Cell-cycle Dependent Regulation of Telomere-associate Proteins

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology and Cancer Biology in the Graduate School of Duke University

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ABSTRACT

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Abstract

Telomeres are protein-DNA structures at the ends of eukaryotic chromosomes. The DNA portion is comprised of double-stranded and single-stranded G-rich repetitive DNA. The protein portion is anchored by the “shelterin complex” composed of six proteins. Inappropriate DNA repair and telomere length dysregulation result in cell cycle arrest, genome instability, and carcinogenesis. Thus, this DNA/protein structure protects telomere ends and regulates telomere length.

The shelterin component TRF1, a double-stranded telomeric DNA binding protein, was found to bind accessory protein PinX1 at mitosis. Given this, I investigated the effect of reducing PinX1 level on cell cycle progression and apoptosis. I found that reducing PinX1 expression with shRNA, as assessed by immunoblot, led to delayed entry into mitosis and elevated levels of apoptosis in human cells. These results indicated that PinX1 plays an important role in mitosis progression and cell viability.

Intriguingly, binding of PinX1 to TRF1 at mitosis increased the stability of the latter. Moreover, PinX1 binds to the same site on TRF1 as the protein TIN2, which can suppress degradation of TRF1 by inhibiting poly ADP-ribosylation of TRF1 by the enzyme tankyrase. Collectively, these results suggested that TIN2 might be released from TRF1 to promote the binding of PinX1 on TRF1 at mitosis. Given that proteins are often regulated in the cell cycle by phosphorylation, I investigated whether TIN2 was
phosphorylated at mitosis. To this end, I performed phospho-proteomic analysis of human TIN2, which revealed two phosphorylated residues, serines 295 and 330. Both sites were phosphorylated specifically during mitosis, as detected by two independent approaches, namely Phos-tag reagent and phosphorylation-specific antibodies. Phosphorylation of serines 295 and 330 appeared to be mediated, at least in part, by the mitotic kinase RSK2 in vitro and in vivo. The identification of these specifically timed post-translational events during the cell cycle demonstrates the mitotic regulation of TIN2 by phosphorylation. However, as expressing non-phosphorylatable mutants of TIN2 failed to reveal any overt phenotypes, the consequences of these phosphorylation events remain to be determined.

Lastly, the TRF1-related double-stranded telomeric DNA binding protein, TRF2, was shown to associate with another shelterin component, POT1. POT1 forms heterodimer with TPP1 to bind single-stranded telomeric DNA. Previous research found that mutations of POT1 with reduced binding affinity to either TRF2 or to TPP1 cause distinct phenotypes. To determine whether similar separation-of-function mutants could be generated to dissect the function of POT1s in mice, which are encoded by two genes, *Pot1a* and *Pot1b*, I screened a panel of substitution mutants of mPOT1a for loss of binding to mTRF2 and mTPP1. These studies revealed that mPOT1a does not bind mTRF2, but the association with mTPP1 could be disrupted.
In summary, the described studies have shed insight into the complexity of shelterin regulation, and in particular, highlighted protein-protein interactions and post-translational modifications.
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1. Introduction

1.1 Overview of Telomere biology

Telomeres are essential protein-DNA structures that cap and protect the ends of eukaryotic linear chromosomes (Palm and de Lange 2008; O'Sullivan and Karlseder 2010). The protein component, termed the “shelterin complex”, includes TRF1, TRF2, POT1, TPPI, TIN2 and RAP1 in human cells (Palm and de Lange 2008). The DNA portion is proposed to be comprised of double-stranded DNA (dsDNA) repeats of TTAGGG, followed by a single-stranded DNA (ssDNA) region that invades the dsDNA to form T-loop, as revealed by electron microscopy (Griffith and Comeau et al. 1999). This protein-DNA structure may help protect telomeres from being detected as damaged DNA and maintain appropriate telomere length (O'Sullivan and Karlseder 2010).

