cellular phenotypes involving telomere dysfunction and are associated with a predisposition to cancer.

The most ubiquitous and devastating disease with its roots in telomere maintenance is cancer. The National Cancer Institute, through the US Institute of Health, cites that more than 41% of people will get cancer at some point in their lifetime, making the full understanding of this disease absolutely critical. The most attention on the relevance of telomeres has been focused towards cancer progression. Telomerase expression, or the activation of the ALT pathway, is essential for the uncontrolled cell proliferation that defines tumorigenesis, and therefore telomere changes are a prerequisite to cancer. In the absence of functional p53 or Rb, telomere instability may lead to chromosome rearrangements, amplifications, or deletions which may contribute to oncogenesis (Artandi and DePinho, 2010). A number of tumor suppressors and oncogenes have been associated with regions of the genome which are more susceptible to these genomic rearrangements (Pinkel and Albertson, 2005). Telomeres need to be maintained by shelterin to regulate their length and to prevent the inappropriate activities of DNA repair machinery at chromosome ends, which is linked to mutagenesis. Telomere dysfunction, and POT1 mutations, have been linked to chronic lymphocytic leukemia (CLL), showing a direct connection between shelterin proteins and tumorigenesis (Lin et al., 2010; Ramsay et al., 2013). Telomerase expression must also

be strictly controlled, with too high or too low levels in variant cell types having devastating physiological consequences.

It is apparent that the precise regulation of telomere length is important to human health. With telomere shortening, comes aging related phenotypes. It has been demonstrated that healthy lifestyles, including exercise, eating lots of fruits and vegetables, and not smoking, are correlated with longer telomeres (Bekaert et al., 2007). It has also been suggested that stress may result in telomere shortening, demonstrating that personal choices may influence telomere length (Epel et al., 2004).

1.2 TRF1

1.2.1 TRF1 and Telomere Maintenance

The most well characterized role of TRF1 is in negatively regulating telomere length. In telomerase positive cells, telomeres shorten with increased TRF1 levels and telomeres are lengthened with the removal of TRF1 from telomeres (Ancelin et al., 2002; Smogorzewska et al., 2000; van Steensel and de Lange, 1997). It has been suggested that TRF1 blocks the access of telomerase at telomere ends, as no effect of TRF1 has been observed on the activity of telomerase (Smogorzewska et al., 2000; van Steensel and de Lange, 1997). It is possible that longer telomeres have more shelterin, and TRF1, and therefore more strongly inhibit telomerase, whereas shorter telomeres have less bound TRF1 and therefore telomerase is more readily able to access and elongate these ends, which may explain why telomerase preferentially elongates short telomeres (Britt-Compton et al., 2009). TRF1 targeted to a particular telomere results in telomere shortening at that particular chromosome end. In this way, TRF1 acts in *cis* to regulate telomere length (Ancelin et al., 2002). The binding of TRF1 to other shelterin components may aid in conveying information about the telomere and in regulating telomere length. The presence of POT1 on ssDNA has been shown to be a transducer of

information from TRF1 regarding the length of telomere DNA (Loayza and De Lange, 2003; Veldman et al., 2004). A mutant POT1 that does not bind telomere DNA completely abrogates the control of telomere length by TRF1, and causes telomere lengthening (Loayza and De Lange, 2003). Many of the protein interactions that have been reported involving TRF1, and also the post translational modifications regulating TRF1, regulate telomere length by modifying TRF1 stability, binding, or localization. The knockout of the *TRF1* gene in mice is embryonic lethal at the blastocyst stage, without an apparent telomere length defect, which suggests for the importance of this gene in ways extending beyond its telomere length regulation (Karlseder et al., 2003).

Telomeres have been described as “fragile sites” for DNA replication. These are sites in the genome that pose a problem for the efficient passage of the replication fork during S phase of the cell cycle (Durkin and Glover, 2007). Repetitive sequences of DNA are often fragile sites and experience replication fork stalling, and subsequent chromosome breakage and translocations (Durkin and Glover, 2007). The G-rich nature of telomeres poses a particular challenge for replication forks as telomeres may form higher order structures, such as G-quartets (telomere sequence folded four times forming 4 parallel strands bound in a quadruplex by Hoogsteen hydrogen bonding) (Tran et al., 2011). Evidence that telomeres are fragile sites includes the appearance of multiple telomere signals per chromosome end when replication is stressed, such as with the addition of aphidicolin (Martinez et al., 2009; Sfeir et al., 2009). It has been demonstrated that TRF1 plays a role in ensuring efficient replication at telomeres. The conditional knockout of TRF1 causes fragile telomeres, which are observed as multitelomeric signals, and can be attributed to a defect in replication (Martinez et al., 2009; Okamoto et al., 2008; Sfeir et al., 2009). These results suggest that TRF1 is important for the replication of telomere ends. The helicases BLM and RTEL1 are recruited by TRF1 and are important in this pathway (Barefield and Karlseder, 2012; Sfeir et al., 2009; Vannier et al., 2012). They are required for the efficient replication of

telomeres as promoted by TRF1, perhaps by unwinding strands which would otherwise be difficult to replicate. TRF1 prevents replication fork stalling and promotes the replication of telomeres, which is another important function of TRF1 at chromosome ends (Sfeir et al., 2009).

The conditional deletion of TRF1 also revealed a role for TRF1 in preventing a DNA damage response at chromosome ends. Depletion of TRF1 has been shown to result in telomere fusions, a mark of unprotected ends (Iwano et al., 2004; Martinez et al., 2009; Okamoto et al., 2008; Sfeir et al., 2009). TRF1 null cells rapidly became senescent, initiating both ATM and ATR signalling cascades, and activating both Chk1 and Chk2, resulting in cell cycle arrest (Martinez et al., 2009).The activation of the ATR dependent DNA damage response in TRF1 null cells was observed predominantly in S phase of the cell cycle (Sfeir et al., 2009). Telomere dysfunction induced foci were observed in cells depleted of TRF1 (Martinez et al., 2009; Sfeir et al., 2009). These results provide support that the presence of TRF1 at telomeres prevents the activation of a DNA damage response. When p53 and RB pathways were disrupted in TRF1 null cells, gross chromosomal instability resulted, and the appearance of sister chromatid fusions was evident (Martinez et al., 2009). Deleting TRF1 in the stratified epithelia of mice (a tissue specific knockout) caused mice to die perinatally (Martinez et al., 2009). Depleting p53 in these TRF1 null mice rescues that lethality, but these mice display a predisposition to cancer and epithelial abnormalities, which is independent of telomere shortening (Martinez et al., 2009). These results show that TRF1 is important in conferring protection at chromosome ends. Overexpressing TRF1 also causes telomere instability and detrimental consequences, and it is interesting to note that TRF1 is naturally upregulated in some human epithelial cancers (Matsutani et al., 2001). Mice overexpressing transgenic TRF1 displayed shorter telomeres, multitelomeric signals, and telomere recombination (Munoz et al., 2009). The levels of TRF1, then, must be precisely regulated within a defined limit for proper telomere maintenance.

1.2.2 TRF1 Stability

The function of TRF1 is directly interconnected with its ability to bind telomere DNA. TRF1 binding to telomere DNA also plays a direct role in regulating its stability. When it is not bound to telomere DNA, TRF1 is accessible for ubiquitination, which targets it for degradation through the proteasome-mediated protein degradation pathway (Chang et al., 2003). There are three E3 ligases which have been shown to ubiquitinate TRF1 and moderate this effect. The first, Fbx4, is a member of the F-box protein family. It was identified as an interacting partner of TRF1 in a yeast two-hybrid screen for proteins interacting with TRF1/PIN2 (Lee et al., 2006). Fbx4 interacts with the homodimerization domain of TRF1, and adds ubiquitin groups onto TRF1 *in vivo* and *in vitro* (Lee et al., 2006; Zeng et al., 2010). The homodimerization domain is the same region of TRF1 which interacts with TIN2, and it has been demonstrated that the binding of TRF1 to TIN2 prevents its ubiquitination by Fbx4 (Lee et al., 2006; Zeng et al., 2010). In this way, TRF1 that is telomere-bound, and therefore associated with TIN2, is protected from degradation. Depletion of Fbx4 increases the amount of TRF1 in the cell and results in telomere shortening, whereas overexpressing Fbx4 reduces TRF1 levels (Lee et al., 2006). Another member of the F-box family of proteins shown to interact with TRF1 in ALT cells is β-TrCP,or beta-transducin repeat-containing protein, an E3 ubiquitin ligase (Wang et al., 2013). The interaction between TRF1 and β-TrCP was determined by mass spectrometry of proteins interacting with tagged TRF1 (Wang et al., 2013). B-TrCP is a component of the SCF ubiquitin ligase complex, and serves as the substrate recognition component (Wu et al., 2003). B-TrCP targets TRF1 for degradation in an ubiquitin dependent manner, and is also important for the recruitment of TRF1 to PML bodies in ALT cells. These results indicate that TRF1 stability may be involved in the formations of APBs (Wang et al., 2013). RLIM was also identified as an E3 ligase capable of ubiquitinating TRF1 (Her and Chung, 2009). RLIM is a RING-H2 zinc-finger protein with intrinsic ubiquitin ligase activity which was identified as a TRF1 interacting partner in a

yeast two-hybrid screen (Her and Chung, 2009). RLIM binds to the linker region of TRF1 and ubiquitinates TRF1 both *in vivo* and *in vitro* (Her and Chung, 2009). Overexpression of RLIM, like Fbx4, decreases TRF1 levels, whereas the depletion of RLIM increases TRF1 levels and results in telomere shortening (Her and Chung, 2009). RLIM and Fbx4 function independently of one another (Her and Chung, 2009).

The accessibility of E3 ligases to ubiquitinate TRF1 is negatively regulated by TRF1 binding to telomere DNA. There is a high rate of exchange between bound and free TRF1, and any modification, then, which regulates the binding of TRF1 to telomeres and shifts this dynamic rate of exchange regulates the stability of TRF1 (Chang et al., 2003; Mattern et al., 2004). Tankyrase 1 and tankyrase 2 were identified as TRF1 interacting factors in a yeast two-hybrid screen with TRF1 as bait (Cook et al., 2002; Smith et al., 1998). Tankyrases are PARPs, which are poly(ADP-ribose) polymerases that add ADP-ribose polymers onto glutamic acid residues of proteins (Hsiao and Smith, 2008). Tankyrase 1 recognizes a region in the N terminal domain of TRF1, specifically an RxxADG motif, as shown through a yeast two-hybrid screen (Smith et al., 1998). Tankyrase 1 and 2 poly(ADP-ribosyl)ate human TRF1 (both *in vivo* and *in vitro*), adding poly-ADP-ribose chains to glutamic acid residues, which reduces the ability of TRF1 to bind telomere DNA (Adaikalakoteswari et al., 2007; Cook et al., 2002; Smith and de Lange, 2000; Smith et al., 1998). The release of TRF1 from telomeres renders it susceptible for degradation through the proteasome pathway. Overexpression or knockdown of tankyrase 1 leads to telomere elongation or shortening, respectively (Cook et al., 2002; Donigian and de Lange, 2007; Smith and de Lange, 2000), presumably by moderating the access of telomerase to telomere ends, as this effect is dependent upon telomerase expression. TIN2 is involved in this process, and forms a ternary complex with TRF1 and tankyrase 1 (Ye and de Lange, 2004). The complex formation with TIN2 protects TRF1 from poly(ADP-ribosyl)ation by tankyrase 1 and stabilizes TRF1 (Ye and de Lange, 2004). These findings have proposed that the poly(ADP-ribosyl)ation

of TRF1 by tankyrase 1 removes TRF1 from telomeres, which allows for its subsequent ubiquitination and degradation by the proteasome pathway. The activity of tankyrase is not required for the ubiquitination and degradation of TRF1 per se, however (Chang et al., 2003).

It has been shown that TRF1 can be de-ubiquitinated, which in turn can regulate its stability. USP22, a member of the SAGA complex, whose functions include the de-ubiquitination of histones, has been shown to affect the stability of TRF1 (Atanassov et al., 2009). Depletion of USP22 in the cell results in decreased levels of TRF1, and overexpression of USP22 similarly increases the levels of TRF1 (Atanassov et al., 2009). In this way, USP22 promotes the stability of TRF1.

Protein interactions may moderate TRF1 stability and provide another level at which TRF1 is regulated. GNL3L, guanine nucleotide binding protein-like 3, a GTP-binding protein, binds to the homodimerization domain of TRF1, and blocks the interaction between TRF1 and Fbx4, while having no effect on the interaction between TRF1 and TIN2 (Tsai, 2009; Zhu et al., 2009). The interaction with GNL3L stabilizes TRF1, as it prevents the Fbx4 mediated ubiquitination of TRF1 (Zhu et al., 2009). The amount of TRF1 dimerization is proportional to the amount of GNL3L in the cell, and it has been suggested that GNL3L protects TRF1 in its unbound form from degradation by Fbx4, and encourages TRF1 dimerization and binding to DNA (Tsai, 2009; Zhu et al., 2009). TRF1 also interacts with nucleostemin (NS), which prevents the dimerization of TRF1 and also reduces the amount of time that TRF1 remains bound to telomere DNA (Meng et al., 2011; Tsai, 2009). The interaction between TRF1 and NS significantly reduces the stability of TRF1, but does not directly affect its ubiquitination (Meng et al., 2011; Zhu et al., 2006).

TRF1 interacts with PinX1, as identified in a yeast two-hybrid screen (Zhou and Lu, 2001). PinX1 has also been shown to interact with telomerase, both the RNA and the

protein subunits, and inhibit its activity (Banik and Counter, 2004). The interaction between PinX1 and TRF1 happens primarily in mitosis, and stabilizes the TRF1 protein (Yonekawa et al., 2012). Without PinX1, cells exhibit delayed mitotic entry and anaphase bridges (Yonekawa et al., 2012). This is an example of another protein-protein interaction that contributes to the regulation of TRF1 stability.

1.2.3 TRF1 and Cell Cycle Regulation

Pin2, an isoform of TRF1, was identified in a yeast two-hybrid screen for proteins interacting with the NIMA (Never in mitosis A) kinase (Lu et al., 1996). Pin2 protein levels have been shown to increase in mitosis, and Pin2 has been demonstrated to localize at the mitotic spindle and have an effect on mitotic progression (Kishi et al., 2001a; Nakamura et al., 2001a; Shen et al., 1997). It was proposed that the increase in Pin2 protein levels in mitosis could be the result of an increase in Pin2 stability (Shen et al., 1997). A GFP-Pin2 fusion protein was demonstrated to localize at telomeres in interphase cells, but move to mitotic spindles in mitotic cells (Nakamura et al., 2001a). These observations were confirmed using antibodies against endogenous proteins. TRF1 binds to microtubules through its C-terminus, and can promote microtubule polymerization *in vitro* (Nakamura et al., 2001a).

TRF1 has been demonstrated to interact with EB1, a microtubule plus-end binding protein (Nakamura et al., 2002). EB1 is located at the mitotic spindle and is important in the assembly of the spindle and for the spindle checkpoint function (Mimori-Kiyosue et al., 2000). The interaction with EB1 has been illustrated to be important for the role of TRF1 in microtubule polymerization, perhaps by bringing TRF1 to microtubule ends (Nakamura et al., 2001b).

The interaction between GNL3L and TRF1 plays a role in cell cycle progression (Zhu et al., 2009). Binding to GNL3L increases the ability of TRF1 to homodimerize, and increases it retention time on telomere DNA *in vivo* (Zhu et al., 2009). The increase in

TRF1 levels in mitosis is abrogated when GNL3L is depleted (Zhu et al., 2009). It is also observed, with high resolution confocal microscopy, that while there is more TRF1 in mitosis, it is not at telomeres (Tsai, 2009). It is noted that the knockdown of either GNL3L or TRF1 results in mitotic spindle defects, specifically multipolarity of spindles (Zhu et al., 2009). The involvement of TRF1 in the formation of mitotic spindles is also apparent with the examination of AT cells, which display a mitotic spindle defect that can be rescued with the depletion of TRF1 (Nakamura et al., 2002). These findings suggest that TRF1 plays an important role in the mitotic transition.

The generation of mice with transgenic TRF1 expression in epithelial tissues has confirmed the participation of TRF1 in mitotic progression. Mice overexpressing TRF1 (termed K5TRF1 mice) displayed shorter telomeres, as expected, and also end to end fusions, multitelomeric signals, and telomere recombination, which confirms the function of TRF1 in maintaining telomere protection and in promoting telomere replication (Munoz et al., 2009). What was also observed in this setting was the involvement of TRF1 in the spindle checkpoint. Mice with overexpressed TRF1 displayed mitotic spindle aberrations, including multipolarity and misaligned chromosomes, and a colocalization between TRF1 and proteins involved in the mitotic spindle checkpoint (BubR1 and Mad2) (Munoz et al., 2009). A partial colocalization was also observed between TRF1 and EB1, Aurora B at kinetochores, and markers for centromeres (Munoz et al., 2009). These results demonstrate a clear involvement for TRF1 in the regulation of mitotic progression.

1.3 The Phosphorylation of TRF1

TRF1 is phosphorylated on multiple residues, at various points in the cell cycle, and under various conditions. These modifications have been shown to impact TRF1 binding to telomeres, stability, and localization (Walker and Zhu, 2012). These effects have direct consequences on the role of TRF1 in telomere protection, telomere length

maintenance, and in functions away from telomere ends. The known and characterized TRF1 phosphorylation sites are depicted in Figure 1.3.

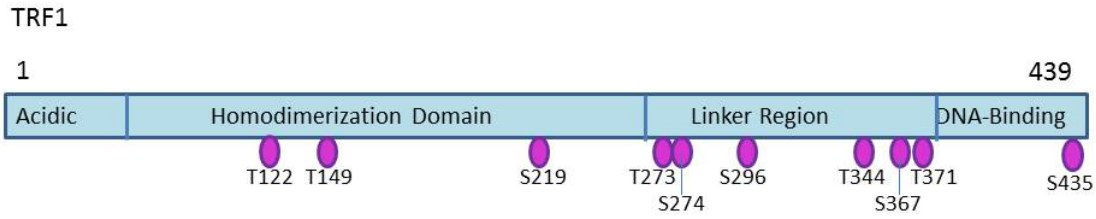

Figure 1.3 TRF1 Phosphorylation Sites
Schematic of TRF1 structure and phosphorylation sites, indicated by purple ovals. Domains and distances are not drawn to scale.

TRF1 is phosphorylated at T122 by casein kinase 2. Casein kinase 2 (CK2) is a serine/ threonine kinase that has roles involved in cell cycle regulation, the DNA damage response, and in cell growth (St-Denis and Litchfield, 2009). TRF1 was shown to interact with the β-subunit of CK2 through immunoprecipitation, GST pull down assays, and, originally, through a yeast two-hybrid screen for proteins interacting with TRF1 in HeLa cells (Kim et al., 2008a). Inhibiting CK2 results in a decreased association of TRF1 with telomere DNA *in vivo* and *in vitro,* as well as a reduction in TRF1 stability (Kim et al., 2008a). These effects were demonstrated with CK2 inhibition and knockdown, and also by using TRF1 constructs mutated at T122 to prevent phosphorylation (Kim et al., 2008a). These findings also revealed that this phosphorylation site may be involved in regulating the ability of TRF1 to form homodimers, perhaps providing an explanation for the reduction in binding capacity (Kim et al., 2008a).

AKT has been demonstrated to phosphorylate TRF1 T273 (Chen et al., 2009). The phosphorylation at T273 has been suggested to promote TRF1 association with telomeres; increasing AKT levels results in increased TRF1 levels and subsequent telomere shortening (Chen et al., 2009). TRF1 and AKT were shown to interact by GST

pull-down assays and also coimmunoprecipitation (Chen et al., 2009). Evidence for this phosphorylation was shown *in vitro,* and *in vivo* data remains to be substantiated.

Serine 435 of TRF1 is phosphorylated by Plk1, and this phosphorylation has been suggested to stimulate TRF1 binding to telomere DNA *in vivo* (Wu et al., 2008b). Polo like kinases are important for mitotic progression and cell division, playing roles in aspects such as spindle formation, chromosome segregation, and centrosome maturation (van Vugt and Medema, 2005). Plk1 was identified as a TRF1 interacting partner initially in a yeast two-hybrid screen for proteins interacting with Plk1, and this interaction was confirmed with GST-pull down assays and immunoprecipitation (Wu et al., 2008b). The *in vitro* phosphorylation of TRF1 by Plk1 was reported, however we have been unable to replicate the observation that TRF1 is a substrate of Plk1 (Wu et al., 2008b). Another group has revealed a role for Plk1 in phosphorylating xenopus TRF1 and increasing the binding of xTRF1 to telomere DNA *in vitro* (Nishiyama et al., 2006). In humans, mutational analysis supports a role for this site in regulating TRF1 binding to telomeres, as a mutation preventing phosphorylation at S435 impairs binding of TRF1 to telomere DNA (Wu et al., 2008b). It was suggested that phosphorylation of T344 TRF1 by Cdk1 serves as a priming site to allow for the phosphorylation of S435 by Plk1 (Wu et al., 2008b). Cdk1 is a kinase which is crucial for mitotic entry (Ferrari, 2006). It recognizes substrates at consensus S/T-P sequences, phosphorylating serine and threonine sites (Fisher et al., 2012).

TRF1 is a substrate of cyclin dependent kinases at multiple residues. Another of these residues, T149, is important for the interaction between PIN1 and TRF1 (Lee et al., 2009). PIN1 is a prolyl isomerase which binds to S/T P motifs in proteins and isomerizes these motifs (Shen et al., 1998). The interaction with PIN1 has been demonstrated by mutating T149 in TRF1, and also by generating an antibody which recognizes TRF1 when phosphorylated at this site (Lee et al., 2009). The interaction between TRF1 and PIN1 is

abrogated by the treatment of cells with phosphatase. Depleting PIN1 results in an enhanced stability of TRF1 and also an increased TRF1 association with telomere DNA, resulting in telomere shortening (Lee et al., 2009). T149 phosphorylation happens primarily in mitosis, and suggests a model where by CDK phosphorylation of TRF1 allows for the regulation of TRF1 by PIN1 interaction (Lee et al., 2009; Shen et al., 1997).

Aurora A has been demonstrated to phosphorylate TRF1 at S296, and investigating mutations at this site has revealed that this modification is important in regulating mitotic abnormalities (Ohishi et al., 2010). Aurora A is known to play important roles in mitotic progression which include regulating centrosome assembly, spindle arrangement, chromosome alignment in metaphase, and cytokinesis (Marumoto et al., 2005). With Aurora A overexpression, abnormalities including misalignment of chromosomes at metaphase, multinucleated cells, and centrosome amplification, are observed (Marumoto et al., 2005). If tankyrase 1 is overexpressed in these cells, the mitotic defects are ablated, which led to the notion that TRF1 may be bridging these effects. Depleting TRF1 (or preventing phosphorylation at S296) in these cells rescues these defects, suggesting that TRF1 phosphorylation by Aurora A promotes mitotic defects (Ohishi et al., 2010). *In vitro* work has demonstrated an interaction between Aurora A and TRF1, and also that TRF1 is a substrate of Aurora A (Ohishi et al., 2010).

TRF1 is additionally phosphorylated on S219 by ATM (Kishi et al., 2001b). ATM is one of the main signal transducers involved in the DNA damage response, by phosphorylating a number of downstream effectors and initiating checkpoint activation or apoptosis (Savitsky et al., 1995a; Savitsky et al., 1995b; Zakian, 1995). TRF1 S219 phosphorylation happens after the induction of DNA damage, specifically ionizing radiation. These findings demonstrate that TRF1 and ATM interact, both *in vivo* and *in vitro* (Kishi et al., 2001b). These researchers have reported that TRF1 overexpression leads to mitotic defects and apoptosis, and they note that phosphorylation at S219

suppresses these phenotypes (Kishi et al., 2001a). AT cells; lacking ATM and demonstrating sensitivity to ionizing radiation, were used to investigate the impact of TRF1 phosphorylation. When S219 is mutated so as to prevent phosphorylation, the sensitivity of AT cells to DNA damage increased, whereas mimicking phosphorylation at this site rescued the sensitivity (Kishi et al., 2001b). These findings suggest that TRF1 plays a role in the response of the cell to DNA double strand breaks.

It has been demonstrated *in vivo* that ATM can phosphorylate TRF1 in undamaged cells, using an inhibitor of ATM, KU55933 (Wu et al., 2007). Phosphorylation of TRF1 by ATM was shown to impair TRF1 binding to telomeres *in vivo* and *in vitro*. Investigation into ATM consensus sites of TRF1 (SQ sites; S219, S274 and S367), revealed that mimicking phosphorylation at either S274 or S367, but not S219, results in a decreased association of TRF1 with telomere DNA *in vitro* (Wu et al., 2007). The phosphorylation of TRF1 by ATM is dependent upon the MRN complex (Wu et al., 2007). These results suggest that the phosphorylation of TRF1 by ATM may participate in controlling TRF1 binding to telomeres.

Overall, it is apparent that there are a number of phosphorylation sites on TRF1, shown in Figure 1.3, which have functional implications in regulating the binding of TRF1 to telomere DNA, the stability of TRF1, or its participation in other functions, such as the DNA damage response. It was of interest to expand on the knowledge of the regulation of TRF1 by post-translational modifications.

1.4 TRF1 and the Separation of Sister Chromatids

1.4.1 The Cohesin Complex

Sister chromatids are tethered together by a protein complex called cohesin from S phase, when the sister chromatids are replicated, until their separation at anaphase (Mehta et al., 2012). The cohesin complex is composed of four protein

subunits, Smc1, Smc3, Rad21, and SA, which can be either SA1 or SA2 in vertebrates, as shown in Figure 1.4 (Nasmyth and Haering, 2009; Onn et al., 2008). The Smc, or Structural Maintenance of Chromosome family proteins, are ATPases which regulate different aspects of chromosome structure (Michaelis et al., 1997). Smc1 and Smc3 interact with each other through their hinge domains (generated on each protein by its folding back upon itself), and the ATPase heads of both Smc1 and Smc3 bind to the Rad21 subunit, which acts as a bridge (Haering et al., 2002; Melby et al., 1998; Schleiffer et al., 2003). This bridging helps to form a closed triangular "ring shape" complex (Mehta et al., 2012; Remeseiro and Losada, 2013). In vertebrate somatic cells, each cohesin complex contains either SA1 or SA2, but not both, and the complexes containing SA2 are approximately three times as abundant (Losada et al., 2000; Sumara et al., 2000). The SA2 containing cohesin complexes have been shown to be important for cohesion along chromosome arms and at centromeres, whereas SA1 containing complexes are involved in cohesion and replication at telomeres (Remeseiro et al., 2012a; Remeseiro et al., 2012b; Remeseiro and Losada, 2013). The depletion of SA1 by siRNA causes a deficiency in the ability of sister telomeres to remain associated after DNA replication, whereas the depletion of SA2 results in defects in the cohesion of centromeres, but maintains telomere and chromatid arm cohesion (Canudas and Smith, 2009). The knockout of SA1 in mice results in early embryonic lethality, which is the result of aneuploidy generated from problems in telomere replication and a loss of telomere cohesion, confirming that SA1 has a specific role at telomeres (Remeseiro et al., 2012a). There are many theories as to how the cohesin complex holds chromatids together; the most popular models include the ring model, which suggests that one cohesin complex wraps around two sister chromatids, tethering them together, or the handcuff model, which proposes that each chromatid is bound by a cohesin ring, and that these two rings interact with one another to bring the strands of DNA together (Campbell and Cohen-Fix, 2002; Huang et al., 2005; Losada and Hirano, 2005; Milutinovich and Koshland, 2003; Nasmyth, 2005).

The ring model is depicted in Figure 1.4. Cohesion is important in ensuring that daughter cells get an equal complement of chromosomes, and holds chromatids together from their replication in S-phase till their split at the metaphase to anaphase transition, ensuring that the orientation and separation of chromatids is accurate (Mehta et al., 2012; Remeseiro and Losada, 2013). Sister chromatids must be held together tightly in mitosis to prevent the microtubules from pulling the sister chromatids apart too early and causing random segregation. These bonds must be quickly broken in anaphase to allow for the appropriate separation of sister chromatids at this point. Some cohesin is removed in prophase by the action of polo like kinase and Cdk1 phosphorylation, and the phosphorylation of the SA2 cohesin subunit, which removes much of the cohesin along chromosome arms (Dreier et al., 2011; Remeseiro and Losada, 2013; Waizenegger et al., 2000). The remaining cohesin is removed in anaphase by the anaphase promoting complex and the action of separase, which proteolytically cleaves Rad21 and releases the remaining cohesin ring structures (Hauf et al., 2001; Oliveira et al., 2010; Remeseiro and Losada, 2013).

Cohesin is also important for homologous recombination, in ensuring that the template strand is within close proximity to be used for repair. After the induction of DNA double strand breaks, the cohesin complex aids in DNA repair by homologous recombination in G2 phase, and the depletion of members of the cohesin complex (SMC1 or Rad21) impairs cell survival (Bauerschmidt et al., 2010). The cohesin complex has been shown to accumulate at sites of laser induced DNA damage, in an Mre11 and Rad50 dependent manner (Kim et al., 2002). Additionally, the cohesion of sister chromatids after DNA damage is supported by γH2AX (Xie et al., 2004).

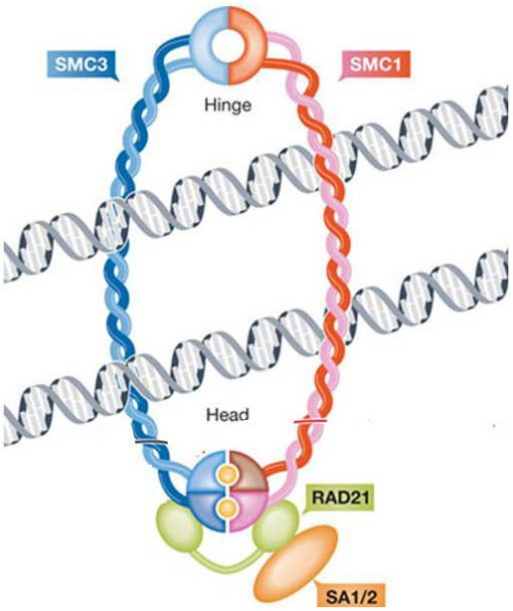

Figure 1.4 The Cohesin Complex
A schematic representation of the human cohesin complex, illustrating the ring model of sister chromatid cohesion. Figure is an adaptation. (Jessberger, 2012)

Typically, sister chromatids are fully resolved by the time that cells complete metaphase. What is interesting, however, is the observation that in cells approaching senescence (and perhaps with shorter or dysfunctional telomeres), persistent sister chromatid association was observed at telomeres (Yalon et al., 2004). These findings indicate that telomeres may be a site of persistent, or sticky, cohesion.

1.4.2 Cohesion at Telomeres

There exist differences in the cohesin complex at telomere ends as compared to that which is present along chromosome arms. Telomeres require a distinct cohesin complex and regulation from chromosome arms to maintain their function, and appear to be sites of more persistent cohesion (Canudas and Smith, 2009).

In cells that have been depleted for tankyrase 1 by siRNA, sister chromatids separate in mitosis across the entire chromatid with the exception of the telomeres,

which remain associated, triggering a mitotic arrest (Dynek and Smith, 2004). Chromosomes are properly aligned at the metaphase plate but fail to separate at their ends (Dynek and Smith, 2004). Telomeres may have a different requirement for the separation of sister chromatids than chromatid arms or centromeres, the separation of which was unaffected by tankyrase 1 knockdown (Dynek and Smith, 2004). In cells that experience persistent cohesion at telomere ends, telomeres become dysfunctional and are recognized as sites of DNA damage. As a result, non-homologous end joining fuses sister chromatid ends together, which is observed as one strong telomere signal in the middle of two chromatids at the end of a chromosome (Hsiao and Smith, 2009).

TRF1, as well as its shelterin-interacting partner TIN2, were both shown to bind to SA1, which is a member of the cohesin complex (Canudas et al., 2007). They do not bind to the SA1 homologue SA2, which is present in a subset of cohesin complexes, indicating a higher level of regulation of this process (Canudas et al., 2007). In cells depleted for tankyrase 1, and unable to resolve their telomeres in mitosis, the depletion of SA1 or TRF1 and TIN2 rescues this defect (Canudas et al., 2007). The depletion of SA1 in cells not depleted for tankyrase 1, however, is detrimental to the cell. In the absence of SA1, and therefore telomere cohesion, sister chromatids are broken and telomere loss is observed (Canudas and Smith, 2009). These results indicate that there is a connection between telomere proteins and the regulation of cohesion at telomeres in mitosis.

The combination of TRF1 and TIN2 is clearly important in regulating the cohesion of telomeres, which is interesting as TIN2 is also directly implicated in the regulation of TRF1 by tankyrase 1. Tankyrase 1 regulates the release of TRF1 from telomere DNA through its poly(ADP-ribosyl)ation (Cook et al., 2002; Smith et al., 1998). TIN2 forms a complex with both TRF1 and tankyrase 1 and shields TRF1 from poly(ADP-ribosyl)ation by tankyrase 1 (Ye and de Lange, 2004). The knockdown of TIN2 by siRNA results in a

loss of TRF1 from telomeres, and telomere elongation (Ye and de Lange, 2004). It is possible that the release of TRF1 from telomeres, mediated by its poly(ADP-ribosyl)ation, is important for the release of the cohesin complex and the separation of sister telomeres (Canudas et al., 2007; Dynek and Smith, 2004). Mediating sister chromatid cohesion provides a function for TRF1 in addition to regulating telomere length and protecting telomere ends. It has been proposed that there may be a relationship between telomere cohesion and telomere elongation by telomerase. Telomeres that are more "cohered" experience more elongation by telomerase, perhaps because the structure of telomerase favours the coordinated extension of two chromatid ends (Houghtaling et al., 2012).

TIN2 has been shown to interact with HP1, and it appears as though this association is important for the cohesion of telomeres in S phase (Canudas et al., 2011). The C-terminus of TIN2 binds HP1 through an HP1 binding motif, PTVML (Canudas et al., 2011). Mutating this site in TIN2, and abrogating the interaction between TIN2 and HP1, results in cohesion defects and a larger distance between sister telomeres, which is indicative of less cohesion (Canudas et al., 2011). These results demonstrate that there are distinct and complicated mechanisms regulating cohesion at chromosome ends.

1.5 The DNA Damage Response

1.5.1 Overview of the DNA Damage Response

DNA damage can result from endogenous or exogenous sources; ultraviolet light, ionizing radiation, reactive oxygen species, mutagenic chemicals or toxins, and from byproducts of cellular metabolism. These agents will cause different forms of lesions in the DNA, including but not limited to single strand DNA breaks, double strand breaks, mismatched bases, or 8-oxo-G residues (Houtgraff,2006). Double strand breaks are

extremely cytotoxic and must be efficiently repaired to prevent detrimental consequences.

Genomic instability resulting in deleterious mutations is one of the main driving forces of tumorigenesis. Cellular mechanisms have evolved to quickly detect DNA lesions and prevent cellular proliferation until these lesions are properly repaired. If these lesions cannot be properly repaired, DNA damage response factors induce apoptosis or cellular senescence to prevent the propagation of these mutated cells and the accumulation of damage (Moynahan and Jasin, 2010; San Filippo et al., 2008).

This section will address the cellular response to DNA double strand breaks (DSBs), as this form of damage is relevant to the situation at chromosome ends. If double strand breaks go unrepaired, they may lead to chromosome breaks and translocations, and overall genome instability.

1.5.2 Homologous Recombination

Homologous recombination (HR) is a process which can be used to repair harmful breaks occurring on both DNA strands (a double strand break), using the sister chromatid as a template for repair. HR happens in S and G2 phases of the cell cycle, when a sister chromatid is available, and is a relatively error free form of DNA repair (Moynahan and Jasin, 2010; San Filippo et al., 2008). A schematic of this process is shown in Figure 1.5.

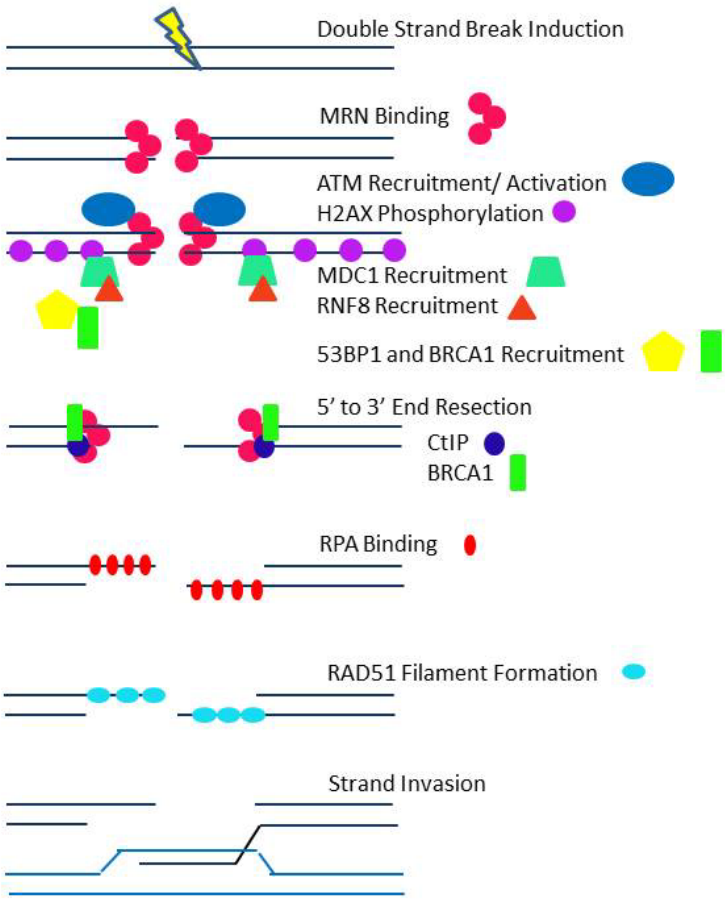

Figure 1.5 Homologous Recombination
A schematic of the cascade of proteins associated with a double strand break that is being repaired through the homologous recombination pathway. This repair pathway is described within the text. Proteins and interactions are representations only and are not drawn to scale. A homologous sister chromatid is shown in blue; whereas the damaged double stranded DNA is shown in black.

For homologous recombination (HR), DNA DSBs recruit the MRN complex (MRE11-RAD50-NBS1), which is one of the first responders (Carson et al., 2003; Lavin, 2007; Petrini, 1999; Petrini and Stracker, 2003). The MRN complex is responsible for tethering the two ends of the DNA break together, as well as for the activation of ATM, which initiates a feedback loop that retains and activates the MRN complex (Durocher and Jackson, 2001; Falck et al., 2005; Jazayeri et al., 2006; Lee and Paull, 2005, 2007; You et

al., 2005). ATM is a phosphoinositide-3-kinase related protein kinase with serine or threonine kinase activity at S/TQ sites, which acts as a transducer of the DNA damage response. ATM phosphorylates H2AX at S139 to form γH2AX around the break site, contributing to the formation of DNA damage foci and the recruitment of numerous DNA damage response proteins (Paull et al., 2000). The sensor of γH2AX, MDC1, is recruited to this chromatin modification. The presence of MDC1 at break sites helps to recruit RNF8, which in turn promotes the accumulation of BRCA1 and 53BP1 at break sites, which work together to decide the fate of repair choice (Bunting et al., 2010; Chapman et al., 2012; Escribano-Diaz et al., 2013; Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007). The surrounding H2A and H2AX histones are ubiquitinated by the ubiquitin ligases RNF8, RNF168, and HERC2, which are recruited by MDC1 (Huen et al., 2007; Mailand et al., 2007). The ubiquitination of histones facilitates changes, and relaxation, in the chromatin structure around the break site (Polo and Jackson, 2011). BRCA1 is an E3 ubiquitin ligase, and its ubiquitination of CtIP helps to promote the binding of CtIP to sites of damage (Hashizume et al., 2001; Morris and Solomon, 2004; Yu et al., 2006). The phosphorylation of the CtIP nuclease by CDKs promotes its interaction with BRCA1, which then work together at DSBs to generate longer regions of single stranded DNA in the next stage of this process (Huertas and Jackson, 2009). CtIP also interacts with the MRN complex, which has endo- and exonuclease functions, to promote HR and end resection (D'Amours and Jackson, 2002; Lavin, 2004; Sartori et al., 2007). The end of the DSB must be processed, or resected in the 5’ to 3’ direction, to generate a 3’ single strand overhang. End resection at sites of DSBs is accomplished by the MRN complex, CtIP, and the EXO1 and BLM nucleases (Gravel et al., 2008; Nimonkar et al., 2011; Nimonkar et al., 2008; Paull and Gellert, 1998, 1999). The single stranded DNA becomes coated with RPA, which prevents its degradation, and acts as a sensor of the accumulation of single stranded DNA (Alani et al., 1992). The presence of RPA on single stranded DNA is thought to prevent the formation of secondary structures and to

stabilize the invasion intermediate (Eggler et al., 2002; Sugiyama et al., 1997). The RPA coated DNA recruits ATR, through its interacting partner ATRIP, and the 9-1-1 complex (RAD9-RAD1-HUS1) (Cortez et al., 2001; Rouse and Jackson, 2002; Zou and Elledge, 2003; Zou et al., 2003). ATR activates Chk1 to initiate cell cycle checkpoints downstream of ATM (Myers and Cortez, 2006). The RPA bound single stranded DNA becomes coated with RAD51 recombinase, and RPA is displaced with the help of BRCA2 and RAD52 (Liu et al., 2010; New et al., 1998; Sugiyama and Kowalczykowski, 2002). RAD51 coated DNA becomes involved in a homology search and invades the complementary strand of DNA, forming a displacement loop by homologous base pairing and strand exchange (Baumann and West, 1998; Krejci et al., 2012; San Filippo et al., 2008). Strand invasion is catalyzed by Rad51, which has formed ssDNA-Rad51 filaments, and completed with contributions from RAD52, RAD55/RAD57, and RAD 54 (Alexeev et al., 2003; Sugawara et al., 2003). Using the homologous sequence as a template, synthesis occurs to replace the nucleotides that are missing due to the initial damage or as a result of the end resection, possibly through PCNA and Polδ, although this involvement is still unclear (Li et al., 2013; Li et al., 2009). The two Holliday junctions are cleaved and resolved by helicase and resolvase activity, the ends are ligated, and the repair of the break is completed (Ip et al., 2008; Liu et al., 2004c; Mimitou and Symington, 2009a, b).

1.5.3 Non-Homologous End Joining

Another form of double strand break repair is non-homologous end joining, which may happen at any stage in the cell cycle as it does not require a template strand. Non-homologous end joining (NHEJ), is much more error prone than HR, albeit the more prominent form of damage repair in mammalian cells (Lieber, 2010). It occurs mainly in G1, when there is no homologous sequence that HR can utilize as a template. A schematic of this process is shown in Figure 1.6.

Chromatin changes occur around the break site, in a manner similar to HR; γH2AX signals the presence of DNA damage in both HR and NHEJ. The doubles strand breaks

are sensed by Ku70 and Ku80, and are held in close proximity by this end binding heterodimer (Falzon et al., 1993; Lieber, 2008). The DNA-dependent protein kinase (DNA-PK) catalytic subunit is activated and recruited by Ku70/Ku80, and forms a complex with the nuclease Artemis, and 5'-3' endonucleolytic resection of the break may occur, if the ends are not compatible for direct ligation (Gottlieb and Jackson, 1993; Ma et al., 2002). If a gap is generated, it is filled in by DNA polymerase before the ends are ligated by DNA ligase IV-XRCC4 (Grawunder et al., 1997). NHEJ often results in translocations, insertions, or deletions of DNA, which can clearly be detrimental to genome stability (Lieber et al., 1997).

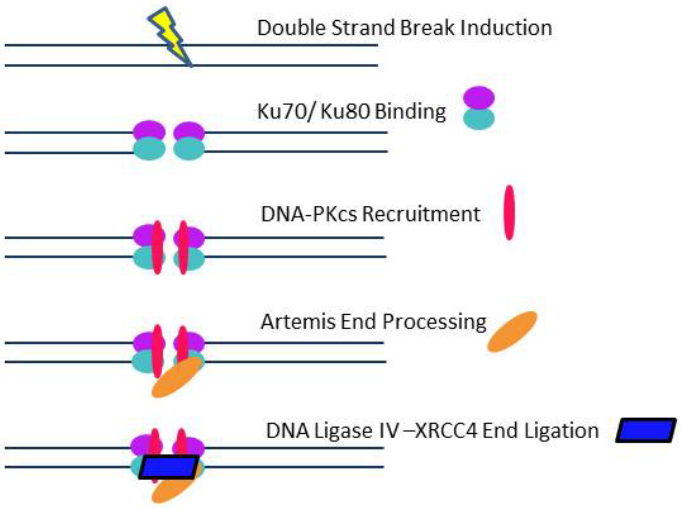

Figure 1.6 Non-Homologous End Joining
A schematic of the cellular response to a DSB by the NHEJ repair pathway. Main proteins involved are depicted, proteins and interactions are a representation only and are not drawn to scale. This process is described within the text.

There is a precise balance and mechanism required to decide the fate of a DNA DSB; whether it will be repaired by HR or NHEJ. HR is the favored option in S or G2 phase cells, when there is a homologous sequence available as a template, as this repair pathway minimizes errors (Haber, 2000). The balance of certain proteins at sites of damage also helps to decide the fate of a break. The presence of 53BP1 may help

promote NHEJ over HR, and one way that it may accomplish this is by increasing the mobility of the broken end, making it more likely to locate a distant end for joining (Bunting et al., 2010; Dimitrova et al., 2008; Escribano-Diaz et al., 2013; Lowndes, 2010). BRCA1 and 53BP1 appear to be the central mechanism of choice control, regulating the fate of repair by either HR or NHEJ, respectively. 53BP1 acts to inhibit end resection, which promotes NHEJ over HR, as end resection is a crucial step in HR (Bunting et al., 2010; Dimitrova et al., 2008; Escribano-Diaz et al., 2013). BRCA1 deficiency impairs HR, and promotes NHEJ. BRCA1 excludes 53BP1 from break sites, and stimulates end resection (Cao et al., 2009; Chapman et al., 2012).

1.5.4 Checkpoint Activation

Following the induction of DNA damage, checkpoints are activated to stop or delay the cell cycle and prevent the propagation of DNA damage. Checkpoints exist at the G1/S boundary, within S phase, and at the G2/M boundary.

In response to DNA damage, the ATM and ATR kinases phosphorylate downstream effector kinases, Chk2 and Chk1 respectively. These act as sensors of the DNA damage, and initiate signalling cascades which result in cell cycle arrest, senescence or apoptosis. In G1 phase of the cell cycle, DNA damage activates p53 through ATM and ATR, which leads to the expression of p21, which inhibits CDK2, preventing the G1-S transition (Agarwal et al., 1995; Bartek and Lukas, 2001a, b). Within S phase, Chk1 and Chk2 can phosphorylate cdc25A, to degrade it, which in turn maintains CDK2/cyclinE in an inactive state (Finn et al., 2012; Houtgraaf et al., 2006). In G2 phase, the Wee1 and Myt1 kinases inactivate cdc25, which is a phosphatase that removes inhibitory phosphorylation from Cdk1-cyclin B complexes, resulting in a G2/M arrest (Fernandez-Capetillo et al., 2002; Finn et al., 2012; Houtgraaf et al., 2006). The G2/M checkpoint prevents the cell from replicating damaged DNA and from propagating damaged DNA to daughter cells, until that damage can be repaired, and the checkpoint signals are removed.

1.5.5 Preventing a DNA Damage Response at Telomeres

Telomeres must avoid being detected by the DNA damage response machinery and recognized as a site which requires repair. TRF2 is important in preventing an ATM mediated response at telomeres (Celli and de Lange, 2005; Karlseder et al., 1999; Karlseder et al., 2004), and POT1 has been shown to be important for repressing the ATR signalling pathway, which is activated upon its deletion (Denchi and de Lange, 2007; Guo et al., 2007). POT1 may prevent a damage signal at telomeres by antagonizing the binding of RPA to single stranded telomere DNA and thus preventing the signalling cascade that RPA would initiate (Flynn et al., 2011).

TRF2 may exert its function of conferring end protection by forming the t-loop structure (Stansel et al., 2001). In the absence of TRF2, t-loops may be disassembled, and the 3' telomere overhangs may be lost as a result of the nuclease activity of XPF/ERCC1 (Wu et al., 2008a; Zhu et al., 2003). The loss of the 3'overhang leads to the fusion of chromosome ends into long tracts by NHEJ (Celli and de Lange, 2005; Konishi and de Lange, 2008; Zhu et al., 2003). The complex of TRF2-Rap1 interacts with DNA-PKcs to aid in the prevention of end joining at telomeres; Rap1 is also very important in preventing NHEJ at telomeres (Bae and Baumann, 2007; Sarthy et al., 2009; Smogorzewska et al., 2002). TRF2 has been suggested to directly interact with ATM, in its autophosphorylation region, and inhibit the activation of ATM following DNA damage, which may be the mechanism by which TRF2 suppresses an ATM dependent response at telomeres (Karlseder et al., 2004). In addition, TRF2 directly binds Chk2, the downstream target of ATM in the DNA damage response cascade. TRF2 binds through the ATM phosphorylation site of Chk2, perhaps preventing the ATM-mediated DNA damage response (Buscemi et al., 2009). TRF2 has been shown to interact with the MRN complex at telomeres, which may contribute to the formation of the t-loop structure (Zhu et al., 2000). The Ku complex also has a substantial presence at telomeres, interacting directly with both TRF1 and TRF2 (Hsu et al., 2000). Ku is important in the

NHEJ pathway of double strand break repair, and mediates end to end fusions in the absence of TRF2 (Celli et al., 2006). Its presence on telomeres has been predicted to repress recombination events at telomeres; in its absence rapid telomere deletion and t-circle formation are observed (Wang et al., 2009).

Homology directed repair is also a threat at telomere ends, and there exists mechanisms to preclude HR at telomeres. T-loops may be resolved in a manner similar to the resolution of Holliday junctions; producing a telomere circle and a cleaved telomere end as products. The basic domain of TRF2 is important for preventing this pathway at telomeres, and mutated TRF2 lacking the basic domain reveals a high degree of telomere loss and t-circle formation (Wang et al., 2004). The depletion of shelterin components leads to the activation of the DNA damage response, cell cycle checkpoint activation, and chromosomal instability (Palm and de Lange, 2008).

The presence of numerous DNA damage response factors at telomeres has long been evident but has not been well understood. It seems counterintuitive that in preventing a DNA damage response, telomeres accumulate a number DNA damage response factors. Some of the DNA repair proteins that associate with telomeres include the MRN complex, Ku, ATM, ATR, BRCA1, and XPF/ERCC1 (Ballal et al., 2009; Denchi and de Lange, 2007; Hsu et al., 1999; Hsu et al., 2000; Karlseder et al., 1999; Karlseder et al., 2004; Petrini and Stracker, 2003; Zhu et al., 2000; Zhu et al., 2003). The precise role for each of these proteins at telomeres has not been fully elucidated, but it is clear that they are required for efficient telomere replication, for the processing of telomere ends and overhang production, and for the formation of protective structures such as t-loops at telomeres (de Lange, 2005).

Telomere structure is dynamic throughout the cell cycle, and adjusts so as to allow for functionality in each cell cycle stage. The formation of the t-loop at telomere ends would pose a problem for the passing replication fork in S-phase, and therefore the t-

loop must be resolved at this point in time (de Lange, 2010). Telomeres become transiently uncapped during each S phase, perhaps to allow for replication, and become reprotected in G2 phase of the cell cycle (Verdun et al., 2005). Telomeres are not recognized as sites of damage at this point; the p53 dependent DNA damage response is diminished in S phase (Gottifredi et al., 2001). In G2 stage of the cell cycle, it has been suggested that telomeres are recognized as sites of DNA damage, and accumulate NBS1 and ATM and release POT1, which allows for the re-formation of t-loops (Verdun et al., 2005). Telomere extension by telomerase appears to be restricted to S and G2 phases of the cell cycle (Jady et al., 2006; Marcand et al., 2000; Tomlinson et al., 2006). Perhaps this helps to coordinate telomerase extension during the time period when t-loops are relaxed. Removal of TRF2 from telomeres in G1 phase results in a damage signal at telomeres and the fusion of chromosome ends through non-homologous end joining (Konishi and de Lange, 2008).

1.5.6 DNA Damage Signalling at Dysfunctional or Shortened Telomeres

As cells age, replicative senescence, and its associated telomere shortening, generate a DNA damage response at telomeres. It has been shown that an accumulation of five telomeres with length-induced dysfunction is sufficient to predict p53-dependent cellular senescence (Kaul et al., 2012). In p53 and Rb proficient cells, telomere shortening results in a cell cycle arrest, senescence, or apoptosis, whereas in p53 deficient cells, telomere shortening results in telomere crisis (Artandi and DePinho, 2010; Chin et al., 1999; Deng et al., 2008; Harrington and Robinson, 2002). Telomere crisis causes non-homologous end joining of chromosome ends, which break, and lead to chromosome rearrangements and genomic instability, referred to as the breakage-fusion-bridge cycle (Murnane, 2012; Murnane and Sabatier, 2004). In situations where telomeres are recognized as sites of damage and are rendered dysfunctional, or they become critically short and enter a crisis stage, cells may become tetraploid (Davoli et al., 2010). Tetraploidy could be due to a failure in effectively completing mitosis

(accumulating a polyploidy complement of chromosomes) or due to endoreduplication, and is viewed as an early step in tumor formation, and underlies another way in which proper telomere maintenance is important in maintaining genomic stability (Davoli and de Lange, 2012).

Telomeres that are critically short display the accumulation of phosphorylated ATM, 53BP1, MRN, and γH2AX (d'Adda di Fagagna et al., 2003; Longhese, 2008; Verdun and Karlseder, 2006). The presence of DNA damage response factors at telomeres is referred to as TIFs (Telomere dysfunction induced foci). TIFs are also evident when a member of the shelterin complex, particularly TRF2, POT1, or TIN2, is inhibited or depleted (Hockemeyer et al., 2005; Kim et al., 2004; Okamoto et al., 2013). Uncapped telomeres may accumulate RNF8, an E3 ubiquitin ligase, which modifies H2A, and results in the accumulation of 53BP1 and ATM at these ends, promoting NHEJ (Peuscher and Jacobs, 2011; Rai et al., 2011). Uncapped telomeres in G2 phase of the cell cycle are prevented from entering mitosis in a p53 dependent manner (Thanasoula et al., 2010). The accumulation of DNA damage foci in cells with dysfunctional telomeres may help to promote senescence or cell cycle arrest of these cells, and prevent proliferation (de Lange, 2005, 2010). The observation that dysfunctional telomeres are detected as damaged DNA provides support that proper telomere maintenance is essential in avoiding a DNA damage response at telomeres. With the knowledge that DNA damage leads to cell cycle arrest, senescence, or apoptosis, it would be detrimental to cell survival and proliferation if telomeres were constantly eliciting DNA damage signals.

Treatment of cells (HeLa and U2OS) with etoposide, which is a topoisomerase II inhibitor that induces DNA double stand breaks, reduces the expression of TRF1, POT1, and tankyrase in the cell, while increasing the expression of Rap1 (Kato et al., 2013). These data suggests that the induction of DNA damage, by a common therapeutic agent used in chemotherapy, impacts the shelterin complex and therefore telomere function

at the transcriptional level, and offers another way by which telomere maintenance and the DNA damage response are intimately linked (Kato et al., 2013).

1.5.7 TRF1 and the DNA Damage Response

It is clear that shelterin proteins contribute to preventing a DNA damage response at chromosome ends, however it is less clear what role these proteins may play at sites of DNA damage away from telomeres. TRF1, in particular, may be involved in the DNA damage response apart from telomeres. TRF1 S219, for instance, is phosphorylated by ATM after ionizing radiation (Kishi et al., 2001b). To further examine a link between TRF1 and the damage response, the impact of TRF1 depletion was evaluated in AT cells. AT cells, lacking functional ATM, display phenotypes which include defects in the activation of the S and G2/M phase checkpoints following DNA damage and also an increased sensitivity to ionizing radiation (Lavin and Shiloh, 1997). Expression of dominant negative mutants of TRF1, which were unable to bind telomere DNA, in AT cells, resulted in a rescue of the G2/M checkpoint and also a recovery of the sensitivity to ionizing radiation (Kishi and Lu, 2002). A rescue of the S phase checkpoint was not observed in this situation (Kishi and Lu, 2002). These observations suggest that TRF1 may have a role in regulating the G2/M checkpoint and cell survival after DNA damage.

Experiments in chicken DT40 cells have suggested a role for TRF1 in the response to DNA damage. TRF1 was deleted by gene targeting, and a significant sensitivity of these cells to ionizing radiation was observed using a clonogenic survival assay (Cooley et al., 2009). The *Saccharomyces cerevisiae* protein Tbf1, which is related to human TRF1 and TRF2, has been demonstrated to play a part in the DNA damage response (Bonetti et al., 2013). Inactivation of Tbf1 results in an increased sensitivity to agents that induce DNA double strand breaks (Bonetti et al., 2013). Tbf1 was demonstrated to be recruited to sites of breaks with an inducible system, and is involved in promoting end resection at

these sites (Bonetti et al., 2013). Tbf1 was suggested to be important for non-homologous end joining mediated repair of these breaks. Additionally, Tbf1 may be influencing the chromatin structure surrounding break sites, as Tbf1 mutant yeast strains show impaired nucleosome eviction and chromatin relaxation at breaks (Bonetti et al., 2013). These findings are consistent with the function for TRF1 and TRF2 that was previously discussed; in moderating telomere chromatin structure.

The binding of TRF1 to nucleostemin, which is a nucleolar GTP-binding protein, prevents the dimerization of TRF1 and reduces the stability of TRF1, but also appears to play a role in the damage response at telomeres (Meng et al., 2011). In cells with dysfunctional telomeres, such as those expressing TRF2ΔBΔM, the interaction between nucleostemin and TRF1 reduces the formation of damage foci, or TIFs, at telomeres (Meng et al., 2011). These findings suggest that TRF1 may be connected to the DNA damage response at dysfunctional telomeres. Taken as a whole, it appears as though TRF1 may be involved in the protection of chromosome ends and also plays a role in the cellular response to DNA damage at sites other than chromosome termini.

1.6 Significance and Objectives

The discussion of telomeres and telomere maintenance has reinforced the fundamental physiological importance of this regulation. When proper telomere maintenance in disrupted, severe cellular stress ensues, which is the root of a number of devastating human conditions. The proteins which associate with telomere DNA, the higher order structures that telomere DNA forms, the regulation of telomere length and transcription, and the prevention of a DNA damage response at telomeres are all crucial aspects in telomere maintenance. As reviewed in this chapter, TRF1 plays a number of roles in regulating telomere metabolism. It serves as a regulator of telomere length, it helps to protect telomere ends, it ensures proper telomere replication, and it plays a role in the transcription of telomeres and in TERRA production.

TRF1 is phosphorylated on many residues, and these modifications greatly impact its stability, its binding to telomere DNA, and appear to modify the functionality of TRF1. This knowledge was the impetus for this research project. Mass spectrometry revealed a number of phosphorylation sites on TRF1, some of which were not documented or characterized. Previous work within the lab had suggested that TRF1 S367 may be involved in regulating the ability of TRF1 to bind to telomere DNA. A phosphorylation site only four amino acids away from this site, T371, was also identified as a phosphorylation site *in vivo*. Investigating the regulation and function of these phosphorylation sites on TRF1 was the purpose of this thesis. With the knowledge surrounding how precisely and specifically TRF1 is controlled, it was proposed that these sites were important in regulating the function of TRF1. This hypothesis formed the foundation of my thesis projects aimed at elucidating the role of TRF1 phosphorylation at S367 and T371 in regulating its stability, its binding to telomere DNA, and its overall function.

Two primary experimental approaches were used to investigate the phosphorylation of these residues throughout this thesis. The first approach involved generating polyclonal antibodies in rabbits against peptides containing TRF1 modified at either S367 or T371. These antibodies were characterized to specifically recognize TRF1 when modified at the appropriate site, as will be discussed throughout this thesis.

The second approach involved generating cell lines expressing TRF1 constructs mutated at TRF1 S367 or T371 to either prevent phosphorylation (with an alanine substitution) or mimic phosphorylation (with an aspartic acid substitution). This experiment was originally done in HT1080 cells, with overexpressed TRF1 constructs in a pLPC-N-Myc vector. It was then considered that the endogenous TRF1 in this setting may be dimerizing with the overexpressed mutated TRF1, and masking potential phenotypes associated with these mutations. The endogenous TRF1 in these cells may be adequate

to perform any TRF1 associated functions, and therefore we were aware that mutations in this set of cell lines may not reveal significant defects. In light of this, we depleted TRF1 in HeLaII cells with shRNA, in a pRS (pRetroSuper) vector. HeLaII cells were chosen for this as we observed the most substantial TRF1 knockdown in this cell line. TRF1 constructs, in a pWZL-N-Myc vector, with alanine or aspartic acid mutations at S367 or T371, were introduced into this background. These constructs had silent mutations introduced so as to avoid knockdown by shRNA. These steps resulted in two full sets of cell lines that were used for experimentation; HT1080 cells with overexpressed pLPC-N-Myc TRF1 and associated TRF1 mutants, and HeLaII with shTRF1, and expressing pWZL-N-Myc TRF1 and associated TRF1 mutants.

These experimental systems were utilized jointly to determine the role of TRF1 phosphorylation at S367 and T371 *in vivo*. *In vitro* data was used throughout this thesis to complement knowledge obtained from *in vivo* work.

Information concerning the regulation of telomere binding proteins is essential to our understanding of telomerase manipulation. The most direct consequence of altered telomere metabolism is cancer or ageing, and these are both by products of telomerase activity. TRF1, a negative regulator of telomerase-dependent telomere elongation, may be important to our understanding of how to alter the efficiency of telomerase mediated telomere synthesis. The full comprehension of how TRF1 is controlled, which is in part by post-translational modifications, such as phosphorylation, may provide insight into how telomerase-mediated telomere lengthening can be regulated. The understanding of this would be of clinical relevance in the treatment of cancer by telomerase inhibition. The goal of this thesis is to explore, in detail, how phosphorylation of TRF1 at S367 or T371 regulates its function.

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Chapter 2

ATM Regulates Proteasome-Dependent Subnuclear Localization of TRF1, Which is Important for Telomere Maintenance

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2.1 Introduction

2.1.1 Publication

The work presented in this chapter was published in *Nucleic Acids Research*, on January 20, 2012. The work was published on pages 3975-3989 of Volume 40, No 9, doi: 10.1093/nar/gks035. The authors listed were as follows: Megan McKerlie, Sichun Lin, and Xu-Dong Zhu.

2.1.2 Contribution

I performed all of the experiments included within this chapter, with the exception of the first three cell lines in Figure 7A (Southern blotting controls; knockdown TRF1 samples and wild type TRF1 samples), which was completed by Sichun Lin. Kajaparan Jeyanthan generated the recombinant wild type TRF1 used within this publication. Xu-Dong Zhu and I conceptualized and designed all of the experiments and wrote the manuscript.

2.1.3 Context and Significance

The purpose of this thesis is to investigate the role that phosphorylation events play in modulating the function of TRF1. This chapter furthers this purpose by considering one phosphorylation site on TRF1, S367, and characterizing the function and regulation of this modification.

This work elucidates a role for the phosphorylation of TRF1 at S367 by ATM in regulating telomere length and stability. This provides information which furthers our understanding on how post-translational events modify the functionality of TRF1 within the cell.

2.2 Publication: ATM regulates proteasome-dependent subnuclear localization of TRF1, which is important for telomere maintenance; ***Nucleic Acids Research*** **(2012),** by permission of Oxford University Press.

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ATM regulates proteasome-dependent subnuclear localization of TRF1, which is important for telomere maintenance

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2.2.1 ABSTRACT

Ataxia telangiectasia mutated (ATM), a PI-3 kinase essential for maintaining genomic stability, has been shown to regulate TRF1, a negative mediator of telomerase-dependent telomere extension. However, little is known about ATM-mediated TRF1 phosphorylation site(s) *in vivo*. Here we report that ATM phosphorylates S367 of TRF1 and that this phosphorylation renders TRF1 free of chromatin. We show that phosphorylated (pS367)TRF1 forms distinct non-telomeric subnuclear foci and that these foci occur predominantly in S and G2 phases, implying that their formation is cell cycle regulated. We show that phosphorylated (pS367)TRF1-containing foci are sensitive to proteasome inhibition. We find that a phosphomimic mutation of S367D abrogates TRF1 binding to telomeric DNA and renders TRF1 susceptible to protein degradation. In addition, we demonstrate that overexpressed TRF1-S367D accumulates in the subnuclear domains containing phosphorylated (pS367)TRF1 and that these subnuclear domains overlap with nuclear proteasome centers. Taken together, these results suggest that phosphorylated (pS367)TRF1-containing foci may represent nuclear sites for TRF1 proteolysis. Furthermore, we show that TRF1 carrying the S367D mutation is unable to inhibit telomerase-dependent telomere lengthening or to suppress the formation of telomere doublets and telomere loss in TRF1-depleted cells, suggesting that S367 phosphorylation by ATM is important for the regulation of telomere length and stability.

2.2.2 INTRODUCTION

Telomeres are specialized heterochromatic structures found at the ends of linear eukaryotic chromosomes. Mammalian telomeric DNA consists of TTAGGG tandem repeats that are coated with shelterin, a telomere-specific protein complex composed of TRF1, TRF2, TIN2, Rap1, TPP1 and POT1 (1-3). The shelterin complex functions not only to protect telomeres from being recognized as double strand breaks (1,4) but also to maintain telomere length homeostasis, which is intimately associated with tumorigenesis and aging. Disruption of shelterin proteins has been shown to induce the de-protection of telomeres, resulting in telomere abnormalities including telomere end-to-end fusions, telomere loss, and telomere doublets/fragile telomeres (more than one telomeric signal at a single chromatid end) (4-12). These dysfunctional telomeres are recognized as damaged DNA (8-10,12,13) and can contribute to genomic instability.

TRF1, a component of the shelterin complex, binds specifically to duplex telomeric DNA (14), and is implicated in telomere replication, telomere protection and telomere length maintenance. Deletion of TRF1 promotes the formation of fragile telomeres in S phase, a phenomena thought to be associated with replication-dependent defects (6,9). It has been suggested that TRF1 is required to prevent fork stalling, allowing efficient replication of telomeric DNA (9). Loss of TRF1 from telomeres has been shown to induce telomerase-dependent telomere lengthening whereas overexpression of TRF1 results in telomere shortening, suggesting that TRF1 negatively regulates telomerase-dependent telomere extension, perhaps by restricting the access of telomerase to the ends of telomeres (15-17).

TRF1 interacts with ataxia telangiectasia mutated (ATM) (18), a PI-3 kinase essential for maintaining genomic stability. Mutations in *ATM* give rise to ataxia-telangiectasia (AT), an autosomal recessive disorder characterized by immunodeficiency, spontaneous chromosomal instability, hypersensitivity to ionizing irradiation and a

predisposition to cancer (19). Primary fibroblasts derived from AT patients accumulate telomere abnormalities and show an elevated rate of telomere shortening as compared to cells from normal individuals (20-25). In agreement with these findings, inhibition of ATM has been shown to induce telomere shortening in telomerase-expressing cancer cells, indicative of the role of ATM as a positive mediator of telomere length maintenance (26). It has been suggested that ATM promotes telomerase-dependent telomere elongation by negatively regulating TRF1 association with telomeric DNA (26). However, little is known about the ATM phosphorylation site(s) of TRF1 important for telomere length maintenance.

In this report, we demonstrate that ATM phosphorylates S367 of TRF1 both *in vivo* and *in vitro*. We show that a phosphomimic mutation of S367D abrogates TRF1 interaction with telomeric DNA. Using a phosphospecific antibody, we demonstrate that phosphorylated (pS367)TRF1 forms distinct subnuclear foci that are not associated with telomeric DNA. These results reveal that the phosphorylation of S367 by ATM prevents TRF1 binding to telomeric DNA. We show that phosphorylated (pS367)TRF1-containing foci predominantly occur in S and G2 phases, implying that the formation of these foci is cell cycle regulated. In addition, we find that phosphorylated (pS367)TRF1-containing foci are sensitive to proteasome inhibition. We show that the phosphomimic mutation of S367D renders mutant TRF1 susceptible to protein degradation and promotes its localization in phosphorylated (pS367)TRF1-containing subnuclear domains, which overlap with nuclear proteasome centers. These results suggest that phosphorylated (pS367)TRF1-containing foci may represent nuclear proteolytic sites for TRF1 degradation. Furthermore, we demonstrate that the phosphomimic mutation of S367D impairs the ability of TRF1 not only to inhibit telomerase-dependent telomere elongation but also to suppress the formation of telomere doublets and telomere loss in TRF1-depleted cells, suggesting that S367 phosphorylation by ATM plays an important role in regulating telomere length and stability.

2.2.3 MATERIALS AND METHODS

DNA constructs, cell culture and retroviral infection

The oligonucleotides encoding siRNAs directed against TRF1 have been previously described (12). The annealed oligonucleotides were ligated into pRetroSuper (pRS) vector (kindly provided by Titia de Lange, Rockefeller University), giving rise to pRetroSuper-shTRF1. The QuickChange site-directed mutagenesis kit (Strategene) was used to create TRF1 mutations of S367A and S367D as well as silent mutations resistant to shTRF1 as previously described (12). Wild type TRF1 and TRF1 mutants (S367A and S367D) carrying silent mutations resistant to shTRF1 were then subcloned into either the retroviral vector pWZL-N-Myc (27) or the bacterial expression vector pHis-Parallel-2 (28).

Cells were grown in DMEM medium with 10% fetal bovine serum (FBS) for GM09607 (Coriell), GM05849 (Coriell), GM16666 (Coriell), GM16667 (Coriell), HeLaI.2.11, HeLaII, GM847, Phoenix cells and 15% FBS for IMR90 cells, supplemented with non-essential amino acids, glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. The ATM-deficient AT22IJE cell line was transfected with either an ATM expression construct or an empty vector, giving rise to ATM-corrected (GM16667) and ATM-deficient (GM16666) stable cell lines, respectively (29). HeLaI.2.11 and HeLaII are two sublines of HeLa cells of different telomere length (4,30). Retroviral gene delivery was carried out as described (31-33) to generate stable cell lines. HeLaII cells expressing pRS/pWZL, shTRF1/pWZL, shTRF1/TRF1, shTRF1/S367A or shTRF1/S367D were maintained in the selection medium containing either puromycin (2 μg/ml) or hygromycin (90 μg/ml) alternating every two weeks for the entirety of the experiments.

Cell-cycle-analysis

Synchronization of HeLaI.2.11 with a double thymidine block was carried out essentially as described (34) with the exception that 2 mM thymidine instead of aphidicolin was used. For FACS analysis, two million cells were fixed in 80% ethanol, digested with RNase A (2 mg/ml), stained with 50 µg/ml propidium iodide, and analyzed using a Becton-Dickinson LSRII located at the SickKids-UHN flow cytometry facility, Toronto, Ontario, Canada.

Protein extracts, differential salt extraction of chromatin and immunoblotting

Differential salt extraction of chromatin was performed as described (35). Protein extracts and immunoblotting were carried out essentially as described (27,34). Rabbit polyclonal anti-pS367 antibody was developed by Biosynthesis Inc. against a TRF1 peptide containing phosphorylated serine 367 (VSK-pS-QPVTPEKHRARKR). Antibodies used were anti-TRF1 (a kind gift from Titia de Lange), anti-proteasome (PA1-1962, Pierce), anti-Myc (9E10, Calbiochem), anti-H3K9m3 (Upstate) and anti-γ-tubulin (GTU88, Sigma).

Production of recombinant TRF1 proteins

Production of 6xHis-tagged TRF1 proteins was carried out as described (10,12). Briefly, induction of wild type and mutant TRF1 proteins was carried out overnight with 0.1 mM IPTG at room temperature. The cell pellet was resuspended in binding buffer (20 mM Tris-HCl [pH 7.9] and 500 mM NaCl) and then lysed by sonication. Following centrifugation, the supernatant was incubated with nickel-resin (Qiagen) for 2 h at 4^{o}C. The beads were washed three times with 60 mM imidazole and bound proteins were eluted with a buffer containing 1 M imidazole, 20 mM Tris-HCl pH 7.9 and 0.5 M NaCl.

Immunoprecipitation of ATM and *in vitro* Kinase Assays

Immunoprecipitation of ATM was performed as previously described (26). Briefly, cell lysates were made in lysis buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 10% Glycerol, 1% Tween-20, 50 mM NaF, 1 mM NaVO4, 0.1 mM DTT, 0.5 mM PMSF, 0.5 µg/ml leupeptin), followed by sonication (50% duty cycle, 9 pulses and output of 3). For each ATM IP, 4 µg anti-ATM (Ab-3) antibody and 300 µl cell lysate (equivalent to $6x10^6$ cells) were used. Following 1 hr incubation on ice, 25 µl protein G sepharose slurry (Pharmacia) was added to each IP and continued incubation for 1 hr at 4^oC. The IP pellet was washed twice in lysis buffer, once in LiCl buffer (0.5 M LiCl and 0.1 M Tris-HCl pH7.5), and twice in kinase buffer (10 mM Hepes pH7.9, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 5 µM ATP and 1mM DTT). For ATM kinase assays, the final IP pellet was resuspended in kinase buffer, mixed with bacterial-derived recombinant wild type TRF1 (2 µg) or TRF1-S367A (2 µg) in the presence of 10 µCi γ-^{32}P-ATP in a final volume of 15 µl. For ATM kinase assays followed by *in vitro* gel shift assays, cold ATP (1.8 mM) was used. For DNA-PKcs kinase assays, recombinant wild type TRF1 (2 µg) or TRF1-S367A (2 µg) was incubated with 20 units of purified DNA-PKcs (Promega, V5811) in the presence of γ-^{32}P-ATP according to the manufacturer's instruction.

Immunofluorescence (IF) and fluorescence *in situ* hybridization (FISH)

Immunofluoresence was performed essentially as described (10,32,34). IF-FISH (fluorescence *in situ* hybridization) analysis was conducted as described (9). Briefly, cells grown on coverslips were fixed at RT for 10 min in PBS-buffered 2% paraformaldehyde, washed in PBS twice for 5 min each, followed by incubation at RT for 30 min in blocking buffer containing 1 mg/ml BSA, 3% goat serum, 0.1% Triton X-100 and 1 mM EDTA in PBS. Blocked coverslips were incubated with anti-pS367 antibody in blocking buffer at RT for 1 hr. After three washes in PBS, coverslips were incubated with FITC-conjugated donkey anti-rabbit (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 TRITC-conjugated-$(TTAGGG)_3$ PNA probe (Biosynthesis Inc.) for 2 hr in the dark at RT. Following incubation, cover slips were washed with 70% formamide and 10 mM Tris-HCl (pH 7.2) twice for 15 min. After 3 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 (4,32). TRF1-depleted HeLaII cells expressing various TRF1 alleles or the vector alone were arrested in nocodazole (0.1 μg/ml) for 90 min. Following arrest, cells were harvested by trypsinization, incubated for 7 min at 37^{o}C in 75 mM KCl, and fixed in freshly-made methanol/glacial acidic acid (3:1). Cells were stored overnight at 4^{o}C, dropped onto slides and air-dried overnight in a chemical hood.

FISH analysis on metaphase chromosome spreads was carried out essentially as described (32,36). Slides with chromosome spreads were incubated with 0.5 μg/ml FITC-conjugated-$(CCCTAA)_3$ PNA probe (Biosynthesis Inc.) for 2 hr 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.

***In Vitro* Gel Shift Assays**

In vitro gel shift assays were done essentially as described (26,37) with an end-labeled 188-bp BglII-XhoI fragment from plasmid pTH12 (37). Bacteria-derived recombinant wild type or mutant TRF1 protein was incubated with the end-labeled DNA (1 ng) at room temperature for 20 min in a 20-µl reaction containing 20 mM HEPES-KOH (pH7.9), 150 mM KCl, 5% (v/v) glycerol, 4% (w/v) Ficoll, 1 mM EDTA, 0.1 mM $MgCl_2$, 0.5 mM DTT, 70 µg BSA, 2 µg sheared *Escherichia coli* DNA and 50 ng β-casein. The DNA-protein complexes were fractionated on a 0.7% agarose gel run in 0.1X TBE (8.9 mM Tris-base, 8.9 mM boric acid, 0.2 mM EDTA) at 130 V for 1 hr at room temperature. Gels were dried and exposed to PhosphorImager screens.

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 0.5xTBE. Blotting for telomeric fragments was carried out according to standard protocols (15). The average telomeric restriction fragment length was determined by PhosphorImager analysis using ImageQuant and MS Excel as described (38).

2.2.4 RESULTS

ATM phosphorylates S367 of TRF1 both *in vivo* and *in vitro*.

Phosphorylation has been shown to play an important role in modulating the function of TRF1 in telomere metabolism (12,26,39-41). In an effort to identify TRF1 phosphorylation sites *in vivo*, we generated an HT1080 cell line stably expressing Flag-tagged TRF1. Mass spectrometry analysis of immunoprecipitated Flag-TRF1 indicated serine at position 367 of TRF1 to be a candidate phosphorylation site *in vivo* (Figure 1A and data not shown).

To further investigate the phosphorylation of S367 of TRF1 *in vivo*, we raised an antibody against a TRF1 peptide containing phosphorylated S367, referred to as anti-pS367. Anti-pS367 antibody specifically recognized the phosphorylated TRF1 peptide but not the unphosphorylated TRF1 peptide (Figure 1B). The ability of anti-pS367 to specifically recognize the phosphorylated (pS367)TRF1 peptide was further demonstrated by peptide competition assays. While pre-incubation of unmodified peptide had no effect on binding of anti-pS367 antibody to the phosphorylated peptide, pre-incubation of the phosphorylated peptide completely abrogated its ability to bind the phosphorylated peptide (Figure 1C). We showed that anti-pS367 antibody predominantly recognized a protein band with an apparent molecular weight indistinguishable from that of endogenous TRF1 in HeLa cell lysate (Figure 1D). Depletion of TRF1 resulted in a loss of TRF1 recognized by anti-pS367 antibody *in vivo* (Figure 1E). Taken together, these results suggest that anti-pS367 antibody specifically recognizes phosphorylated S367 of TRF1 *in vivo*.

S367 ($S^{367}Q$) of TRF1 matches the consensus sequence for ATM and we decided to investigate whether ATM may be involved in phosphorylating S367 of TRF1. To address this question, we performed *in vitro* ATM kinase assays with bacterial-derived recombinant wild type TRF1 or mutant TRF1 carrying a single amino acid substitution at S367 (TRF1-S367A). We found that while ATM immunoprecipitated from HeLa cells was able to phosphorylate wild type TRF1, consistent with previous findings (18,26), the alanine substitution of S367 impaired TRF1 phosphorylation by ATM *in vitro* (Figure 1F). However, the S367A mutation had no effect on TRF1 phosphorylation by DNA-PKcs *in vitro* (Figure 1F), indicative of the specificity of S367 phosphorylation by ATM.

Using the phospho-specific anti-pS367 antibody, we examined the effect of ATM inhibition on S367 phosphorylation *in vivo*. HeLa cells were treated with either DMSO, KU55933, a highly specific inhibitor of ATM (42), or NU7026, a specific inhibitor of DNA-PKcs (43). We found that treatment with KU55933 but not NU7026 led to a reduction in

S367 phosphorylation (Figure 1G). Furthermore, we detected a substantial reduction in S367 phosphorylation in ATM-deficient GM09607 and GM05849 cells compared to ATM-proficient IMR90 and HeLaI.2.11 cells (Figure 1H). Treatment of GM09607 cells with the DNA-PKcs inhibitor NU7026 led to a further decrease in S367 phosphorylation and such decrease was not observed in KU55933-treated GM09607 cells (Figure 1I), implying that DNA-PKcs may phosphorylate S367 *in vivo* in the absence of functional ATM. Taken together, these results demonstrate that ATM phosphorylates S367 of TRF1 both *in vitro* and *in vivo*.

Phosphorylated (pS367)TRF1 forms distinct non-telomeric nuclear foci that are cell cycle regulated.

We have shown that TRF1 is phosphorylated at S367 *in vivo* and we decided to examine the nuclear localization of phosphorylated (pS367)TRF1. Using the phospho-specific anti-pS367 antibody, we performed indirect immunofluorescence on both primary and transformed cell lines. We found that in interphase cells, anti-pS367 staining formed distinct nuclear foci in both primary and transformed cell lines (Figure 2A) and that these foci were very heterogenous in size, a feature distinct from telomere staining. We found that depletion of TRF1 led to a severe reduction in anti-pS367 staining and the formation of anti-pS367-containing foci (Figure 2B). On the other hand, anti-pS367-containing foci were fully restored in TRF1-depleted cells expressing wild type TRF1 but not in TRF1-depleted cells expressing TRF1 carrying either a nonphosphorylatable mutation (S367A) or a phosphomimic mutation (S367D) (Figure 2B), suggesting that these foci represent phosphorylated (pS367)TRF1. The lack of rescue of anti-pS367-containing foci by TRF1 mutants demonstrates that phospho-specific anti-pS367 antibody does not recognize TRF1 carrying a single amino acid substitution of S367. Phosphorylated (pS367)TRF1-containing foci were also visible in early prophase cells (Figure 2C), however anti-pS367 staining became very diffuse in the rest of mitosis from metaphase to telophase (Figure

2C). These results suggest that the formation of phosphorylated (pS367)TRF1-containing foci may be cell cycle regulated.

To further investigate the nature of phosphorylated (pS367)TRF1-containing foci, we conducted IF-FISH analysis with anti-pS367 antibody in conjunction with a TRITC-conjugated telomeric DNA-containing PNA probe. We observed very little overlap between the majority of bright foci stained by anti-pS367 and telomeric DNA (Figure 2D). Occasionally we observed an overlap between one or two bright anti-pS367 foci and telomeric DNA (Figure 2D). While the nature of this overlap is unknown, it may be insignificant due to the high number of anti-pS367 foci (Figure 2D). Analysis of a differential salt extraction of chromatin revealed that while TRF1 was predominantly found in the chromatin-bound fraction (420 mM KCl), consistent with previous findings (35), phosphorylated (pS367)TRF1 was overwhelmingly associated with the chromatin-free fraction (150 mM KCl) (Figure 2E). Taken together, these results demonstrate that phosphorylated (pS367)TRF1 is not associated with telomere chromatin. We estimated that 1-5% of endogenous TRF1 may be phosphorylated at S367 (Figure 2E).

To address the cell cycle-dependent nature of anti-pS367 staining, we arrested HeLaI.2.11 and GM847 cells at the G1/S boundary with a double thymidine block and then released them into fresh media for 0 hr, 2 hr, 4 hr, 6 hr, 8 hr, 10 hr or 16 hrs. Consistent with our previous findings (34), we found that HeLa cells progressed through S phase 2-6 hrs post release and enter mitosis 10 hrs post release (Figure 3A). Analysis of immunofluoresence showed that the bright foci stained by anti-pS367 antibody were predominantly seen in cells fixed 2-8 hrs post release from a double thymidine block whereas very few cells arrested at G1/S or in G1 (16 hrs post release from a double thymidine block) displayed such foci (Figure 3B and 3C). These results suggest that phosphorylated (pS367)TRF1-containing foci are cell cycle regulated, occuring in S and G2 phase.

ATM is required for the subnuclear localization of phosphorylated (pS367)TRF1.

To investigate whether phosphorylated (pS367)TRF1-containing foci might be ATM-dependent, indirect immunofluorescence with anti-pS367 antibody was performed on HeLaI.2.11 cells treated with either DMSO or KU55933. We observed a severe loss of anti-pS367 staining along with the disappearance of distinct nuclear foci of phosphorylated (pS367)TRF1 in KU55933-treated cells as compared to DMSO-treated cells (Figure 4A). Treatment with KU55933 led to a 6-fold reduction in the percentage of cells exhibiting phosphorylated (pS367)TRF1-containing foci (Figure 4B). The absence of distinct anti-pS367 nuclear foci was also evident in ATM-deficient GM09607 and GM05849 cells when compared to ATM-proficient IMR90 cells (Figure 4C and 4D). In addition, we found that introduction of ATM into ATM-deficient cells restored phosphorylated (pS367)TRF1-containing foci (Figure 4E and 4F). Taken together, these results suggest that the subnuclear localization of phosphorylated (pS367)TRF1 is dependent upon functional ATM.

The phosphomimic mutation S367D abrogates TRF1 association with telomeres and renders it susceptible to degradation.

We have shown that phosphorylation of S367 by ATM renders TRF1 free of telomere chromatin *in vivo*. Therefore we decided to investigate whether prephosphorylation of TRF1 by ATM might affect TRF1 binding to telomeric DNA *in vitro*. Bacteria-derived recombinant wild type TRF1 was pre-incubated with ATM either in the presence or absence of cold ATP prior to gel shift assays. We found that preincubation of TRF1 with ATM in the presence of cold ATP completely abrogated TRF1 binding to telomeric DNA (Figure 5A), consistent with our previous findings that ATM negatively regulates TRF1 binding to telomeric DNA (26).

To further investigate the role of S367 phosphorylation in TRF1 binding and stability, we changed S367 of TRF1 to either alanine (S367A, non-phosphorylatable) or

aspartic acid (S367D, phosphomimic). Using bacterial-derived recombinant wild type TRF1, TRF1-S367A and TRF1-S367D, we performed *in vitro* gel shift assays and found that while TRF1-S367A bound telomeric DNA at a level indistinguishable from wild type TRF1, TRF1-S367D displayed a severe defect in its interaction with telomeric DNA (Figure 5B). To gain evidence that the phosphomimic mutation of S367D might also affect TRF1 association with telomeres *in vivo*, we performed differential salt extraction of chromatin on TRF1-depleted HeLaII cells stably expressing Myc-TRF1, Myc-TRF1-S367A or Myc-TRF1-S367D. We found that the majority of overexpressed Myc-TRF1 or Myc-TRF1-S367A was associated with chromatin whereas very little of overexpressed Myc-TRF1-S367D was associated with chromatin (Figure 5C), consistent with our earlier finding that phosphorylated (pS367)TRF1 is not associated with chromatin (Figure 2E). Expression of Myc-TRF1-S367D was comparable to that of Myc-TRF1 or Myc-TRF1-S367A (Figure 5D), suggesting that it is unlikely that the lack of Myc-TRF1-T367D association with chromatin might have arisen from a difference in protein expression. These results support the notion that S367 phosphorylation by ATM prevents TRF1 association with telomeric DNA.

To investigate whether S367 phosphorylation might be involved in regulating TRF1 stability, we used cycloheximide to inhibit protein translation in TRF1-depleted HeLaII cells stably expressing various TRF1 alleles. We found that Myc-TRF1-S367D was less stable than Myc-TRF1 or Myc-TRF1-T367A (Figure 5E). At 8 h post-cycloheximide chase, about 50% of Myc-TRF1-S367D was degraded whereas less than 30 % of degradation was observed for Myc-TRF1-T367A or Myc-TRF1 (Figure 5F). Myc-TRF1-S367D was also observed to be less stable than Myc-TRF1 or Myc-TRF1-S367A when overexpressed in HT1080 cells (Figure 5G). These results suggest that S367 phosphorylation by ATM renders TRF1 susceptible to protein degradation.

The subnuclear localization of phosphorylated (pS367)TRF1 is sensitive to proteasome inhibition.

Analysis of dual indirect immunofluorescence with anti-pS367 antibody in conjunction with anti-Myc antibody revealed that when transiently expressed in HeLa cells, both Myc-TRF1 and Myc-TRF1-S367A exhibited punctate nuclear staining (Figure 6A), indicative of their association with telomeric DNA (Figure 6A). While overexpressed Myc-TRF1 or Myc-TRF1-S367A did not exhibit any overlap with endogenous phosphorylated (pS367)TRF1 (Figure 6A), overexpressed Myc-TRF1-S367D was found to co-localize with phosphorylated (pS367)TRF1 (Figure 6A). Earlier we have shown that a very small amount (1-5%) of TRF1 is phosphorylated at S367 (Figure 2E) and therefore it is likely that the vast majority of overexpressed Myc-TRF1 is not phosphorylated at S367, which may account for the apparent lack of an overlap between anti-Myc and anti-pS367 staining in Myc-TRF1-overexpressing HeLaII cells. In addition, we observed an overlap between Myc-TRF1-S367D-containing foci and proteasome-containing nuclear centers (Figure 6B), suggesting that Myc-TRF1-S367D-containing foci are part of nuclear proteasome centers. Consistent with this notion, we found that treatment of cells with MG132, a potent proteasome inhibitor, completely abrogated the formation of phosphorylated (pS367)TRF1-containing foci (Figure 6C). Taken together, these results suggest that phosphorylated (pS367)TRF1-containing foci may represent the nuclear proteolytic sites for TRF1 degradation.

TRF1 carrying a phosphomimic mutation of S367D is unable to inhibit telomerase-dependent telomere lengthening.

To investigate the role of S367 phosphorylation in telomere maintenance, we infected TRF1-depleted HeLaII cells with retrovirus expressing the vector pWZL alone, Myc-tagged wild type TRF1, Myc-tagged TRF1-S367A or Myc-tagged TRF1-S367D, giving rise to four stable cell lines (shTRF1/pWZL, shTRF1/TRF1, shTRF1/S367A, shTRF1/S367D).

These stable cell lines contained pools of cells and they were not single cell clones. As a control, we also generated a HeLaII cell line stably expressing two vectors pRetroSuper (pRS) and pWZL. Myc-tagged wild type TRF1, Myc-tagged TRF1-S367A and Myc-tagged TRF1-S367D were resistant to shTRF1 due to engineered silent mutations. We chose to use TRF1-depleted cells for analysis of TRF1 mutants to minimize the interference from endogenous TRF1.

HeLaII cells stably expressing pRS/pWZL, shTRF1/pWZL, shTRF1/TRF1, shTRF1/S367A, shTRF1/S367D were cultured for over 66 population doublings (PDs). Depletion of TRF1 resulted in telomere elongation (Figure 7A and 7B). Introduction of shTRF1-resistant wild type TRF1 into TRF1-depleted HeLaII cells fully reversed shTRF1-mediated telomere lengthening (Figure 7A-7C). We found that TRF1-S367A was able to reverse shTRF1-mediated telomere lengthening in a manner similar to wild type TRF1 (Figure 7A-7C). In contrast, a severe defect in suppressing shTRF1-mediated telomere lengthening was reproducibly observed in TRF1-depleted cells overexpressing TRF1-S367D (Figure 7A-7C). This defect was unlikely due to the lack of protein expression (Figure 7D). TRF1-S367D-expressing cells grew at a rate indistinguishable from TRF1-S367A-expressing cells (Figure 7E), arguing against the possibility that the difference in cell proliferation may account for the inability of TRF1-S367D to inhibit telomere length maintenance. These results suggest that S367 phosphorylation negatively regulates the ability of TRF1 to modulate telomerase-dependent telomere lengthening.

TRF1 carrying a phosphomimic mutation of S367D fails to suppress shTRF1-induced telomere doublets and telomere loss.

TRF1 has been shown to be important for telomere replication (6,9), the defect of which can give rise to fragile telomeres (6,9), also known as telomere doublets. Previously we have shown that depletion of TRF1 induces the formation of telomere doublets and telomere loss, both of which can be suppressed by wild type TRF1 (12). We asked

whether S367 phosphorylation might affect the ability of TRF1 to suppress these abnormalities. FISH analysis was performed on metaphase cells derived from HeLaII cells stably expressing shTRF1/pWZL, shTRF1/TRF1, shTRF1/S367A, or shTRF1/S367D. Consistent with previous findings (12), we found that overexpression of wild type TRF1 was able to suppress the formation of both telomere doublets and telomere loss in TRF1-depleted cells (Figure 8A-8C). Introduction of TRF1-S367A into TRF1-depleted cells was also able to suppress these telomere abnormalities (Figure 8A-8C). On the other hand, we found that overexpression of TRF1-S367D failed to result in any reduction in telomere doublets or telomere loss in TRF1-depleted HeLaII cells (Figure 8B and 8C). In fact, we detected a further increase in the formation of telomere doublets as a result of TRF1-S367D expression in TRF1-depleted HeLaII cells (Figure 8B). Aphidicolin, an inhibitor of DNA replication, has been shown to induce telomere doublets (6,9). We observed a substantial increase in telomere doublets in TRF1-depleted cells upon treatment with aphidicolin (Figure 8D), consistent with a previous report that the effect of aphidicolin was additive with the loss of TRF1 (9). Such a increase was also observed in TRF1-S367D-expressing cells (Figure 8D). Taken together, these results suggest that the phosphomimic mutation of S367D impairs TRF1 function in telomere replication.

2.2.5 DISCUSSION

In this report, we have demonstrated that ATM phosphorylates S367 of TRF1 both *in vivo* and *in vitro*. Using multiple approaches including immunofluorescence, differential salt extraction and mutational analysis, we have shown that the phosphorylation of S367 by ATM prevents TRF1 association with telomere chromatin, consistent with previous findings that ATM acts as a negative mediator of TRF1 binding to telomeric DNA (26). These results suggest that S367 may be a major phosphorylation site for ATM to control TRF1 interaction with telomeric DNA *in vivo*.

Previous work suggests that TRF1 undergoes phosphorylation at S219 by ATM in response to ionizing irradiation (18). We did not observe any increase in the level of S367 phosphorylation in response to ionizing irradiation, nor any association of S367 phosphorylation with sites of DNA damage (Unpublished data, attached as Figure 2.3.1). These findings suggest that ATM may regulate TRF1 function through phosphorylation at distinctive sites.

Using a phospho-specific antibody, we have shown that phosphorylated (pS367)TRF1 forms proteasome-dependent subnuclear foci. Our finding that TRF1 carrying the phosphomimic mutation of S367D accumulates in these foci and overlaps with nuclear proteasome centers suggests that these foci may represent the nuclear proteolytic sites for TRF1 degradation. Our findings are consistent with previous studies that several nuclear proteins are found to be detectable at proteasome-associated nuclear foci while being degraded including histones, splicing factor SC35, splicesomal components (U1-70k, SmB/B') and promyelocytic leukemia protein (PML) (44). Further studies are required to investigate the kinetics of TRF1 turnover in these centers.

Proteasomes are known to localize in both the cytoplasm and the nucleus (45). It has been reported that proteasome-mediated nuclear proteolysis takes place in distinct nucleoplasmic foci that can partially overlap with subnuclear structures such as PML and nuclear speckles (46). However, we observed little overlap between phosphorylated (pS367)TRF1-containing foci and PML bodies, nuclear speckles or Cajal bodies (Unpublished data, attached as Figure 2.3.1), suggesting that these foci may represent a novel type of subnuclear domain.

Phosphorylation plays an important role in regulating TRF1 interaction with telomeric DNA (12,26,39-41). Previously we have shown that TRF1 is phosphorylated on T371 by Cdk1 during mitosis and this phosphorylation plays an important role in the resolution of sister chromatids (12). S367 is located four amino acids upstream from T371 in the linker region immediately preceding the DNA binding domain (Myb domain)

of TRF1 (Figure 1A). The fact that S367 phosphorylation prevents TRF1 from binding to telomeric DNA, along with our previous finding that phosphorylation of T371 also keeps TRF1 away from telomere chromatin (12) suggest that phosphorylation in this portion of the linker region may interfere with TRF1 binding to telomeric DNA, perhaps due to the creation of unfavorable electrostatic interactions. We have shown that S367 phosphorylation by ATM targets TRF1 to proteasome-dependent nuclear foci, which is associated with its increased susceptibility to protein degradation. This contrasts with Cdk1-mediated T371 phosphorylation, which has been shown to increase the half-life of TRF1 (12). Perhaps the temporal separation of these two phosphorylation events (at different stages of the cell cycle) may account for the differential role that these two closely-positioned sites play at telomeres. These results highlight the tight regulation of TRF1 function and the important role of posttranslational modifications in this control.