The telomere DNA-protein structure is conserved through the evolution of eukaryotes, including ciliates, fungi, green plants, and animals (Fajkus and Sykorova et al. 2005). Although the repetitive DNA sequence and the structure of telomere-associate proteins is not exactly the same among species, capping chromosome ends with a special DNA-protein structure remains important during evolution (Fajkus and Sykorova et al. 2005; Teixeira and Gilson 2005). Recent studies have identified several novel telomere-associate proteins in mammalian genome which are homologues to the telomere-binding proteins in yeast, suggesting that multiple telomere-associate protein complexes
may exist and some of them have undertaken different functions during evolution (Linger and Price 2009).

Telomere dysfunction has been associated with cancer, aging, and anemia. When telomere length becomes critically short, or the protection of telomeres by shelterin complex is compromised, telomere ends can be detected by DNA damage response machinery, followed by inappropriate DNA repair (O'Sullivan and Karlseder 2010). These illegitimate repairs then lead to chromosome instability and mutations inside oncogenes or tumor suppressor genes through break-fusion-bridge (BFB) cycles, a key step during carcinogenesis (O'Sullivan and Karlseder 2010). Mammalian telomere length attrition is accompanied with normal aging, resulted in somatic cell apoptosis or senescence (Lopez-Otin and Blasco et al. 2013; Armanios 2013). One of the premature aging diseases related to the telomere dysfunction is dyskeratosis congenita, which manifests short telomeres and bone marrow failure. The causes of this disease have been linked to the genetic mutations of telomere-associate proteins and components of telomerase, the enzyme that specifically elongates telomeres (Mason and Bessler 2011).

In summary, telomere DNA-protein structure is conserved component of eukaryotic chromosomes, the disruption of which leads genomic instability and telomere length deregulation.
1.2 Telomeric DNA

Telomeric DNA is composed of tandem repeats of TTAGGG sequence. In human somatic cells, the length of telomeric DNA becomes shorter after each cell cycle due to the end-replication problem (Watson 1972; Olovnikov 1971). However, telomere length is maintained in the germline (Gunes and Rudolph 2013; Flores and Blasco 2010) by telomerase, a reverse transcriptase responsible for de novo elongating telomeres. T-loops, a telomeric DNA structure formed by 3’ overhang invading the upstream dsDNA region, are found to restrict the access of telomerase to telomere ends. It has been proposed that longer telomeres recruited more telomere associate proteins, which could then promote T-loop formation and prevent telomere elongation by blocking the access of telomerase (Ye and Hockemeyer et al. 2004; Loayza and De Lange 2003). Although the molecular mechanism behind T-loop formation and its maintenance is still unclear, it is suggested that during normal cell cycle, telomere structures need to switch between T-loop and open/linear states to enable telomere protection in interphase and replication during S phase, respectively (Wei and Price 2003).

The G-rich telomeric DNA can also form a G-quadruplex. This secondary DNA structure can also restrict telomerase access to telomere ends in vitro (Tang and Kan et al. 2008; Xu and Sato et al. 2007). Further, recent studies have identified a telomeric repeat-containing RNA (TERRA), transcribed from telomeric repeats sequence at telomere that can hybridize to the RNA template (Redon and Reichenbach et al. 2010; Luke and
Lingner 2009) and protein component (Redon and Zemp et al. 2013) of telomerase and inhibit its activity. The discovery of TERRA adds another component to telomere length regulation (Figure 1).

In summary, T-loop formation, G-quadruplex structure, and transcription of TERRA from telomeric DNA all contribute to telomere length regulation and maintenance.
Figure 1: Telomere structure and associated proteins

Telomere DNA structure change between linear state and T-loop state, facilitated by various proteins. Telomere-binding protein complex (Shelterin) were illustrated on the figure, along with telomerase and TERRA. (O’Sullivan and Karlseder 2010)
1.3 Overview of shelterin protein complex

Among the six members of shelterin proteins in human cells, two proteins have the unique ability to bind dsDNA, namely telomeric repeat binding factor 1 (TERF1, hereafter referred to as TRF1) (Zhong and Shiue et al. 1992) and telomeric repeat binding factor 2 (TERF2, hereafter referred to as TRF2) (Bilaud and Koering et al. 1996) through their C-terminal Myb domain. Both proteins function as homodimers via their N-terminal TRFH domain (Broccoli and Smogorzewska et al. 1997). Although TRFH domains in TRF1 and TRF2 are structurally similar, the functions and binding partners of these two proteins are different (Fairall and Chapman et al. 2001).