We have shown that phosphorylated (pS367)TRF1-containing foci are cell cycle regulated, appearing predominantly in S and G2 phases. ATM has been shown to be associated with human telomeres in a cell cycle regulated manner, peaking in S and G2 phases (47,48). Perhaps when ATM is recruited to telomeres in S and G2 phase, it phosphorylates TRF1 at S367, promoting its dissociation from telomeric DNA and its accumulation in proteasome-dependent subnuclear domains. However, we did not observe any significant change in the level of S367 phosphorylation throughout the cell cycle (data not shown), raising the possibility that phosphorylation of S367 by ATM per se may not be cell cycle regulated. How phosphorylated (pS367)TRF1-containing foci are predominantly formed in S and G2 phases requires further investigation.

We have shown that TRF1 carrying the phosphomimic mutation of S367D (TRF1-S367D) fails to suppress the formation of telomere doublets and telomere loss in TRF1-depleted cells. While telomere doublets are thought to arise from a defect in telomere replication (6,9), telomere loss can arise from homologous recombination-mediated events (8). Our findings suggest that S367 phosphorylation might play a role in

modulating the pathway(s) responsible for the formation of telomere doublets or telomere loss.

TRF1 is required for efficient telomere replication (6,9), however it needs to be removed from telomeres to support telomerase-dependent telomere extension (15-17,26). We have shown that TRF1-S367D is not only defective in supporting telomere replication but also unable to inhibit telomerase-dependent telomere extension. We have estimated that 1-5% of endogenous TRF1 is phosphorylated at S367 in a cell cycle-dependent manner. These results raise the possibility that the action of ATM on S367 of TRF1 may occur only at a few telomeres, which might have a severe replication defect and require the repair from telomerase. Taken together, these results suggest that S367 phosphorylation by ATM is important for the regulation of telomere length and stability.

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Conflict of Interest Statement. None declared.

2.2.6 FIGURES AND FIGURE LEGENDS

Figure 1 McKerlie et al.

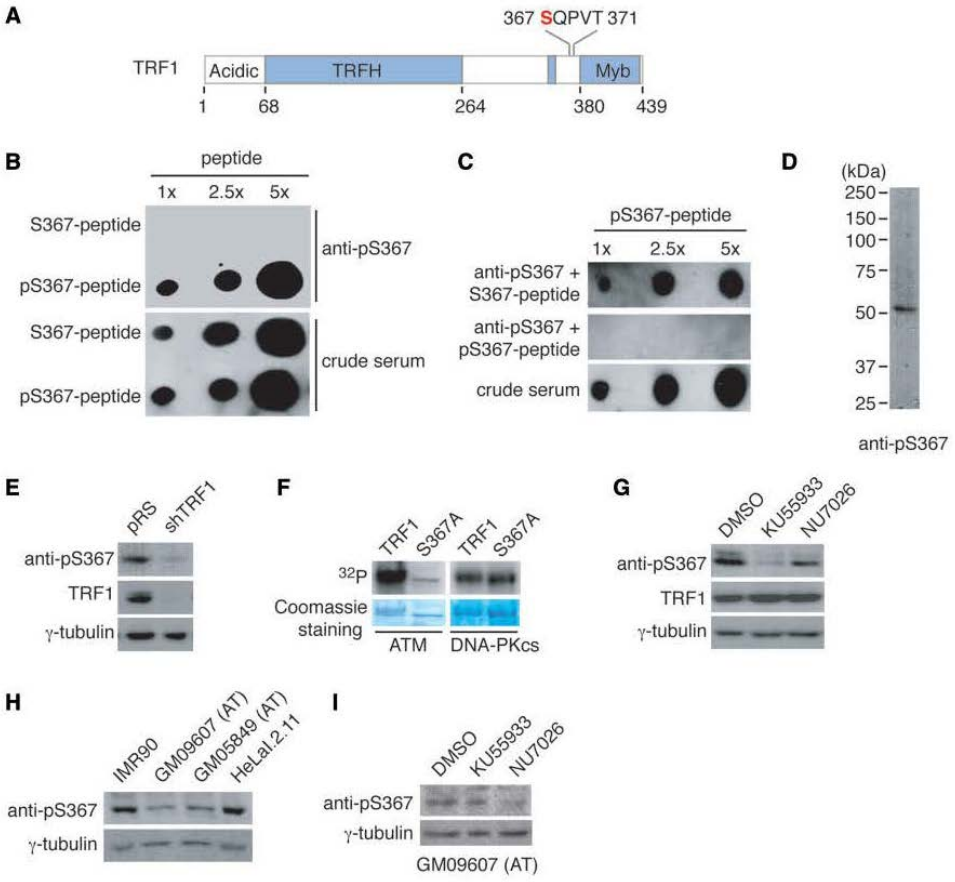

Figure 1. ATM phosphorylates S367 of TRF1 both *in vivo* and *in vitro*. (**A**) Schematic diagram of TRF1 domain structures. S367 of TRF1 in red was identified by LC/MS/MS analysis to be a candidate phosphorylation site. (**B**) Affinity-purified anti-pS367 antibody specifically recognizes TRF1 peptide containing phosphorylated S367 (pS367-peptide). An increasing amount of peptide either carrying unmodified S367 (S367-peptide) or phosphorylated S367 (pS367-peptide) was spotted on a nitrocellulose membrane, followed by immunoblotting with affinity-purified anti-pS367 antibody or crude serum. The amount of peptide spotted from left to right is 0.7, 1.75 and 3.5 µg. (**C**) Peptide competition assays. Affinity-purified anti-pS367 antibody was incubated with 5.5 µg of either unmodified (S367-peptide) or phosphorylated peptide (pS367-peptide) prior to immunoblotting. Crude serum was used to show the presence of pS367-peptide on the nitrocellulose membrane. The amount of pS367-peptide spotted from left to right is 0.7, 1.75 and 3.5 µg. (**D**) Western analysis of phosphorylated TRF1. The whole cell extract (20 µg) from HeLaII cells was immunoblotted with affinity-purified anti-pS367 antibody. (**E**) Depletion of endogenous TRF1 leads to loss of phosphorylated TRF1 recognized by anti-pS367 antibody. HeLaII cells were infected with retrovirus expressing shTRF1 or the vector pRS alone. Western analysis was performed with anti-pS367 or anti-TRF1 antibody. The γ-tubulin blot was used as a loading control. (**F**) *In vitro* kinase assays. Bacterial-expressed his-tagged wild type TRF1 (2 µg) or TRF1 mutant S367A (2 µg) was incubated with either ATM immunoprecipitated from HeLa cells or purified DNA-PKcs in the presence of γ-^{32}P-ATP. (**G**) ATM inhibition leads to a diminished anti-pS367 staining. HeLaI.2.11 cells were treated DMSO, KU55933 (an ATM inhibitor) or NU7026 (a DNA-PKcs inhibitor) for 90 min, followed by western analysis. Immunoblotting was performed with anti-pS367, anti-TRF1 or anti-γ-tubulin antibody. (**H**) Loss of anti-pS367 staining in ATM-deficient cells. Western analysis of cell extracts from IMR90 cells, HeLaI.2.11 cells and ATM-deficient cells (GM09607 and GM05849). Immunoblotting was performed with anti-pS367 or anti-γ-tubulin antibody. (**I**) NU7026 leads to a further reduction in S367 phosphorylation in ATM-deficient cells. GM09607 cells were treated

with DMSO, KU55933 or NU7026 for 90 min, following by immunoblotting with anti-pS367 or anti-γ-tubulin antibody.

Figure 2 McKerlie et al.

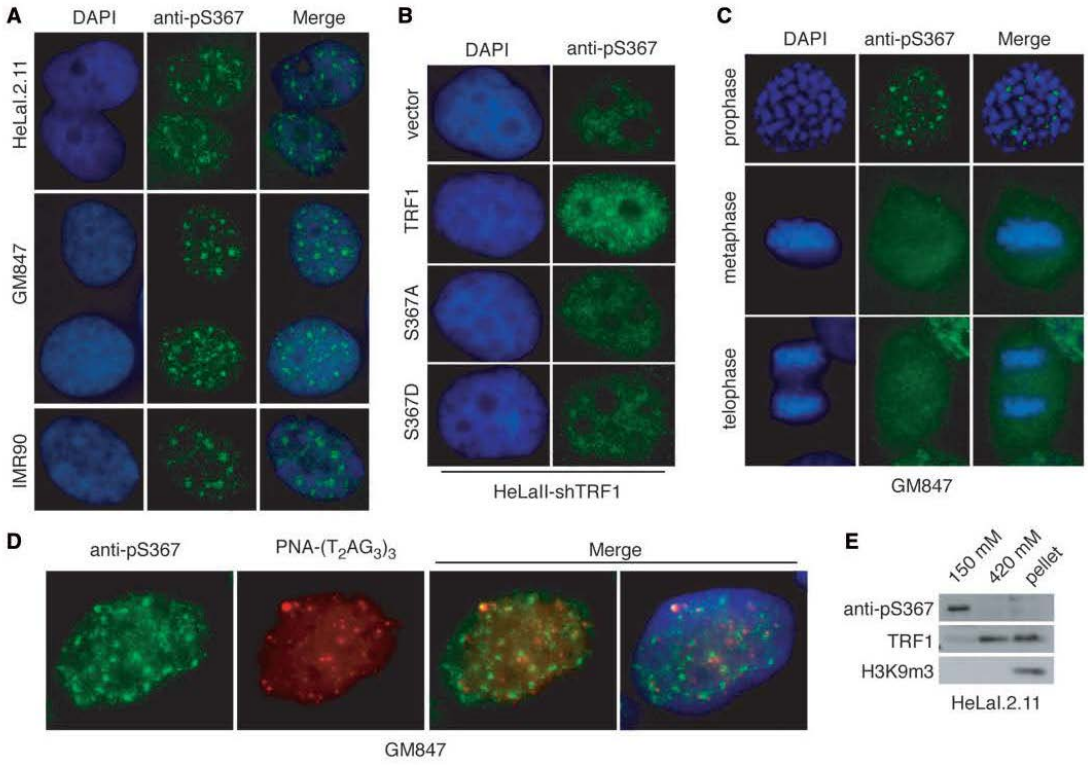

Figure 2. Phosphorylated (pS367)TRF1 forms distinct nuclear foci that are not associated with telomere chromatin. (**A**) Indirect immunofluorescence (IF) using anti-pS367 antibody was performed on human primary IMR90 cells as well as transformed HeLaI.2.11 and GM847 cells. Cell nuclei were stained with DAPI shown in blue. (**B**) Indirect immunofluorescence using anti-pS367 antibody was performed on TRF1-depleted HeLaII cells expressing the vector alone, wild type TRF1, TRF1-S367A or TRF1-S367D. Wild type TRF1, TRF1-S367A or TRF1-S367D were engineered to be resistant to shTRF1. Cell nuclei were stained with DAPI shown in blue. (**C**) Phosphorylated (pS367)TRF1-containing foci are visible in early prophase but become diffuse in the rest of mitosis. Indirect immunofluorescence using anti-pS367 antibody was performed on GM847 cells. Cell nuclei were stained with DAPI shown in blue. (**D**) Phosphorylated (pS367)TRF1 is not associated with telomeric DNA. IF-FISH analysis was performed on fixed GM847 cells with anti-pS367 antibody (green) in conjunction with TRITC-conjugated telomeric DNA-containing PNA probe (red). Cell nuclei were stained with DAPI shown in blue. (**E**) Phosphorylated (pS367)TRF1 is predominantly free of chromatin. Differential salt (KCl) extraction of chromatin was performed on HeLaI.2.11 cells. Immunoblotting was carried out with anti-pS367, anti-TRF1 or anti-H3K9m3 antibody. The anti-H3K9m3 blot was used as control to assess the extraction of chromatin.

Figure 3 McKerlie et al.

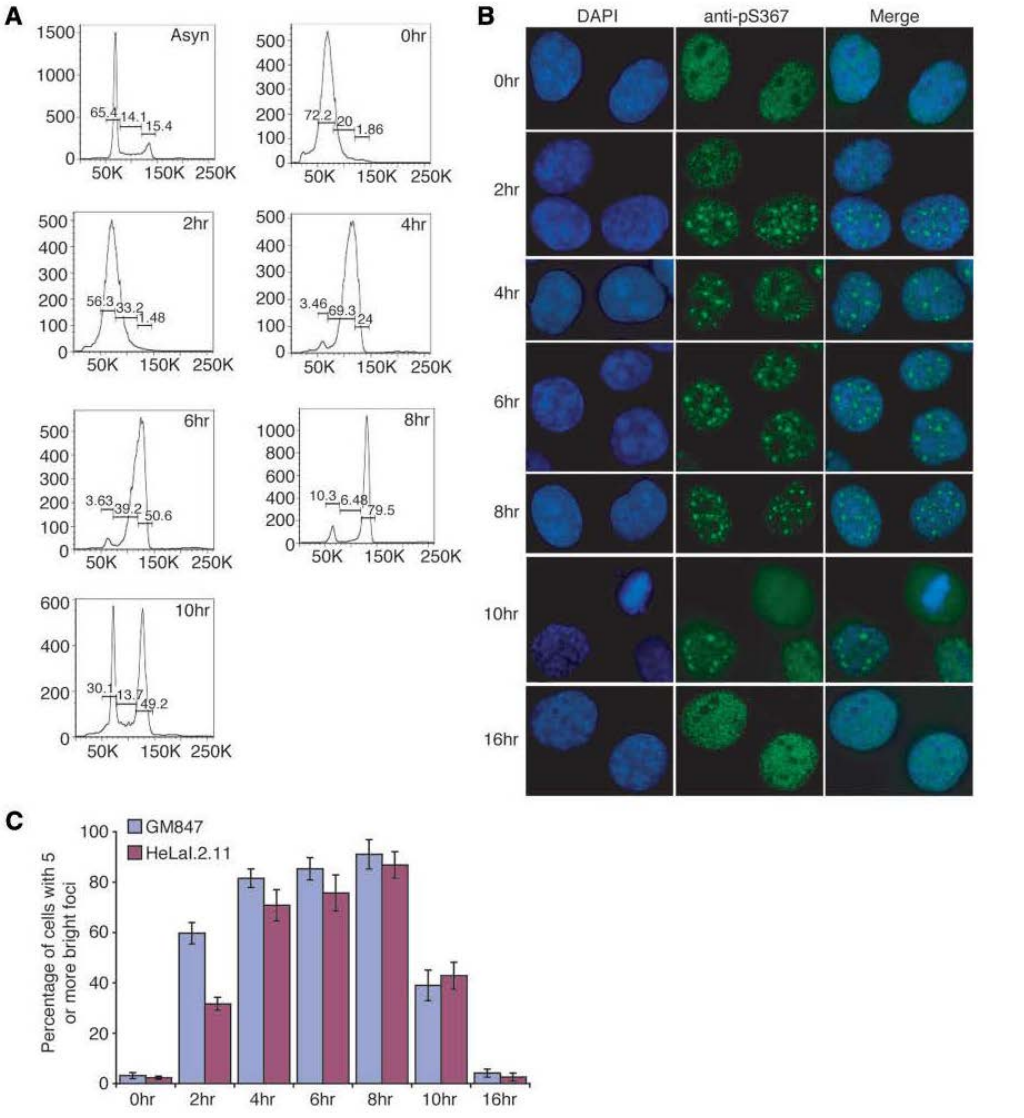

Figure 3. Phosphorylated (pS367)TRF1 forms distinct non-telomeric foci in a cell cycle regulated manner, enriched in S and G2 phases. (**A**) FACS analysis of synchronized HeLaI.2.11 cells. *y* axis, cell numbers; *x* axis, relative DNA content on the basis of staining with propidium iodine. Asyn, asynchronous population; 0-10hr, cells were released for 0-10 hr from a double thymidine block. (**B**) Indirect immunofluorescence using anti-pS367 antibody was performed on GM847 cells released for 0-16 hr from a double thymidine block. Cell nuclei were stained with DAPI shown in blue. (**C**) Quantification of percentage of GM847 and HeLaI.2.11 cells exhibiting five or more phosphorylated (pS367)TRF1-containing foci. For each of the indicated time points post release from a double thymidine block, a total of at least 1500 cells from three independent experiments were scored in blind for both GM847 and HeLaI.2.11 cells. Standard deviations from three independent experiments are indicated.

Figure 4 McKerlie et al.

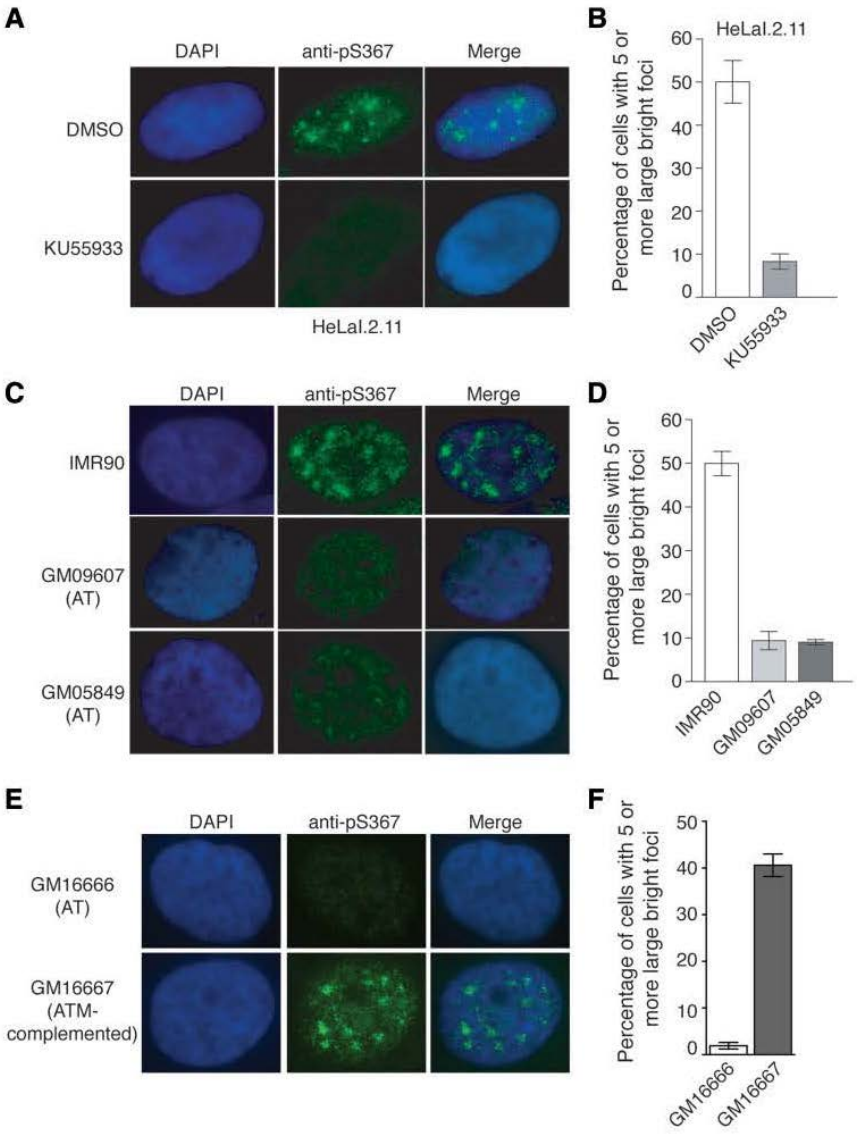

Figure 4. ATM is required for the subnuclear localization of phosphorylated (pS367)TRF1. (**A**) ATM inhibition leads to loss of phosphorylated (pS367)TRF1-containing foci. Indirect immunofluorescence (IF) was performed with anti-pS367 antibody on HeLaI.2.11 cells treated with either DMSO or KU55933. Cell nuclei were stained with DAPI in blue. (**B**) Quantification of DMSO-treated or KU55933-treated HeLaI.2.11 cells showing five or more phosphorylated (pS367)TRF1-containing foci. A total of at least 1500 cells from three independent experiments were scored in blind for both DMSO-treated and KU55933-treated cells. Standard deviations from three independent experiments are indicated. (**C**) ATM-deficient cells lack phosphorylated (pS367)TRF1-containing foci. IF was performed with anti-pS367 antibody on IMR90 cells as well as ATM-deficient GM09607 and GM05849 cells. Cell nuclei were stained with DAPI in blue. (**D**) Quantification of ATM-proficient or ATM-deficient cells showing five or more phosphorylated (pS367)TRF1-containing foci. A total of at least 1500 cells from three independent experiments were scored in blind for IMR90, GM09607 and GM05849 cells. Standard deviations from three independent experiments are indicated. (**E**) Introduction of ATM into ATM-deficient cells restores phosphorylated (pS367)TRF1-containing foci. IF was conducted with anti-pS367 antibody on ATM-deficient cells expressing either the vector alone (GM16666) or complemented with ATM (GM16667). Cell nuclei were stained with DAPI in blue. (**F**) Quantification of GM16666 or GM16667 cells showing five or more phosphorylated (pS367)TRF1-containing foci. A total of 1500 cells from three independent experiments were scored. Standard deviations from three independent experiments are indicated.

Figure 5 McKerlie et al.

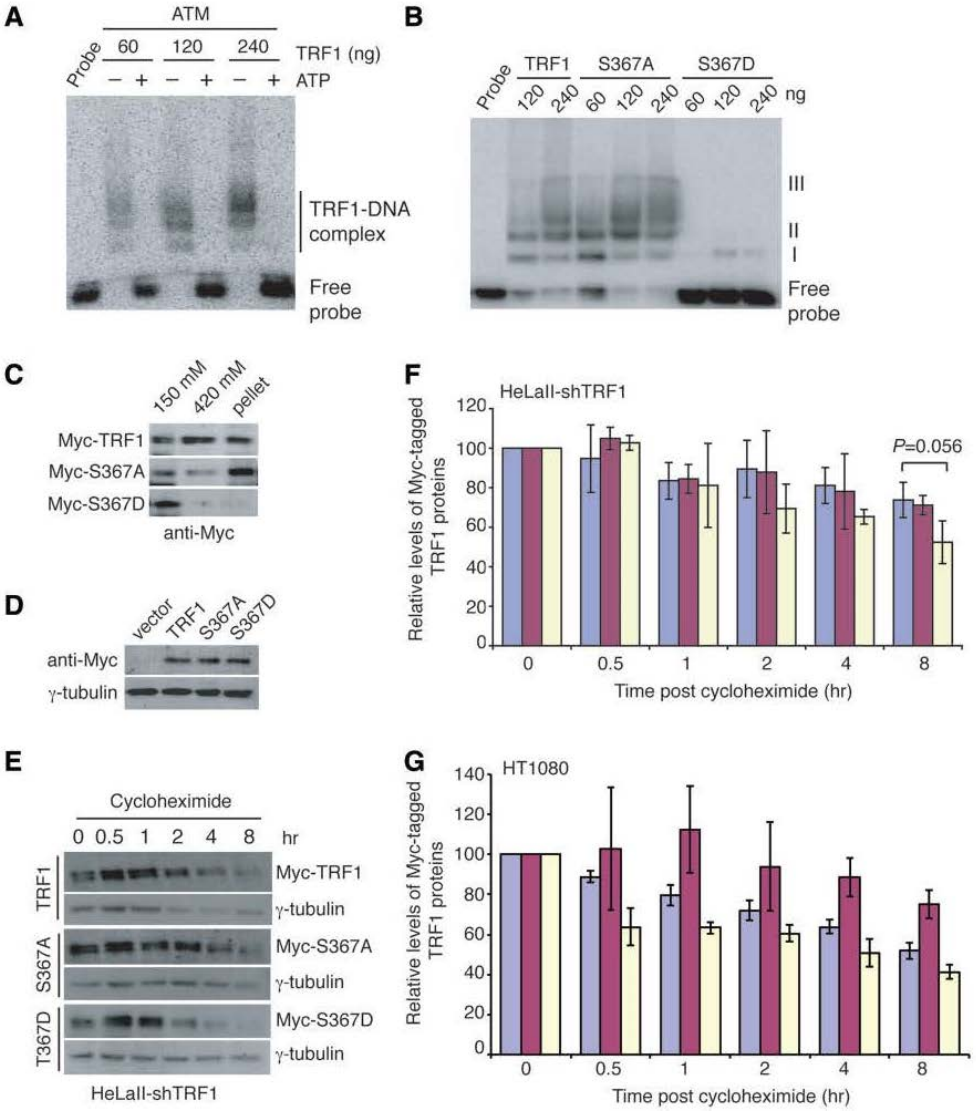

Figure 5. A phosphomimic mutation of S367D not only impairs the ability of TRF1 to interact with telomeric DNA but also renders it susceptible to degradation. (A) Prephosphorylation of TRF1 by ATM abrogates its ability to bind telomeric DNA. Bacterial-derived recombinant TRF1 of varying amouts as indicated above the lanes was preincubated with ATM immunoprecipiated from HeLa cells in the presence or absence of cold ATP, followed by *in vitro* gel shift assays. (**B**) TRF1-S367D is defective in binding to telomeric DNA. Bacterial-derived recombinant TRF1 proteins were used in the gel-shift assays as indicated above the lanes. The positions of three TRF1-containing complexes (I, II and III) are indicated on the right. The concentrations of recombinant TRF1 used were indicated above the lanes. (**C**) Differential salt (KCl) extraction of chromatin was performed on TRF1-depleted HeLaII cells expressing Myc-tagged wild type TRF1, Myc-tagged TRF1-S367A or Myc-tagged TRF1-T367D. Immunoblotting was carried out with anti-Myc antibody. (**D)** Western analysis of expression of various Myc-tagged TRF1 proteins in TRF1-depleted HeLaII cells. Immunoblotting was carried out with anti-Myc or anti-γ-tubulin antibody. (**E**) Cycloheximide chase experiments. TRF1-depleted HeLaII cells stably expressing Myc-TRF1, Myc-TRF1-S367A or Myc-TRF1-S367D were treated with 100 μg/ml cycloheximide for the indicated times, followed by immunoblotting of the lysates with anti-Myc or anti-γ-tubulin antibody. (**F**) Quantification of Myc-tagged wild type TRF1 (blue bars), Myc-tagged TRF1-S367A (burgundy bars) and Myc-tagged TRF1-S367D (light yellow bars) from (E). The signals from the western blots were quantified with densitometry. The level of Myc-tagged TRF1 proteins is represented in arbitrary units after their signals were normalized relative to those of γ-tubulin. Standard deviations from three independent experiments are indicated. (**G**) Quantification of cycloheximide chase experiments from HT1080 cells stably expressing Myc-tagged wild type (blue bars), Myc-tagged TRF1-S367A (burgundy bars) and Myc-tagged TRF1-S367D (light yellow bars). Quantification was conducted as described in 5F. Standard deviations from three independent experiments are indicated.

Figure 6 McKerlie et al.

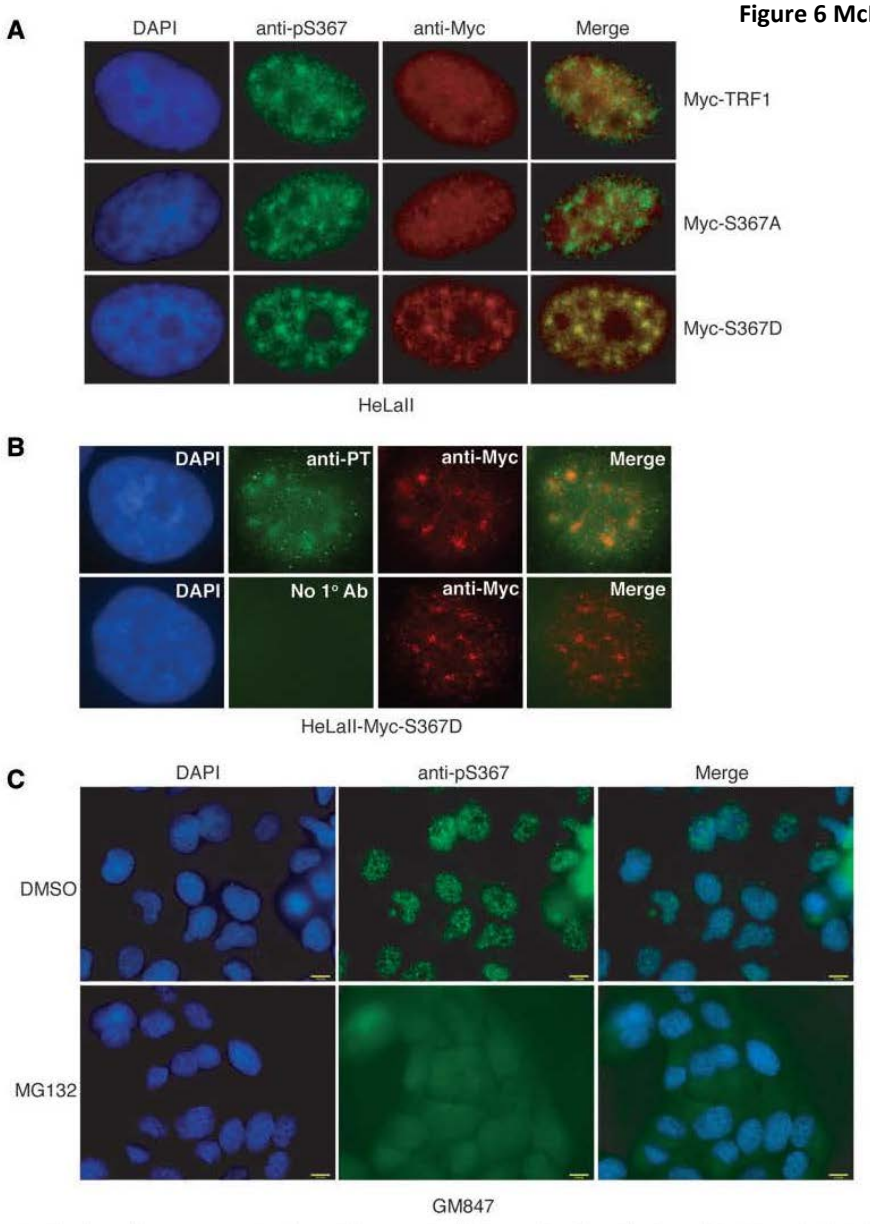

Figure 6. (**A**) Nuclear localization of overexpressed various Myc-tagged TRF1 proteins. Dual indirect immunofluorescence with anti-pS367 in conjunction with anti-Myc antibody was performed on HeLa cells transiently overexpressing Myc-TRF1, Myc-TRF1-S367A or Myc-TRF1-S367D. Cell nuclei were stained with DAPI shown in blue. (**B**) Overexpressed TRF1-S367D colocalizes with nuclear proteasomes. Dual IF was conducted on HeLaII cells transiently expressing Myc-S367D with anti-Myc antibody (red) in conjunction with either rabbit anti-proteasome antibody (green) or no primary antibody (no 1^{o} Ab). Cell nuclei were stained with DAPI shown in blue. (**C**) Phosphorylated (pS367)TRF1-containing foci are sensitive to proteasome inhibition. GM847 cells were treated with either DMSO or MG132 (12.5 μM) for 4 hr prior to IF analysis with anti-pS367 antibody. Cell nuclei were stained with DAPI shown in blue.

Figure 7 McKerlie et al.

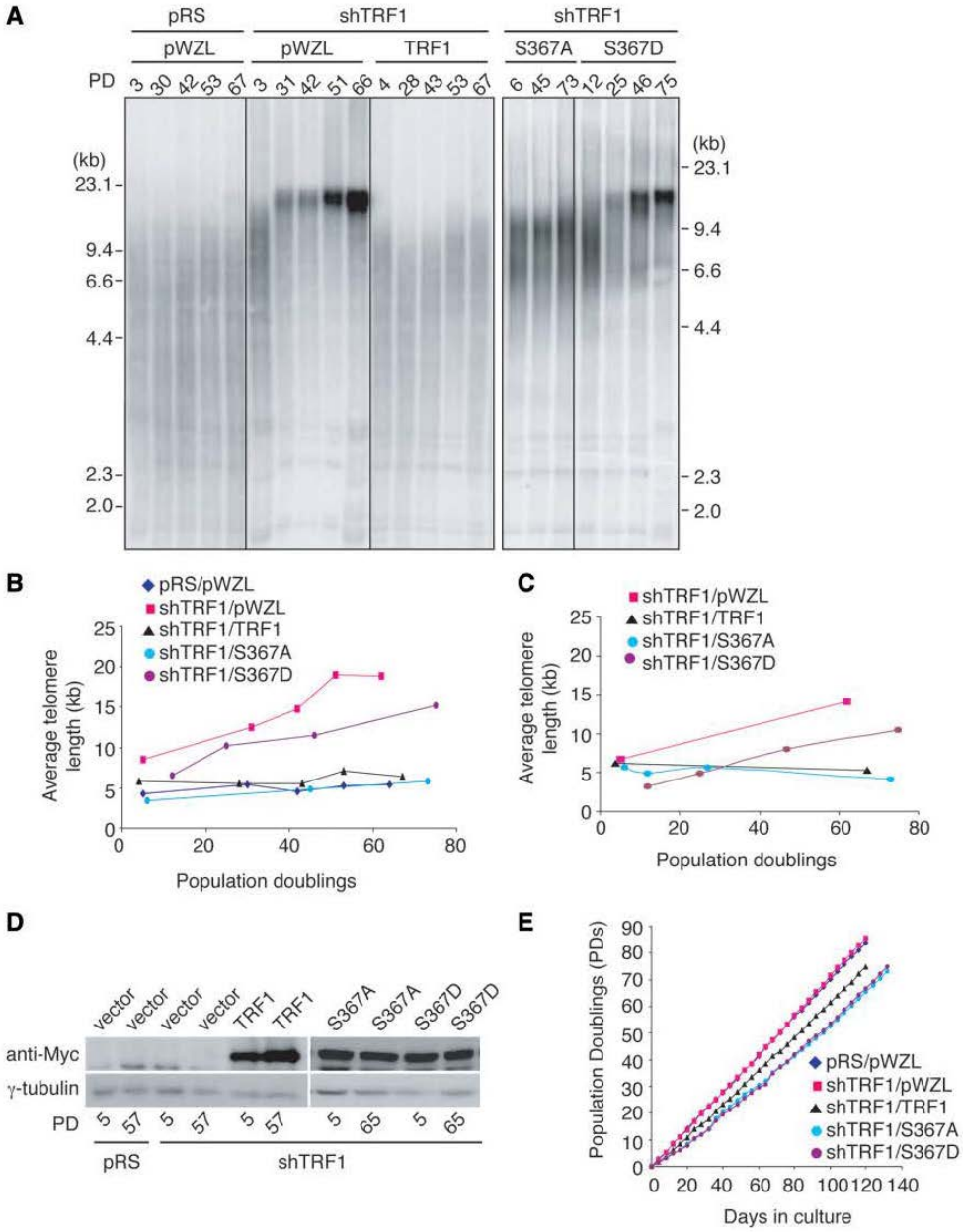

Figure 7. TRF1 carrying a phosphomimic mutation is defective in the inhibition of telomerase-dependent telomere lengthening. (**A**) Genomic blots of telomeric restriction fragments from TRF1-depleted HeLaII cells (shTRF1) expressing either the vector alone (pWZL) or various TRF1 alleles as indicated above the lanes. HeLaII cells expressing both pRS and pWZL were used as a control. Population doublings (PD) are indicated above the lanes whereas DNA molecular weight markers are shown on both left and right of the blots. About 3 µg of *Rsa*I/*Hinf*I-digested genomic DNA from each sample was used for gel electrophoresis. (**B**) Average telomere length of indicated cell lines was plotted against population doublings. (**C**) Average telomere length of indicated cell lines was plotted against population doublings from a repeated experiment. (**D**) Western analysis of expression of various Myc-tagged TRF1 proteins in early and late PDs. Immunoblotting was performed with anti-Myc antibody. The anti-γ-tubulin blots were used as a loading control. (**E**) Growth curve of HeLaII cells expressing various constructs as indicated. The number of PDs was plotted against days in culture.

Figure 8 McKerlie et al.

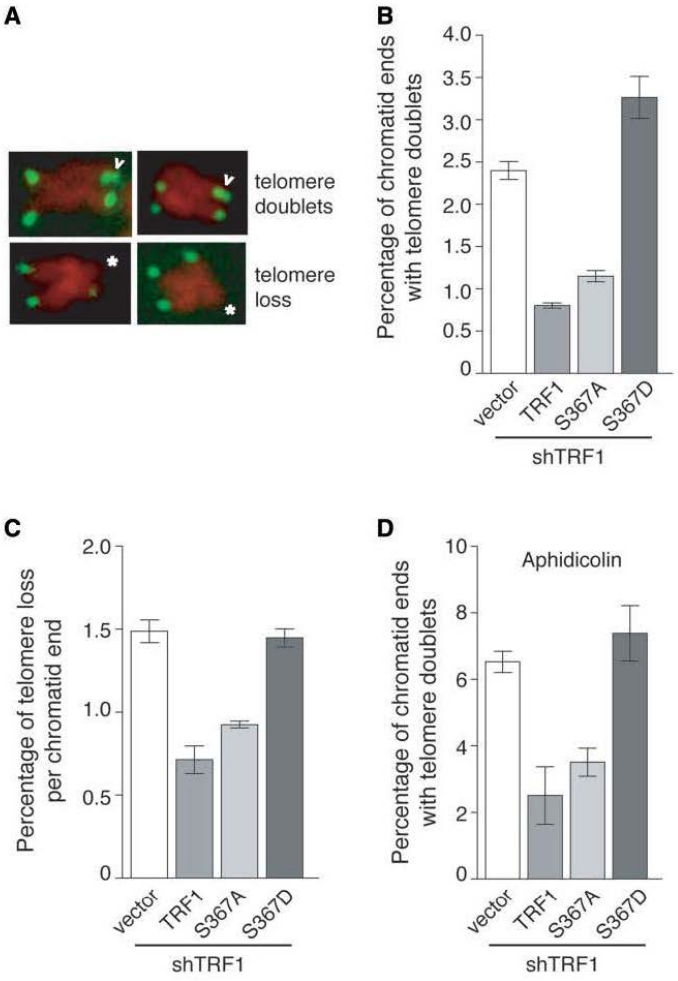

Figure 8. Overexpression of TRF1-S367D promotes the formation of telomere doublets in TRF1-depleted HeLaII cells. (**A**) Images of metaphase chromosomes depicting telomere abnormalities. Metaphase chromosomes were stained with DAPI and false-colored in red. Telomeric DNA was detected by FISH using a FITC-conjugated $(CCCTAA)_3$-containing PNA probe in green. Open arrows represent telomere doublets whereas asterisks indicate telomere loss. (**B**) Quantification of telomere doublets from TRF1-depleted cells expressing indicated constructs. For each cell line, a total of 3150 to 3750 chromosomes from 60 to 69 metaphase cells were scored in a blind manner for the presence of telomere doublets in (B & D) and telomere loss in (C). Standard deviations derived from three independent experiments are indicated. (**C**) Quantification of telomere loss from TRF1-depleted cells expressing indicated constructs. Telomere loss refers to chromatids without a detectable telomere signal. The total number of chromatid ends without a detectable telomere signal was divided by the total number of chromatid ends scored, giving rise to the percentage of telomere loss per chromatid end. (**D)** Quantification of telomere doublets from aphidicolin-treated cells. TRF1-depleted cells expressing indicated constructs were treated with aphidicolin (0.2 μM) for 16 h prior to FISH analysis.

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2.3 Complementary Data

2.3.1 (pS367)TRF1 Does Not Form Foci That Colocalize with Cajal Bodies, PML Bodies, Nuclear Speckles, or Sites of DNA Damage

Indirect immunofluorescence with anti-pS367 TRF1 demonstrated that the foci observed with the anti-pS367 TRF1 antibody do not colocalize with cajal bodies (as marked by coilin), PML bodies, nuclear speckles (as marked by SC-35), or sites of DNA damage (as marked by γH2AX) (Figure 2.3.1). Ruling out these nuclear structures as possible sites of localization allowed us to narrow in on the localization of this particular form of TRF1 at sites of proteasome degradation. The lack of colocalization at these structures also indicates that this focus formation is specific in nature, and likely tightly regulated. This data was referred to within Section 2.2, as data which was not shown. It is included here to provide a full complement of information.

Figure 2.3.1 pS367 TRF1 does not go to nuclear speckles, cajal bodies, PML bodies, or sites of DNA damage

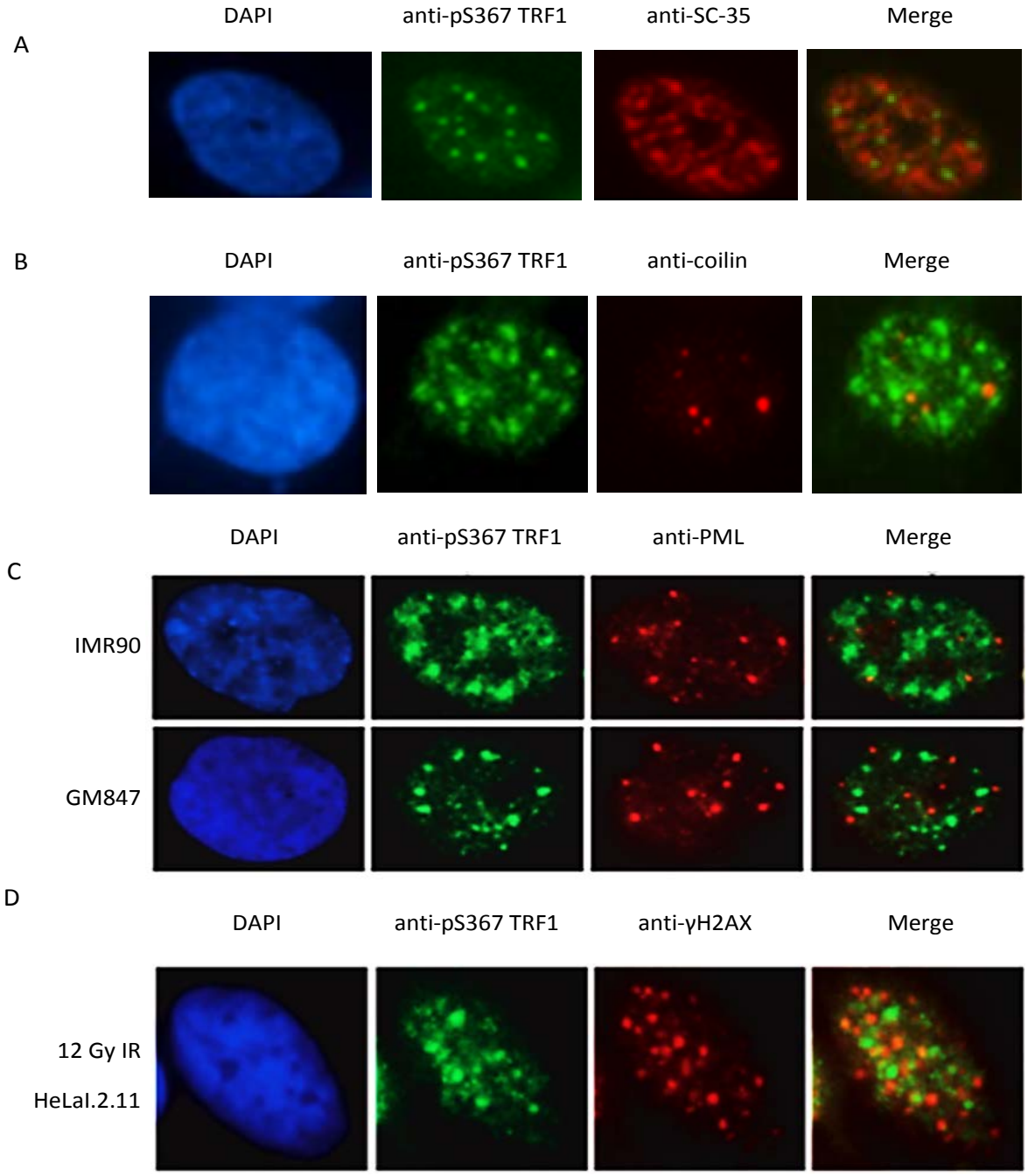

Figure 2.3.1 (pS367)TRF1 does not go to nuclear speckles, cajal bodies, PML bodies, or sites of DNA damage. Indirect immunofluorescence with dual staining to investigate the identity of (pS367)TRF1 foci. Anti-pS367 TRF1 is illustrated in green within each panel, and DNA is stained with DAPI. All cells were fixed with a standard fixation protocol, as described. A) Indirect immunofluorescence in GM847 cells illustrating that (pS367)TRF1 is not associated with nuclear speckles, as stained with anti-SC-35, illustrated in red. B) Indirect immunofluorescence in GM847 cells with anti-coilin, used as a marker for cajal bodies, and displayed in red. Anti-pS367 TRF1 does not colocalize with cajal bodies. C) Indirect immunofluorescence with anti-PML antibody, illustrated in red, in the indicated cell lines. PML bodies do not colocalize with anti-pS367 TRF1 foci. D) HeLa1.2.11 cells were treated with 12Gy of ionizing radiation and fixed 8 hours later. Indirect immunofluorescence was performed with anti-γH2AX, a marker for sites of DNA damage, shown in red. TRF1 phosphorylated at S367 does not form foci that colocalize with sites of DNA damage after IR.

Chapter 3

Cyclin B-Dependent Kinase 1 Regulates Human TRF1 to Modulate the Resolution of Sister Telomeres

Megan McKerlie and Xu-Dong Zhu

3.1 Introduction

3.1.1 Publication

The work presented in this chapter was published in *Nature Communications*, on June 2 2011. The work was published in Volume 2, Issue 371, doi: 10.1038/ncomms1372. The authors listed were as follows: Megan McKerlie and Xu-Dong Zhu.

3.1.2 Contribution

I performed all of the experiments included within this publication. Xu-Dong Zhu and I conceived the project, designed the experiments and wrote the manuscript.

3.1.3 Context and Significance

This chapter advances our understanding of how post-translational events control the function of TRF1. Within this chapter the phosphorylation of TRF1 at T371 and the role that this modification plays in cell cycle progression are investigated. This contributes to the purpose of this thesis in gaining a better appreciation of the diverse functions of TRF1 and the role of phosphorylation events in regulating TRF1 function.

This work furthers our understanding of the importance of the telomere protein TRF1 and is important in understanding the levels of control involved in mitotic progression and in the separation of sister chromatids in anaphase. Demonstrating the importance of pT371 TRF1 in the resolution of sister chromatids brings us closer to defining the full functionality of TRF1 and the role of phosphorylation events in this repertoire of function.

3.2 Publication: Cyclin B-dependent kinase 1 regulates human TRF1 to modulate the resolution of sister telomeres; *Nature Communications* (2011),

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Cyclin B-dependent kinase 1 regulates human TRF1 to modulate the resolution of sister telomeres

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3.2.1 ABSTRACT

Cyclin B-Cdk1 is a key mediator of mitotic entry, however little is known about its role in the separation of sister chromatids. Here we report that upon mitotic entry, Cdk1 specifically phosphorylates threonine 371 of TRF1, a telomere binding protein implicated in the regulation of sister telomere cohesion. Such phosphorylation is removed in late mitosis when Cdk1 activity is inhibited, indicative of a tight regulation of T371 phosphorylation. We show that T371 phosphorylation by Cdk1 keeps TRF1 free of chromatin and this phosphorylation is associated with loss of telomere-bound TRF1 and TIN2, and a reduction in telomere heterochromatin. We find that a phosphomimic mutation at T371 of TRF1 induces telomere deprotection, resulting in telomere loss and the formation of telomere fusions whereas a non-phosphorylatable substitution of T371 blocks sister telomere resolution, promotes micronuclei formation and impairs cell proliferation. Our work suggests that Cdk1 controls TRF1 association with telomeres to facilitate temporal telomere de-protection essential for sister telomere resolution.

3.2.2 INTRODUCTION

Telomeres are specialized heterochromatic structures that function to maintain the integrity and stability of natural chromosome ends in eukaryotic cells. Mammalian telomeric DNA is coated with shelterin, a telomere-specific protein complex composed of TRF1, TRF2, TIN2, Rap1, TPP1 and POT1[1-3]. Disruption of shelterin proteins or the telomere heterochromatic state has been shown to induce de-protection of telomeres, resulting in telomere abnormalities such as telomere end-to-end fusions, telomere loss and telomere doublets/fragile telomeres (more than one telomeric signal at a single chromatid end)[4-11]. These dysfunctional telomeres are recognized as damaged DNA and are associated with DNA damage response factors such as γ-H2AX and 53BP1[7-9,12].

TRF1, a duplex telomeric DNA binding protein[13], plays a crucial role in telomere metabolism. Knockout of TRF1 leads to early embryonic lethality[14], indicative of an essential role of TRF1 in development. Conditional deletion of TRF1 induces the formation of fragile telomeres in S phase, a phenomena associated with replication-dependent defects[5,8], suggesting that TRF1 is required to prevent fork stalling, allowing efficient replication of telomeric DNA[8]. Removal of TRF1 from telomeres promotes telomerase-dependent telomere lengthening whereas overexpression of TRF1 leads to telomere shortening, implying that binding of TRF1 to telomeric DNA negatively regulates the access of telomerase to the ends of telomeres[15-17]. Aside from its role in telomere length maintenance and telomere replication, TRF1 has also been implicated in regulating sister telomere cohesion[18].

Human sister telomeres are fully resolved by the time cells reach metaphase[19,20], and their resolution has been shown to require the action of tankyrase 1[21], a poly(ADP-ribose) polymerase known to ribosylate TRF1[22]. Poly(ADP-ribosyl)ation by tankyrase 1 has been reported to release TRF1 from telomeric DNA[22], rendering TRF1 susceptible to proteosome-dependent degradation[23,24]. TRF1 has been shown to physically interact with SA1[18,25], a component of the cohesin complex. It has been suggested that the release of TRF1 from telomeres through tankyrase 1-dependent poly(ADP-ribosyl)ation may provide a mechanism to allow for the dissociation of cohesins from telomeres[18]. Depletion of tankyrase 1 has been reported to result in unresolved sister telomeres in mitosis[21] and such a defect can be fully rescued by depletion of SA1 or TIN2 but not TRF1[18]. Thus the role of TRF1 in the separation of sister telomeres has so far been elusive.

Cyclin B-dependent kinase 1 (Cdk1) is a key regulator of mitotic entry[26,27] and little is known about its role in the separation of sister chromatids. Here, we report that Cdk1 specifically phosphorylates unbound TRF1 at T371 at the time of mitotic entry and

that this phosphorylation sequesters TRF1 from binding to telomeres. Such sequestration is associated with loss of telomere-bound TRF1 and TIN2 as well as an accumulation of γ-H2AX, indicating that telomeres undergo temporal de-protection in early mitosis. We show that the lack of T371 phosphorylation by Cdk1 leads to a blockage of sister telomere resolution. Our findings suggest that Cdk1 regulates TRF1 binding to telomeres to facilitate temporal telomere de-protection needed for sister telomere resolution.

3.2.3 MATERIALS AND METHODS

Plasmids and Antibodies

The QuickChange site-directed mutagenesis kit (Strategene) was used to generate TRF1 mutants (T371A, T371D and R425V) and wild type TRF1 carrying silent mutations and resistant to shTRF1 from pLPC-N-myc-TRF1. Wild type TRF1 and TRF1 mutants (T371A and T371D) were then subcloned into either the retroviral vector pWZL-N-myc[35] or the bacterial expression vector pHis-Parallel-2[36]. The annealed oligonucleotides encoding shTRF1 (5'-GAATATTTGGTGATCCAAA-3') were ligated into pRetroSuper vector[9], giving rise to pRetroSuper-shTRF1. The BglII-XhoI fragment containing telomeric DNA in pTH12 was replaced with a BglII-XhoI fragment containing non-telomeric DNA, giving rise to the pTH12-NT plasmid.

Rabbit polyclonal anti-pT371 antibody was developed by Biosynthesis Inc. against a TRF1 peptide containing phosphorylated threonine 371 (VSKSQPV-pT-PEKHRARKR). Antibodies to TRF1, TRF2 and TIN2 were kind gifts from Titia de Lange, Rockefeller University. Commercially obtained antibodies included γ-H2AX (Upstate), H3K9m3 (Upstate), H3-pS10 (Cell Signaling) and anti-γ-tubulin (GTU88, Sigma).

Cell Culture and Synchronization

Cells were grown in DMEM medium with 10% fetal bovine serum (FBS) for HeLaI.2.11, HeLaII, GM637, Phoenix cells and 15% FBS for hTERT-RPE, 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[37,38].

Cell synchronization at G1/S boundary was carried out as previously described[28] with modifications. HeLaI.2.11 cells were first arrested with thymidine (2 mM) for 14 hrs, followed by washing in PBS for three times and then released into refresh media for 11 hrs. Subsequently, cells were arrested again with 2 mM thymidine for 14 hrs and washed in PBS for three times before their release into fresh media for 0-16 hrs.

For mitotic synchronization, HeLaI.2.11 cells were treated with 100 ng/ml nocodazole (Sigma) for 16 hrs. To inhibit Cdk1 activity in mitosis, either 20 μM Roscovitin (Sigma) or 2 μM CGP74541A (Sigma) was added to cell cultures in the last two hours of nocodazole treatment. For inhibition of DNA replication, cells were treated with aphidicolin (0.2 μM) for 16 hr as previously described[8].

Differential Salt Extraction of Chromatin

Differential salt extraction of chromatin was performed as described[31] except that cells were treated with either DMSO or nocodazole for 16 hrs before proceeding with the extraction. Protein extracts were prepared essentially as described[28,35], with the exceptions that extracts used for the detection of γ-H2AX and H3K9m3 were prepared by dissolving cell pellets directly in 2X Laemmli buffer. Immunoblotting and immunoprecipitation were carried out essentially as described[28,35].

Recombinant TRF1 Proteins and *in vitro* Kinase Assays

Production of 6xHis-tagged TRF1 proteins was carried out essentially as described[9]. Induction of wild type and mutant TRF1 proteins was carried out overnight with 0.1 mM

IPTG at room temperature. The cell pellet was resuspended in binding buffer (20 mM Tris-HCl [pH 7.9] and 500 mM NaCl) and then lysed by sonication. Following centrifugation, the supernatant was incubated with nickel-resin (Qiagen) for 2h at 4^{o}C. The beads were washed three times with 60 mM imidazole and bound proteins were eluted with a buffer containing 1 M imidazole, 20 mM Tris-HCl pH 7.9 and 0.5 M NaCl. For Cdk1 kinase assays, two micrograms of His-tagged wild type or mutant TRF1 pre-incubated with either DNA or no DNA was incubated with 40 units of Cdk1-cyclin B (NEB, P6020) in the presence of γ-^{32}P-ATP according to the manufacturer. For Plk1 kinase assays, two micrograms of either His-tagged wild type TRF1 or β-casein (Sigma) were incubated with Plk1 (Millipore) in the presence of γ-^{32}P-ATP according to the manufacturer. The sequential kinase assays were performed essentially as described[29]. Two micrograms of His-tagged wild type or mutant TRF1 were first incubated with or without Cdk1 (NEB) in the presence of cold 1 mM ATP, followed by addition of γ-^{32}P-ATP in conjunction with either Plk1 (Millipore) or no Plk1.

Chromatin Immunoprecipitations (ChIPs)

ChIPs were carried out essentially as described[39,40]. Cells were treated with nocodazole (Sigma) or DMSO for 16 h prior to the treatment with 1% formaldehyde in PBS. For experiments involving the release of cells from a nocodazole arrest, cells were treated with DMSO or 1 μM okadaic acid for either 30 min or 90 min before addition of PBS-buffered 1% formaldehyde. Otherwise, cells were directly fixed with 1% formaldehyde in 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 2x10^{6} cells) was used. For the total telomeric DNA, two aliquots of 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 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 average of the two input signals was used for quantification of telomeric DNA recovered. The ratio of the signal from each ChIP relative to the average signal from the two input lanes 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. Detection of telomeric DNA was done with a radioactively-labeled 800-bp TTAGGG repeat-containing fragment as previously described[39].

Immunofluorescence and Chromosome Specific FISH

Immunofluorescence was performed as described[9,28,38] except for that cover slips for scoring of anaphase bridges and lagging chromosomes were stained directly with DAPI.

Chromosome specific FISH was carried out as described[21] with modifications. Cells were treated with 100 ng/ml nocodazole for 1 hr, harvested by shake-off and then fixed twice in 3:1 methanol acetic acid for 15 min each. On the following day, cells were dropped onto slides and dried overnight at RT. Slides were washed in PBS twice for 5 min each, followed by dehydration in a series of 70%, 85% and 100% ethanol. The dried slides were denatured at 80^{o}C for 10 min and hybridized with either the FITC-labeled subtelomere specific probe (16pter; Cytocell) alone or a combination of both TRITC-labeled Alagille probe (JAG1/D20S1091) and FITC-labeled subtelomeric specific probe (20qter; Cytocell) at RT for 2 hr in the dark according to the manufacturer's instruction. Slides were washed twice in 70% formamide and 10 mM Tris (pH 7.2) for 15 min. Following three times wash in PBS for 5 min, slides were 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.

Metaphase Chromosome Spreads

Metaphase chromosome spreads were essentially prepared as described[10,38]. TRF1-depleted HeLaII cells expressing various TRF1 alleles or the vector alone were arrested in nocodazole (0.1 μg/ml) for 90 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 slides and air-dried overnight in a chemical hood.

FISH analysis on metaphase chromosome spreads was carried out essentially as described[38,41]. Slides with chromosome spreads were incubated with 0.5 μg/ml FITC-conjugated-$(CCCTAA)_3$ PNA probe (Biosynthesis Inc.) for 2 hr 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).

***In Vitro* Gel Shift Assays**

In vitro gel shift assays were done essentially as described[40,42] with an end-labeled 188-bp BglII-XhoI fragment from plasmid pTH12[42]. Bacteria-derived recombinant wild type or mutant TRF1 protein was incubated with the end-labeled DNA (1 ng) at room temperature for 20 min in a 20-μl reaction containing 20 mM HEPES-KOH (pH7.9), 150 mM KCl, 5% (v/v) glycerol, 4% (w/v) Ficoll, 1 mM EDTA, 0.1 mM $MgCl_2$, 0.5 mM DTT, 500 ng sheared *Escherichia coli* DNA and 50 ng β-casein. The DNA-protein complexes were fractionated on a 0.7% agarose gel run in 0.1X TBE (8.9 mM Tris-base, 8.9 mM boric acid, 0.2 mM EDTA) at 130 V for 1 hr at room temperature. Gels were dried and exposed to PhosphorImager screens and the signals on the gels were quantified by ImageQuant analysis. The combined signal from three TRF1-DNA complexes was normalized to the total input signal in the corresponding lane to give the percentage of total DNA converted to TRF1-DNA complexes.

Growth Curve and TUNEL Assays

Growth curve was conducted as described[9,43]. For TRF1-depleted HeLaII cells expressing the vector, wild type TRF1 or TRF1 mutants, 2 x 10^4 cells per well were seeded in duplicates onto 12-well plates and experiments were done in triplicates. Cells were counted every three days and media was changed every four days. Cells expressing wild type TRF1 or the vector alone were split 1:16 on the ninth day after seeding whereas cells expressing TRF1-T371A were split 1:16 on the twelfth day after seeding.

The TUNEL assays were performed using the *In Situ* Cell Death Detection kit (Roche) according to the manufacturer's protocol.

3.2.4 RESULTS

Cdk1 phosphorylates T371 of TRF1 in early mitosis

Mass spectrometry analysis of immunoprecipitated Flag-TRF1 revealed threonine 371 of TRF1 to be a candidate phosphorylation site in vivo (Supplementary Fig. S1a). We raised an antibody specifically against a TRF1 peptide containing phosphorylated T371 (Supplementary Fig. S1b and S1c) and showed that anti-pT371 antibody predominantly recognized a protein band with an apparent molecular weight indistinguishable from that of endogenous TRF1 (Fig. 1a). Depletion of TRF1 resulted in a loss of TRF1 recognized by anti-pT371 antibody (Fig. 1b). The antibody also recognized Myc-tagged wild type TRF1 but not Myc-tagged TRF1 carrying a single amino acid substitution of T371 to either alanine (T371A) or aspartic acid (T371D) (Fig. 1c), which was not due to a lack of protein expression (Fig. 1c). These results demonstrate that anti-pT371 antibody specifically recognizes phosphorylated T371 of TRF1.

Analysis of indirect immunofluorescence showed that anti-pT371 antibody stained mitotic cells much more intensely than interphase cells (Fig. 1d, Supplementary Fig. S2). Western analysis also revealed an increase in T371 phosphorylation in cells 10 hrs post release from a double thymidine block (Fig. 1e). Previously we have shown that cells entered mitosis 10 hrs post release[28]. Examination of the sub-stages of mitosis showed that T371 phosphorylation was specifically up regulated in prophase and metaphase and such up-regulation was completely abolished when cells entered telophase (Fig. 1f and Supplementary Fig. S2). These results reveal that T371 phosphorylation is cell cycle regulated, peaking in early mitosis.

T371 (T^{371}PEK) matches the consensus sequence for Cdk1, a cyclin-dependent kinase essential for the G2/M transition. An alanine substitution of T371 drastically abrogated TRF1 phosphorylation by Cdk1 in vitro (Fig. 1g), consistent with previous findings[29]. Treatment with Cdk1 inhibitors (CGP74514A or Roscovitine) completely abolished the increase of TRF1 phosphorylation associated with the nocodazole arrest but it had no effect on phosphorylation of serine 10 of H3, a substrate of Aurora B (Fig. 1h), suggesting that Cdk1 is the main kinase responsible for T371 phosphorylation in early mitosis.

T371 phosphorylation by Cdk1 keeps TRF1 free of chromatin

Pre-incubation of TRF1 with telomeric DNA completely abolished TRF1 phosphorylation by Cdk1 (Fig. 2a). The inhibitory effect of telomeric DNA on TRF1 phosphorylation by Cdk1 was not observed with a TRF1 mutant (TRF1-R425V) (Fig. 2a), which has been previously reported to be unable to bind to telomeric DNA[30]. Furthermore, pre-incubation with telomeric DNA did not inhibit phosphorylation of H1 by Cdk1 (Fig. 2a). These results suggest that Cdk1 specifically phosphorylates unbound TRF1 and that when bound to telomeric DNA, TRF1 may become inaccessible for phosphorylation by Cdk1, perhaps due to a conformational change.

Analysis of differential salt extraction of chromatin revealed that TRF1 was chromatin-bound (420 mM KCl fraction) and very little TRF1 was detected in the chromatin-free fraction (150 mM KCl) in DMSO-treated cells (Fig. 2b), consistent with previous findings[31]. However, upon nocodazole treatment, we observed a reduction in the amount of chromatin-bound TRF1 and the appearance of chromatin-free TRF1, which coincided with a sharp increase in T371 phosphorylation that was predominantly (if not all) chromatin free (Fig. 2b). Nocodazole treatment resulted in an enrichment of mitotic cells (Supplementary Fig. S3a and Fig. 2c) and no release of the heterochromatic mark H3K9m3 from chromatin was observed in mitosis (Fig. 2b and Supplementary Fig. S3b). The reduction of chromatin-bound TRF1 in nocodazole-treated cells was also observed in our ChIP analysis. While nocodazole treatment did not affect the level of total TRF1 (Fig. 2c and 2d), it resulted in at least a 50% decrease in telomeric association of total TRF1 (Fig. 2e and 2f). Such a decrease was also seen in nocodazole-treated hTERT-immortalized normal cells (hTERT-RPE) (Fig. 2g). Nocodazole treatment led to a four-fold increase in the level of T371 phosphorylation (Fig. 2c and 2d) and yet no increase in the association of phosphorylated TRF1 with telomeric DNA was observed (Fig. 2e and 2f). These results together with our earlier finding that most of anti-pT371 staining in mitotic cells did not overlap with DAPI staining suggest that T371 phosphorylation by Cdk1 sequesters TRF1 from associating with telomere chromatin.

Consistent with our in vivo findings, pre-phosphorylation of the recombinant wild type TRF1 by Cdk1 resulted in a reduction in TRF1 binding to telomeric DNA whereas telomeric DNA binding of TRF1-T371A was not impaired by pretreatment with Cdk1 and ATP (Fig. 2h). BSA was included in the gel shift assays to minimize the nonspecific stimulation of the in vitro DNA binding activity of TRF1 by Cdk1 and such nonspecific stimulation has also been observed for a number of other proteins[13,22]. These results suggest that T371 phosphorylation by Cdk1 negatively regulates TRF1 binding to telomeric DNA.

Previously it has been suggested that phosphorylation of T344 and T371 by Cdk1 serves as a docking site for TRF1 phosphorylation by Plk1[29], which has been reported to stimulate TRF1 binding to telomeric DNA[29]. However we did not observe any TRF1 phosphorylation by Plk1 despite the fact Plk1 readily phosphorylated β-casein (Fig. 2i), a positive control for Plk1. Furthermore, we failed to detect any TRF1 phosphorylation by Plk1 even when TRF1 was preincubated with Cdk1 and cold ATP (Fig. 2j). The phosphorylation signal observed in the lane containing both Cdk1 and Plk1 was likely due to the action of Cdk1 alone since a similar level of TRF1 phosphorylation was also detected in the lane containing only Cdk1 and no Plk1 (Fig. 2j). Such signal was completely absent when TRF1-T371A was used in the reaction (Fig. 2j). While we cannot rule out the possibility that the difference in the source of recombinant proteins used might have contributed to the lack of TRF1 phosphorylation by Plk1, our finding that Plk1 can readily phosphorylate β-casein but not TRF1 suggests that the lack of TRF1 phosphorylation signal is unlikely due to Plk1 activity. Our findings that T371 phosphorylation by Cdk1 negatively regulates TRF1 interaction with telomeric DNA both in vivo and in vitro, along with the lack of a detectable stimulation of Plk1-dependent TRF1 phosphorylation by Cdk1, strongly argue against the previously-suggested possibility that T371 phosphorylation by Cdk1 may serve as a positive regulator of TRF1 binding[29].

Dephosphorylation of T371 allows TRF1 binding to telomeres

To examine whether the loss of T371 phosphorylation in telophase cells (Fig. 1f) might be due to phosphatase activity, we arrested HeLaI.2.11 cells in nocodazole and then released them into media containing either DMSO or okadaic acid, the latter being a potent phosphatase inhibitor[32]. Cells remained in mitosis 30-min post release from the nocodazole arrest but they started to exit mitosis 90-min post release (Fig. 3a). T371 phosphorylation persisted in okadaic acid-treated cells but was diminished in DMSO-

treated cells 90-min post release (Fig. 3b). The level of total TRF1 was not affected by treatment with okadaic acid (Fig. 3b). These results suggest that T371 undergoes dephosphorylation in late mitosis.

Anti-TRF1 ChIP analysis revealed that 30-min post release, telomeric association of TRF1 in cells grown in DMSO-containing media was indistinguishable from that in cells grown in okadaic acid-containing media (Fig. 3c and 3d). However, 90-min post release, cells grown in DMSO-containing media exhibited a 3.9-fold increase in TRF1 association with telomeric DNA compared to cells cultured in okadaic acid-containing media (Fig. 3c and 3d). While TRF1 remained phosphorylated at T371 30-min post release, T371 was dephosphorylated in cells grown in DMSO-containing media by 90 min post release (Fig. 3b). Treatment with okadaic acid did not affect the total level of TRF1 (Fig. 3b), nor did it affect telomeric association of TRF2 (Fig. 3c). These results suggest that TRF1 is re-associated with telomeric DNA in late mitosis as a result of T371 dephosphorylation.

Telomeres are recognized as damaged DNA in early mitosis

To investigate whether the loss of telomere-bound TRF1 in early mitosis might affect telomeric association of other shelterin proteins, we arrested cells with nocodazole and performed ChIPs with anti-TIN2 or anti-TRF2 antibody. Nocodazole treatment caused a 35% reduction in telomeric association of TIN2 (Fig. 4a and 4b), but had no effect on telomeric association of TRF2 (Fig. 4a and 4b). The total level of TIN2 or TRF2 was not affected by nocodazole treatment (Fig. 4c).

Anti-H3K9m3 ChIP analysis revealed that nocodazole treatment resulted in a 50% reduction in telomeric association of the heterochromatic mark H3K9 trimethylation (Fig. 4d and 4e) in HeLaI.2.11 cells. Along with the loss of telomere-bound H3K9m3, we also observed a substantial increase in γ-H2AX association with telomeres in both HeLaI.2.11 and hTERT-RPE cells treated with nocodazole (Fig. 4f, 4g and 4h), indicative of

telomere uncapping in early mitosis. The reduction in the amount of H3K9m3 at telomeres is unlikely due to a change in the total level of trimethylated H3K9 (Fig. 4i) or its ability to associate with chromatin in mitosis (Fig. 2b and Supplementary Fig. S3b). Our finding that γ-H2AX was accumulated at telomeres in nocodazole-treated hTERT-RPE cells suggests that the observed telomere uncapping in early mitosis is unlikely solely the result of escaped damage from the previous G2 due to a non-functional p53 pathway as reported[33]. These results suggest that telomeres lose their heterochromatic state and are temporally recognized as damaged DNA in early mitosis.

T371 phosphorylation protects unbound TRF1 from degradation

To examine whether T371 phosphorylation might be involved in regulating TRF1 stability, we subjected nocodazole-treated HeLa cells to cycloheximide experiments in the presence of okadaic acid, which was used to prevent dephosphorylation of TRF1. Phosphorylated (pT371)TRF1 displayed a slower rate of degradation than total TRF1 (Fig. 5a and 5b). Consistent with this finding, the phosphomimic TRF1-T371D mutant was significantly more stable than wild type TRF1 (Fig. 5c and 5d). At 8hr post cycloheximide chase, about 61% of Myc-tagged wild type TRF1 was degraded whereas no degradation was observed for Myc-tagged TRF1-T371D (Fig. 5d). These results suggest that T371 phosphorylation by Cdk1 protects the pool of unbound TRF1 from being targeted for degradation.

Persistent T371 phosphorylation promotes telomere uncapping

TRF1-depleted HeLaII cells were infected with retrovirus expressing the vector alone, Myc-tagged wild type TRF1 (resistant to shTRF1 due to engineered silent mutations), Myc-tagged TRF1-T371A or Myc-tagged TRF1-T371D, giving rise to four stable cell lines (shTRF1-vector, shTRF1-TRF1, shTRF1-T371A, shTRF1-T371D). We chose to use TRF1-

depleted cells for analysis of TRF1 mutants to minimize the interference from endogenous TRF1.

ChIP analysis showed that Myc-TRF1-T371D exhibited a 41% decrease in its association with telomeric DNA compared to wild type TRF1 whereas binding of Myc-TRF1-T371A to telomeric DNA was similar to that of wild type TRF1 (Fig. 6a and 6b). Expression of TRF1 mutants was indistinguishable from that of wild type TRF1 (Fig. 6c). The impaired ability of the phosphomimic mutant to associate with telomeric DNA was also demonstrated through in vitro gel-shift assays using bacteria-expressed recombinant TRF1 proteins (Fig. 6d and 6e). Furthermore, analysis of differential salt extraction revealed that the amount of chromatin-bound Myc-TRF1-T371D was severely reduced compared to that of chromatin-bound wild type TRF1 or Myc-TRF1-T371A (Fig. 6f). The amount of chromatin-bound TRF2 was not reduced in Myc-TRF1-T371D-expressing cells (Fig. 6f), suggesting that the loss of chromatin-bound Myc-TRF1-T371D was specific. We also observed a slight decrease in the amount of chromatin-bound TIN2 in Myc-TRF1-T371D-expressing cells (Fig. 6f), consistent with our earlier finding that T371 phosphorylation in mitosis was associated with loss of TIN2 at telomeres (Fig. 4a and 4b). Overexpression of TRF1-T371D resulted in a 46% loss of trimethylated H3K9 at telomeres compared to wild type TRF1 although overexpression of TRF1-T371A had little impact on H3K9m3 at telomeres (Fig. 6g and 6h). Expression of TRF1 mutants did not affect the total level of H3K9 trimethylation (Fig. 6i). These results suggest that T371D mimics T371 phosphorylation and that persistent phosphorylation of T371 induces loss of telomere heterochromatin.

Overexpression of TRF1-T371D promoted the formation of lagging chromosomes and anaphase bridges (Supplementary Fig. S4), the latter of which suggests the formation of telomere fusions. Depletion of TRF1 did not promote the formation of telomere-containing extra-chromosomal DNA but resulted in the induction of telomere

doublets, telomere loss and sister telomere fusions (Supplementary Fig. S5), consistent with previous findings[5,8]. Overexpression of wild type TRF1 was able to suppress the formation of all these abnormalities in TRF1-depleted cells (Figs. 7a-7f). Introduction of either TRF1-T371A or TRF1-T371D into TRF1-depleted cells suppressed the formation of telomere doublets, similar to that of wild type TRF1 (Fig. 7b and Supplementary Fig. S6). Aphidicolin, an inhibitor of DNA replication, has been shown to induce telomere doublets, which are thought to arise from a defect in telomere replication[5,8]. We observed a striking increase in telomere doublets in TRF1-depleted cells upon treatment with aphidicolin (Fig. 7c), consistent with a previous report[8] that the effect of aphidicolin was additive with loss of TRF1. Such an increase was not observed in TRF1-depleted cells expressing wild type TRF1, TRF1-T371A or TRF1-T371D (Fig. 7c). These results suggest that T371 may not be required for the function of TRF1 in telomere replication.

In contrast to the phenotype of telomere doublets, overexpression of either TRF1-T371A or TRF1-T371D further promoted telomere loss in TRF1-depleted cells (Fig. 7d and Supplementary Fig. S6). Compared to the vector alone in TRF1-depleted cells, an increase of 1.8-fold and 2.1-fold in telomere loss was observed in cells expressing either TRF1-T371A or TRF1-T371D, respectively (Fig. 7d). In addition, overexpression of TRF1-T371D further induced the formation of sister telomere fusions in TRF1-depleted cells (Figs. 7e and 7f and Supplementary Fig. S6). It is unlikely that these fusions were mistaken for chromosomes that are missing a detectable telomere signal on one sister chromatid since they were suppressed in TRF1-T371A-expressing cells that exhibited an accumulation of telomere loss (Fig. 7d). Compared to the vector alone, the average number of sister telomere fusions nearly tripled in TRF1-T371D-expressing cells (Fig. 7e), suggesting that persistent phosphorylation of T371 leads to telomere uncapping.

Cell proliferation assays revealed that cells overexpressing TRF1-T371D failed to proliferate compared to the vector-expressing cells (Fig. 7g). Such a failure was

associated with a high rate of apoptosis as shown by TUNEL assays (Fig. 7h and Supplementary Fig. S7). Compared to the vector alone, a sharp increase of 8.7-fold in cell death was observed in TRF1-depleted cells expressing TRF1-T371D (Fig. 7h). These results suggest that TRF1-T371D-induced telomere uncapping promotes the induction of apoptosis.

Lack of T371 phosphorylation blocks sister telomere resolution

Anti-Myc ChIP analysis revealed that in nocodazole-arrested cells, TRF1-T371A exhibited a 1.9-fold increase in its association with telomeric DNA compared to wild type TRF1 (Fig. 8a and 8b) whereas the level of Myc-TRF1-T371A was indistinguishable from that of Myc-tagged wild type TRF1 (Fig. 8c), suggesting that the lack of T371 phosphorylation leads to an increase in TRF1 binding to telomeric DNA in early mitosis.

Analysis of chromosome-specific FISH using FITC-conjugated subtelomeric probe 16pter revealed that 95% of sister telomeres in TRF1-depleted cells expressing the vector alone were fully resolved, giving rise to two telomere signals seen in metaphase cells (Fig. 8d and 8e). Depletion of TRF1 had no effect on the resolution of sister telomeres (Fig. 8e). Similar to the vector alone, overexpression of wild type TRF1 or the phosphomimic mutant TRF1-T371D did not affect the resolution of sister telomeres (Fig. 8d and 8e). Full resolution of 96% and 94% of sister telomeres was observed in TRF1-depleted cells expressing either wild type TRF1 or TRF1-T371D, respectively (Fig. 8e). In contrast, 91% of sister telomeres remained associated in TRF1-depleted cells expressing the non-phosphorylatable mutant TRF1-T371A (Fig. 8d and 8e), giving rise to a single telomere signal seen in metaphase cells (Fig. 8d). The failure of TRF1-T371A to resolve sister telomeres was also observed with a FITC-conjugated subtelomeric probe 20qter (Fig. 8f). However no defect in the ability of TRF1-T371A to separate chromosome arms was detected (Fig. 8f and 8g), suggesting that the T371A mutation specifically abrogates the ability of TRF1 to resolve sister telomeres.

In addition to its defect in the resolution of sister telomeres, we also observed a 2.2-fold increase in the number of micronuclei in the cell line expressing TRF1-T371A compared to that in the cell line expressing wild type TRF1 (Fig. 8h). Such increase likely contributed to mild apoptosis and slow growth detected in TRF1-depleted cells expressing TRF1-T371A (Fig. 7g and 7h). These results, taken together, suggest that T371 phosphorylation by Cdk1 is required for the resolution of sister telomeres in early mitosis and that the blockage in sister telomere resolution may lead to chromosome missegregation and impaired cell proliferation.

3.2.5 DISCUSSION

It has been a long-standing question as to whether Cdk1, a key mediator of mitotic entry, plays a role in the separation of sister chromatids. In this report, we have uncovered that Cdk1 regulates the separation of sister telomeres by controlling TRF1, a shelterin protein known to interact with SA1[18]. Our work raises an interesting question as to whether Cdk1 might regulate the separation of chromosome arms by modulating proteins that interact with cohesin proteins. Our finding that failure to resolve sister telomeres is associated with the induction of micronuclei formation as well as impaired cell proliferation suggests that sister telomere resolution may be crucial for chromosome segregation and cell growth.

T371 of TRF1 undergoes Cdk1-dependent phoshorylation upon mitotic entry and this phosphorylation is removed in late mitosis to allow re-association of TRF1 with telomeres. Using multiple approaches including IF, ChIP, differential salt extraction of chromatin and in vitro gel shift assays, we have demonstrated that T371 phosphorylation by Cdk1 negatively regulates TRF1 interaction with telomeric DNA, arguing against the previously-suggested possibility that T371 phosphorylation by Cdk1 serves as a positive regulator of TRF1 binding to telomeric DNA[29]. The weak anti-pT371

staining in interphase cells is not sensitive to treatment with Cdk1 inhibitors (Fig. 1h), suggesting that cyclin-dependent kinase(s) might not be involved in T371 phosphorylation in interphase cells.

Although T371 of TRF1 does not appear to be involved in telomere replication, the phosphorylation status of T371 is essential for telomere protection and the separation of sister telomeres. Persistent phosphorylation of T371 promotes loss of telomere heterochromatin and telomere-bound TRF1, leading to induction of telomere loss and the formation of sister telomere fusions. The lack of a significant accumulation of micronuclei in TRF1-T371D-expressing cells is unclear. It is possible that sister telomere fusions might have promoted micronuclei formation but these micronuclei might have escaped detection due to the rapid cell death. On the other hand, the lack of T371 phosphorylation promotes TRF1 association with telomeres in early mitosis, resulting in near blockage of sister telomere resolution. Accumulation of telomere loss was also observed in cells expressing TRF1-T371A and yet there was no loss of telomere heterochromatin, suggesting that TRF1-T371A-induced telomere loss may occur by a distinct pathway from that of TRF-T371D. These results further imply that telomere loss might arise from forced separation of unresolved sister telomeres.

Knockout of TRF1 in either mouse embryonic fibroblasts or mouse embryonic stem cells results in the formation of telomere fusions, indicative of telomere de-protection[4,5]. The lack of a severe telomere de-protection phenotype in TRF1-depleted cells may be due to the fact that shRNA-mediated knockdown did not completely eliminate endogenous TRF1 (Fig. 1b). Perhaps the residual amount of endogenous TRF1 was able to provide sufficient telomere protection needed to sustain cell growth in TRF1-depleted cells. TRF1-T371D promotes telomere uncapping only in TRF1-depleted cells but not in TRF1-proficient cells, indicative of it being a loss of function mutation. The level of TRF1-T371D expressed in TRF1-depleted cells was over 10-fold higher than

the residual amount of endogenous TRF1. Conceivably the excess amount of TRF1-T371D might titrate away the residual amount of endogenous TRF1 in TRF1-depleted cells. Such titration may in part account for the difference in phenotypes between TRF1-depleted cells expressing TRF1-T371D and TRF1-depleted cells. Mutations at T371 do not affect TRF1 interaction with TIN2 or tankyrase 1 (McKerlie and Zhu, unpublished). Collectively, these results suggest that phenotypes associated with either T371A or T371D are likely due to an altered association of TRF1 with telomeres.

We observed a net loss of total TRF1 at telomeres in mitosis, which is likely caused by a combined effect of the sequestration of unbound TRF1 by Cdk1 and the release of telomere-bound TRF1 (Supplementary Fig. S8). It has been shown that poly(ADP-ribosyl)ation by tankyrase 1 releases TRF1 from telomeres, rendering it susceptible to degradation[23]. Perhaps the release of TRF1 from telomeres by tankyrase 1, in conjunction with T371 phosphorylation, may in part lead to the net loss of total TRF1 at telomeres in early mitosis.

TRF1 binding to telomeres is very dynamic and there is a high exchange rate between bound TRF1 and free TRF1[34]. When not bound to telomeric DNA, TRF1 is susceptible for degradation[23,24]. Although phosphorylated (pT371)TRF1 is excluded from associating with chromatin, it is more stable than the total TRF1. Similarly, the phosphomimic mutant TRF1-T371D is more stable than both wild type TRF1 and the non-phosphorylable mutant TRF1-T371A despite the fact it has an impaired affinity for telomeric DNA. These results led us to propose that T371 phosphorylation by Cdk1 may temporally safeguard the pool of unbound TRF1 from being degraded in early mitosis when the resolution of sister telomeres occurs. Such temporal safeguard may provide the pool of TRF1 needed to re-establish telomere protection once chromosomes are pulled apart in late mitosis (Supplementary Fig. S8).

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AUTHOR CONTRIBUTIONS

M.M. performed the experiments. M.M. and X.D.Z. conceived and designed the experiments as well as wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

3.2.6 FIGURES AND FIGURE LEGENDS

Figure 1 McKerlie and Zhu

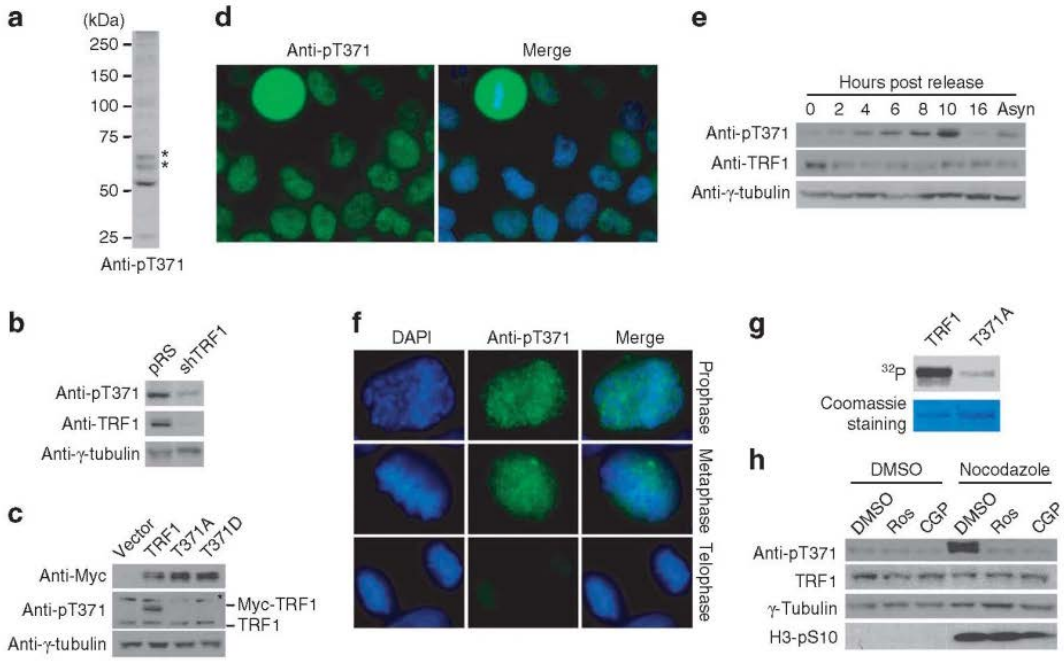


Figure 1. T371 of TRF1 is phosphorylated in early mitosis. (**a**) Western analysis with anti-pT371 antibody. Asterisks indicate non-specific bands that do not respond to shTRF1 knockdown. (**b**) Western analysis of HeLaII cells depleted for endogenous TRF1. Immunoblotting was performed with anti-pT371 or anti-TRF1 antibody. The γ-tubulin blot was used as a loading control in this experiment and all following westerns shown in this article. (**c**) Western analysis of various Myc-tagged TRF1 proteins as indicated. Immunoblotting was performed with anti-Myc, anti-pT371 or anti-γ-tubulin antibody. Myc-tagged TRF1 proteins migrate slower than endogenous TRF1. Asterisks indicate non-specific bands that do not respond to shTRF1 knockdown. (**d**) Indirect immunofluorescence with anti-pT371 antibody on transformed GM637 cells. (**e**) Western analysis of cell extracts from asynchronous HeLaI.2.11 cells (Asyn) or cells released for 0-16 h from a double thymidine block. (**f**) Indirect immunofluorescence with anti-pT371 antibody on HeLaI.2.11 cells. (**g**) *In vitro* Cdk1 kinase assays. Recombinant cyclin B-Cdk1 was incubated with bacteria-expressed his-tagged wild type TRF1 or TRF1 mutant T371A in the presence of γ-^{32}P-ATP. (**h**) Western analysis. HeLaI.2.11 cells were treated with either DMSO or nocodazole, followed by incubation with or without Cdk1 inhibitors Roscovitine (Ros) or CGP74514A (CGP). Immunoblotting was performed with anti-pT371, anti-TRF1, anti-H3pS10 or anti-γ-tubulin antibody.

Figure 2 McKerlie and Zhu

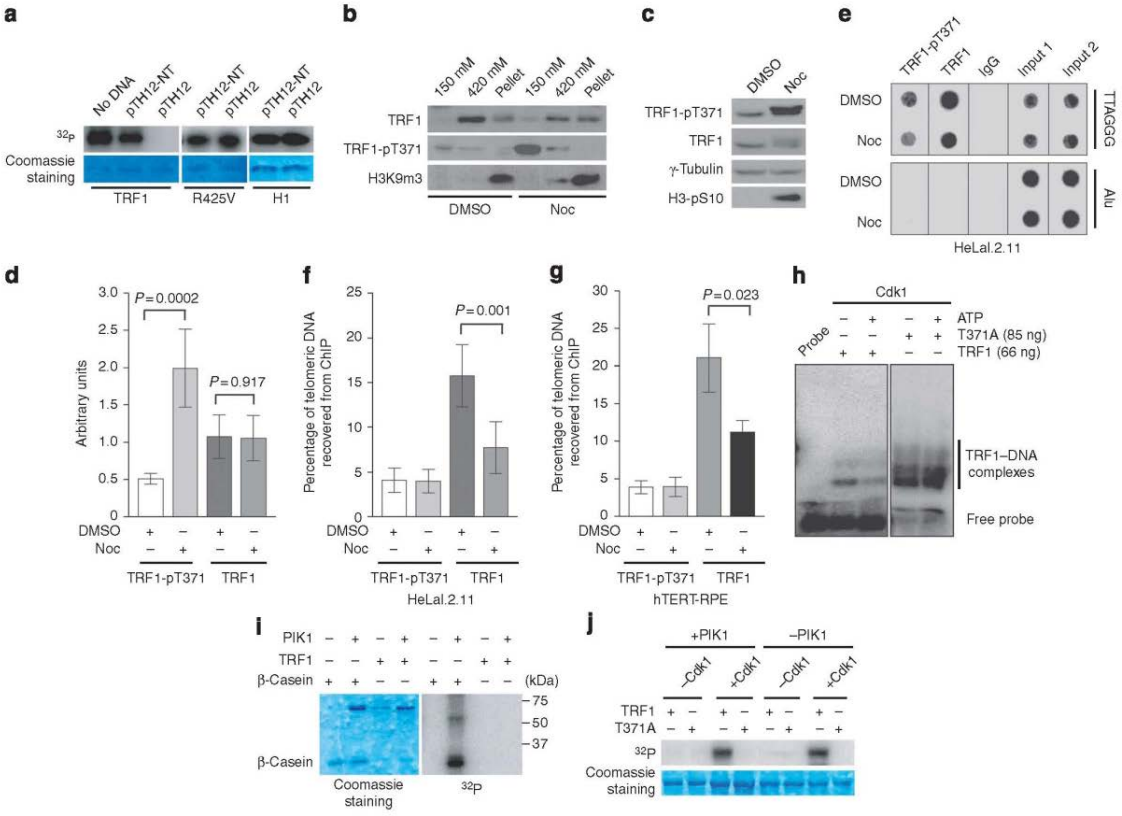


Figure 2. Cdk1 specifically phosphorylates unbound TRF1 and this phosphorylation keeps TRF1 free of chromatin. (**a**) Two micrograms of recombinant wild type TRF1, mutant TRF1-R425V or H1 was pre-incubated with no DNA or 5 μg of pTH12-NT or pTH12, followed by Cdk1 kinase assays. (**b**) Differential salt extraction of chromatin on HeLaI.2.11 cells treated with DMSO or nocodazole (Noc). (**c**) Western analysis. (**d**) Quantification of total TRF1 and phosphorylated TRF1 from (c). The signals from the western blots were quantified with densitometry. The level of total TRF1 and phosphorylated TRF1 are represented in arbitrary units after their signals were normalized relative to those of γ-tubulin. A Student's two-tailed t test was used to derive all *P* values. Standard deviations (SDs) from three independent experiments are indicated. (**e**) Dot blots of ChIPs with anti-pT371 or anti-TRF1 antibody. HeLaI.2.11 cells were treated with DMSO or nocodazole. Anti-IgG antibody was used as a negative control in this experiment and all the following ChIPs shown in this article. (**f**) Quantification of ChIPs from (e). ImageQuant analysis was used for quantification in this experiment and all the following ChIPs shown in this article. The *P* value was calculated using a Student's two-tailed t test. SDs are derived from at least four independent experiments. (**g**) Quantification of ChIPs with anti-pT371 or anti-TRF1 antibody from hTERT-RPE cell extracts. The *P* value was calculated using a Student's two-tailed t test. SDs from three independent experiments are indicated. (**h**) DNA binding assays. Prior to gel shift assays, recombinant wild type TRF1 (66 ng) or T371A (85 ng**)** was incubated with BSA (1 μg) and Cdk1 with or without 5 mM cold ATP. BSA was used as a competitor for the nonspecific activity of Cdk1. (**i**) Recombinant Plk1 was incubated with recombinant wild type TRF1 or β-casein in the presence of γ-^{32}P-ATP. Recombinant TRF1 comigrates with Plk1 on an 8% SDS-polyacrylamide gel. (**j**) Sequential kinase assays. Recombinant wild type TRF1 or TRF1-T371A was incubated with cold ATP with or without Cdk1, followed by addition of γ-^{32}P-ATP in the presence of Plk1 or no Plk1 as indicated above the lanes.

Figure 3 McKerlie and Zhu

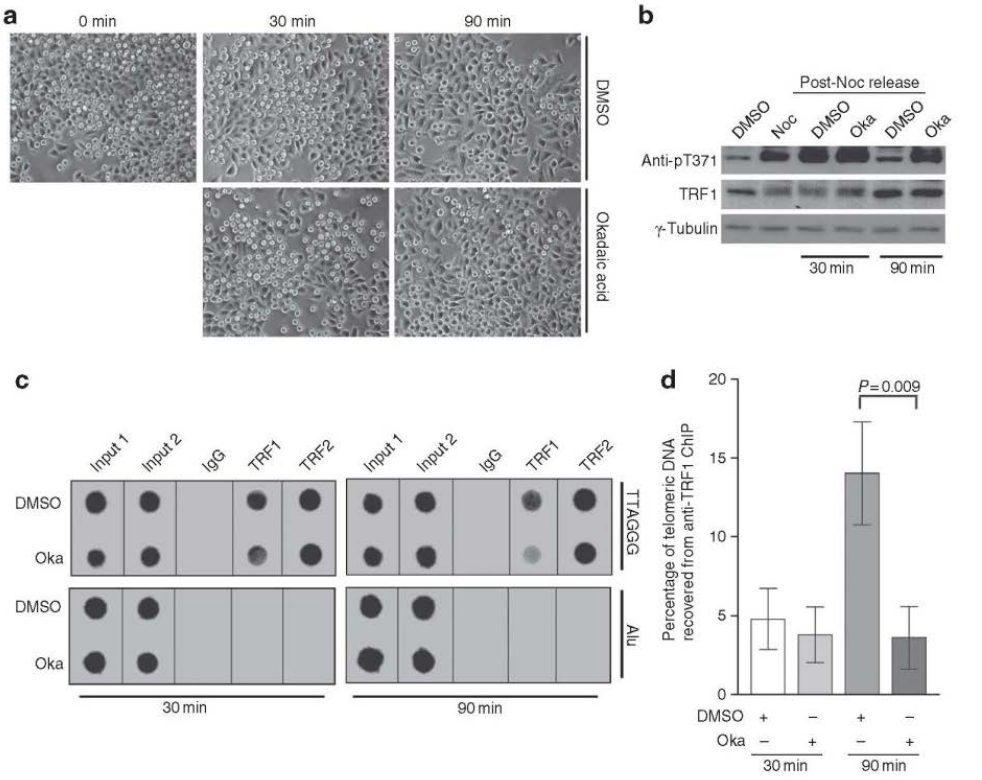


Figure 3. T371 of TRF1 is dephosphorylated in late mitosis and this dephosphorylation allows the re-association of TRF1 with telomeric DNA. (**a**) Live cell images for HeLaI.2.11 cells released from a nocodazole arrest into fresh media containing either DMSO or okadaic acid (1 μM) for 0-90 min. (**b**) Western analysis performed with anti-pT371, anti-TRF1 or anti-γ-tubulin antibody. (**c**) Dot blots of ChIPs performed with anti-TRF1 or anti-TRF2 antibody. Nocodazole-arrested HeLaI.2.11 cells were released into fresh media containing either DMSO or okadaic acid (Oka) for either 30 min or 90 min. (**d**) Quantification of anti-TRF1 ChIPs from (c). The *P* value was calculated using a Student's two-tailed t test. Standard deviations derived from at least three independent experiments are indicated.

Figure 4 McKerlie and Zhu

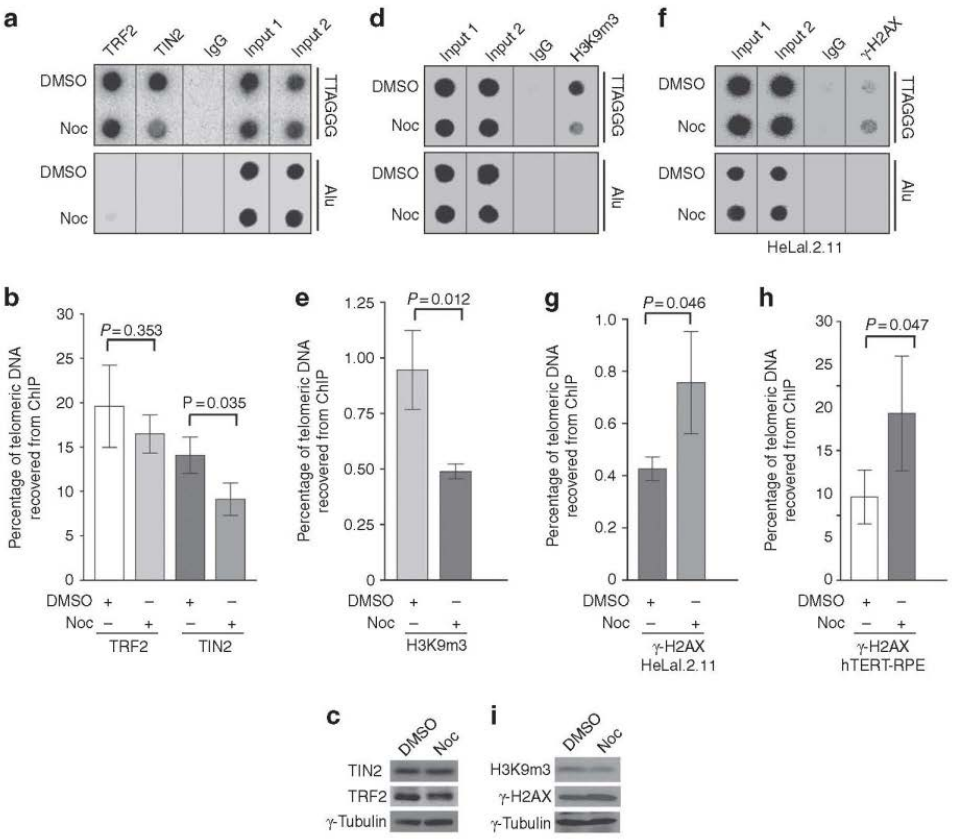


Figure 4. Nocodazole arrest results in not only the loss of TIN2 and the heterochromatic mark at telomeres but also an accumulation of γ-H2AX at telomeres. (**a**) Dot blots of anti-TIN2 and anti-TRF2 ChIPs from HeLaI.2.11 cells treated with either DMSO or nocodazole. (**b**) Quantification of anti-TRF2 and anti-TIN2 ChIPs from (a). The *P* value was calculated using a Student's two-tailed t test. Standard deviations from three independent experiments are indicated. (**c**) Western analysis with anti-TIN2, anti-TRF2 or anti-γ-tubulin antibody. (**d**) Dot blots of anti-H3K9m3 ChIPs from HeLaI.2.11 cells treated with either DMSO or nocodazole. (**e**) Quantification of anti-H3K9m3 ChIPs. The *P* value was calculated using a Student's two-tailed t test. Standard deviations from three independent experiments are indicated. (**f**) Dot blots of anti-γ-H2AX ChIPs from HeLaI.2.11 cells treated with either DMSO or nocodazole. (**g**) Quantification of anti-γ-H2AX ChIPs from (f). The *P* value was calculated using a Student's two-tailed t test. Standard deviations from three independent experiments are indicated. (**h**) Quantification of anti-γ-H2AX ChIPs for hTERT-RPE cells. The *P* value was calculated using a Student's two-tailed t test. Standard deviations from three independent experiments are indicated. (**i**) Western analysis with anti-H3K9m3, anti-γ-H2AX or anti-γ-tubulin antibody.

Figure 5 McKerlie and Zhu

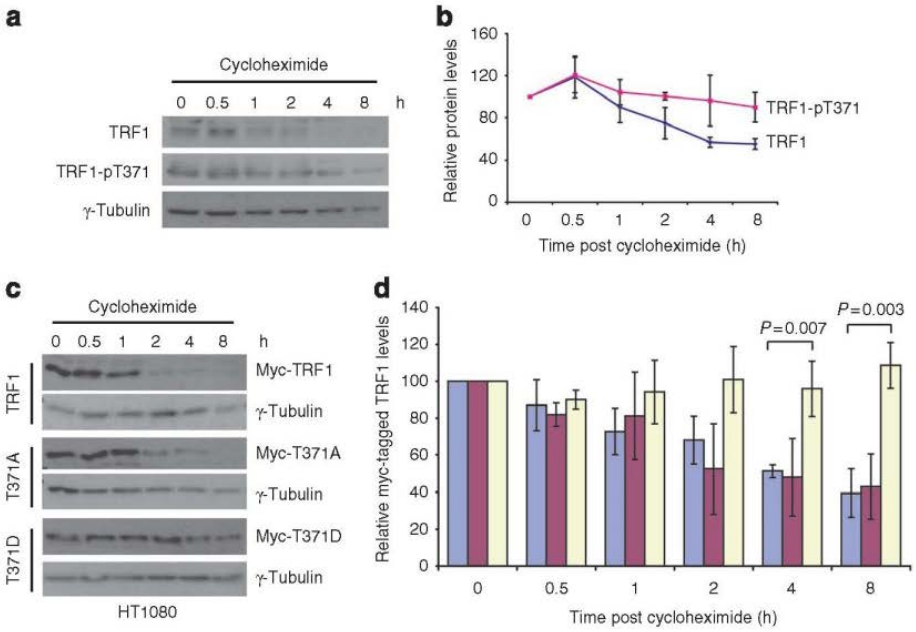


Figure 5. Phosphorylation of T371 protects TRF1 from being targeted for degradaton. (**a**) Cycloheximide chase experiments. Nocodazole-arrested HeLaI.2.11 cells were treated with cycloheximide (100 μg/ml) in the presence of 1 μM okadaic acid for the indicated times. Okadaic acid was used to prevent dephosphorylation of T371. Immunoblotting was performed with anti-TRF1, anti-pT371 or anti-γ-tubulin antibody. (**b**) Quantification of total TRF1 and phosphorylated (pT371)TRF1 from (a). The signals from the western blots were quantified with densitometry. The level of total TRF1 or phosphorylated (pT371)TRF1 is represented in arbitrary units after their signals were normalized relative to those of γ-tubulin. Standard deviations from three independent experiments are indicated. (**c**) Cycloheximide chase experiments. HT1080 cells expressing Myc-TRF1, Myc-TRF1-T371A or Myc-TRF1-T371D were treated with 100 μg/ml cycloheximide for the indicated times, followed by immunoblotting of the lysates with anti-Myc or anti-γ-tubulin antibody. (**d**) Quantification of Myc-tagged wild type TRF1 (blue bars), Myc-tagged TRF1-T371A (purple bars) and Myc-tagged TRF1-T371D (light yellow bars) from (c). Quantification was performed as described in (b). All *P* values were calculated using a Student's two-tailed t test. Standard deviations from three independent experiments are indicated.

Figure 6 McKerlie and Zhu

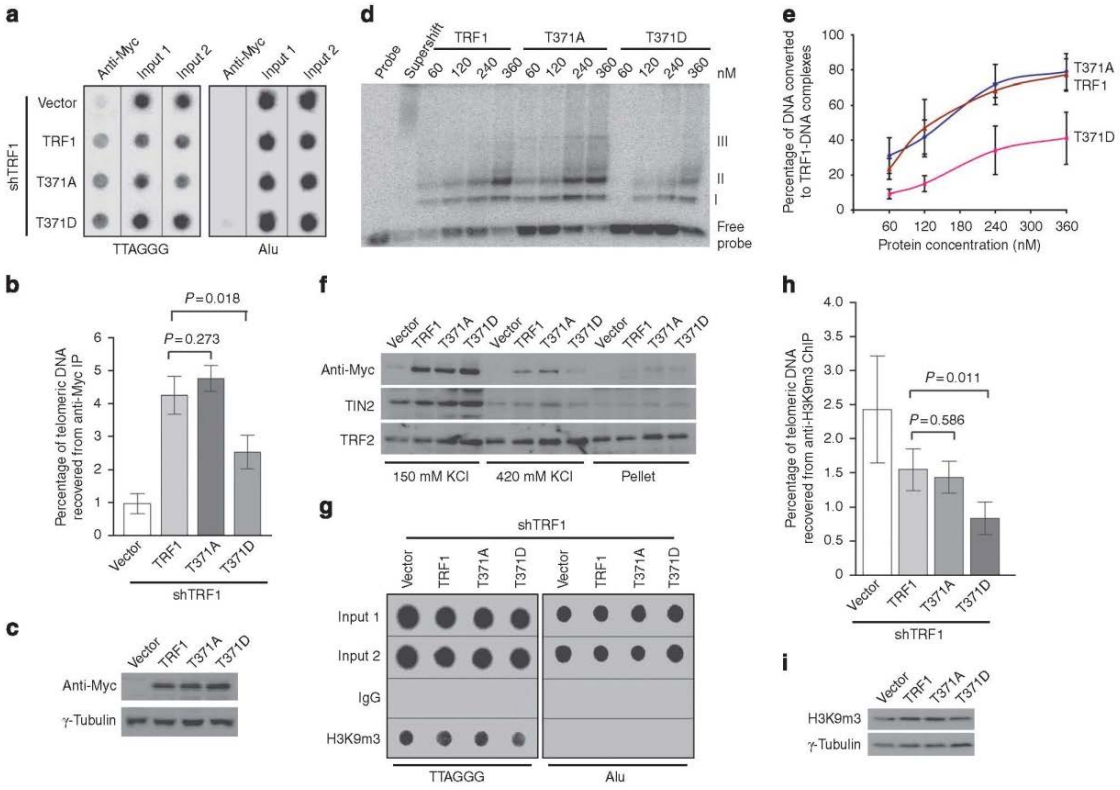


Figure 6. A phosphomimic mutation at T371 (T371D) not only impairs TRF1 interaction with telomeres but also promotes loss of telomere heterochromatin. (**a**) Dot blots of anti-Myc ChIPs from TRF1-depleted HeLaII cells expressing various constructs as indicated on the left. (**b**) Quantification of anti-Myc ChIPs from (a). The *P* values were calculated using a Student's two-tailed t test. Standard deviations from three independent experiments are indicated. (**c**) Western analysis of Myc-tagged TRF1 proteins. (**d**) *In vitro* gel shift assays. Bacteria-derived recombinant TRF1 proteins were used in the gel-shift assays as indicated above the lanes. The positions of three TRF1-containing complexes (I, II and III) are indicated on the right. All TRF1-containing complexes can be supershifted by anti-TRF1 antibody. The concentrations of recombinant TRF1 used were indicated above the lanes. (**e**) Quantification of percentage of total DNA converted to TRF1-DNA complexes. Standard deviations for TRF1 mutants (T371A and T371D) were derived from three independent experiments whereas standard deviations for wild type TRF1 were derived from at least two independent experiments. (**f**) Differential salt extraction of chromatin on TRF1-depleted cells expressing various constructs as indicated above the lanes. (**g**) Dot blots of anti-H3K9m3 ChIPs from TRF1-depleted HeLaII cells expressing various constructs as indicated. (**h**) Quantification of anti-H3K9m3 ChIPs. The *P* values were calculated using a Student's two-tailed t test.Standard deviations derived from four independent experiments are indicated. **(i)** Western analysis of H3K9m3 expression.

Figure 7 McKerlie and Zhu

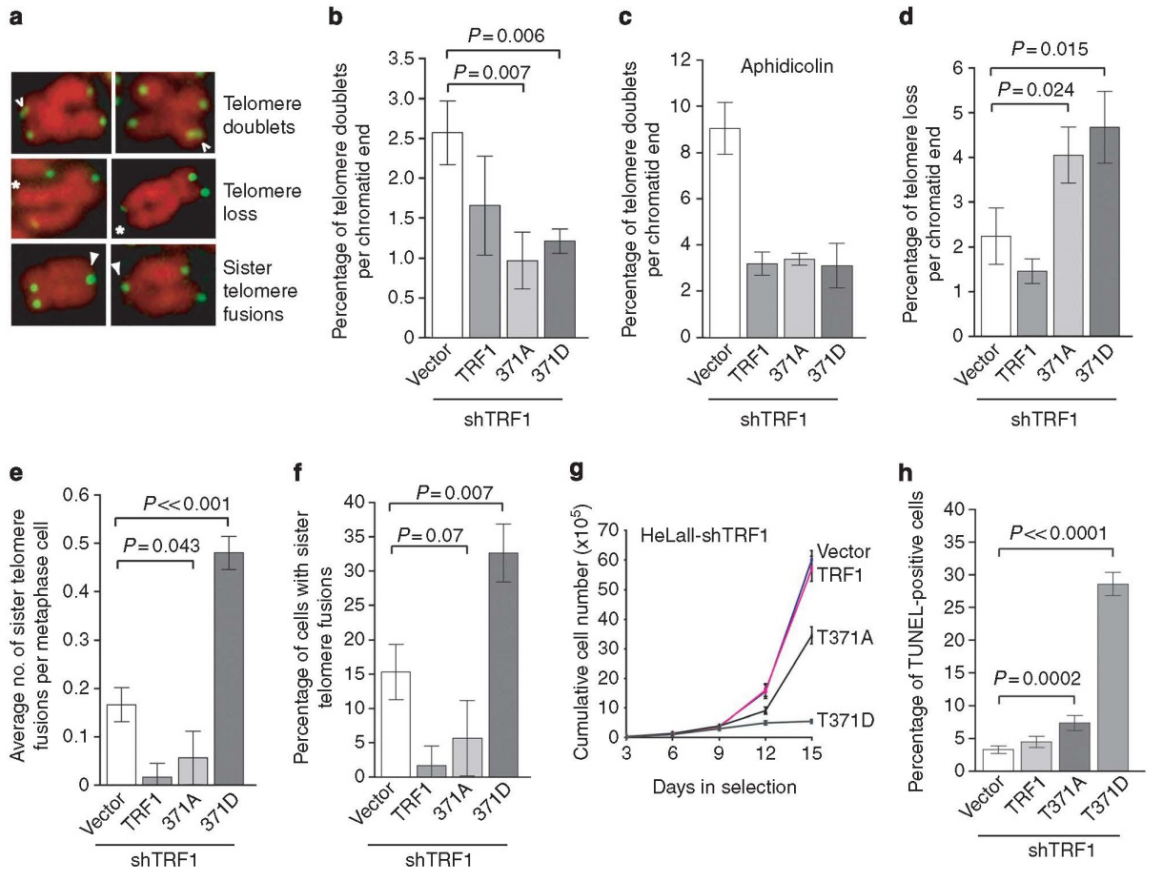


Figure 7. Overexpression of TRF1-T371D promotes telomere uncapping, resulting in the induction of apoptosis. (**a**) Images of metaphase chromosomes depicting telomere abnormalities. Metaphase chromosomes were stained with DAPI and false-colored in red. Telomeric DNA was detected by FISH using a FITC-conjugated $(CCCTAA)_3$-containing PNA probe in green. Open arrows: telomere doublets. Asterisks represent telomere loss whereas filled arrows indicate sister telomere fusions. (**b**) Quantification of telomere doublets from TRF1-depleted cells expressing indicated constructs. For each cell line, a total of 3,486 to 4,696 chromosomes from 52 to 71 metaphase cells were scored in a blind manner for the presence of telomere doublets in (b & c) as well as telomere loss in (d) and sister telomere fusions in (e & f). All *P* values shown in (7b-7f and 7h) were calculated using a Student's two-tailed t test. Standard deviations derived from three independent experiments are indicated. (**c**) Quantification of telomere doublets from aphidicolin-treated cells. (**d**) Quantification of telomere loss from TRF1-depleted cells expressing indicated constructs. Telomere loss refers to chromatids without a detectable telomere signal. The total number of chromatid ends without a detectable telomere signal was divided by the total number of chromatid ends scored, giving rise to the percentage of telomere loss per chromatid end. (**e)** Quantification of average number of sister telomere fusions per metaphase cell from TRF1-depleted cells expressing indicated constructs. **(f**) Quantification of percentage of cells with sister telomere fusions from TRF1-depleted cells expressing indicated constructs. (**g**) Proliferation assays of TRF1-depleted cells expressing various constructs as indicated. Standard deviations from three independent experiments are indicated. (**h**) Quantification of TUNEL assays. Scoring of TRF1-depleted HeLaII cells expressing various constructs as indicated was done in a blind manner. A total of 1000 cells from each of four independent experiments were scored. Standard deviations are derived from four independent experiments.

Figure 8 McKerlie and Zhu

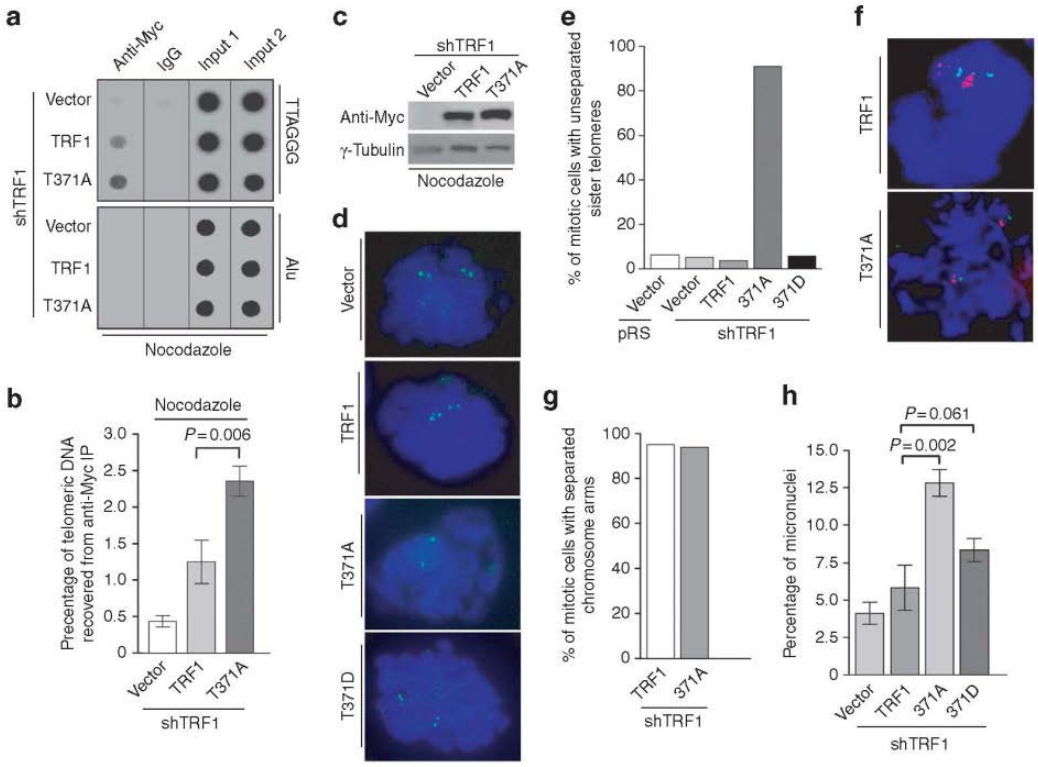
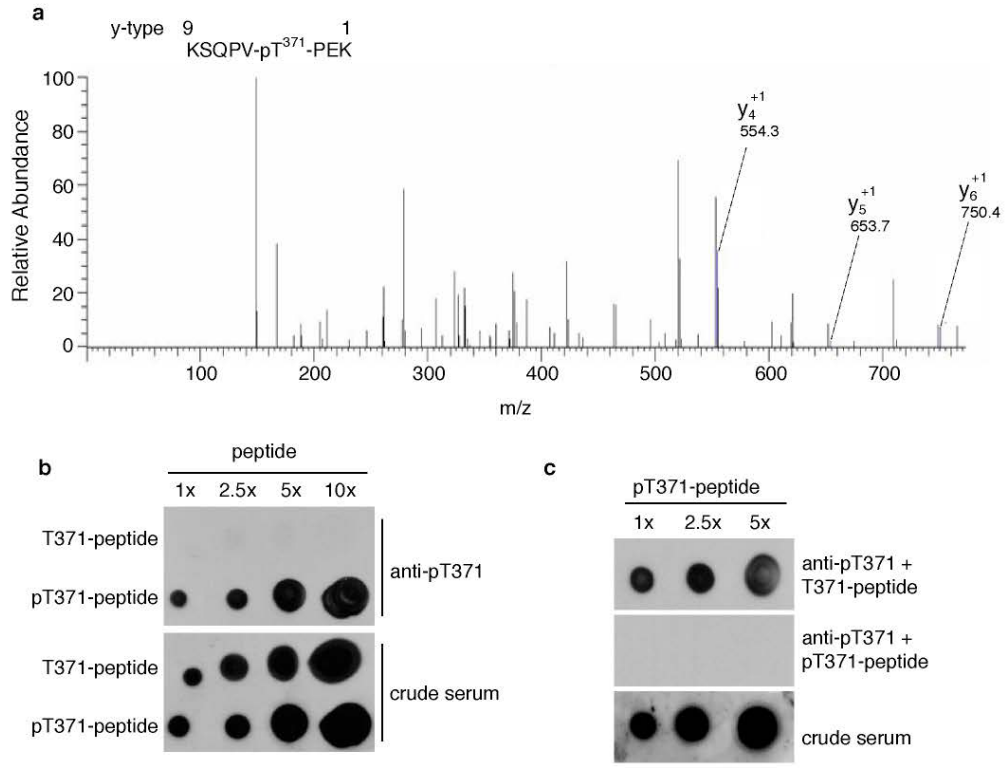


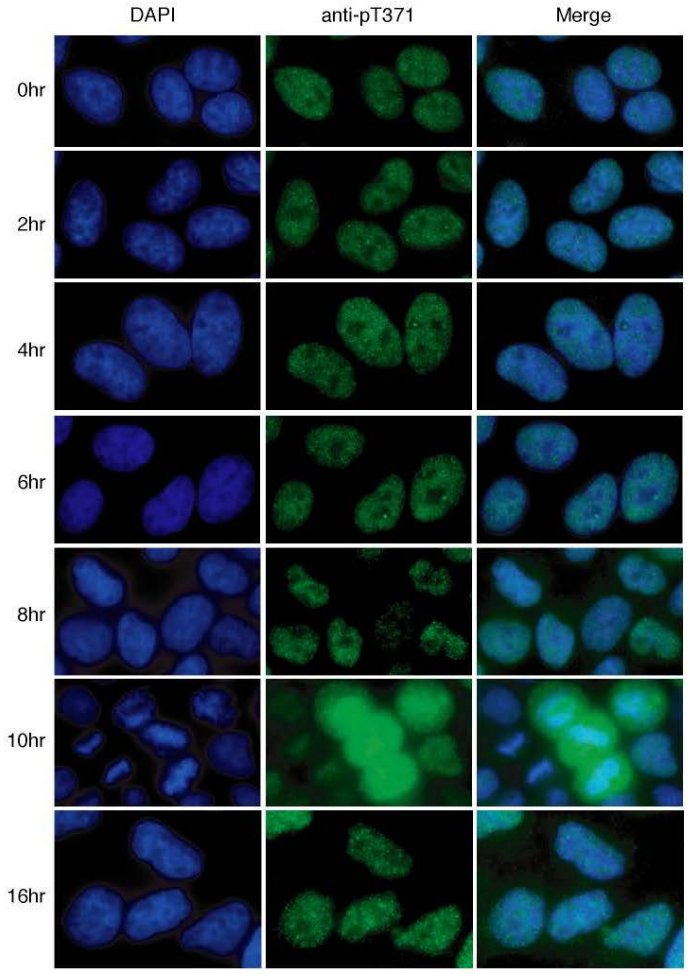
Figure 8. The lack of phosphorylation at T371 leads to near complete blockage of the resolution of sister telomeres. **(a)** Dot blots of anti-Myc ChIPs. TRF1-depleted HeLaII cells expressing various constructs as indicated were treated with nocodazole prior to ChIP analysis. (**b**) Quantification of anti-Myc ChIPs. Standard deviations derived from three independent experiments are indicated. (**c**) Western analysis of Myc-tagged TRF1 expression. (**d**) Chromosome-specific FISH with a FITC-labeled subtelomere specific probe (16pter; Cytocell) in green. DNA was stained with DAPI shown in blue. (**e**) Quantification of metaphase cells carrying unresolved sister telomeres. Metaphase cells carrying two sets of a green doublet were scored positive for resolved sister telomeres whereas metaphase cells carrying two sets of a green singlet were scored positive for unresolved sister telomeres. Approximately 100 metaphase cells were scored for each cell line in blind. (**f**) Chromosome-specific FISH using a TRITC-labeled Alagille probe (JAG1/D20S1091) in red in conjunction with a FITC-labeled subtelomeric specific probe (20qter; Cytocell) in green. DNA was stained with DAPI shown in blue. (**g**) Quantification of metaphase cells carrying the separated chromosome arms. Metaphase cells carrying two sets of a red doublet were scored positive for the separated chromosome arms. Approximately 100 metaphase cells were scored for each cell line in blind. (**h**) Quantification of micronuclei formation in TRF1-depleted HeLaII cells expressing various constructs as indicated. Scoring of each cell line was done in a blind manner. For each cell line, at least a total of 3060 cells from three independent experiments were scored. Standard deviations derived from three independent experiments are indicated. The *P* values were calculated using a Student's two-tailed t test.

Supplementary Figure S1 McKerlie & Zhu



Supplementary Figure. S1. T371 of TRF1 is phosphorylated *in vivo*. **(a)** LC/MS/MS analysis was performed on TRF1 immunoprecipitated from HT1080 cells. The spectrum of the peptide identified to contain phosphorylated threonine at position 371 is shown with the relative abundance plotted against the monoisotopic mass (m/z). The m/z peaks from the y-type ions are indicated. Mass spectrometry analysis of Flag-tagged TRF1 was done through service provided by WEMB Biochem. Inc., Toronto, Canada (**b**) Affinity-purified anti-pT371 antibody specifically recognizes TRF1 peptide containing phosphorylated T371 (pT371-peptide). An increasing amount of peptide either carrying unmodified T371 (T371-peptide) or phosphorylated T371 (pT371-peptide) was spotted on a nitrocellulose membrane, followed by immunoblotting with affinity-purified anti-pT371 antibody or crude serum. The amount of peptide spotted from left to right is 0.7 μg, 1.75 μg, 3.5 μg and 7 μg. (**c**) Peptide competition assays. Affinity-purified anti-pT371 antibody was incubated with 5.5 μg of either unmodified (T371-peptide) or phosphorylated peptide (pT371-peptide) prior to immunoblotting. Crude serum was used to show the presence of pT371-peptide on the nitrocellulose membrane. The amount of pT371-peptide spotted from left to right is 0.7 μg, 1.75 μg and 3.5 μg.

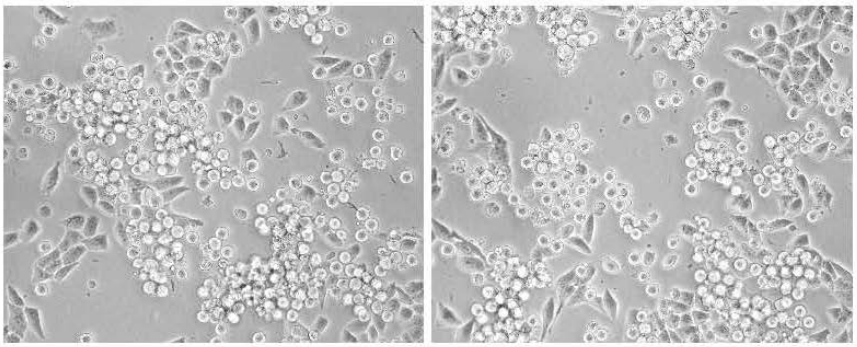
Supplementary Figure S2 McKerlie & Zhu



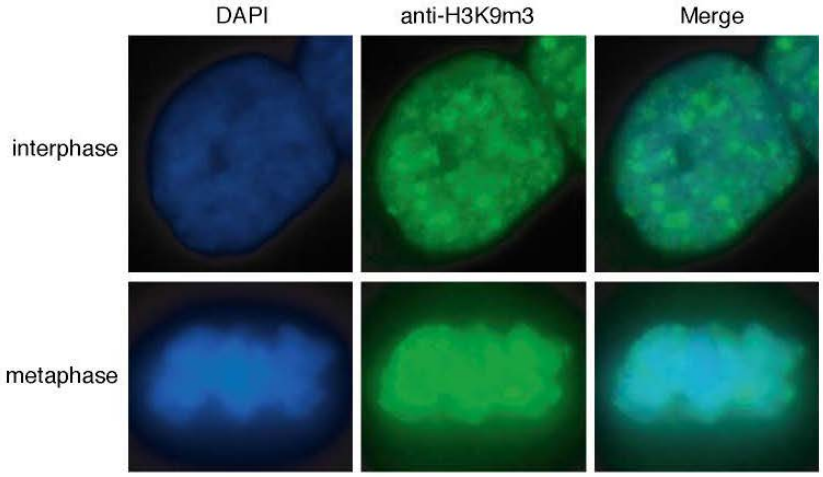
Supplementary Figure S2. Phosphorylation of T371 of TRF1 is specifically up regulated in mitosis. Indirect immunofluorescence using anti-pT371 antibody was performed on HeLaI.2.11 cells released for 0-16 h from a double thymidine block. Cell nuclei were stained with DAPI, shown in blue.

Supplementary Figure S3 McKerlie & Zhu

a

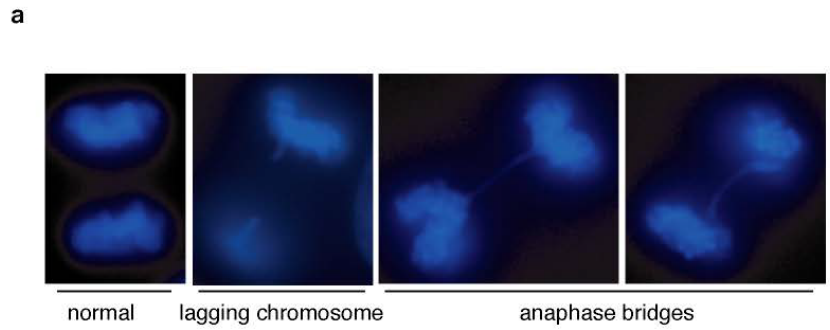


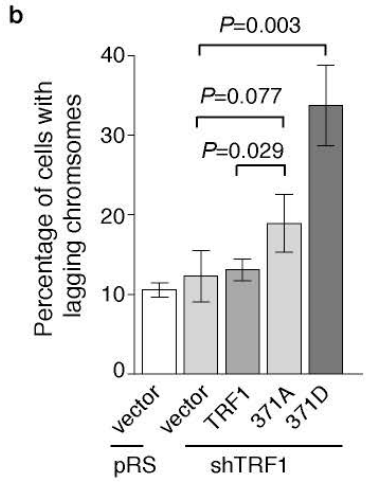
b

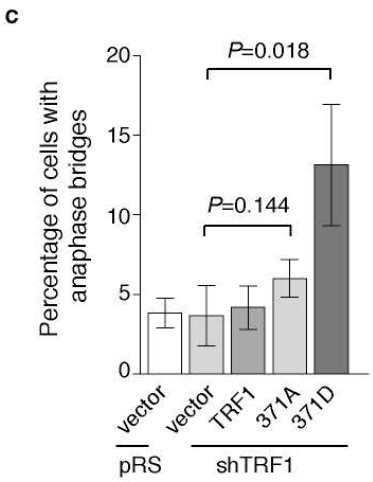


Supplementary Figure S3. Analysis of nocodazole treatment and chromatin association of the heterochromatic mark H3K9m3 in mitosis. (**a**) Live cell images were taken for HeLaI.2.11 cells that had been treated with nocodazole for 16 hr. (**b**) Indirect immunofluorescence using anti-H3K9m3 antibody was performed on HeLaI.2.11 cells. Cell nuclei were stained with DAPI shown in blue.

Supplementary Figure S4 McKerlie & Zhu

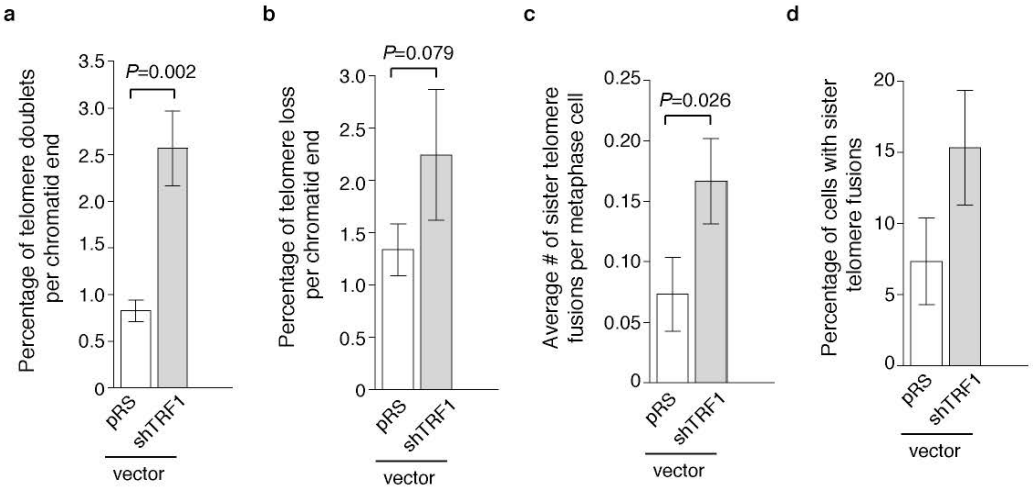






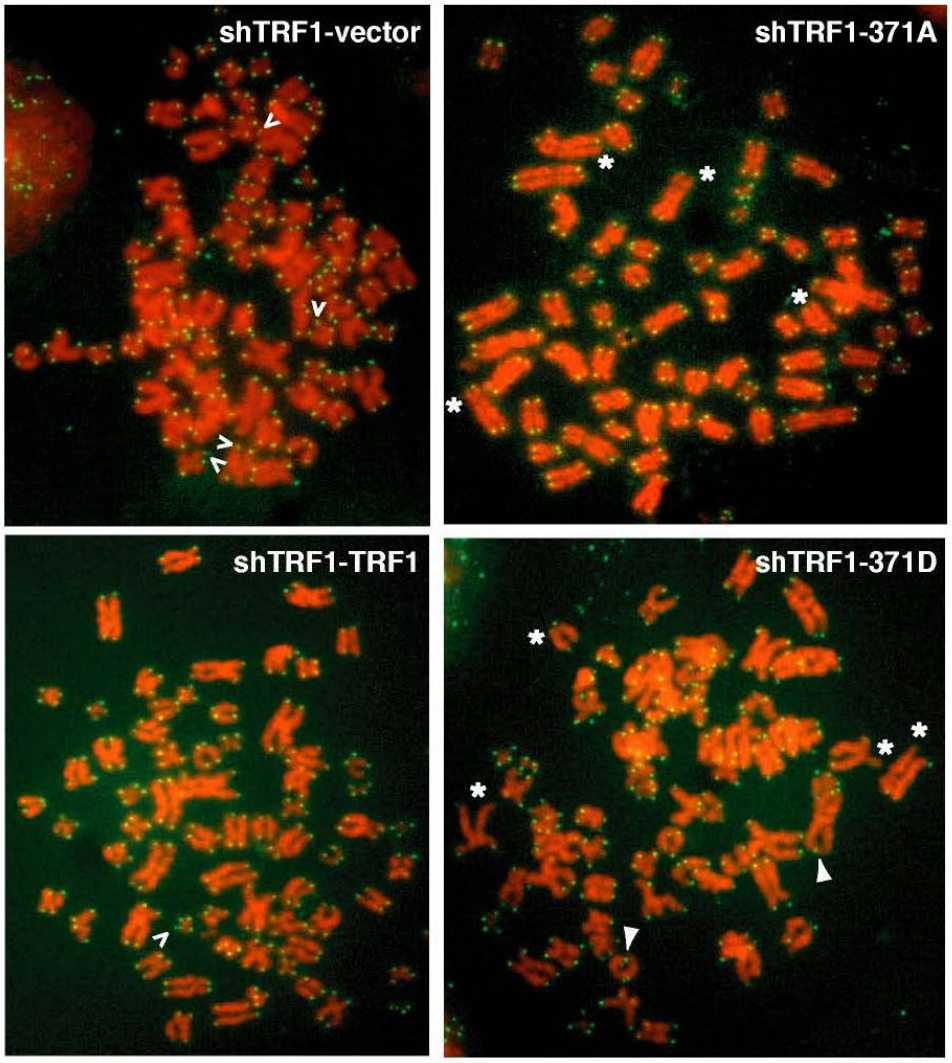
Supplementary Figure S4. Occurrence of lagging chromosomes and anaphase bridges in TRF1-depleted HeLaII cells expressing various TRF1 alleles. (**a**) Images of cells either normal or carrying lagging chromosomes or anaphase bridges. TRF1-depleted HeLaII cells expressing various constructs were fixed and stained directly with DAPI. Anaphase cells were inspected for the presence of lagging chromosomes or anaphase bridges. (**b**) & (**c**) Percentage of anaphase cells bearing lagging chromosomes and anaphase bridges respectively. Scoring of each cell line was done in a blind manner. A total of 300 anaphase cells from each of three independent experiments were scored. Standard deviations derived from three independent experiments are indicated.

Supplementary Figure S5 McKerlie & Zhu



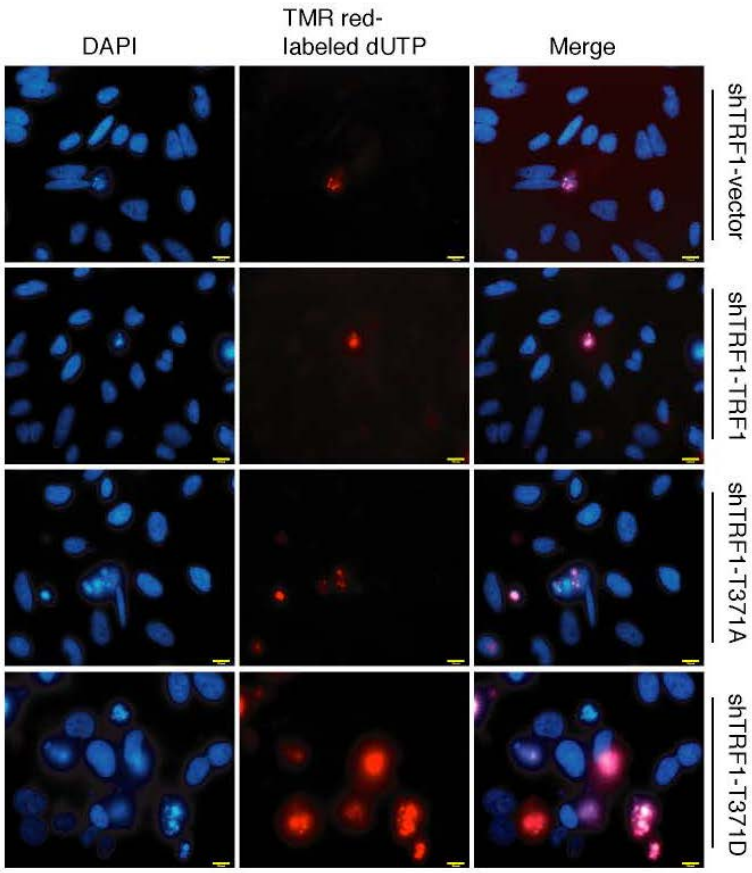
Supplementary Figure S5. Depletion of TRF1 leads to an accumulation of telomere doublets, telomere loss and sister telomere fusions. (**a-d**) Quantification of telomere abnormalities in HeLaII cells stably expressing either shTRF1/vector pWZL-N-myc or pRS/vector pWZL-N-myc. pWZL-N-myc is the vector used to express various Myc-tagged TRF1 proteins. For each cell line, a total of 69-71 metaphase cells were scored. Standard deviations derived from three independent experiments are indicated.

Supplementary Figure S6 McKerlie & Zhu



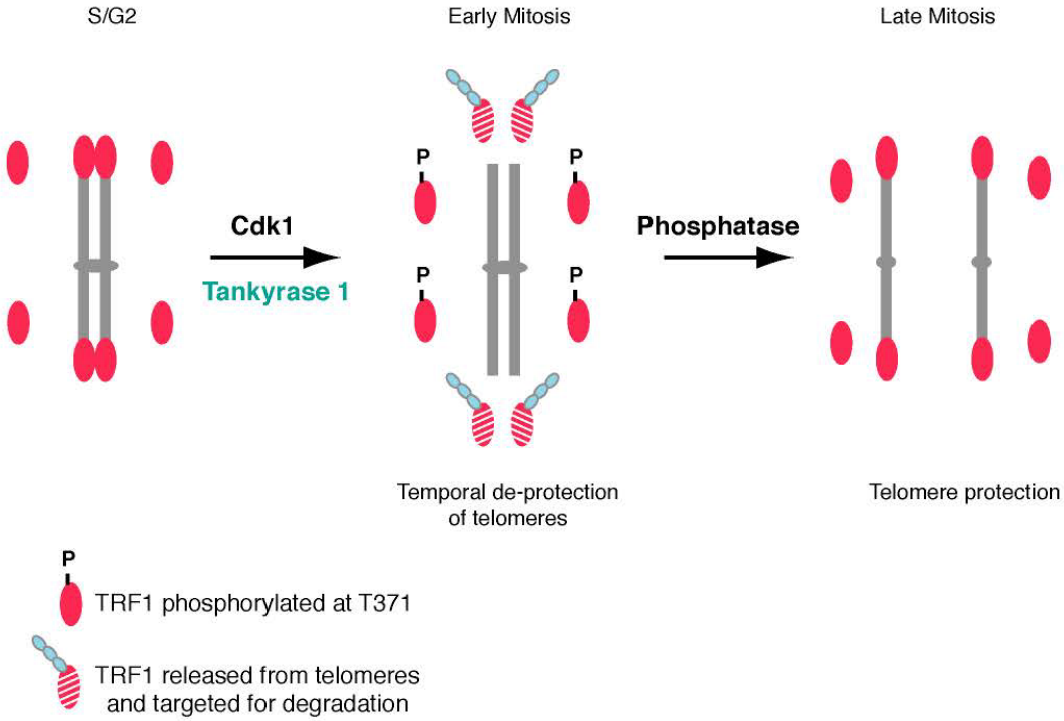
Supplementary Figure S6. Amino acid substitutions at position T371 of TRF1 induce telomere instability. Metaphase spreads of TRF1-depleted HeLaII cells expressing various constructs as indicated. Metaphase chromosomes were stained with DAPI and false-colored in red. Telomeric DNA was detected by FISH using a FITC-conjugated $(CCCTAA)_3$-containing PNA probe in green. Open arrows: telomere doublets. Asterisks represent telomere loss whereas filled arrows indicate sister telomere fusions.

Supplementary Figure S7 McKerlie & Zhu



Supplementary Figure S7. TUNEL assay. TRF1-depleted HeLaII cells expressing various constructs as indicated were stained with TMR-red, which distinguishes apoptotic cells, and counterstained with DAPI in blue.

Supplementary Figure S8 McKerlie & Zhu



Supplementary Figure S8. Model for the role of Cdk1 in controlling the resolution of sister telomeres. We propose that upon entry into mitosis, Cdk1 phosphorylates unbound TRF1 at T371 and this phosphorylation sequesters it from associating with telomeres. Such phosphorylation, perhaps in conjunction with the action of tankyrase 1, results in a net loss of TRF1 from telomeres, promoting the temporal de-protection of telomeres as well as the resolution of sister telomeres. In late mitosis, TRF1 undergoes dephosphorylation at T371 and this dephosphorylation allows re-association of TRF1 with telomeres and re-establishment of telomere protection.

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3.3 Complementary Data

3.3.1 (pT371)TRF1 Impacts Progression Through Mitotic Substages

We have observed that changes in TRF1 status, and mutating TRF1 at T371, results in an altered mitotic progression. We see that there are more cells in mitosis (by pS10 H3 staining) in cells expressing TRF1 T371D as compared to wild type TRF1, and fewer mitotic cells in cells expressing TRF1 T371A, as discussed in Section 3.2. To examine more closely the distribution of cells at different substages of mitosis, I evaluated DAPI staining (Figure 3.3.1). This revealed that, when mutating TRF1 T371 to mimic phosphorylation, there is a higher number of cells in anaphase as compared to both vector control cells and cells expressing wild type TRF1 (Figure 3.3.1D). There is also a significant decrease in cells at the telophase stage of mitosis in cells expressing TRF1 T371D, as compared to vector control and wild type TRF1 expressing cells (Figure 3.3.1E). This suggests that not only is the number of cells in mitosis altered by disrupting the regulation of TRF1 T371 phosphorylation, but the progression through mitosis is affected. The other TRF1 cell lines that I examined exhibited a similar pattern of mitotic distribution to one another, contrary to TRF1 T371D. This indicates that, even though the overall percentage of cells in mitosis may differ, for instance with the expression of TRF1 T371A, which prevents phosphorylation at T371, the progression from one substage to another within mitosis may not be affected.

Figure 3.3.1 (pT371)TRF1 impacts mitotic progression

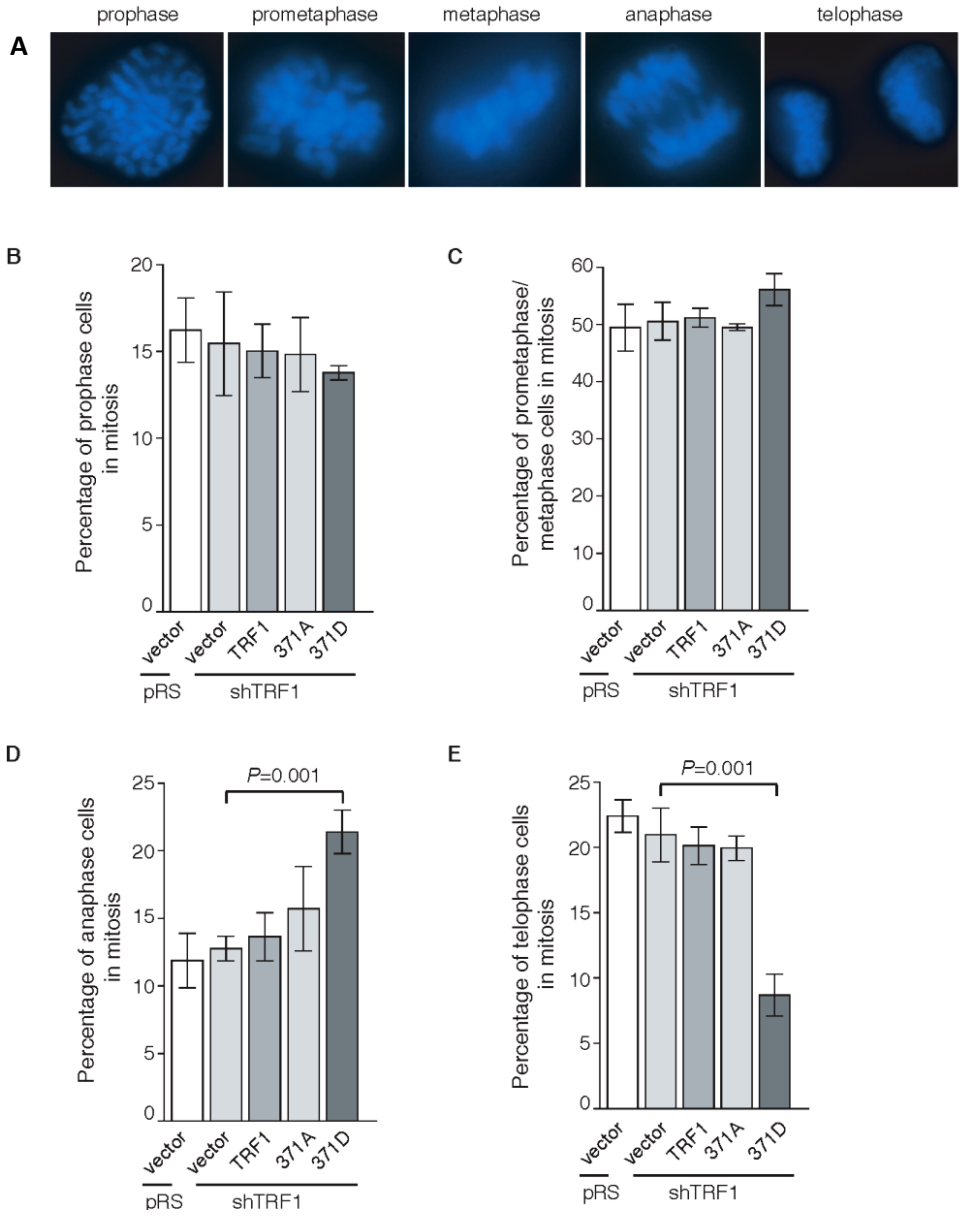

Figure 3.3.1 (pT371)TRF1 impacts mitotic progression A) An example of DAPI stained HeLaII cells illustrating the staining observed at each substage of mitosis. The depictions illustrate cells in prophase, prometaphase, metaphase, anaphase, and telophase, from left to right. Cells were fixed with a standard fixation protocol, blocked with PBG, and stained with DAPI for ten minutes. **B-E)** Quantification of the proportion of mitotic cells that were in **B)** prophase, **C)** prometaphase or metaphase, **D)** anaphase, or **E)** telophase, as determined visually with DAPI staining. HeLa II cells depleted for TRF1 and expressing the indicated TRF1 constructs were scored. This experiment was performed in blind, in triplicates. 500 cells were scored per replicate. Standard deviations are indicated. Where P-values are indicated, a two-tailed student's t-test was performed.

3.3.2 (pT371)TRF1 Colocalizes with γ-tubulin in Mitosis

Indirect immunofluorescence with anti-pT371 TRF1 and anti-γ-tubulin antibodies in mitotic cells revealed that pT371 TRF1 goes to sites that are consistent with centrosome localization in mitosis (Figure 3.3.2). The bright spots which form at poles symmetrically opposing one another, separated by the metaphase place, and distinguished by γ-tubulin staining, are centrosomes. This localization is consistent with TRF1 being an important player in mitotic progression, as many proteins involved in mitosis are found at centrosomes. To name a few, Cdk1, Plk1, MAPK, NuMa, Casein kinase 1, PP2A, BRCA1, and Aurora A kinase are all found at centrosomes (Schatten 2008). Centrosomes are an important docking site for proteins involved in mitotic progression, and serve as a central scaffold for their various functions. The staining pattern observed with anti-pT371 TRF1 would be consistent with the presence of TRF1 at these sites, and an additional indicator that TRF1 plays an important role in regulating mitotic progression. A similar colocalization can also be observed with pT371 TRF1 and α-tubulin. The localization of TRF1 at these sites coincides with the model presented in Section 3.2 that suggests, in mitosis, TRF1 is released from telomeres and is stabilized in this form. This data provides a possible alternative localization for TRF1 at this time; at centrosomes, which would require further confirmation. TRF1 has been previously shown to localize at mitotic spindles, as reviewed in Chapter 1, and this data provides a suggestion as to how this localization may be regulated.

Figure 3.3.2 (pT371)TRF1 staining overlaps with γ-tubulin staining in mitosis

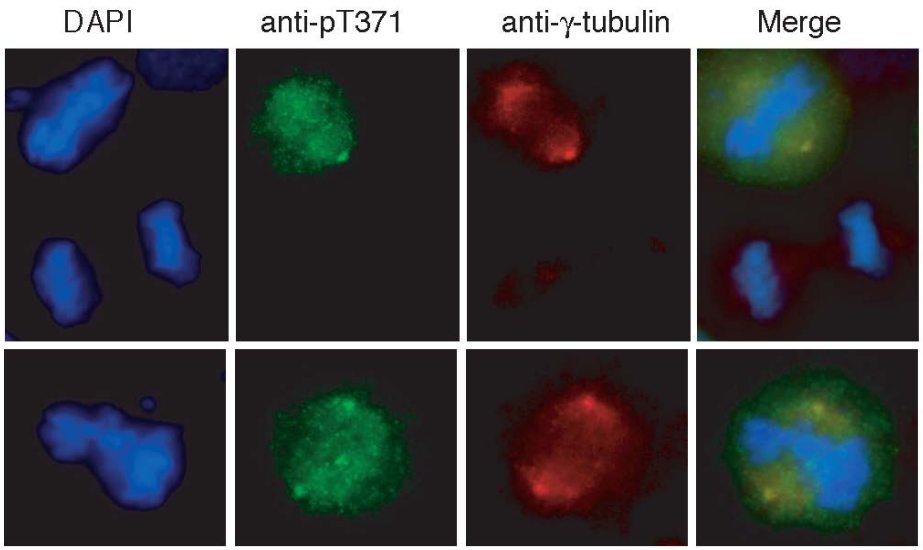

Figure 3.3.2 (pT371)TRF1 staining overlaps with γ-tubulin staining in mitosis. Indirect immunofluorescence on HeLaII cells stained with anti-pT371 TRF1, which is depicted in green, and anti-γ-tubulin, depicted in red. DNA is stained with DAPI. Cells were fixed with a standard fixation protocol. Examples of cells in different stages of mitosis are depicted. Merged images of all three layers are shown.

Chapter 4

Phosphorylated (pT371)TRF1 is Recruited to Sites of DNA Damage to Facilitate Homologous Recombination and Checkpoint Activation

Megan McKerlie, John R. Walker, Taylor R.H. Mitchell, Florence Wilson, and Xu-Dong Zhu

4.1 Introduction

4.1.1 Publication

The work presented in this chapter has been accepted for publication in Nucleic Acids Research.

4.1.2 Contribution

I performed all of the experiments included within this publication. John R. Walker, Taylor R.H. Mitchell, and Florence Wilson were responsible for generating knockdown (shRNA) constructs as well as expression constructs for various Nbs1 alleles. Xu-Dong Zhu, John R. Walker, and I conceived and designed the experiments and wrote the manuscript.

4.1.3 Context and Significance

The work included within this manuscript serves to further expand on the multiple functions of TRF1 and the role that post-translational modifications, specifically phosphorylation, may play in regulating these functions. This work demonstrates a function for the phosphorylation of T371 TRF1 in interphase cells. This builds on the information presented in Chapter 3, which describes a role for (pT371)TRF1 in mitotic progression. This contributes to the broader understanding of TRF1 function, and demonstrates the importance of phosphorylation in regulating TRF1 function.

4.2 Manuscript: Phosphorylated (pT371)TRF1 is Recruited to Sites of DNA Damage to Facilitate Homologous Recombination and Checkpoint Activation; Accepted for publication in *Nucleic Acids Research.*

Phosphorylated (pT371)TRF1 is recruited to sites of DNA damage to facilitate homologous recombination and checkpoint activation

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4.2.1 ABSTRACT

TRF1, a duplex telomeric DNA binding protein, plays an important role in telomere metabolism. We have previously reported that a fraction of endogenous TRF1 can stably exist free of telomere chromatin when it is phosphorylated at T371 by Cdk1, however the role of this telomere-free (pT371)TRF1 has yet to be fully characterized. Here we show that phosphorylated (pT371)TRF1 is recruited to sites of DNA damage, forming damage-induced foci in response to ionizing radiation, etoposide and camptothecin. We find that IR-induced (pT371)TRF1 foci formation is dependent upon the ATM- and Mre11/Rad50/Nbs1-mediated DNA damage response. While loss of functional BRCA1 impairs the formation of IR-induced (pT371)TRF1 foci, depletion of either 53BP1 or Rif1 stimulates IR-induced (pT371)TRF1 foci formation. In addition, we show that TRF1 depletion or the lack of its phosphorylation at T371 impairs DNA end resection and repair of non-telomeric DNA double strand breaks by homologous recombination. The lack of TRF1 phosphorylation at T371 also hampers the activation of G2/M checkpoint and sensitizes cells to PARP inhibition, ionizing radiation and camptothecin. Collectively, these results reveal a novel but important function of phosphorylated (pT371)TRF1 in facilitating DNA double strand break repair and in the maintenance of genome integrity.

4.2.2 INTRODUCTION

DNA double strand breaks (DSBs), a lethal form of DNA damage, can promote tumorigenesis if not repaired properly. Sensing of DSBs is mediated by ATM, a PI-3 kinase which transduces the DNA damage signal through phosphorylation of many proteins essential for the activation of the DNA damage checkpoint, cell cycle arrest, DNA repair or apoptosis (1,2). Following the induction of DSBs, γH2AX, resulting from the phosphorylation of histone variant H2AX at serine 139 by ATM (3,4), marks damaged chromatin and directs the recruitment of many DNA damage signaling and DNA repair proteins into repair centers, also known as "foci" (2,5).

Repair of DSBs is mediated by two major repair pathways: nonhomologous end joining (NHEJ) and homologous recombination (HR) (5). NHEJ, error-prone, can ligate two broken ends in the absence of sequence homology whereas HR, largely error-free, requires sequence homology and is often restricted to the S and G2 phases of the cell cycle during which sister chromatids are present. An error in the choice of the DNA DSB repair pathway can lead to genomic instability. The tumor suppressor proteins 53BP1 and BRCA1 have been shown to play pivotal roles in influencing the fate of the repair of DSBs by either NHEJ or HR (5). While 53BP1 is found to inhibit HR and to promote NHEJ, BRCA1 antagonizes 53BP1 at DSBs, allowing HR to proceed (6-9). BRCA1 is thought to facilitate DNA end resection (6), an early step of HR marked by the generation of RPA-coated single-stranded DNA (RPA-ssDNA).

TRF1, a duplex telomeric DNA binding protein (10), is a component of the six-subunit shelterin complex essential for maintaining telomere length and integrity (11). TRF1 is best known for its role in telomere metabolism (11), but it has also been found to interact with proteins involved in the DNA damage response, such as ATM (12,13) and Mre11/Rad50/Nbs1 (14). Whether and how TRF1 may play a role in the DNA damage response and DNA repair is poorly understood. TRF1 is predominantly found at human

telomeres (15), however, a fraction of endogenous TRF1 can also stably exist free of telomere chromatin in the nucleus (16). We have previously reported that TRF1 is phosphorylated at T371 by Cdk1 and that this phosphorylation keeps TRF1 free of telomere chromatin and protects it from proteasome-mediated protein degradation (16). While T371 phosphorylation is up regulated in mitosis to facilitate the separation of sister telomeres (16), a low level of phosphorylated (pT371)TRF1 is also detected in interphase cells (16). However the role of this unbound (pT371)TRF1 in interphase has yet to be characterized.

In this report, using a phospho-specific anti-pT371 antibody, we have shown that telomere-free phosphorylated (pT371)TRF1 forms damage-induced foci in response to ionizing radiation (IR), camptothecin and etoposide, indicative of its association with DSBs. We have shown that inhibition of Cdk activity severely impairs the formation of IR-induced (pT371)TRF1 foci, consistent with our previous finding that Cdk1 phosphorylates TRF1 at T371 (16). We have demonstrated that an amino acid substitution abrogating TRF1 binding to telomeric DNA stimulates the recruitment of exogenously-expressed Myc-tagged TRF1 to sites of DNA damage in a manner dependent upon T371 phosphorylation, further supporting the notion that it is telomere-free phosphorylated (pT371)TRF1 that is recruited to sites of DNA damage. We have found that the recruitment of phosphorylated (pT371)TRF1 to sites of DNA damage requires the ATM- and Mre11/Rad50/Nbs1-dependent DNA damage response. While the formation of IR-induced (pT371)TRF1 foci is impaired by loss or depletion of BRCA1, it is stimulated by knockdown of 53BP1 or its downstream effector Rif1 (17-21). Furthermore, we have demonstrated that phosphorylated (pT371)TRF1 not only facilitates DNA end resection and homologous recombination, but also activates the G2/M checkpoint and confers cell survival following the induction of DSBs. Taken together, these results have uncovered an important role of phosphorylated (pT371)TRF1 in DNA double strand break repair.

4.2.3 MATERIALS AND METHODS

Plasmids and antibodies

Expression constructs for shTRF1 and various TRF1 mutant alleles (T371A, T371D, R425V) have been previously described (16). Wild type Nbs1 was cloned into pLPC retroviral vector with a Myc epitope tag replacing the start codon. Nbs1 deletion constructs were generated through PCR using wild type Nbs1 as a template. The sequence of primers for cloning Nbs1 deletion alleles will be made available upon request. The annealed oligonucleotides encoding small interfering RNAs previously described for ATM (22), BRCA1 (23), and 53BP1 (24) were ligated into the pRetroSuper vector, giving rise to knockdown expression constructs used in this study. Small interference RNA against Rif1 was a generous gift from Daniel Durocher, Samuel Lunenfeld Research Institute.

Phospho-specific anti-pT371-TRF1 has been previously described (16). Other antibodies used include TRF1 (a gift from Titia de Lange, Rockefeller University); Nbs1 (kindly provided by John Petrini, Memorial Sloan-Kettering Cancer Center); Rif1 (kindly provided by Daniel Durocher, Samuel Lunenfeld Research Institute); BRCA1 (MS110, Abcam); RPA32 (9H8, Abcam); Chk1-pS317 (Bethyl); RPA32-pS4/pS8 (Bethyl); ATM-pS1981 (Cell Signaling); H3-pS10 (Cell Signaling); Chk1 (FL-476, Santa Cruz); γ-H2AX (Milipore); 53BP1 (BD Biosciences); ATM (Novus) and γ-tubulin (GTU88, Sigma).

Cell Culture and Treatments

Cells were grown in DMEM medium with 10% fetal bovine serum supplemented with non-essential amino acids, L-glutamine, 100 U/ml penicilin and 0.1 mg/ml streptomycin. HT1080, MCF7 and HCC1937 are cell lines from ATCC. GM637, GM16666 (AT-deficient) and GM16667 (ATM-complemented) are cell lines from Coriell. Pheonix cells, NBS-ILB1

and two subclones of HeLa cells (HeLaI.2.11 and HeLaII) were gifts from Titia de Lange, Rockefeller University. Retroviral gene delivery was carried out as described (16,25).

To inhibit Cdk1 activity, 20 μM Roscovitine (Sigma) or 2 μM CGP74541A (Sigma) was added to cells 4 hr prior to IR treatment. For inhibition of ATM or Mre11, cells were treated with either KU55933 (20 μM, Sigma) or Mirin (50 μM, Sigma) for 1 hr before IR treatment. For damage induction, cells were treated with etoposide (1 μM, Sigma) or camptothecin (1 μM, Sigma) for 1 hr. Ionizing radiation was delivered from a Cs-137 source at McMaster University (Gammacell 1000).

Differential Salt Extraction of Chromatin and Immunoblotting

Protein extracts, differential salt extraction of chromatin and immunoblotting were performed as described (16,25). For IR-treated cells, protein extracts were in general prepared 8 hr post IR except where specified.

Immunofluorescence and Fluorescence *in situ* hybridization

Immunofluorescence was performed as described (25,26). In brief, cells seeded on coverslips were fixed with 3% paraformaldehyde/2% sucrose in PBS for 10 min at RT, followed by permeabilization with Triton X-100 buffer (0.5% Triton X-100, 20 mM Hepes-KOH (pH7.9), 50 mM NaCl, 3 mM $MgCl_2$ and 300 mM sucrose) for 10 min at RT. For IR-treated cells, cells were fixed in general 8 hr post IR except where specified. For each treatment or time point, a total of 500-1000 cells were scored in blind for a single experiment and a minimum of three independent experiments were performed.

Immunofluorescence (IF)-fluorescence *in situ* hybridization (FISH) analyses were conducted as described (27). Briefly, cells grown on coverslips were mock treated or treated with 12 Gy IR. Eight hours later, cells 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 (1 mg/ml bovine serum albumin, 3% goat serum, 0.1% Triton X-100, 1 mM EDTA in PBS). Blocked coverslips were incubated with anti-pT371 antibody (1:200) in blocking solution at RT for 2 h. Following three washes in PBS, coverslips were incubated with FITC-conjugated donkey anti-rabbit (1:100, Jackson Laboratories) at RT for 30 min. Subsequently, cells on coverslips were fixed again in PBS buffered 2% para-formaldehyde at RT for 5 min, followed by dehydration in a series of 70, 85 and 100% ethanol. The air-dried coverslips were denatured at 80^{o}C for 10 min and hybridized with Tamara-conjugated-$(TTAGGG)_3$ PNA probe (Biosynthesis Inc.) overnight in dark at 4^{o}C. Coverslips were then washed with 70% formamide and 10 mM Tris-HCl (pH7.2) 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 prepared as described (28). Analysis of chromosome aberration was carried out as described (29) with modifications. Cells were treated with 1 Gy IR and allowed to recover in the incubator for 1 hr prior to the addition of 100 ng/ml nocodazole. Following a 4 hr incubation in the presence of nocodazole, cells were collected by shake-off, incubated for 7 min at 37^{o}C in 75 mM KCl and then fixed in freshly-made methanol/acetic acid (3:1). On the following day, cells were dropped onto slides and air-dried overnight at RT in a chemical hood. The dried slides with chromosome spreads were subsequently processed according to the FISH protocol as described (27,28) except that the steps of the denaturation and the incubation with PNA probe were omitted. The slides were then counter-stained with 0.2 µg/ml DAPI and embedded in 90% glycerol/10% PBS containing 1 mg/ml *p*-

phenylenediamine (Sigma). All cell images were recorded on a Zeiss Axioplan 2 microscope with a Hammamatsu C4742-95 camera and processed in Open Lab.

DNA Recombinational Repair Assays

Three reporter plasmids pDR-GFP, pSA-GFP and pEGFP-Pem1-Ad2, generously provided by Eric Hendrickson, University of Minnesota, were used to assess homologous recombination, single-strand annealing and nonhomologous end joining, respectively, as described (30). In brief, lipofectamine LTX plus reagent (Invitrogen) was used to transfect cells with *I-Sce*I-expressing plasmid, pCherry and either pDR-GFP, pSA-GFP or pEGFP-Pem1-Ad2 in a ratio of 1:0.5:1 according to manufacture's instructions. Fourty-eight hours post transfection, cells were harvested and subjected to FACS analysis. The number of cells positive for both GFP and pCherry was normalized to the total number of pCherry-positive cells, giving rise to percentage of GFP-positive cells. For FACS analysis, cells were harvested, washed in 1X PBS and fixed in PBS-buffered 4% paraformaldehyde. FACS analysis was performed using a Becton-Dickinson LSRII located at the SickKids-UHN flow cytometry facility, Toronto, ON, Canada.

Clonogenic Survival and G2/M checkpoint assays

For clonogenic survival assays, 4 hr prior to camptothecin (CPT) treatment, HT1080 and TRF1-depeleted HeLaII cells stably expressing various TRF1 alleles were seeded in duplicates at 120 cells (0-50nM CPT) or 720 cells (100 nM CPT) per 6-cm plate. After 1 hr of CPT treatment, the drug was washed off with PBS and fresh growth medium was added. For IR treatment, cells were counted, irradiated, and seeded in duplicates at 120 cells (0-3 Gy) or at 720 cells (5 Gy) per 6-cm plate, followed by replacement with fresh media after 24 hr incubation. For PARP inhibitor treatment, 24 hr post seeding, cells were treated with Olaparib (Selleck Chemicals) and allowed to grow in the presence of Olaparib for the entirety of the experiments. Ten days later, colonies were fixed and

stained at RT for 10 min with a solution containing 50% methanol, 7% acetic acid and 0.1% Coomassie blue. Colonies consisting of more than 32 cells were scored.

For the G2/M checkpoint assay, cells seeded on coverslips were treated with 12 Gy IR and allowed to recover in the incubator. Following 1 hr incubation, cells were gently washed with PBS, fixed with paraformaldehyde and then processed for immunofluorescence with anti-H3-pS10 antibody.

Statistical Analysis

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

4.2.4 RESULTS

Phosphorylated (pT371)TRF1 is recruited to sites of DNA double strand breaks.

Previously we have reported that Cdk1 phosphorylates T371 of TRF1 and that this phosphorylation prevents TRF1 from associating with telomeric DNA both *in vivo* and *in vitro* (16). To investigate the biological function of TRF1 phosphorylation at T371 in interphase, we treated HeLaII cells with ionizing radiation, followed by analysis of differential salt extraction of chromatin with our previously-characterized phospho-specific anti-pT371 antibody (16). We found that treatment with 12 Gy IR had little effect on the chromatin association of total TRF1 (Fig. 1A), which was found mainly in the chromatin-bound fraction (Fig. 1A), consistent with previous findings (16). In contrast, we observed a pronounced shift of phosphorylated (pT371)TRF1 from the chromatin-free fraction to the chromatin-bound fraction following 12 Gy radiation (Fig. 1A), suggesting that phosphorylated (pT371)TRF1 may be associated with damaged chromatin.

To further investigate the damage-induced chromatin association of phosphorylated (pT371)TRF1, we performed indirect immunofluorescence with phospho-specific anti-pT371 antibody and found that phosphorylated (pT371)TRF1 formed IR-induced foci in a number of human cell lines including HeLa, GM637 and hTERT-BJ cells (Fig. 1B and Supplementary Fig. 1A and 1B), suggesting that IR-induced (pT371)TRF1 foci formation is not cell type specific. The formation of IR-induced (pT371)TRF1 foci was dose-dependent (Fig. 1C) and peaked eight hours post IR (Fig. 1D).

Analysis of IF-FISH with anti-pT371 antibody in conjunction with telomeric DNA-containing PNA probe revealed little overlap between IR-induced (pT371)TRF1 foci and human telomeres (Fig. 1E), suggesting that damage-induced chromatin association of (pT371)TRF1 is independent of the presence of telomeric DNA. On the other hand, we found that IR-induced (pT371)TRF1 foci merged well with IR-induced foci of 53BP1 (Fig. 1F) or γ-H2AX (Fig. 1G), both of which are known to accumulate at sites of DSBs (2). Aside from IR, we also observed damage-induced phosphorylated (pT371)TRF1 foci in cells treated with DSB-inducing agents such as camptothecin or etoposide (Supplementary Fig. 1C and 1D). Collectively, these results suggest that phosphorylated (pT371)TRF1 is recruited to sites of DSBs.

To address the possiblity that our phospho-specific anti-pT371 antibody might cross react with other DNA damage response factors, we depleted endogenous TRF1 in HeLaII cells (Fig. 2A) and then complemented TRF1-depleted HeLaII cells with Myc-tagged shTRF1-resistant wild type TRF1 or TRF1 carrying a nonphosphorylatable T371A mutation (Fig. 2B). We found that TRF1 depletion significantly impaired the formation of IR-induced (pT371)TRF1 foci (Fig. 2C and 2D). While the introduction of Myc-tagged wild type TRF1 was able to rescue the formation of IR-induced (pT371)TRF1 foci in TRF1-depleted HeLaII cells (Fig. 2C and 2D), no rescue in the formation of these foci was observed when TRF1-depleted HeLaII cells were complemented with Myc-tagged TRF1

carrying the nonphosphorylatable T371A mutation (Fig. 2C and 2D). The level of expression of Myc-tagged TRF1-T371A was indistinguishable from that of Myc-tagged wild type TRF1 (Fig. 2B). These results argue against the possibility that the observed IR-induced (pT371)TRF1 foci are due to a cross-reactivity of the phospho-specific anti-pT371 antibody with other DNA damage response proteins.

To further demonstrate the association of phosphorylated (pT371)TRF1 with damaged DNA, we examined the ability of exogenously expressed Myc-tagged wild type TRF1, TRF1 carrying a nonphosphorylatable T371A mutation, or TRF1 with a phosphomimic T371D mutation to form IR-induced foci. Analysis of indirect immunofluorescence with anti-Myc antibody revealed that no IR-induced anti-Myc-containing foci was observed for Myc-TRF1-T371A-expressing HT1080 cells whereas at least 9% of HT1080 cells overexpressing Myc-TRF1-T371D exhibited IR-induced anti-Myc-containing foci (Fig. 3A and 3B). Less than 2% of HT1080 cells overexpressing Myc-tagged wild type TRF1 exhibited IR-induced anti-Myc containing foci (Fig. 3B), indicating that Myc-tagged TRF1 was also able to form IR-induced foci albeit at a much lower frequency than Myc-TRF1-T371D. Previously we have reported that 1-5% of endogenous TRF1 in cells is phosphorylated at T371 (16). The low abundance of T371 phosphorylation may in part account for the observed low frequency of IR-induced Myc-TRF1-containing foci. Futhermore, analysis of differential salt extraction of chromatin revealed that while Myc-tagged TRF1-T371D was predominantly found in the chromatin-free fraction of untreated cells (Fig. 3C), consistent with our previous finding (16), it became largely associated with chromatin following ionizing radiation (Fig. 3C). Taken together, these results suggest that phosphorylation at T371 directs TRF1 to sites of DNA damage. In support of this notion, we also found that the treatment of HeLaII cells with roscovitine or a second selective Cdk1 inhibitor CGP74541A significantly impaired the formation of IR-induced (pT371)TRF1 foci (Fig. 3D and 3E), consistent with our previously finding that

T371 is a target of Cdk1 (16). These results suggest that phosphorylation of T371 by Cdk1 is required for the association of TRF1 with damaged DNA.

Being telomere free is necessary for (pT371)TRF1 recruitment to sites of DNA damage.

TRF1 is a duplex telomeric DNA binding protein (10) and therefore we decided to investigate whether binding to telomeric DNA might affect TRF1 association with DSBs. Previously we and others have reported that a single amino acid substitution of R425V in the Myb-like DNA binding domain of TRF1 completely abrogates the ability of TRF1 to bind telomeric DNA both *in vivo* and *in vitro* (16,32). We therefore generated human fibroblastoma HT1080 cell lines stably expressing Myc-tagged wild type TRF1 or Myc-tagged TRF1-R425V. We found that Myc-tagged TRF1-R425V was able to form IR-induced damaged foci that colocalized with not only endogenous (pT371)TRF1 (Fig. 3A) but also γH2AX (Fig. 3F). In addition, we observed a six-fold induction of IR-induced anti-Myc containing foci in Myc-TRF1-R425V-expressing cells compared to Myc-TRF1-expressing cells (Fig. 3A and 3B). Furthermore, analysis of differential salt extraction of chromatin revealed that Myc-TRF1-R425V was predominantly found in the chromatin-free fraction in untreated cells (Fig. 3C), consistent with previous findings that it is defective in binding to telomeric DNA (16,32), however, a substantial amount of it became associated with chromatin following IR treatment (Fig. 3C). Introduction of a nonphosphorylatable T371A mutation into Myc-TRF1-R425V completely abrogated not only its ability to form IR-induced foci (Fig. 3A and 3B) but also its IR-induced chromatin association (Fig. 3C). On the other hand, a phosphomimic T371D mutation had little effect on the ability of Myc-TRF1-R425 to form IR-induced foci (Fig. 3A and 3B), nor did it affect its IR-induced chromatin association (Fig. 3C). Taken together, these results suggest that phosphorylation of T371 directs telomere-free TRF1 to sites of DNA damage.

(pT371)TRF1 recruitment to sites of DNA double strand breaks requires the ATM- and Mre11/Rad50/Nbs1-dependent DNA damage response.

Upon the induction of DSBs, a number of DNA damage response factors are recruited to sites of DNA damage including ATM, γH2AX, Mre11/Rad50/Nbs1, BRCA1 and 53BP1. Recruitment of these proteins to sites of DSBs following ionizing radiation was not affected by depletion of TRF1 or overexpression of TRF1 carrying a nonphosphorylatable T371A mutation (Supplementary Figure 6), suggesting that T371 phosphorylation is not involved in sensing DSBs. On the other hand, we found that treatment of cells with KU55933, a specific inhibitor of ATM, completely abrogated the ability of (pT371)TRF1 to form IR-induced foci (Fig. 4A and 4B). The defect in the ability of (pT371)TRF1 to form IR-induced foci was also observed in HeLaII cells knocked down for ATM (Supplementary Fig. 2A and 2B) as well as in cells derived from an AT patient (Supplementary Fig. 2D and 2E). Inhibition, knockdown or loss of ATM had little effect on the level of T371 phosphorylation (Fig. 4C and Supplementary Fig. 2C and 2F). These results suggest that ATM is required for the recruitment of (pT371)TRF1 to DSBs.

It has been reported that the Mre11/Rad50/Nbs1 complex is required for ATM activation following the induction of DSBs (33,34). Therefore we also examined the role of the Mre11/Rad50/Nbs1 complex in recruiting (pT371)TRF1 to DSBs. We found that treatment of HeLa cells with Mirin, a specific inhibitor of Mre11 (35), impaired IR-induced ATM-pS1981 foci formation (Supplementary Fig. 3A and 3B), in agreement with the previous finding that Mirin prevents ATM autophosphorylation on S1981 (35). Analysis of indirect immunofluorescence with anti-pT371 antibody revealed that treatment with Mirin also significantly impaired the ability of (pT371)TRF1 to form IR-induced foci (Fig. 4D and Supplementary Fig. 3C), although it had little effect on the level of T371 phosphorylation (Supplementary Fig. 3D). In addition, we found that the complementation of wild type Nbs1 into Nbs1-deficient cells led to at least a 3-fold

increase ($P<<0.001$) in the formation of IR-induced (pT371)TRF1 foci (Fig. 4E and Supplementary Fig. 4), suggesting that loss of Nbs1 impairs IR-induced (pT371)TRF1 foci formation.

The C-terminal 220 amino acids of Nbs1 has also been reported to bind TRF1 in a yeast two-hybrid assay (14). Nbs1 contains forkhead associated (FHA) and BRCT-repeat domains (Fig. 4F), both of which are known to bind phosphorylated threonine/serine. These findings prompted us to examine whether a particular domain(s) of Nbs1 might mediate the recruitment of (pT371)TRF1 to sites of DSBs. To address this question, we generated Myc-tagged Nbs1 lacking the FHA domain alone (Myc-Nbs1-ΔFHA), both FHA and BRCT domains (Myc-Nbs1-ΔFHA-ΔBRCT) or the C-terminal TRF1-interacting domain (Myc-Nbs1-ΔC) (Fig. 4F). These alleles were examined for their ability to rescue IR-induced (pT371)TRF1 foci in Nbs1-deficient cells. We found that while full-length Nbs1 rescued the formation of IR-induced (pT371)TRF1 foci in Nbs1-deficient cells (Fig. 4E and Supplementary Fig. 4), Nbs1 lacking either the N-terminal FHA domain (Myc-Nbs1-ΔFHA) or the C-terminal domain (Myc-Nbs1-ΔC) failed to rescue IR-induced (pT371)TRF1 foci in Nbs1-deficient cells (Fig. 4E and Supplementary Fig. 4). The inability of Myc-Nbs1-ΔFHA and Myc-Nbs1-ΔC to rescue IR-induced (pT371)TRF1 foci was unlikely due to a lack of their expression (Fig. 4G). Deletion of the C-terminal domain of Nbs1 also failed to activate ATM as evidenced by the lack of IR-induced ATM phosphorylation at S1981 (Fig. 4G). Expression of full-length or various Nbs1 deletion alleles had little effect on the level of TRF1 phosphorylation at T371 (Fig. 4G). Together, these results suggest that the ATM- and Mre11/Rad50/Nbs1-mediated DNA damage response is required for (pT371)TRF1 recruitment to sites of DSBs. These results further imply that multiple domains of Nbs1 may mediate the recruitment of (pT371)TRF1 to sites of DNA damage.

BRCA1 and 53BP1 play opposing roles in recruiting (pT371)TRF1 to DSBs.

Analysis of indirect immunofluorescence with anti-pT371 antibody revealed that while the breast cancer cell line MCF7 expressing wild type BRCA1 was competent in forming IR-induced (pT371)TRF1 foci (Fig. 5A and 5B), little formation of IR-induced (pT371)TRF1 foci was observed in the breast cancer HCC1937 cells expressing truncated BRCA1 lacking its C-terminal BRCT domain (Fig. 5A and 5B). The level of T371 phosphorylation in HCC1937 cells was comparable to that in MCF7 cells (Fig. 5C). In addition, the defect of (pT371)TRF1 in forming IR-induced foci was also detected in HeLaII cells depleted for BRCA1 (Fig. 5D and 5E). BRCA1 knockdown did not affect the level of T371 phosphorylation (Fig. 5F). These results suggest that BRCA1 is required for (pT371)TRF1 recruitment to sites of DSBs.

BRCA1 is a tumor suppressor protein that facilitates repair of DSBs by homologous recombination (HR) and its action is antagonized by 53BP1 (6-9). Therefore we also examined the role of 53BP1 in the formation of IR-induced (pT371)TRF1 foci. We found that depletion of 53BP1 with either sh53BP1-1 or sh53BP1-2, two independent shRNA against 53BP1 (24), stimulated the formation of IR-induced (pT371)TRF1 foci (Fig. 5D and 5E) and this stimulation was also observed in cells knocked down for Rif1, an effector of 53BP1 (17,19,20) (Fig. 5G). Knockdown of either 53BP1 or Rif1 had little effect on the level of TRF1 or (pT371)TRF1 (Fig. 5H and 5I). These results suggest that 53BP1 and Rif1 act as antagonists of (pT371)TRF1 recruitment to DSBs.

T371 phosphorylation of TRF1 facilitates DNA end resection and homologous recombination.

We have shown that BRCA1 and 53BP1 play opposing roles in the formation of IR-induced (pT371)TRF1 foci, suggesting a role for (pT371)TRF1 in DSB repair. It has been reported that HR is a slow repair process taking 7 hr or longer to complete, whereas NHEJ can be completed in about 30 min following the induction of DSBs (36). Our earlier finding that IR-induced (pT371)TRF1 foci formation peaked 8 hr post IR suggests that

(pT371)TRF1 may be involved in HR-mediated DNA repair. To investigate this possibility, we examined the formation of IR-induced RPA32-pS4/pS8 foci, a readout commonly used for DNA end resection (37,38), in TRF1-depleted HeLaII cells expressing wild type TRF1 or TRF1 carrying either a nonphosphorylatable or a phosphomimic mutation at T371. We found that depletion of TRF1 or overexpression of various TRF1 alleles did not significantly alter the percentage of cells in S and G2 phases as measured by cyclin A staining (Fig. 6A and 6B). Analysis of dual indirect immunoflorescence with anti-RPA32-pS4/pS8 antibody in conjunction with anti-cyclin A antibody revealed that knockdown of TRF1 led to a signficant reduction in the number of cyclin A-positive cells exhibiting IR-induced RPA32-pS4/S8 foci (Fig. 6A and 6C). IR-induced RPA foci in TRF1-depleted cells were rescued by overexpression of shTRF1-resistant wild type TRF1 or TRF1 carrying a T371D mutation (Fig. 6A and 6C). On the other hand, no rescue for IR-induced RPA32-pS4/S8 foci was observed in TRF1-depleted cells overexpressing TRF1-T371A (Fig. 6A and 6C). Western analysis revealed that depletion of TRF1 resulted in a loss of IR-induced phosphorylation of S4/S8 of RPA32 (Fig. 6D). This loss was suppressed by shTRF1-resistant wild type TRF1 or TRF1 carrying the phosphomimic T371D mutation (Fig. 6D). In contrast, TRF1 carrying the T371A mutation failed to restore S4/S8 phosphorylation of RPA32 in TRF1-depleted cells (Fig. 6D). Taken together, these results suggest that TRF1 phosphorylation at T371 plays an important role in facilitating DNA end resection.

To further investigate the role of (pT371)TRF1 in homologous recombination, we employed three well-established reporter plasmids; pDR-GFP (39), pSA-GFP (40) and pEGFP-Pem1-Ad2 (41). These reporter plasmids contain GFP disrupted by the insertion of an I-SceI site. HR-mediated repair of I-SceI-induced DSBs restores GFP expression in pDR-GFP whereas repair of I-SceI-induced DSBs by single-strand annealing (SSA), a subpathway of HR, restores GFP expression in pSA-GFP. On the other hand, NHEJ-mediated repair of I-SceI-induced DSBs restores GFP expression in pEGFP-Pem1-Ad2. We found that depletion of TRF1 in HeLaII cells impaired HR- or SSA-mediated restoration of

GFP expression (Fig. 6E and 6F) whereas it had little effect on NHEJ-mediated restoration of GFP expression (Fig. 6G). We observed that when introduced into TRF1-depleted cells, shTRF1-resistant wild type TRF1 or TRF1 carrying a phosphomimic T371D mutation was able to rescue HR- or SSA-mediated restoration of GFP expression, whereas shTRF1-resistant TRF1 carrying a nonphosphorylatable T371A mutation failed to do so (Fig. 6E and 6F). Overexpression of wild type TRF1 or TRF1 carrying either a T371A or a T371D mutation had little effect on NHEJ-mediated restoration of GFP (Fig. 6G). Furthermore, we found that depletion of TRF1 sensitized HeLaII cells to Olaparib, a PARP inhibitor known to be toxic to HR-deficient cells (17) (Fig. 6H). This sensitivity was suppressed when TRF1-depleted HeLaII cells were complemented with shTRF1-resistant wild type TRF1 (Fig. 6H). In constrast, overexpression of shTRF1-resistant TRF1 carrying a mutation of T371A was not able to reverse the sensitivity of TRF1-depleted cells to Olaparib (Fig. 6H). Taken together, these results reveal that TRF1 phosphorylation at T371 promotes the repair of DSBs by homologous recombination.

TRF1 faciliates the activation of the G2/M checkpoint and the maintenance of genome integrity.

We found that depletion of TRF1 abrogated ATR-dependent phosphorylation of S317 of Chk1 following ionizing radiation (Fig. 6D). Introduction of wild type TRF1 or TRF1 carrying a phosphomimic T371D mutation was able to rescue IR-induced S317 phosphorylation of Chk1 in TRF1-depleted cells, whereas no rescue of IR-induced S317 phosphorylation was observed in TRF1-depleted cells complemented with TRF1 carrying a nonphosphorylatable T371A mutation (Fig. 6D). In addition, analysis of the mitotic index following ionizing radiation revealed that TRF1-depleted HeLaII cells overexpressing TRF1 carrying a nonphosphorylatable T371A mutation failed to undergo a G2/M arrest whereas the IR-induced G2/M arrest was observed in TRF1-depleted cells overexpressing wild type TRF1 or TRF1 carrying a phosphomimic T371D mutation (Fig.

7A). Overexpression of TRF1-T371A was also observed to mitigate the IR-induced G2/M arrest in HT1080 cells (Supplementary Fig. 5A). These results suggest that TRF1 phosphorylation at T371 is important for facilitating the activation of the ATR-Chk1-mediated G2/M checkpoint.

Analysis of metaphase chromosome spreads revealed that depletion of TRF1 promoted an accumulation of IR-induced chromosome/chromatid breaks and gaps (Fig. 7B and Supplementary Figure 7), in line with the notion that TRF1 facilitates DSB repair by HR. Overexpression of shTRF1-resistant wild type TRF1 or TRF1 carrying a phosphomimic T371D mutation was able to suppress the level of IR-induced chromosome/chromatid breaks and gaps in TRF1-depleted cells (Fig. 7B), which was not reduced when TRF1-depleted cells were complemented with TRF1 carrying the T371A mutation (Fig. 7B). Furthermore, we found that depletion of TRF1 increased the sensitivity of cells to IR and camptothecin (Fig. 7C and 7D), indicative of its role in promoting cell survival following the induction of DNA double strand breaks. The hypersensitivity of TRF1-depleted cells to IR or camptothecin was rescued by wild type TRF1 but not by TRF1 carrying a T371A mutation (Fig. 7C and 7D). The inability of TRF1 carrying a T371A mutation to facilitate cell survival was also observed in HT1080 cells treated with IR or camptothecin (Supplementary Fig. 5B and 5C). Expression of TRF1-T371A was comparable to that of wild type TRF1 or TRF1-T371D (Supplementary Fig. 5D). Together, these results demonstrate an important function of phosphorylated (pT371)TRF1 in maintaining genome integrity and promoting cell survival.

4.2.5 DISCUSSION

Many DNA repair proteins are found to be associated with human telomeres and participate in the maintenance of telomere length and integrity. However whether the process of DNA repair may involve telomere binding proteins has been poorly

understood. In this report, we have uncovered that TRF1 phosphorylated at T371 is recruited to sites of DNA damage and that this recruitment is inhibited by 53BP1 and Rif1 but it is dependent upon BRCA1 as well as the ATM- and MRN-mediated DNA damage response (Fig. 7E). We have demonstrated that phosphorylated (pT371)TRF1 facilitates DNA end resection and homologous recombination. Our data presented here reveal a novel and important function of phosphorylated (pT371)TRF1 in facilitating DNA double strand break repair and the maintenance of genome integrity.

Our finding that a nonphosphorylatable T371A mutation of TRF1 fails to rescue HR- and SSA-mediated repair of I-SceI-induced DSBs in both pDR-GFP and SSA-GFP reporter constructs demonstrates that phosphorylated (pT371)TRF1 is important for facilitating homologous recombination-mediated repair of non-telomeric DNA double strand breaks. This feature of TRF1 appears to be shared with its related proteins TRF2 and Tbf1. TRF2, another shelterin protein similar to TRF1 that also binds sequence-specifically to duplex telomeric DNA (42,43), has been reported to be recruited to sites of laser-induced DNA damage (44,45) and to promote homologous recombinational repair of non-telomeric DNA double strand breaks (46). Both TRF1 and TRF2 are related to the *Sacchromyces cerevisiae* Tbf1 protein, known as a yeast TTAGGG repeat-binding protein (47). Recently it has been reported that Tbf1 is recruited to sites of HO-induced DSBs and promotes end resection at these sites (48). Our finding that Cdk activity is required for the recruitment of (pT371)TRF1 to sites of DSBs raises an interesting question as to whether the role of TRF2 and Tbf1 in DSB repair may be regulated by Cdk activity.

Although TRF1 is predominantly located at telomeres (10), we have previously reported that a fraction of endogenous TRF1 can also stably exist free of telomeres in the nucleus and that this pool of TRF1, but not telomere-bound TRF1, is phosphorylated at T371 by Cdk1 (16). Several lines of evidence suggest that it is this telomere-free pool

of TRF1 that participates in DNA double strand break repair. Firstly, we did not detect any change in the level of telomere-bound TRF1 in response to ionizing radiation through both analysis of differential salt extraction (Fig. 1a) and chromatin immunoprecipitation (M. McKerlie and X.-D. Zhu, unpublished data), consistent with previous findings that DNA damage reagents do not release TRF1 from telomeres (49). Secondly, we have shown that overexpression of a mutant allele of TRF1 (TRF1-R425V) (16,32), which is defective in binding to telomeric DNA, promotes TRF1 recruitment to sites of DSBs, consistent with the notion that being telomere-free allows TRF1 to participate in DNA double strand break repair. Thirdly, we have shown that inhibition of the Cdk1 activity or the lack of TRF1 phosphorylation at T371 impairs TRF1 association with DSBs whereas a phosphomimic mutation at T371 supports its recruitment to sites of DSBs. These results suggest that TRF1 needs to be not only free of telomeric DNA, but also to be phosphorylated at T371 by Cdk1 in order to facilitate HR-mediated DSBs repair.

We have shown that (pT371)TRF1 recruitment to sites of DNA damage is dependent upon both the Mre11/Rad50/Nbs1 complex and BRCA1. TRF1 has been reported in a yeast two-hybrid assay to interact physically with Nbs1 (14), however we have not been able to detect a physical interaction between TRF1 and Nbs1 when both of them were tagged and overexpressed in 293T cells (T. R. H. Mitchell and X.-D. Zhu, unpublished data). It is possible that TRF1 interaction with Nbs1 may be very transient in cells. It has been reported that TRF1 interacts with BRCA1 in a manner dependent upon the Mre11/Rad50/Nbs1 complex (49). Whether coordination between Nbs1 and BRCA1 is needed to recruit (pT371)TRF1 to sites of DSBs requires further investigation.

BRCA1 and 53BP1 are known to compete with each other to direct the choice of DSB repair by either HR or NHEJ (5). Our finding that BRCA1 and 53BP1 play opposing roles in recruiting phosphorylated (pT371)TRF1 to sites of DSBs raises the question as to

whether (pT371)TRF1 might be involved in regulating the choice of DSB repair, although TRF1 depletion or the lack of its phosphorylation at T371 only impairs HR- or SSA-mediated DSB repair and has little impact on NHEJ-mediated DSB repair. We have shown that the lack of TRF1 phosphorylation at T371 impairs the formation of IR-induced RPA foci, suggesting that (pT371)TRF1 is important for facilitating DNA end resection needed for homologous recombination. How (pT371)TRF1 may facilitate DNA end resection requires further investigation.

HR is regulated by Cdks (37,38), the activity of which promotes efficient DNA end resection to generate RPA-coated ssDNA. Several DSB repair proteins, such as Nbs1 and CtIP, have been shown to be phosphorylated by Cdks and their phosphorylation either activates DNA end resection (37,38) or mediates the complex formation important for DNA end resection (50,51). Our work reveals TRF1 to be a new player in the regulation of DNA end resection by Cdk1, adding further complexity to this process essential for homologous recombination. Furthermore, we have shown that loss of phosphorylated (pT371)TRF1 sensitizes cells to Olaparib, a PARP inhibitor potent in killing HR-deficient cancer cells (17), raising the possibility that phosphorylated (pT371)TRF1 might be exploited for cancer therapeutics.

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AUTHOR CONTRIBUTIONS

M.M. performed the experiments. J.R.W., T.R.H.M., and F.R.W. were responsible for generating knockdown (shRNA) constructs as well as expression constructs for various Nbs1 alleles. M.M., J.R.W. and X.-D.Z. conceived and designed the experiments as well as wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

4.2.6 FIGURES and FIGURE LEGENDS

Figure 1 McKerlie et al.

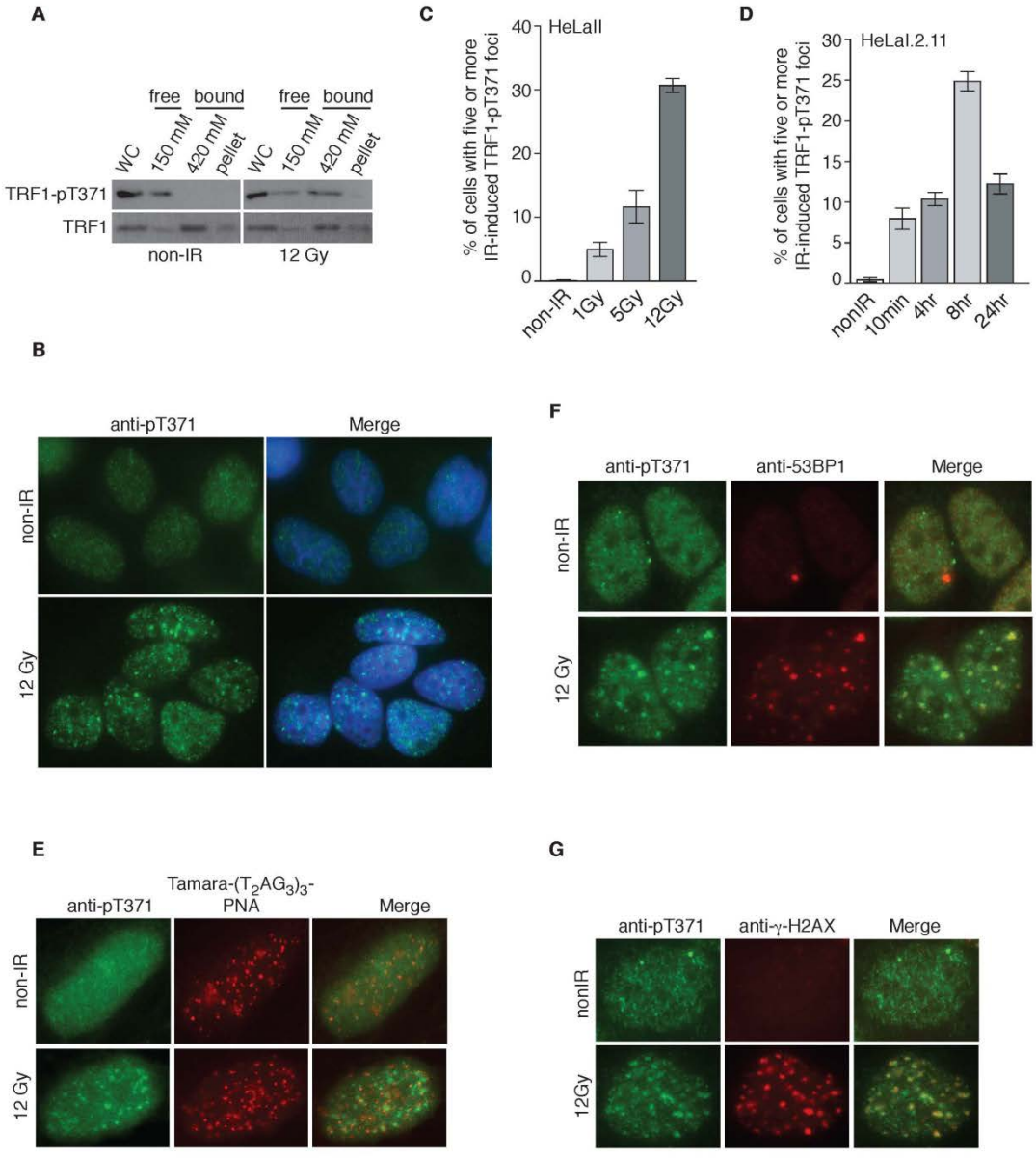


Figure 1. Phosphorylated (pT371)TRF1 forms IR-induced damage foci. (**A**) Analysis of differential salt extraction of chromatin of mock- or 12 Gy IR-treated HeLaII cells. Western analysis was performed with anti-pT371 or anti-TRF1 antibody. WC: whole cell lysate. (**B**) Indirect immunofluorescence with anti-pT371 antibody on HeLaII cells with or without 12 Gy IR treatment. Cell nuclei were stained with DAPI in blue. (**C**) Quantification of the percentage of HeLaII cells with five or more IR-induced (pT371)TRF1 foci. HeLaII cells were treated with a varying dose of IR as indicated. Standard deviation from three independent experiments are indicated. (**D**) Quantification of the percentage of HeLaI.2.11 cells with five or more IR-induced (pT371)TRF1 foci. HeLaI.2.11 were treated with 12 Gy IR and then fixed at various time points as indicated. Standard deviation from three independent experiments are indicated. (**E**) IF-FISH with anti-pT371 antibody (green) in conjunction with Tamara-conjugated-$(T_2AG_3)_3$ PNA probe (red). HeLaII cells were mock treated or treated with 12 Gy IR. (**F**) Indirect immunofluorescence with anti-pT371 in conjunction with anti-53BP1 antibody on HeLaII cells with or without IR treatment. Cell nuclei were stained with DAPI in blue. (**G**) Indirect immunofluorescence with anti-pT371 in conjunction with anti-γH2AX antibodies on HeLaII cells treated with or without IR treatment. Cell nuclei were stained with DAPI in blue.

Figure 2 McKerlie et al.

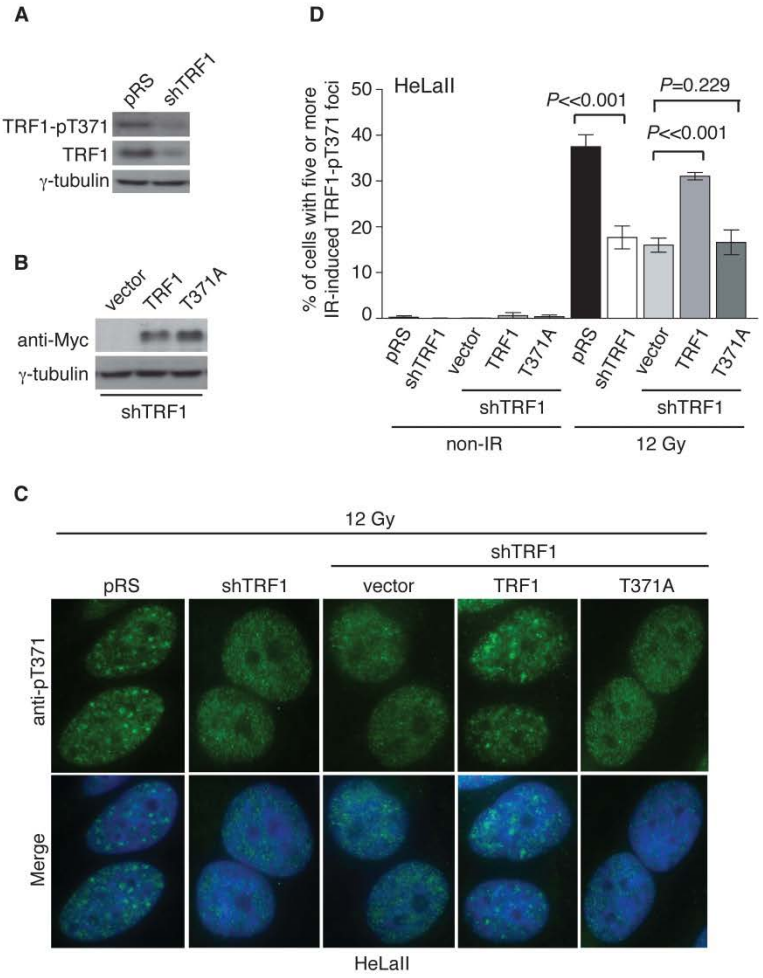


Figure 2. Depletion of TRF1 impairs the formation of IR-induced (pT371)TRF1 foci. (**A**) Western analysis of TRF1 depletion in HeLaII cells. Immunoblotting was performed with anti-pT371 or anti-TRF1 antibody. The γ-tubulin blot was used as a loading control in this and the following experiments. (**B**) Western analysis of shTRF1-resistant Myc-tagged wild type or mutant TRF1 proteins in TRF1-depleted HeLaII cells. (**C**) Indirect immunofluorescence with anti-pT371 antibody. HeLaII cells expressing various constructs as indicated were treated with 12 Gy IR. Cell nuclei were stained with DAPI in blue. (**D**) Quantification of the percentage of cells containing five or more IR-induced (pT371)TRF1 foci from (C). Standard deviations from six independent experiments are indicated.

Figure 3 McKerlie et al.

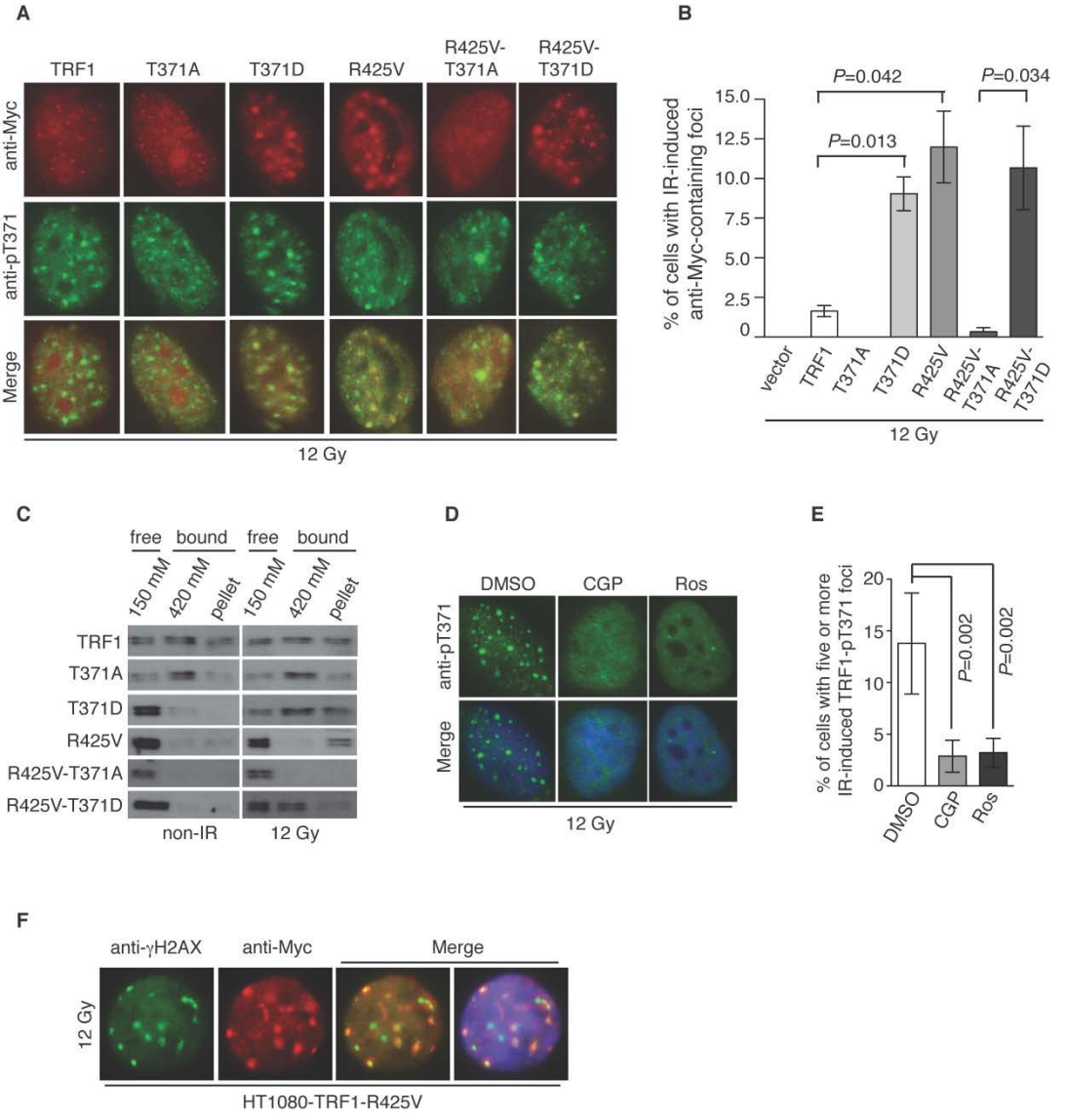


Figure 3. Phosphorylation of T371 by Cdk1 directs telomere-free TRF1 to sites of DSBs. (**A**) Indirect immunofluorescence with mouse anti-Myc antibody (red) in conjunction with rabbit anti-pT371 antibody (green). HT1080 cells stably expressing various Myc-tagged TRF1 alleles as indicated were treated with 12 Gy IR. (**B**) Quantification of the percentage of cells containing five or more IR-induced anti-Myc-containing foci from (A). Standard deviations from three independent experiments are indicated. (**C**) Analysis of differential salt extraction of chromatin. HT1080 cells stably expressing various Myc-tagged TRF1 alleles as indicated were mock treated or treated with 12 Gy IR. Immunoblotting was performed with anti-Myc antibody. (**D**) Indirect immunofluorescence with anti-pT371 antibody. HeLaII cells pretreated with DMSO, CGP74514A (CGP) or Roscovitine (Ros) were subjected to IR and fixed 8 hr post IR. Cell nuclei were stained with DAPI in blue. (**E**) Quantification of the percentage of cells with five or more IR-induced (pT371)TRF1 foci from (D). Standard deviations from at least three independent experiments are indicated. (**F**) Indirect immunofluorescence with rabbit anti-γH2AX antibody in conjunction with mouse anti-Myc antibody. HT1080 cells overexpressing Myc-tagged TRF1 carrying the R425V mutation were treated with 12 Gy IR and fixed 8 hr post IR.

Figure 4 McKerlie et al.

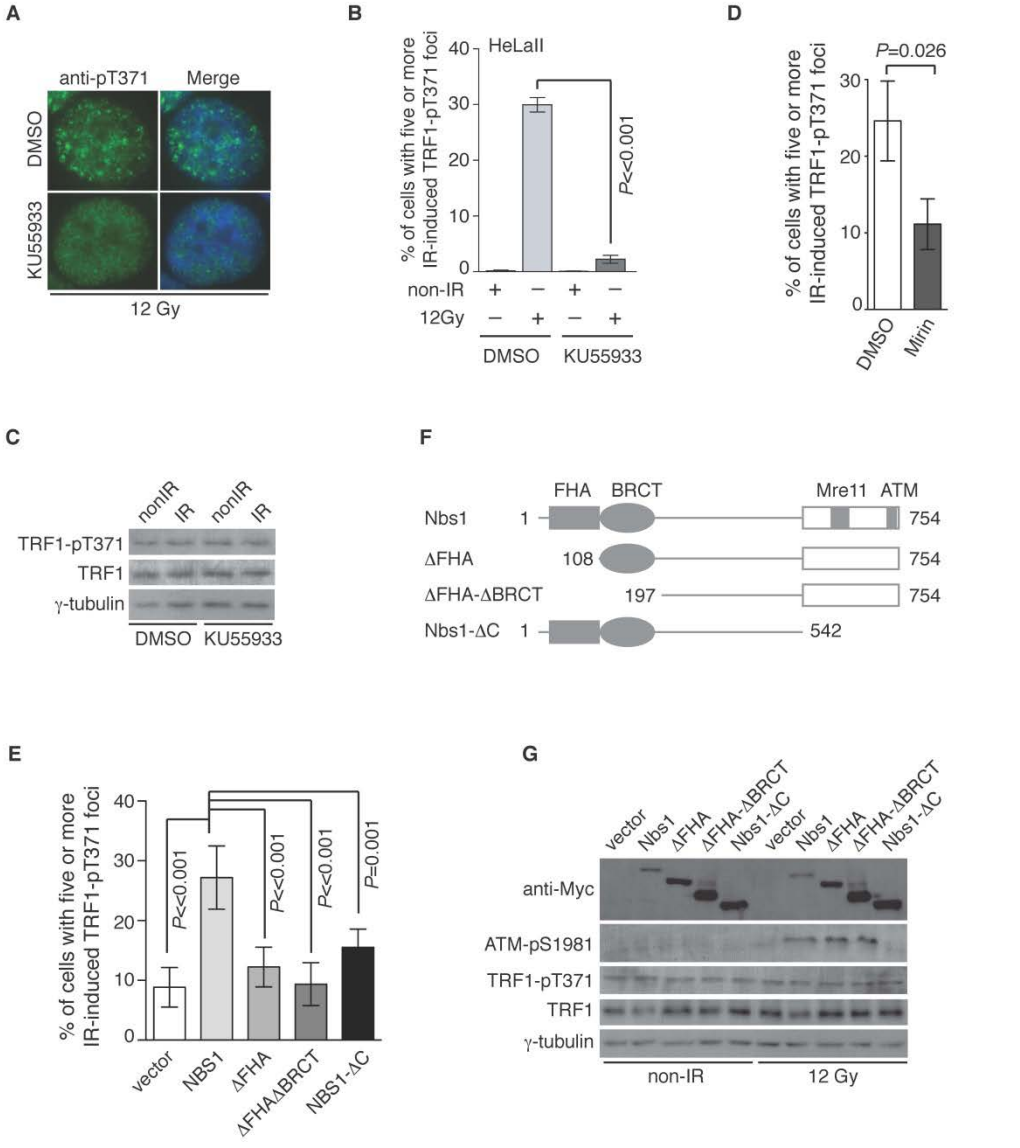


Figure 4. The formation of IR-induced (pT371)TRF1 foci is dependent upon the ATM- and Mre11/Rad50/Nbs1-mediated DNA damage response. (**A**) Indirect immunofluorescence with anti-pT371 antibody. Prior to IR, HeLaII cells were treated with DMSO or KU55933. Cell nuclei were stained with DAPI in blue. (**B**) Quantification of the percentage of cells with five or more IR-induced (pT371)TRF1 foci from (A). Standard deviations from three independent experiments are indicated. (**C**) Western analysis. HeLaII cells treated with DMSO or KU55933 were subjected to mock treatment or 12 Gy IR. Immunoblotting was carried out with anti-pT371, anti-TRF1 and anti-γ-tubuliln antibody. (**D**) Quantification of the percentage of DMSO- or Mirin-treated HeLaII cells with five or more IR-induced (pT371)TRF1 foci. Standard deviations from three independent experiments are shown. (**E**) Quantification of the percentage of cells with five or more IR-induced (pT371)TRF1 foci. Standard deviations from seven independent experiments are indicated. Nbs1-deficient cells (NBS-ILB1) complemented with the vector alone or various Myc-tagged Nbs1 alleles as indicated were mock treated or treated with 12 Gy IR. (**F**) Schematic diagram of Nbs1 alleles. (**G**) Western analysis. Nbs1-deficient cells (NBS-ILB1) complemented with the vector alone or various Myc-tagged Nbs1 alleles as indicated were mock treated or treated with 12 Gy IR. Immunoblotting was done with anti-Myc, anti-ATM-pS1981, anti-pT371, anti-TRF1 or anti-γ-tubulin antibody.

Figure 5 McKerlie et al.

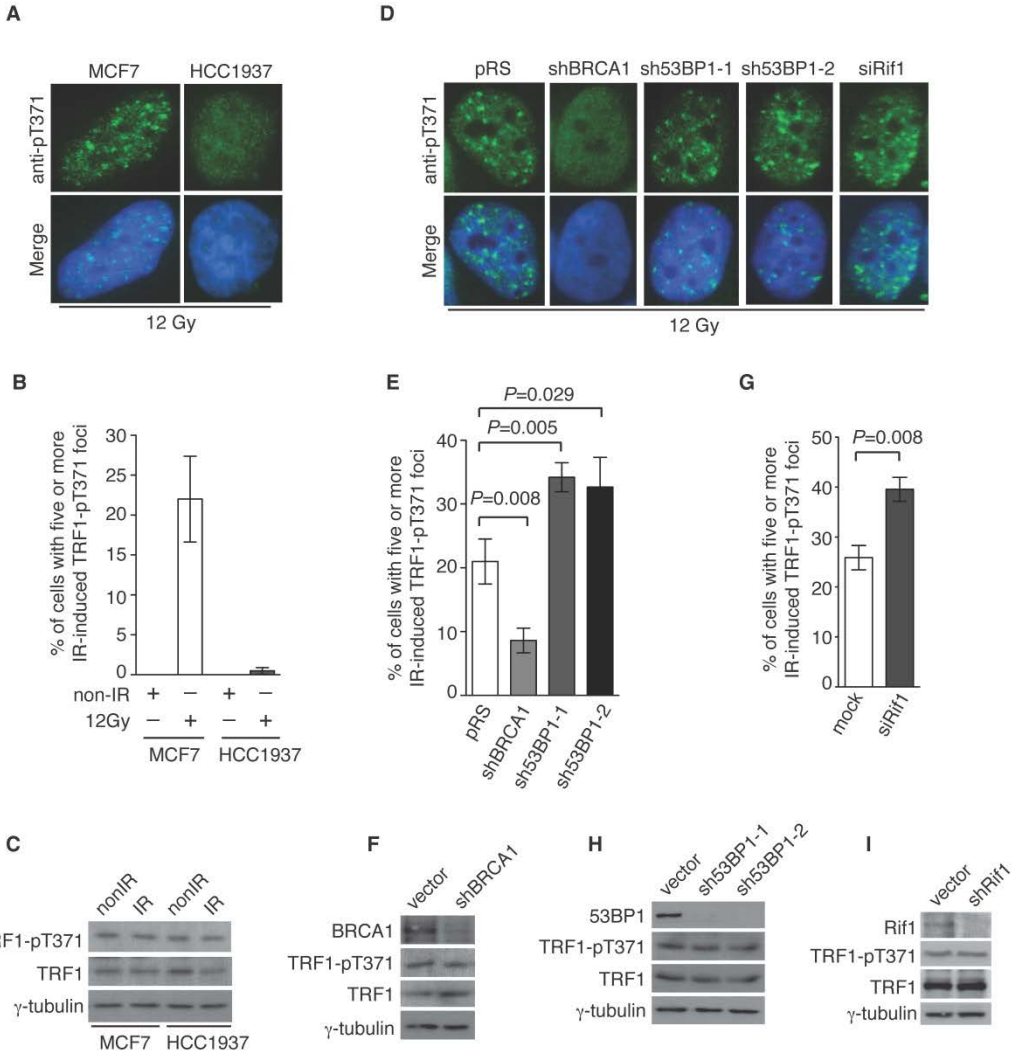


Figure 5. The formation of IR-induced (pT371)TRF1 foci requires BRCA1 but is inhibited by 53BP1 and Rif1. (**A**) Indirect immunofluorescence with anti-pT371 antibody on IR-treated MCF7 (expressing functional BRCA1) or HCC1937 (lacking functional BRCA1) cells. Cell nuclei were stained with DAPI in blue. (**B**) Quantification of the percentage of cells with five or more IR-induced (pT371)TRF1 foci from (A). Standard deviations from three independent experiments are indicated. (**C**) Western analysis of MCF7 and HCC1937 cell extracts with anti-pT371, anti-TRF1, or anti-γ-tubulin antibody. (**D**) Indirect immunofluorescence with anti-pT371 antibody on 12 Gy IR-treated HeLaII cells depleted for BRCA1 or 53BP1. Knockdown of 53BP1 was performed with two independent shRNA (sh53BP1-1 and sh53BP1-2). Cell nuclei were stained with DAPI in blue. (**E**) Quantification of the percentage of cells with five or more IR-induced (pT371)TRF1 foci in HeLaII cells depleted for BRCA1 or 53BP1 from (D). Standard deviations from three independent experiments are indicated. (**F**) Western analysis of HeLaII cell extracts with or without depletion of BRCA1. Immunoblotting was performed with anti-BRCA1, anti-pT371, anti-TRF1 or anti-γ-tubulin antibody. (**G**) Quantification of the percentage of cells with five or more IR-induced (pT371)TRF1 foci in HeLaII cells knocked down for Rif1. Standard deviations from three independent experiments are indicated. (**H**) Western analysis of HeLaII cell extracts with or without depletion of 53BP1. Immunoblotting was performed with anti-53BP1, anti-pT371, anti-TRF1 or anti-γ-tubulin antibody. (**I**) Western analysis of HeLaII cell extracts with or without depletion of Rif1. Immunoblotting was performed with anti-Rif1, anti-pT371, anti-TRF1 or anti-γ-tubulin antibody.

Figure 6 McKerlie et al.

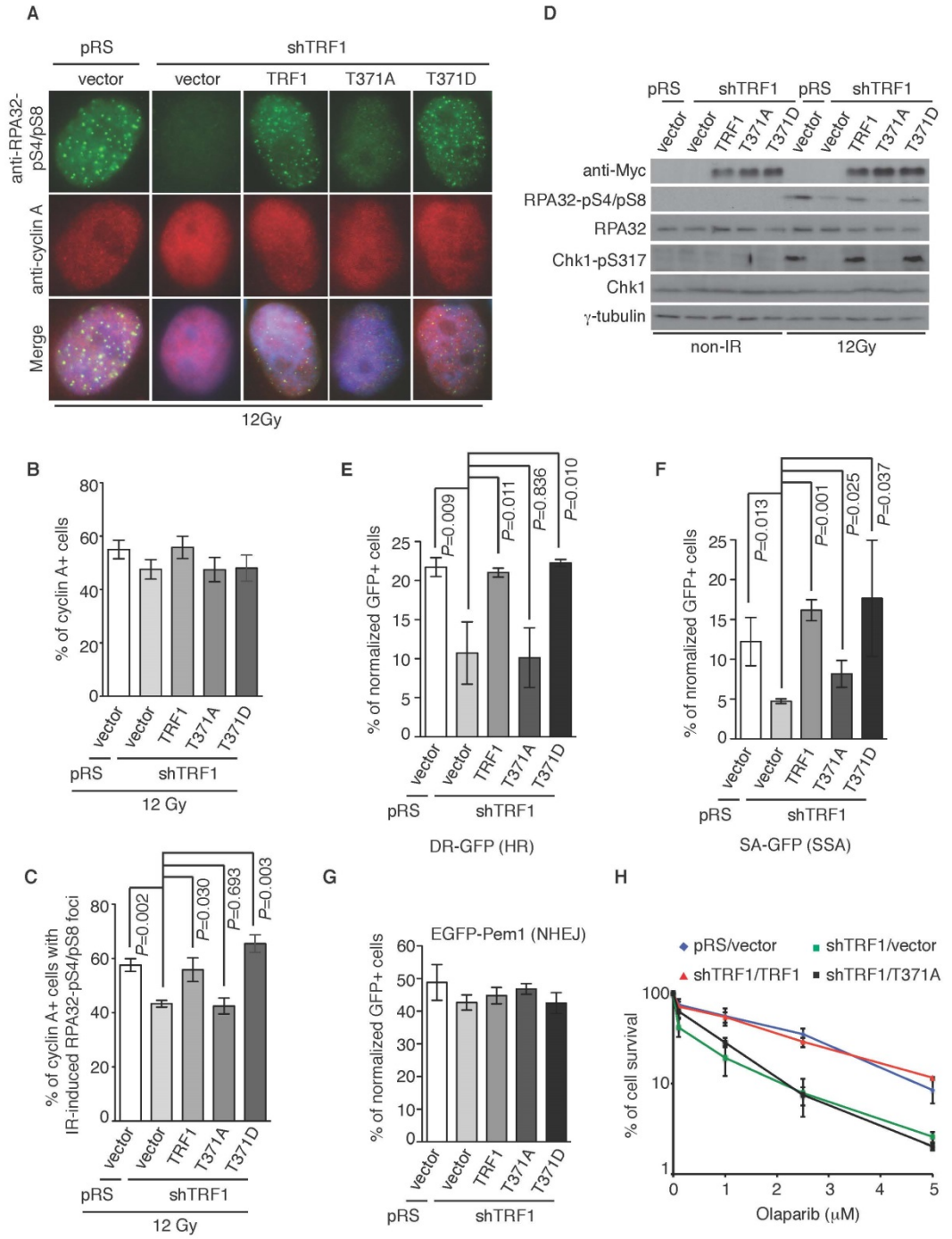


Figure 6. TRF1 phosphorylation at T371 facilitates DNA end resection and homologous recombination. (**A**) Indirect immunofluorescence with anti-RPA32-pS4/pS8 in conjunction with anti-cyclin A antibody. TRF1-depleted HeLaII cells stably expressing the vector alone or various TRF1 alleles as indicated were treated with 12 Gy and fixed 8 hr later. Cell nuclei were stained with DAPI in blue. (**B**) Quantification of cyclin A-positive cells in TRF1-depleted cells expressing various alleles as indicated. Standard deviations from six independent experiments are indicated. (**C**) Quantification of the percentage of cyclin A-positive cells with five or more IR-induced RPA32-pS4/pS8 foci from (A). Standard deviations from six independent experiments are indicated. (**D**) Western analysis of mock- or IR-treated TRF1-depleted HeLaII cells stably expressing the vector alone or various TRF1 alleles as indicated. Immunoblotting was carried out with anti-RPA32-pS4/pS8, anti-RPA32, anti-Chk1-pS317, anti-Chk1 or anti-γ-tubulin. (**E**) HR-mediated repair of I-SceI-induced DSBs. TRF1-depleted HeLaII cells expressing various TRF1 alleles as indicated were cotransfected with pDR-GFP, pCherry and I-SceI expression constructs. The number of cells positive for both GFP and pCherry was normalized to the total number of pCherry-positive cells, giving rise to the percentage of normalized GFP-positive cells. Standard deviations from four independent experiments are indicated. (**F**) SSA-mediated repair of I-SceI-induced DSBs. TRF1-depleted HeLaII cells expressing various TRF1 alleles as indicated were cotransfected with pSA-GFP, pCherry and I-SceI expression constructs. The quantification was done as described in (E). Standard deviations from three independent experiments are indicated. (**G**) NHEJ-mediated repair of I-SceI-induced DSBs. TRF1-depleted HeLaII cells expressing various TRF1 alleles as indicated were cotransfected with pEGFP-Pem1-Ad2, pCherry and I-SceI expression constructs. The quantification was done as described in (E). Standard deviations from three independent experiments are indicated. (**H**) Clonogenic survival assays of Olaparib-treated HeLaII cells stably expressing various alleles as indicated. Standard deviations from three independent experiments are indicated.

Figure 7 McKerlie et al.

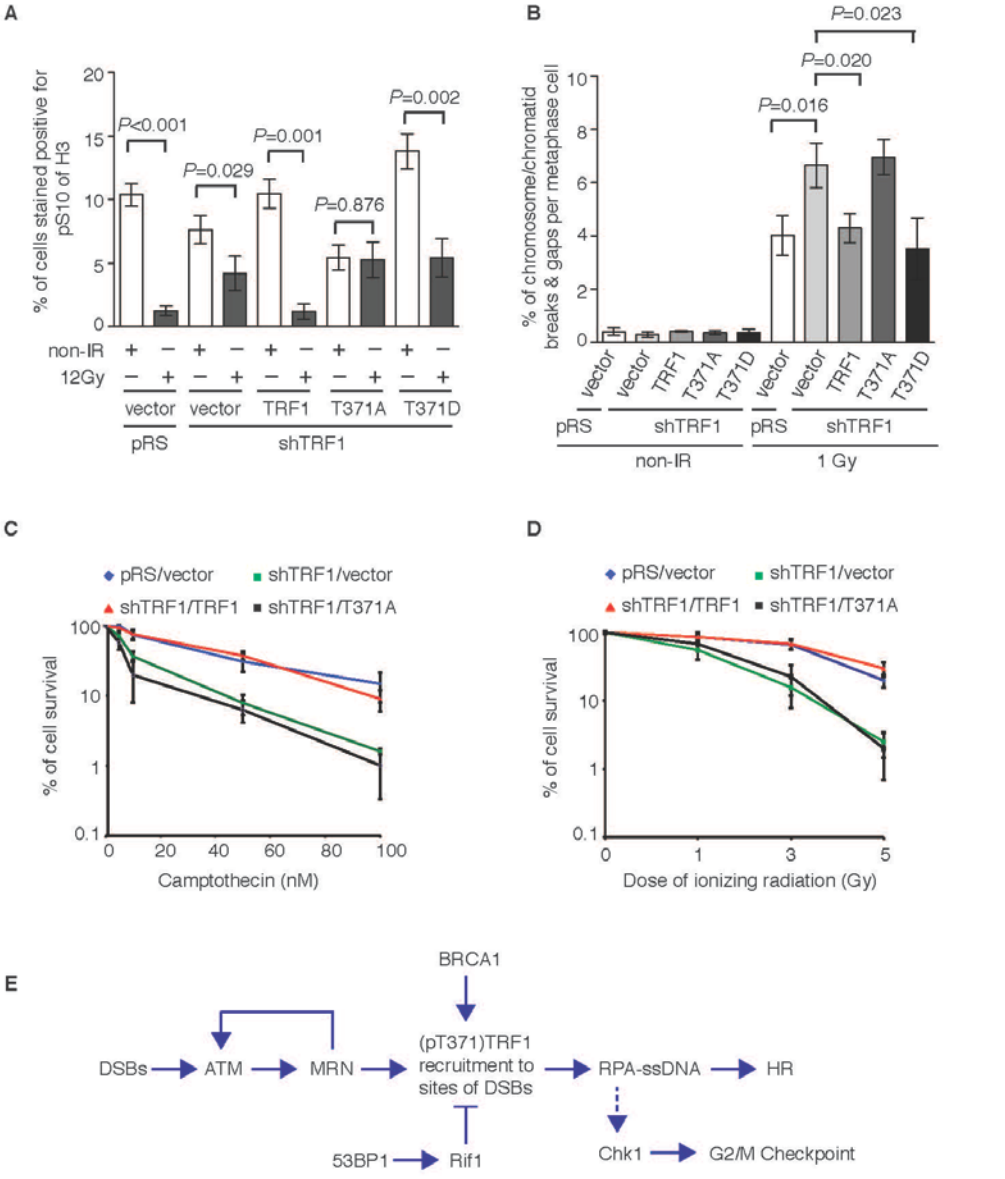
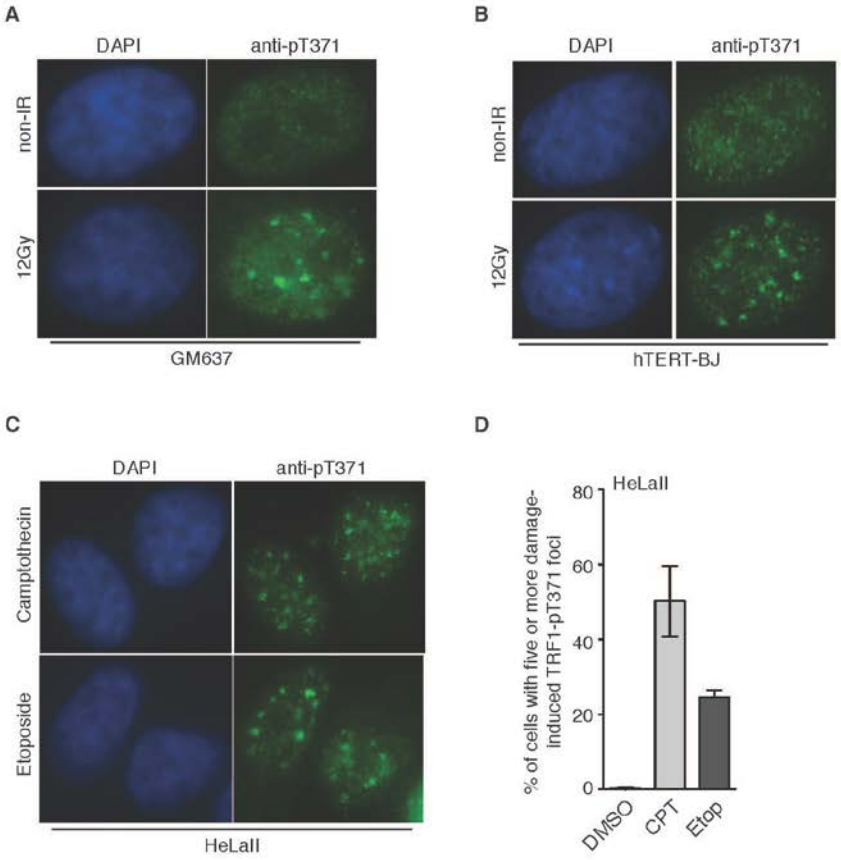


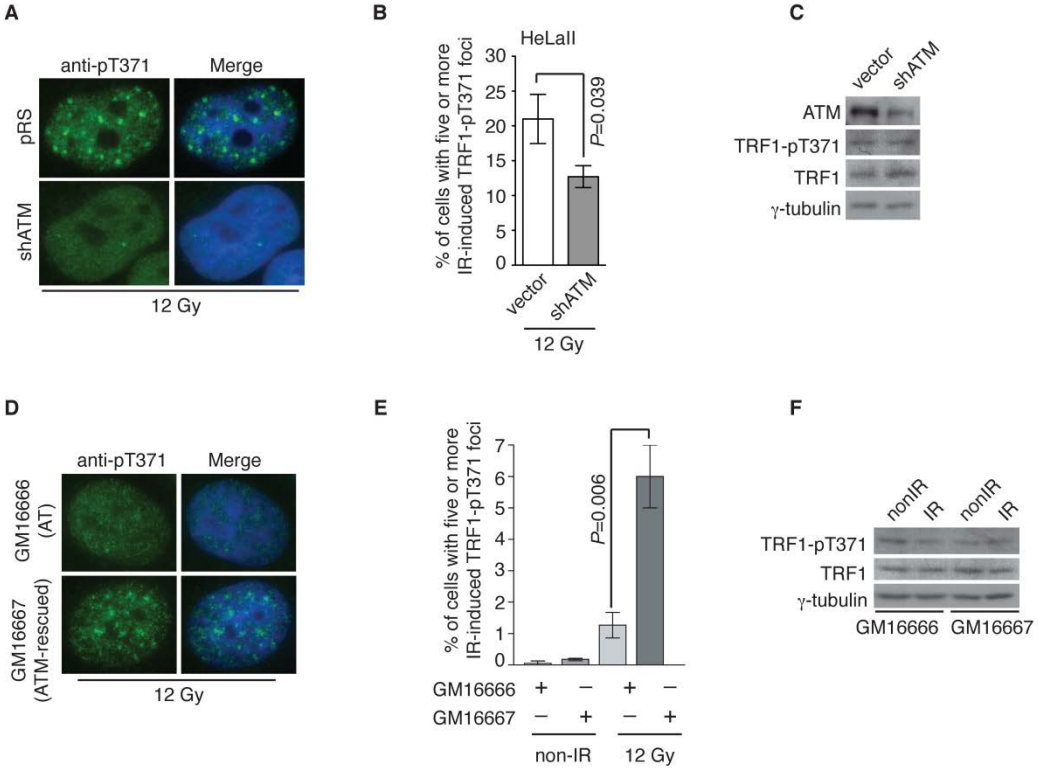
Figure 7. The lack of TRF1 phosphorylation at T371 impairs the activation of the G2/M checkpoint and hampers the maintenance of genome integrity. (**A**) Quantification of the percentage of cells stained positive for H3-pS10. For each cell line, a total of 3000 cells were scored in blind. Standard deviations from four independent experiments are indicated. (**B**) Quantification of the percentage of chromosome/chromatid breaks and gaps per metaphase cell. TRF1-depleted HeLaII cells stably expressing the vector alone or various TRF1 alleles as indicated were mock treated or treated with 1 Gy IR. For each cell line, a total of 60 metaphase cells were scored in blind. Standard deviations from three independent experiments are indicated. (**C & D**) Clonogenic survival assays following various doses of camptothecin (C) or IR (D). The colony forming assays were performed with TRF1-depleted HeLaII cells stably expressing the vector alone or various TRF1 alleles as indicated. HeLaII cells expressing both pRS and pWZL vectors were also included as a control. Standard deviations from six independent experiments are indicated. (**E**) Model for the role of (pT371)TRF1 in facilitating homologous recombination and checkpoint activation. See the text for more information.

Supplementary Figure 1 McKerlie et al.



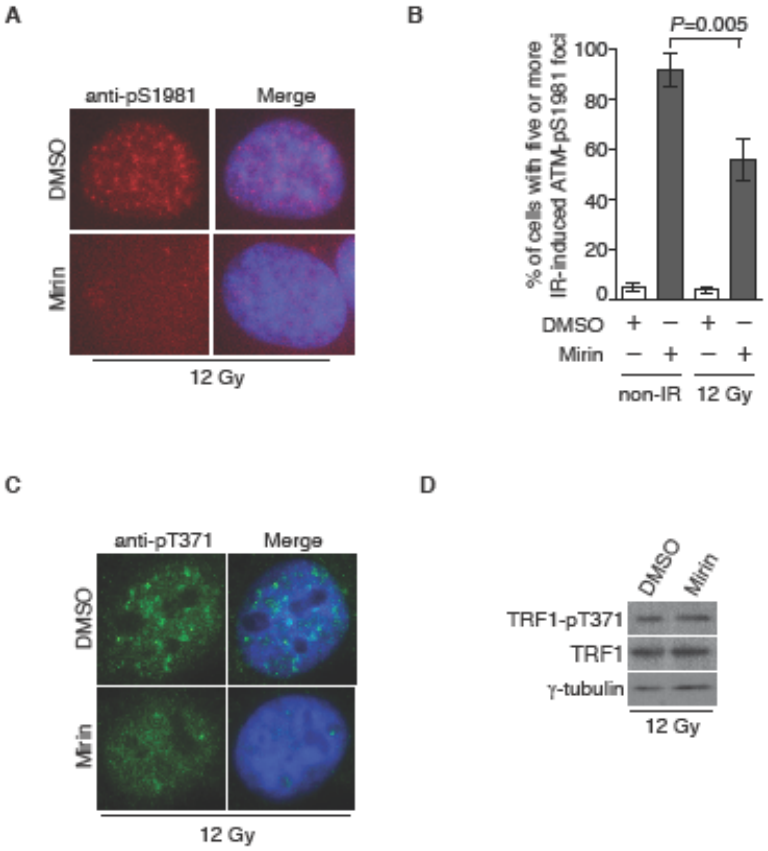
Supplementary Figure 1. Phosphorylated (pT371)TRF1 forms damage-induced foci in cells treated with IR, camptothecin or etoposide. (**A**) Indirect immunofluorescence with anti-pT371 antibody. SV40- transformed GM637 skin fibroblasts were mock treated or treated with 12 Gy IR. Cell nuclei were stained with DAPI in blue. (**B**) Indirect immunufluorescence with anti-pT371 antibody. Telomerase-immortalized BJ (hTERT-BJ) cells were mock treated or treated with 12 Gy IR. Cell nuclei were stained with DAPI in blue. (**C**) Indirect immunofluorescence with anti-pT371 antibody on HeLaII cells treated with camptothecin or etoposide. Cell nuclei were stained with DAPI in blue. (**D**) Quantification of the percentage of HeLaII cells with five or more damage-induced (pT371)TRF1 foci. Standard deviation from three independent experiments are indicated. CPT: camptothecin; Etop: etoposide.

Supplementary Figure 2 McKerlie et al.



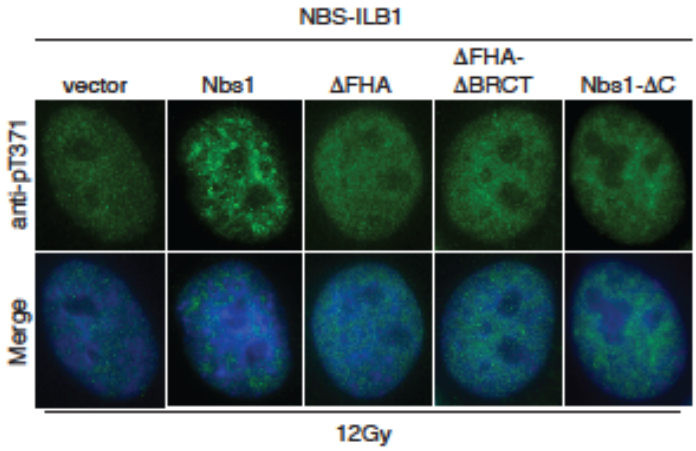
Supplementary Figure 2. Depletion or loss of ATM impairs the formation of IR-induced (pT371)TRF1 foci. (**A**) Indirect immunofluorescence with anti-pT371 antibody on IR-treated HeLaII cells knocked down for ATM. Cell nuclei were stained with DAPI in blue. (**B**) Quantification of the percentage of cells with five or more IR- induced (pT371)TRF1 foci in HeLaII cells with or without depletion of ATM from (A). Standard deviation from three independent experiments are indicated. (**C**) Western analysis of HeLaII cells with or without knockdown of ATM. Immunoblotting was performed with anti-ATM, anti-pT371, anti-TRF1 or anti-γ-tubulin antibody. (**D**) Indirect immunofluorescence with anti-pT371 antibody on IR-treated ATM- deficient cells complemented with either the vector alone (GM16666) or ATM (GM16667). Cell nuclei were stained with DAPI in blue. (**E**) Quantification of the percentage of cells with five or more IR- induced (pT371)TRF1 foci from (D). Standard deviations from three independent experiments are indicated. (**F**) Western analysis of ATM-deficient cells complemented with the vector alone or ATM. Immunobloting was performed with anti-pT371, anti-TRF1 or anti-γ-tubulin.

Supplementary Figure 3 McKerlie et al.



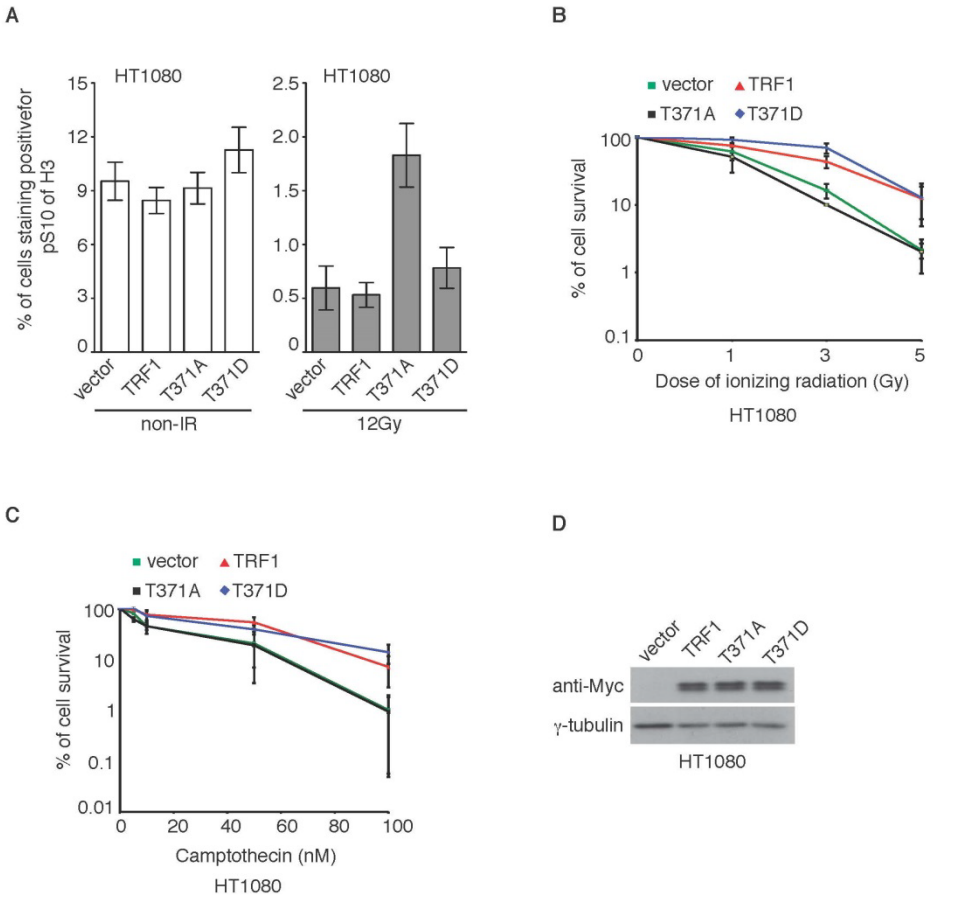
Supplementary Figure 3. The Mre11/Rad50/Nbs1 is required for the formation of IR-induced foci of ATM-pS1981 and (pT371)TRF1. (**A**) Indirect immunofluorescence with anti-ATM-pS1981 antibody. HeLaII cells pretreated with DMSO or Mirin were radiated with 12 Gy IR and then fixed 1 hr post IR. (**B**) Quantification of the percentage of cells with five or more IR-induced ATM-pS1981 foci (A). Standard deviations from three independent experiments are indicated. (**C**) Indirect immunofluorescence with anti-pT371 antibody. HeLaII cells pretreated with DMSO or Mirin were radiated with 12 Gy IR and then fixed 8 hr post IR. (**D**) Western analysis. Prior to 12 Gy IR, HeLaII cell extracts were treated with DMSO or Mirin. Immunoblotting was performed with anti-pT371, anti-TRF1 or anti-γ-tubulin antibody.

Supplementary Figure 4 McKerlie et al.



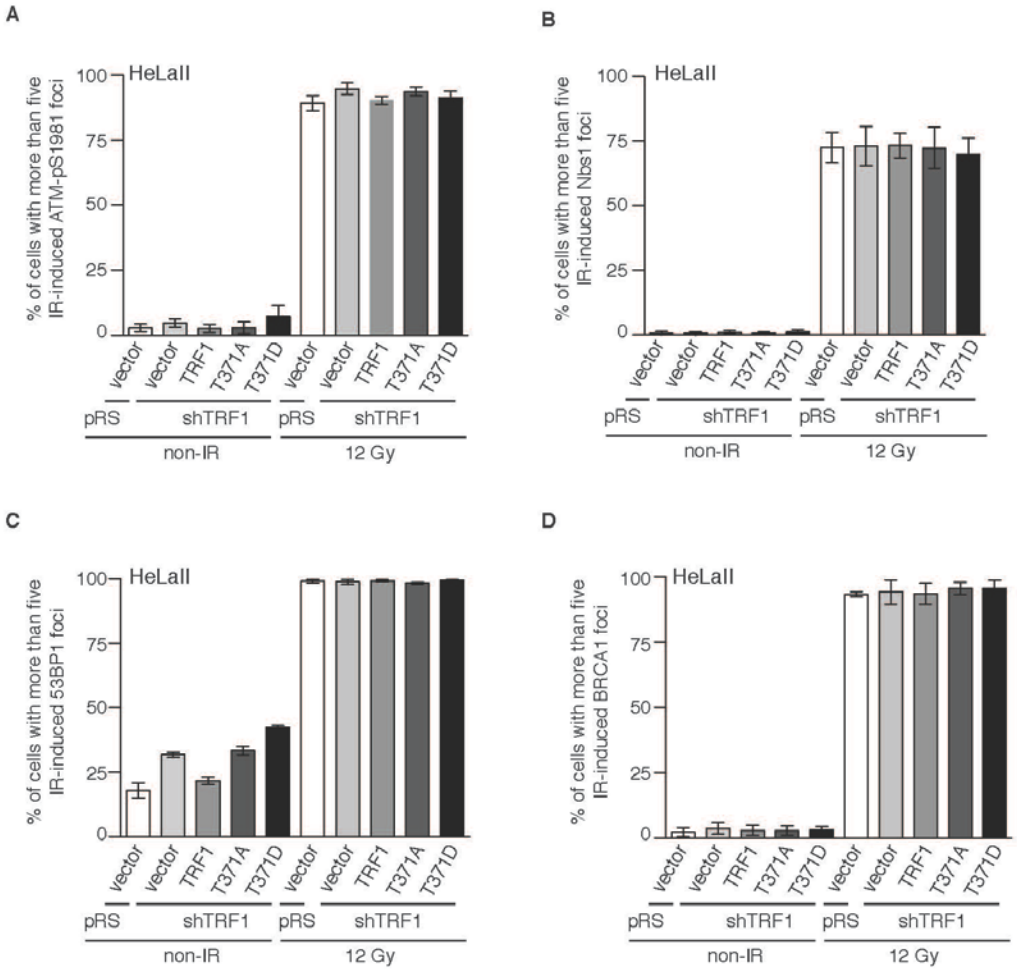
Supplementary Figure 4. Indirect immunofluorescence with anti-pT371 antibody on IR-treated Nbs1-deficient cells (NBS-ILB1) complemented with the vector alone or various Myc-tagged Nbs1 alleles as indicated. Cell nuclei were stained with DAPI in blue.

Supplementary Figure 5 McKerlie et al.



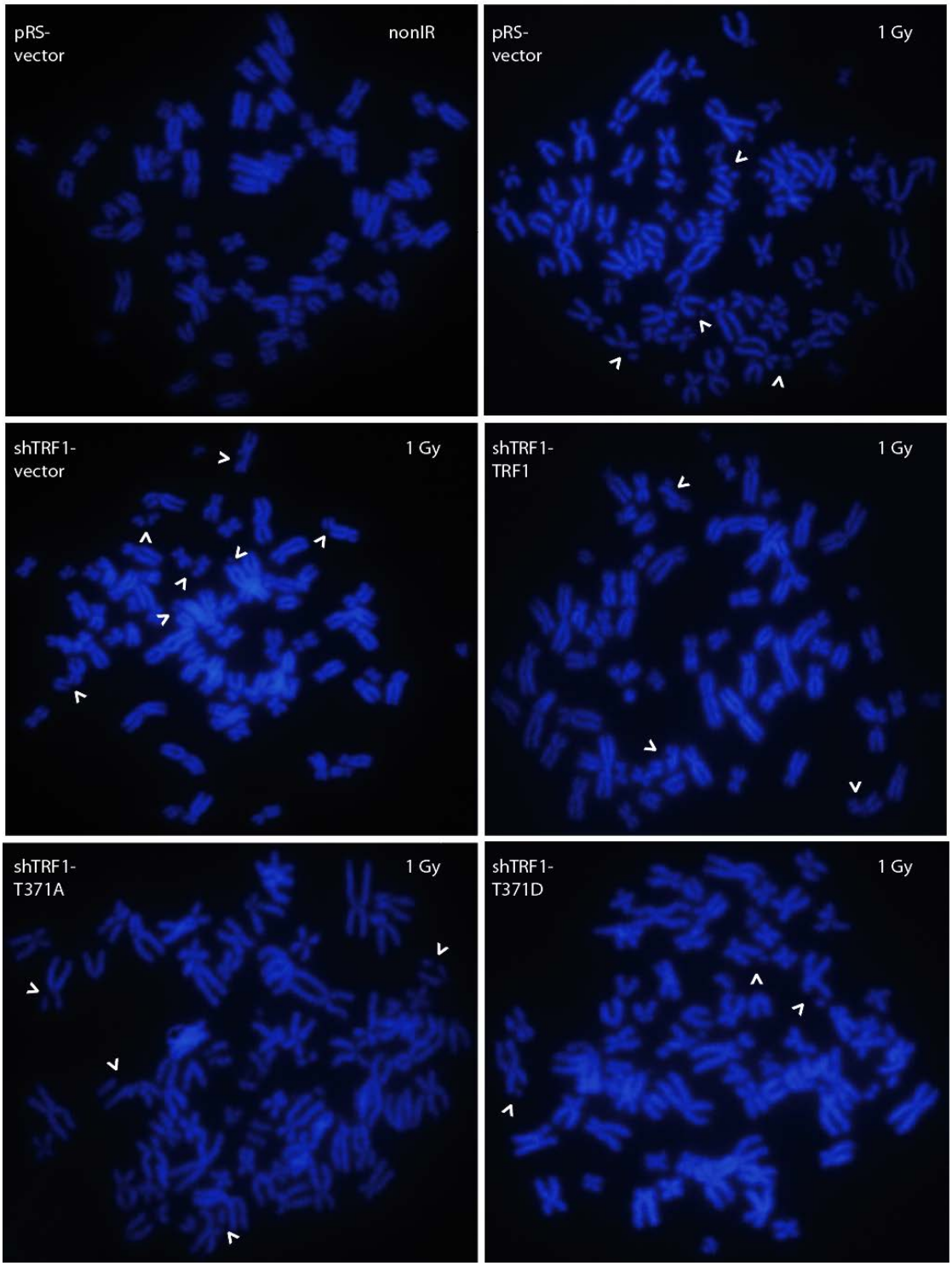
Supplementary Figure 5. Overexpression of TRF1 carrying a nonphosphorylatable mutation of T371A impairs the activation of the G2/M checkpoint and fails to promote cell survival following the treatment with DSB- inducing agents. (**A**) Quantification of the percentage of cells stained positive for H3-pS10. HT1080 cells stably expressing the vector alone or various TRF1 alleles as indicated were mock treated or treated with 12 Gy IR. For each cell line, a total of 3000 cells were scored in blind. Standard deviations from three independent experiments are indicated. (**B & C**) Clonogenic survival assays following various doses of IR (B) or camptothecin (C). The colony forming assays were performed with HT1080 cells overexpressing the vector alone or various Myc-tagged TRF1 alleles as indicated. Standard deviations from at least three independent experiments are indicated. (**D**) Western analysis of HT1080 cells overexpressing the vector alone or various Myc-tagged TRF1 alleles as indicated. Immunoblotting was performed with anti-Myc or anti-γ-tubulin antibody.

Supplementary Figure 6 McKerlie et al.



Supplementary Figure 6. TRF1 does not impact the sensing of DNA double strand breaks. A-D) HeLaII cells depleted for TRF1 and expressing the indicated TRF1 alleles were treated with 12Gy IR and fixed with a standard IF fixation protocol 1 hour later. Cells were stained with antibodies against **(A)** ATM-pS1981, **(B)** Nbs1, **(C)** 53BP1, or **(D)** BRCA1, and the number of cells exhibiting foci in untreated or IR-treated cells is indicated. Cells with more than 5 foci were scored as positive. 500 cells were scored for each experiment, in triplicates, blind. Standard deviations are indicated.

Supplementary Figure 7 McKerlie et al.



Supplementary Figure 7. The lack of TRF1 phosphorylation at T371 impairs the maintenance of genome integrity. DAPI staining of metaphase chromosome spreads of HeLaII cells expressing the indicated TRF1 alleles. Cells were treated with 1Gy IR or not treated. Arrows indicate chromosome gaps or breaks, which were scored, the quantification of which is depicted in Figure 7.

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Chapter 5

Discussion

5.1 Overview of Findings

Within this thesis I have explored the regulation of TRF1 by phosphorylation events at two residues; S367 and T371. My conclusions have revealed a tight regulation of TRF1 function and have highlighted detrimental consequences associated with the deregulation of phosphorylation at these sites. I have used two central approaches throughout this work to investigate the functionality of these post translational modifications; mutational analysis of TRF1 to prevent or mimic phosphorylation at these sites, and the employment of antibodies that specifically recognize TRF1 when it is phosphorylated at either S367 or T371. My work has evaluated the regulation of TRF1 in human cell lines, at different phases of the cell cycle and after exposure to DNA-damage inducing agents. These findings have exposed a novel function of TRF1 in the DNA damage response.

I have demonstrated that TRF1 is phosphorylated at S367 by ATM, and that this phosphorylation decreases the binding of TRF1 to telomere DNA. The phosphorylation at S367 results in the localization of TRF1 to discrete nuclear sites which are associated with proteolysis. Foci representing proteasome centres are visible with our anti-pS367 TRF1 antibody in S and G2 phases of the cell cycle. Phosphorylation at S367 makes TRF1 susceptible to protein degradation, and therefore decreases the stability of TRF1. Mimicking phosphorylation at S367 TRF1 results in telomere lengthening, and the expression of this construct does not rescue defects associated with TRF1 depletion, such as telomere doublets and telomere loss. I demonstrate, then, that the ATM-dependent phosphorylation of TRF1 S367 regulates TRF1 stability and localization, which impacts telomere maintenance.

Another phosphorylation site on TRF1, T371, has been the focus of most of this thesis. I have demonstrated multiple roles for TRF1 that is modified at T371. First, I show that the phosphorylation of T371 in mitosis by Cdk1 stabilizes an unbound fraction of

TRF1; preventing it from being degraded by the proteasome pathway. Upon mitotic exit, this site is dephosphorylated and TRF1 resumes telomere binding, which allows for the separation of sister chromatids in mitosis. Preventing the phosphorylation at T371 results in the failure of sister chromatids to separate. Conversely, mimicking TRF1 phosphorylation at T371 prevents the re-association of TRF1 with telomeres upon mitotic exit, which results in a state in which telomeres are deprotected. The deprotection of telomeres with the expression of TRF1 T371D leads to chromosome instability and apoptosis.

A second role for (pT371)TRF1 that I have discovered is in the repair of damaged DNA. I have shown that after ionizing radiation, (pT371)TRF1 forms DNA-damage induced foci. The localization of TRF1 to sites of DNA damage after IR is dependent upon ATM, the MRN complex, and BRCA1. The presence of TRF1 at these sites is important for efficient end resection and homologous recombination. TRF1, when phosphorylated at T371, promotes cell survival after ionizing radiation and is involved in the G2/M checkpoint.

I have demonstrated that the function of TRF1 is modulated by its phosphorylation at both S367 and T371. These post-translational modifications impact on the stability of TRF1, the binding of TRF1 to telomere DNA, and the localization of TRF1 to other structures within the nucleus. The phosphorylation of TRF1 at T371 has revealed a novel function for TRF1; in the repair of DNA double strand breaks. The examination of this phosphorylation site has also helped to delineate the involvement of TRF1 in the resolution of sister chromatids in mitosis.

5.2 TRF1 Phosphorylation at S367

I have shown that TRF1 is phosphorylated at S367 by ATM and that this modification removes TRF1 from telomeres. The regulation of TRF1 function by ATM has been suggested previously (Wu et al., 2007). Inhibiting ATM increases the binding of

TRF1 to telomere DNA *in vivo* and leads to telomere shortening in telomerase positive cells, which suggests that ATM regulates telomere length maintenance through TRF1 (Wu et al., 2007). There are three ATM consensus SQ sites in TRF1; S219, S274, and S367. ATM has been shown to phosphorylate TRF1 at S219 in response to ionizing radiation, which plays a role in the cellular response to DNA damage (Kishi and Lu, 2002; Kishi et al., 2001b). Mutating this site to prevent phosphorylation increases the sensitivity of cells to ionizing radiation, indicating a functional role for TRF1 in DNA repair (Kishi and Lu, 2002; Kishi et al., 2001b). *In vitro* gel shift assays suggest that the phosphorylation of TRF1 S219 by ATM does not impact on the ability of TRF1 to bind to telomere DNA, and telomere length is not altered with the mutation of this site (Kishi et al., 2001b; Wu et al., 2007). These results suggest that there may be additional ATM sites on TRF1 which are responsible for regulating TRF1 binding to telomere DNA. While TRF1 phosphorylation at S219 occurs in response to DNA damage, evidence has suggested that TRF1 is phosphorylated by ATM in cells that have not been exposed to DNA damage, which further supports the notion that an additional TRF1 residue may be a substrate of ATM *in vivo* (Wu et al., 2007). The other candidate ATM phosphorylation sites in TRF1, S274 and S367, were individually mutated from serine to aspartate to mimic phosphorylation. *In vitro* data demonstrated that mimicking phosphorylation at either S274 or S367 reduced the ability of TRF1 to bind telomere DNA (Wu et al., 2007). Whether either of these sites may regulate telomere maintenance *in vivo* was not clear. I have shown that S367 is phosphorylated by ATM *in vivo* and that this phosphorylation removes TRF1 from telomere DNA, promoting telomere elongation (McKerlie et al., 2012). It is possible that the increase in TRF1 binding to telomere DNA with ATM inhibition observed previously is a result of the lack of phosphorylation occurring at TRF1 S367, which would remove TRF1 and target it to sites of proteasome degradation (Wu et al., 2007). I observe that (pS367)TRF1 is removed from telomeres and goes to sites of proteasome degradation in S and G2 phases of the cell cycle, which is consistent with an

increased presence of ATM and a decreased association of TRF1 with telomeres during these stages of the cell cycle (Verdun et al., 2005). My results, then, are consistent with previous work detailing the regulation of TRF1 binding to telomeres by ATM in undamaged cells. We have expanded on the understanding of how ATM might remove TRF1 from telomeres and contribute to telomere length maintenance.

5.3 TRF1 Phosphorylation at T371

I have evaluated the function of TRF1 phosphorylated at T371 and observed that it is involved in two distinct roles; in the resolution of sister chromatids in mitosis and in the repair of damaged DNA. Mass spectrometry revealed TRF1 T371 as a candidate phosphorylation site and sequence analysis uncovered it as a consensus CDK phosphorylation site. I wished to address whether there was an *in vivo* role of TRF1 phosphorylation at T371.

There are four consensus CDK sites (S/T P) in TRF1, which are S11, T149, T344, and T371. The phosphorylation of TRF1 T149 by CDK in mitosis has been shown to promote the interaction between TRF1 and PIN1, the result of which decreases TRF1 binding to telomere DNA and also decreases TRF1 stability (Lee et al., 2009). TRF1 phosphorylation at T344 has been suggested to promote Plk1 phosphorylation of TRF1 at S435, which promotes TRF1 binding to telomere DNA (Wu et al., 2008). The same report by Liu and colleagues has suggested that TRF1 T371 is an *in vitro* substrate of Cdk1, and proposes that this site may function with T344 as a priming site for Plk1 phosphorylation (Wu et al., 2008). They fail to distinguish between effects mediated by TRF1 T344 and T371, as they employ the use of double mutants to investigate the effect of the phosphorylation of these sites by Cdk1 in promoting the phosphorylation of S435 by Plk1 (Wu et al., 2008). Our work contradicts the assertion that (pT371)TRF1 promotes binding to telomeres, and we have not been able to replicate the observed phosphorylation of TRF1 by Plk1. We have demonstrated that the phosphorylation of

TRF1 at T371 by Cdk1 decreases the binding of TRF1 to telomeres, by differential salt extraction, immunofluorescence, chromatin immunoprecipitation, and gel shift assays. Taken together, our data supports that this site is not serving as a docking site for Plk1 phosphorylation at S435. There does appear to be a substantial level of TRF1 regulation by cyclin dependent kinase activity, and it was of interest to evaluate the specific function of TRF1 phosphorylation at T371.

I noted initially that TRF1 T371 is phosphorylated in mitosis by Cdk1. There has been previous work suggesting roles for TRF1 in cell cycle progression; in spindle assembly and also in the separation of sister chromatids. The transgenic overexpression of TRF1 in mice results in mitotic spindle aberrations, multipolarity, and misaligned chromosomes in mitosis (Munoz et al., 2009). The knockdown of TRF1 has also been shown to cause mitotic spindle defects (Zhu et al., 2009). Many reports have suggested for the association of TRF1 with microtubules and mitotic spindles in mitotic cells, the function of which has remained unclear (Munoz et al., 2009; Nakamura et al., 2001a; Nakamura et al., 2002; Nakamura et al., 2001b). Another function of TRF1 in mitotic cells is in the separation of sister chromatids. TRF1 binds directly to SA1, a member of the cohesin complex at telomere ends (Canudas et al., 2007). Tankyrase 1 activity is required for the separation of sister telomeres in mitosis, and the depletion of tankyrase 1 results in the inability of cells to resolve their telomeres in mitosis (Canudas et al., 2007; Cook et al., 2002; Dynek and Smith, 2004). Tankyrase 1 poly(ADP-ribosyl)ates TRF1, removing it from telomere DNA, and the depletion of TRF1 and TIN2, or SA1, rescues the defect in the resolution of sister chromatids observed with tankyrase 1 knockdown (Canudas et al., 2007; Canudas and Smith, 2009). These findings suggest that in mitosis TRF1 may be removed from telomeres by tankyrase 1 activity, which contributes to the release of the SA1-containing cohesin complex and allows for the resolution of sister telomeres. I have discovered that the phosphorylation of TRF1 at T371 by Cdk1 in mitosis stabilizes an unbound fraction of TRF1 in the cell, while the sister telomeres separate. The

phosphorylation on TRF1 T371 is removed upon mitotic exit, TRF1 resumes telomere binding, and telomeres are restored to a protected state. Preventing the phosphorylation of TRF1 T371 blocks the resolution of sister telomeres. My work is consistent with the known function of TRF1 in the resolution of sister telomeres; it is possible that tankyrase 1 activity removes TRF1 from telomeres in mitosis and that the phosphorylation of TRF1 at T371 stabilizes this protein, ensuring that it is available for reassociation with telomere DNA upon the completion of mitosis. There have been multiple reports which have described an increase in TRF1 levels in mitosis, and have suggested that this increase may be due to an increase in protein stability, which may be explained by our findings that (pT371)TRF1 is a stable form of TRF1 in mitosis (Kishi et al., 2001a; Nakamura et al., 2001a; Shen et al., 1997; Zhu et al., 2009). There is another CDK phosphorylation site on TRF1, T149, which indirectly reduces the binding of TRF1 to telomere DNA in mitosis, which confirms the importance of the release of TRF1 from telomere DNA at this time (Lee et al., 2009). Elucidating the function of (pT371)TRF1 expands on our knowledge of mechanism by which TRF1 regulates the resolution of sister telomeres in mitosis, and also expands on our understanding of the role of TRF1 in mitotic progression.

In addition to being an important player in the resolution of sister chromatids in mitosis, (pT371)TRF1 is important in the repair of DNA double strand breaks. Phosphorylated (pT371)TRF1 localizes to sites of DNA damage after ionizing radiation, camptothecin treatment, or etoposide treatment, and is important in the repair of DNA damage by homologous recombination. TRF1 has previously been suggested to play a functional role in the cellular response to DNA damage. TRF1 has been shown to be phosphorylated by ATM at S219 in response to ionizing radiation (Kishi and Lu, 2002; Kishi et al., 2001b). In AT cells, which lack functional ATM and exhibit a survival and checkpoint defect after IR, the phosphorylation of TRF1 at S219 was shown to be involved in the cellular response to DNA damage (Kishi and Lu, 2002; Kishi et al., 2001b).

Preventing phosphorylation at S219 TRF1 increased the sensitivity to IR, whereas mimicking phosphorylation at S219 TRF1 rescued the IR-induced survival sensitivity and checkpoint defect observed in AT cells (Kishi and Lu, 2002; Kishi et al., 2001b). I have also shown that TRF1 is important for survival following IR as well as in the activation of the G2/M checkpoint. The requirement for TRF1 in cell survival following IR has also been observed in chicken DT40 cells, and the TRF1 related protein, Tbf1, is similarly important in *Saccharomyces cerevisiae* (Bonetti et al., 2013; Cooley et al., 2009). These results suggest that TRF1 may be involved in the DNA damage response in an evolutionarily conserved manner. The site that we have shown to be involved in the role of TRF1 in the DNA damage response, T371, is conserved in murine TRF1, and it would be of interest to investigate the involvement of TRF1 in DNA repair in mouse cells. Future directions to further explore the role of p(T371)TRF1 in the DNA damage response should include an investigation into whether the involvement of TRF1 is limited to sites of double strand breaks, or if TRF1 may play a role at other forms of DNA damage, such as single strand breaks, pyrimidine dimers, or oxidation. The involvement of TRF1 at different forms of DNA damage may reveal a broader role for TRF1 in the maintenance of genomic integrity. It would also be of interest to determine the precise mechanism of TRF1 action at sites of damage. Determining if TRF1 is required for protein complex formation, for enhancing the processivity of another damage response factor, or for a more direct functional activity may help to address this. These directions will expand on the understanding of what TRF1 is contributing at sites of DNA damage.

One of the interacting partners of TRF1 at telomeres, nucleostemin (NS), is a nucleolar GTP-binding protein that interacts with the linker region of TRF1 (Meng et al., 2011; Zhu et al., 2006). The interaction between NS and TRF1 prevents the dimerization of TRF1 and shortens the association time of TRF1 with telomere DNA, without affecting the total level of telomere bound TRF1 protein (Meng et al., 2011). In cells with dysfunctional telomeres, generated by the expression of TRF2ΔBΔM, the overexpression

of NS reduces the level of TIF formation in U2OS cells (Meng et al., 2011). These results suggest that reducing the association time of TRF1 with telomeres and preventing the dimerization of TRF1 reduces damage signalling at deprotected telomeres, although the mechanism through which this occurs is unclear (Meng et al., 2011). U2OS cells use the ALT pathway, and lengthen and repair their telomeres through HR-based events. It is possible that TRF1, existing as a monomer in the presence of NS, promotes these HR-based occurrences at dysfunctional telomeres in ALT cells, reducing the level of detectable damage. These findings provide support for the involvement of TRF1 in DNA repair, although the way in which this is accomplished at dysfunctional telomeres in ALT cells requires further investigation.

I find that (pT371)TRF1 forms DNA damage-induced foci maximally at 8 hours after IR. A peak at 8 hours is much later than that observed with many well characterized DNA damage response factors, such as ATM, γH2AX, or MDC1, however a number of HR specific factors have shown delayed and persistent recruitment to sites of DNA damage (Doil et al., 2009; Kim et al., 2005; Mailand et al., 2007; Mari et al., 2006; Polo and Jackson, 2011; Uematsu et al., 2007; Yano et al., 2008). Proteomic studies investigating the kinetic regulation of phosphorylation sites after ionizing radiation have identified clusters of proteins which have modifications that increase after IR, peaking around 8 hours, and associated with a late DNA damage response (Bennetzen et al., 2010; Polo and Jackson, 2011). Some of the proteins that have phosphorylation events increasing up to 8 hours or have stabilized levels of protein phosphorylation at 8 hours include NBS1, Rad50, and 53BP1 (Bennetzen et al., 2010). Rad50 foci have been observed by immunofluorescence to appear more predominantly 6-8 hours post IR (Paull et al., 2000). Another component of the MRN complex, Mre11, forms IR-induced foci very quickly after IR, but these foci change in shape over time, and by 8 hours they coalesce into fewer, larger, foci, which dissipate by 24 hours (Mirzoeva and Petrini, 2001). These sites, with an altered immunofluorescence staining pattern, may represent

sites of slow repair, as has been observed with a number of DNA damage response factors, including Rad50 and γH2AX (Gatei et al., 2011; Mirzoeva and Petrini, 2001; Paull et al., 2000). Staining patterns associated with sites of slow repair appear very similar to what I observe with anti-pT371 TRF1, which is consistent with (pT371)TRF1 being dependent upon the MRN complex and appearing at a later time point after IR, and having a functional role in promoting HR, as these other late-responding proteins do.

TRF1 and TRF2 both bind to double stranded telomere DNA. TRF2, like TRF1, has been shown to have an involvement in the DNA damage response. TRF2 goes to non-telomeric sites of double strand breaks both after the induction of DNA damage by laser or X-rays (Bradshaw et al., 2005; Huda et al., 2012; Mao et al., 2007). TRF2 is phosphorylated at T188 in response to ionizing radiation by ATM, and is not bound to telomeres after this modification (Huda et al., 2009; Tanaka et al., 2005). TRF2, and its phosphorylation at T188, supports efficient homologous recombination at DSBs, inhibiting NHEJ, although the phosphorylation is not required for its recruitment to these sites (Bradshaw et al., 2005; Huda et al., 2012; Huda et al., 2009; Mao et al., 2007; Tanaka et al., 2005). There are parallels between TRF1 and TRF2, both in the way in which they bind to telomere DNA to prevent the activation of a DNA damage response at telomere DNA, and in the way in which the phosphorylated forms of both of these proteins localize to sites of DNA damage and promote homologous recombination. It would be interesting to examine if there were a common interacting partner of TRF1 and TRF2 at telomeres and at sites of DNA damage; both proteins have been suggested to interact with ATM and the MRN complex, so these may be good candidates for investigation (Bradshaw et al., 2005; Karlseder et al., 2004; Kishi and Lu, 2002; Kishi et al., 2001b; McKerlie et al., 2012; Wu et al., 2000; Zhu et al., 2000). Another of the shelterin proteins, Rap1, may also be involved in the DNA damage response. Two separate proteomic studies have suggested that Rap1 is phosphorylated in response to DNA damage, although the function of this modification remains to be seen (Bennetzen

et al., 2010; Matsuoka et al., 2007). It may be informative to evaluate whether there is crosstalk or redundancy between the effect of TRF1 and TRF2, or other shelterin proteins, on the repair of damaged DNA. It is important to understand how proteins that prevent a DNA damage response at telomeres can help to promote DNA repair at other genomic sites, and the mechanism by which this process may be regulated.

5.4 Multiple Functions of One Phosphorylation Site

It is interesting to consider that one phosphorylation site may be associated with many different functions. I have demonstrated a role for (pT371)TRF1 in the resolution of sister chromatids in mitosis and also in the repair of damaged DNA after ionizing radiation. There are other proteins which display a similar regulation; with one phosphorylation site regulating distinct roles in cell cycle progression and in the DNA damage response. One such protein is replication protein A subunit 32, or RPA32. Two phosphorylation sites on RPA32, S23 and S29, are phosphorylated by cyclinB-Cdk1 in mitosis, in cells that have not been exposed to DNA damage and are not stressed (Fang and Newport, 1993; Oakley et al., 2003; Stephan et al., 2009). RPA32 phosphorylated at these sites is not bound to chromatin, and does not play a role in the repair of damaged DNA. RPA32 pS23/pS29 may be modified at additional sites in response to DNA damage in mitosis, at which point it helps to promote the release of damaged cells from mitosis into G1 phase, but it remains chromatin soluble and does not participate in DNA repair (Anantha and Borowiec, 2009; Anantha et al., 2008; Fang and Newport, 1993; Oakley et al., 2003; Stephan et al., 2009). The regulation of RPA32 pS23/pS29 in mitosis is in contrast to that which is observed in interphase cells, which occurs in response to DNA damage, and is mediated partially by CDK2 (Binz et al., 2004; Dutta and Stillman, 1992; Niu et al., 1997; Oakley and Patrick, 2010). In this setting, these modifications facilitate the phosphorylation of RPA32 by DNA-PKcs at S4, S8, and T21 (Anantha et al., 2007; Niu et al., 1997; Zernik-Kobak et al., 1997). In response to DNA damage, RPA

phosphorylation is important for the repair of damaged DNA and for functional checkpoint activation; the mutation of S23 and S29 results in a DNA repair defect in interphase cells (Anantha et al., 2007; Binz et al., 2004; Oakley and Patrick, 2010; Vassin et al., 2009; Zernik-Kobak et al., 1997). It appears as though the same phosphorylation sites on RPA32, S23 and S29, are regulating different functions of RPA in mitotic versus interphase cells. The same phenomena may be evident with the examination of histone H2AX. The phosphorylation of H2AX at S139 in response to DNA damage by either ATM, ATR, or DNA-PKcs is a well characterized modification in the DNA damage response, and the modified form of H2AX has been termed γH2AX (Kinner et al., 2008; Yuan et al., 2010). It has been shown that γH2AX is important for signalling at sites of DNA damage, protein recruitment, and for the repair of damaged sites (Kinner et al., 2008; Yuan et al., 2010). In cells that have not been treated with DNA damage inducing agents, γH2AX foci increase in number as cells progress from G2 phase to mitosis, and are not found at sites of DNA damage (Huang et al., 2006a; Ichijima et al., 2005; McManus and Hendzel, 2005). Rather, γH2AX in mitotic cells has been shown to be involved in chromosome condensation, and not in the repair of DNA damage (Huang et al., 2006a; Huang et al., 2006b; Ichijima et al., 2005; Kurose et al., 2005; MacPhail et al., 2003; McManus and Hendzel, 2005). It is not unique, then, that (pT371)TRF1 may be involved in multiple functions; in the DNA damage response and in mitotic progression. There are a few possible explanations as to how (pT371)TRF1 may be participating in multiple functions.

I have characterized (pT371)TRF1 as being a stabilized form of TRF1 that is not degraded by the proteasome pathway or bound to telomere DNA. It is possible that this post-translational modification provides a pool of TRF1 protein that is consistently accessible for functional re-localization to other places in the nucleus, such as sites of DNA damage. TRF1 protein, when not phosphorylated at T371, is typically degraded upon its disassociation from telomeres. Unmodified TRF1 may not be stable for durations significant enough for its participation in other functions when it is not bound

to telomere DNA. In this way, (pT371)TRF1 may provide a pool of available TRF1 for functions away from the telomere. The way in which this fraction of protein is localized to different subnuclear places is unclear; however there are a number of possible explanations.

The phosphate group addition at T371 TRF1 could be mediating direct protein-protein interactions at sites of DNA damage, for instance with BRCA1 or NBS1. In this way, variant protein complex formation within the cell at different stages of the cell cycle or in response to different stimuli, such as the induction of DNA damage, may be responsible for the altered localization and functional implications of (pT371)TRF1. Performing immunoprecipitations to evaluate an interaction between TRF1 and proteins localized at sites of DNA damage may test this idea, and may provide clues as to other proteins important for the function of TRF1 in the repair of damaged DNA. As we have noted that the formation of (pT371)TRF1 foci is dependent upon NBS1, Mre11, and BRCA1, these may be likely candidates for initial investigation. It is a possibility that there are additional phosphorylation sites on TRF1 that are modified in conjunction with T371 and jointly regulate TRF1 localization and function. A model whereby additional post-translational modifications occur to support different functions would be in line with the way in which RPA32 pS23/pS29 is regulated. The fraction of unbound TRF1 that is phosphorylated at T371 and stabilized in the nucleoplasm may be modified in different stages of the cell cycle or in response to DNA damage at additional residues, contributing to its function. Mutating more than one TRF1 phosphorylation site at a time and examining combined effects; whether additive or synergistic would be one way in which this model could be investigated. It may also be useful to perform mass spectrometry on (pT371)TRF1 after the induction of DNA damage to evaluate additional post-translational modifications that are present on this population of TRF1, which may be involved in promoting this function. In summary, it is possible that (pT371)TRF1 is stabilized in the nucleoplasm and available for the functional localization to sites of DNA

damage, which may be the result of a direct protein-protein interaction promoted by the phosphorylation group, or the result of an additional modification on TRF1.

It may also be that the variant functions of (pT371)TRF1 can be explained by cell cycle regulation and are modulated by cyclin dependent kinase (CDK) activity, in a manner similar to the regulation of RPA32 by CDK activity. We have shown that, in mitosis, the function of (pT371)TRF1 in the resolution of sister chromatids is a direct consequence of Cdk1 activity, which phosphorylates this site upon mitotic entry. It is possible that this site may be a substrate of another CDK in response to DNA damage in interphase cells, which would require further investigation. A good candidate for the regulation of (pT371)TRF1 after DNA damage may be CDK2, which is present in S and G2 phases of the cell cycle. CDK2 has been linked to the DNA damage response in many ways; it phosphorylates NBS1, interacts with MRN, and phosphorylates CtIP which is important for the interaction between CtIP and BRCA1 and the MRN complex (Buis et al., 2012; Wohlbold et al., 2012). It has been demonstrated that CDK2 may regulate the choice of repair between HR and NHEJ; promoting HR in S and G2 cells, and that CDK2 promotes cell survival following IR (Buis et al., 2012; Deans et al., 2006; Wohlbold et al., 2012) (Chung and Bunz, 2010; Deans et al., 2006). A model suggesting the regulation of (pT371)TRF1 by CDK2 would explain the various functions of TRF1 phosphorylation in terms of the cell cycle stage in which the phosphorylation occurs. This possibility would require investigation into the effect of CDK2 depletion on TRF1 function, localization, and phosphorylation.

Regardless of the exact mechanism responsible for regulating the localization of (pT371)TRF1 to different sites within the nucleus and supporting its function at those sites, it is clear that our anti-pT371 TRF1 antibody has provided an extremely useful tool. I have been able to utilize this tool to visualize a small fraction of the total TRF1 in the cell, which is not associated with telomere DNA. These observations have allowed for a

more clear view of what this small fraction of TRF1 may be doing, and where it may be going, which has allowed us to characterize novel regulations and functions of TRF1.

5.5 The Regulation of TRF1 by Phosphorylation Events

TRF1 is phosphorylated on multiple residues, many of which have been shown to play important roles in regulating TRF1 function. Numerous kinases interact with and phosphorylate TRF1, in different stages of the cell cycle and in response to different circumstances. More than ten phosphorylation sites on TRF1 have been examined in detail, which has shed light on the intricately controlled nature of TRF1 regulation.

Many of the TRF1 phosphorylation sites evaluated have been shown to affect the binding of TRF1 to telomere DNA. TRF1 T122, T344, and S435 have been suggested to positively regulate TRF1 binding to telomere DNA, whereas T149, S274, S367, and T371 have been proposed to negatively regulate binding (Kim et al., 2008; Lee et al., 2009; McKerlie et al., 2012; McKerlie and Zhu, 2011; Wu et al., 2007; Wu et al., 2008). In studies which have evaluated both TRF1 stability and the binding of TRF1 to telomere DNA, it is often the case that when TRF1 is not bound to telomere DNA, it is less stable, and vice versa, as is observed with T122, T149, and S367 (Kim et al., 2008; Lee et al., 2009; McKerlie et al., 2012). These findings are in agreement with the model suggesting that TRF1 is ubiquitinated and targeted for proteasome-mediated protein degradation upon its release from telomere DNA (Chang et al., 2003; Her and Chung, 2009; Lee et al., 2006). An exception to this trend is TRF1 T371, which we have shown promotes TRF1 stability upon its phosphorylation, and remains free of telomeres. These results suggest that some aspect of (pT371)TRF1 must prevent its ubiquitination or targeting to the proteasome centres. RLIM, one of the ubiquitin ligases responsible for regulating the degradation of TRF1, interacts with TRF1 through its linker region, where T371 lies (Her and Chung, 2009). It would be of interest to know whether the phosphorylation of TRF1 at T371 is preventing the interaction between RLIM and TRF1. The other ubiquitin ligase

responsible for modifying TRF1 and targeting it for degradation, Fbx4, interacts with TRF1 in the homodimerization domain (Lee et al., 2006). The TRF1 phosphorylation sites that have been shown to affect TRF1 stability are all found within either the TRFH domain or the linker region of TRF1, suggesting that perhaps the effects observed on TRF1 stability by different phosphorylation events are the result of the regulation of E3 ubiquitin ligase activity or interaction. It would be interesting to evaluate whether the interaction between TRF1 and Fbx4 or RLIM is impacted when TRF1 is mutated at the phosphorylation sites which impact on its stability.

Although the phosphorylation of TRF2 has been less thoroughly investigated than TRF1, there is evidence that the binding of TRF2 to telomere DNA may also be regulated by phosphorylation events. TRF2 S20 has been shown to be phosphorylated by Chk2, which reduces the binding of TRF2 to telomeres (Buscemi et al., 2009). It would be useful to evaluate other potential TRF2 phosphorylation sites, such as T358, for their role in regulating TRF2 binding and function, to determine if TRF2 is regulated by phosphorylation in a similar way as TRF1 (Spengler, 2007).

The two phosphorylation sites examined within this thesis, S367 and T371, reside within the linker, or flexible, region of TRF1. The linker region of TRF1 has not been crystallized and eludes a thorough understanding. It is possible that modifications within the linker region of TRF1 have a significant impact on the tertiary structure of TRF1, and may mediate its function in this way. Phosphorylation events at both TRF1 S367 and T371 have been shown to decrease the association of TRF1 with telomere DNA. Whether these modifications may impact on the structure of TRF1 or its affinity for DNA requires further investigation.

The close proximity of S367 and T371 to each other raises the question of whether these sites may be interconnected in some way. I have discovered that the phosphorylation at both of these sites affects TRF1 stability, although in opposite ways;

S367 phosphorylation results in TRF1 degradation and T371 phosphorylation increases TRF1 stability. The phosphorylation of these sites, S367 and T371, also differentially regulates the localization of TRF1, to sites of proteasome dependent degradation or to sites of DNA damage, respectively. Whether there exists cross talk between the phosphorylation at these two sites requires more extensive examination.

The opposing nature of the regulation of TRF1 by the phosphorylation at S367 and T371 reinforces the idea that TRF1 is very tightly regulated. The fact that there are six TRF1 phosphorylation sites which have been shown to have individual roles in regulating the binding of TRF1 to telomere DNA, often affecting the stability of the protein, implies that the levels of TRF1 must be finely tuned for proper TRF1 function. The levels of TRF1 in the cell are critical in regulating telomere length and protection, and having two phosphorylation sites, S367 and T371, so close together which inversely influence TRF1 levels may help in maintaining this equilibrium. Higher expression levels of TRF1 have been linked to diseases such as chronic myeloid leukemia and MGUS (monoclonal gammopathy of undetermined significance) (Campbell et al., 2006; Panero et al., 2010). Conversely, a lower level of TRF1 expression has been observed in non-small cell lung cancer tissues, B-chronic lymphocytic leukemia, kidney cancer and breast cancer, and has been correlated with a poor prognosis in oral squamous cell carcinoma (Chuang et al., 2011; Kishi et al., 2001a; Lin et al., 2006; Poncet et al., 2008; Shi et al., 2004). Although the reason behind altered TRF1 expression levels in these diseased cells has not been fully elucidated, the correlation clarifies the importance of the strict control of TRF1 levels. We have demonstrated a clear role for phosphorylation events in mediating this process.

5.6 Shelterin Proteins in Non-Telomere Roles

The components of the shelterin complex have well elucidated roles in telomere maintenance and it is understood that their localization to telomere ends is crucial.

What is becoming apparent is the requirement for these proteins at other locations within the cell, and the importance of their functions at these sites. I have shown that TRF1 goes to sites of DNA damage and that it is important for HR, and also that an unbound form of TRF1 is important for the separation of sister chromatids in mitosis. TRF1 has been shown to have at least one other role away from telomeres, and that function is in the proper assembly of the mitotic spindle apparatus in mitosis, although the mechanism by which this occurs is not clear (Kishi et al., 2001a; Munoz et al., 2009; Nakamura et al., 2001a; Nakamura et al., 2002; Ohishi et al., 2010; Shen et al., 1997). The other double stranded telomere DNA binding protein, TRF2, also participates in functions apart from telomeres. Through its basic domain, TRF2 is able to bind to Holliday junctions and replication intermediates (fork-like structures), and stabilize these intermediates, preventing their cleavage (Fouche et al., 2006; Kachouri-Lafond et al., 2009; Poulet et al., 2009). TRF2 also localizes at sites of DNA damage, and may be involved in promoting homologous recombination (Bradshaw et al., 2005; Huda et al., 2012; Huda et al., 2009; Mao et al., 2007).

Other components of the shelterin complex, TIN2 and Rap1, have been shown to play functional roles at places other than telomere ends. TIN2 has been shown to localize to mitochondria through its N-terminus, and to impact on cellular metabolism (Chen et al., 2012). Depleting TIN2 in the cell inhibits glycolysis and also reduces the production of reactive oxygen species (Chen et al., 2012). TIN2 has additionally been shown to have an involvement in tethering telomere ends to the nuclear matrix (Kaminker et al., 2009; Smith, 2009). Rap1, which is associated with the shelterin complex through its interaction with TRF2, can be found bound to non-telomere DNA throughout the genome, and has been suggested to play a role in transcriptional control through its binding to promoter regions (Martinez et al., 2010). Rap1 may also play a cytoplasmic role in inducing the signalling of the NF-kappaB pathway (Teo et al., 2010). Our observation of the involvement of TRF1 in non-telomere functions, then, is not

unprecedented. Novel, non-telomere roles are currently being elucidated for many of the shelterin proteins originally characterized as having exclusively telomere functions.

5.7 Implications and Significance

The role of TRF1 in telomere maintenance has been thoroughly investigated and its important role at telomeres has been widely accepted. The shelterin complex is a crucial contributor to genomic maintenance; the disruption of shelterin can result in chromosome fusions, telomere loss, telomere doublets, and the recognition of telomeres as sites of DNA damage and subsequent genomic instability. TRF1 plays an important role in regulating telomere length in telomerase positive cells, which is linked to tumorigenesis and ageing. The disruption of TRF1 affects the efficiency of telomere replication and the protection of telomere ends. TRF1 is an important player in telomere maintenance, and, by extension, in the maintenance of genomic integrity. The importance of TRF1 at telomeres is not contested; however the involvement of TRF1 in functions at sites other than the telomere has been controversial. My work strongly supports for a role of TRF1 in the repair of damaged DNA away from telomere ends. This work may provoke further investigation into the function of shelterin proteins apart from telomeres, and open the door for further research into the broader regulation of these proteins, which may ultimately widen the breadth of information. Expanding the scope of research in this field can only benefit the progress on cancer and ageing related disorders.

The roles of TRF1 that I have described have strong implications for cell survival and proliferation. The removal of TRF1 from telomeres in mitosis and the reassociation of TRF1 with telomere DNA upon mitotic exit is a crucial process in normal cellular function. The disruption of this regulation results in micronuclei formation, apoptosis, and a failure of the chromatids to separate in mitosis. The function of TRF1 in the repair of damaged DNA has obvious implications in the preservation of genomic integrity.

Disrupting the capacity of TRF1 to promote the repair of DNA double strand breaks increases the accumulation of DNA damage, which would result in mutagenesis and genomic instability. It follows that TRF1 has a clear role in both maintaining telomere function, and in upholding overall genomic integrity. The knowledge that has been gained here may be potentially exploited, and applied to the field of tumorigenesis. Preventing the single modification on TRF1, pT371, would simultaneously disrupt the DNA damage response by decreasing the efficiency of HR, disrupt mitotic progression and the separation of sister chromatids, and reduce telomerase-mediated telomere extension because of the increased association of TRF1 with telomeres. The ability to target these changes would aid in targeting tumor cells therapeutically. The information presented is an important extension of the current knowledge on the way in which telomeres and the DNA damage response are interconnected.

It is important to thoroughly understand the regulation of telomere proteins and the repair of damaged DNA to fully comprehend the parameters of tumorigenesis and ageing. The full elucidation of each step in these processes may reveal details that can be exploited for therapeutic purposes. Drug targets for the treatment of cancer or ageing associated disorders may be revealed by exposing mechanisms involved in maintaining genomic stability and telomere function. I have uncovered novel functions of TRF1 in the repair of DNA double strand breaks, in the resolution of sister chromatids in mitosis, and an additional level of regulation of TRF1 in telomere maintenance. Knowledge of the way in which genomic integrity is maintained is significantly enhanced by the information contributed by this thesis.

5.8 Future Directions

I have shown a role for the phosphorylation of TRF1 at S367 in the removal of TRF1 from telomeres and in the localization of TRF1 to sites of proteasome degradation. I have demonstrated that this occurs predominantly in S and G2 phases of the cell cycle,

although the reason behind this cell-cycle dependency is not clear, and requires further investigation. The explanation as to why (pS367)TRF1 is targeted to degradation centres remains unclear, and may be due to other connections that TRF1 makes through its linker region, where S367 lies. The ubiquitin ligase RLIM interacts with TRF1 through its linker region, and NS also interacts in this area, decreasing the retention time of TRF1 on telomere DNA (Her and Chung, 2009; Meng et al., 2011). Whether these interactions are impacted when TRF1 is phosphorylated at S367, or if these connections are mediating the function of (pS367)TRF1, would be important to evaluate. Co-immunoprecipitations evaluating the interaction between TRF1 and NS or RLIM when TRF1 is mutated at S367 to prevent or mimic phosphorylation would shed light on the involvement of this site in regulating these interactions. Depleting RLIM or NS and monitoring the localization of (pS367)TRF1 within the cell would also be informative, and indicate whether these interactions play a role in the localization of (pS367)TRF1 to sites of proteasome degradation. Whether these interactions, or other interactions that occur through the linker region of TRF1, impact on the ability of TRF1 to bind to telomere DNA when it is phosphorylated at S367, or T371, would be interesting to investigate. Since (pT371)TRF1 is also not bound to telomere DNA, and is also modified in the linker region, performing these experiments to evaluate the way in which (pT371)TRF1 binding to telomeres is modulated would provide useful information. It is possible that TRF1 phosphorylation at either S367 or T371 may affect the interaction with RLIM, or the addition of ubiquitin chains, and it this way regulate the stability of TRF1. TRF1 phosphorylated at either S367 or T371 may differentially regulate this association, which could explain the observed difference in stability of (pS367)TRF1 and (pT371)TRF1. To further this understanding, it would be useful to examine if these regulations, and the functions of (pS367)TRF1 and (pT371)TRF1 are evolutionarily conserved. TRF1 T371 is present in mice, so it would be interesting to see if this site is also involved in sister telomere resolution or in the DNA damage response in mouse cells.

To advance our understanding of the involvement of (pT371)TRF1 in the DNA damage response, it is important to consider how TRF1 is recruited to these sites. It would be useful to evaluate if there are direct protein-protein interactions occurring between (pT371)TRF1 and components of the DNA damage response that are important for its localization. Candidates to consider would include BRCA1 and components of the MRN complex, as the recruitment of (pT371)TRF1 to sites of DNA damage is dependent upon these proteins. Another factor that may be influencing the localization of (pT371)TRF1 to sites of damage is the presence of additional post-translational modifications on TRF1 in response to DNA damage. This could be evaluated using mass spectrometry on (pT371)TRF1 after exposure to DNA damage inducing agents, or by mutating candidate TRF1 residues, such as S219, in conjunction with T371, to evaluate how multiple phosphorylation sites may work collaboratively to regulate this function.

It would also be interesting to further explore the precise mechanism of action of TRF1 at sites of DNA damage. That is, what exactly it is that TRF1 is doing that is promoting homologous recombination. It may be involved in the recruitment of DNA damage response factors to sites of breaks, which could be evaluated by observing the localization of various factors to breaks when TRF1 is depleted or mutated. It may also be involved in enhancing the processivity of repair proteins, such as those involved in end resection. TRF1 phosphorylated at T371 appears to impact on end resection, so evaluating the efficiency of different endonucleases in the presence of TRF1 mutants may help to delineate the involvement of TRF1 in this step of the repair process. Understanding the exact mechanism of (pT371)TRF1 action and the way in which phosphorylation events at S367 and T371 affect the stability and telomere binding capabilities of TRF1 would further clarify the function of these modifications in telomere maintenance and in the DNA damage response.

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