TRF1 and TRF2 have been shown to interact with TIN2 (TRF1)-interacting nuclear factor 2 (TINF2, hereafter referred to as TIN2) simultaneously (Ye and Donigian et al. 2004), while TIN2 also interacts with the ssDNA-binding protein heterodimer POT1 (Protection Of Telomeres 1)/TPP1 (adrenocortical dysplasia homolog, hereafter referred to as TPP1) in the shelterin complex (O’Connor and Safari et al. 2006). Therefore, TIN2 has a central role in the assembly of shelterin complex.

POT1 has the unique role in coating ssDNA at the 3’ overhang via its OB-fold domain in N-terminus and interacting with TPP1 via its C-terminus, presumably to protect this overhang from being detected as DNA damage sites (Barrientos and Kendellen et al. 2008). TPP1 was shown to facilitate telomerase recruitment (Xin and Liu
et al. 2007; Baumann and Cech 2001) and enhance the activity and processivity of this enzyme (Latrick and Cech 2010).

The last member of shelterin complex is the telomeric repeat binding factor 2 interacting protein, TERF2IP (hereafter referred to as RAP1), a binding partner of TRF2. The function of RAP1 was originally thought to prevent DNA damage responses (Sarthy and Bae et al. 2009; Bae and Baumann 2007). Recently however, the role of RAP1 expanded to transcriptional regulation of genes outside of telomere region. Also, a direct DNA-binding ability of RAP1 is discovered recently (Yeung and Ramirez et al. 2013; Martinez and Gomez-Lopez et al. 2013; Teo and Ghosh et al. 2010; Arat and Griffith 2012).

The functions of shelterin complex encompass various physiological processes in cells. Deletion of any component of shelterin complex results in deprotection of telomere, with elevated DNA damage signaling on telomeres and inappropriate DNA repair (discussed below). The recruitment of telomerase to telomere largely replies on the interaction between telomerase and POT1/TPP1 heterodimer (Xin and Liu et al. 2007; Baumann and Cech 2001), while TRF1 serves as a negative regulator of telomere length (Loayza and De Lange 2003). The stability of 3’ overhang and formation of T-loop structure is facilitated by POT1/TPP1 and TRF2, respectively (Stansel and de Lange et al. 2001; Kendellen and Barrientos et al. 2009). Further, the interaction between TIN2 and
TRF1 is also involved in sister chromatids separation during mitosis (Canudas and Houghtaling et al. 2007).

In summary, all six components of the shelterin complex contribute to telomere protection, while they also have unique roles in telomere length maintenance and chromosome segregation.

1.4 TRF1 and PinX1

Human TRF1 (hTRF1) is a 56-kDa protein, comprised of N-terminal TRFH domain that promotes homodimerization, and C-terminal Myb domain, which is responsible for the dsDNA-binding activity of the protein (Broccoli and Smogorzewska et al. 1997; Bianchi and Smith et al. 1997).

TRF1 has been recognized as negative regulator of telomere length, as overexpression of this protein in human cell lines led to shortened telomere, while a dominate-negative mutant of hTRF1 induced telomere lengthening (van Steensel and de Lange 1997). hTRF1 had also been shown to help load human POT1 (hPOT1) on the 3’ overhang via interaction with human TIN2 (hTIN2), which may explain how TRF1 represses telomere length by excluding telomerase from telomere ends (Loayza and De Lange 2003). Since hPOT1 proved to help recruit telomerase to telomeres and enhance telomerase processivity in conjunction with human TPP1 (hTPP1) (Xin and Liu et al. 2007; Wang and Podell et al. 2007), the role of TRF1/TIN2/POT1/TPP1 in telomere length control is likely complicated and will require further investigation.
Although knockdown of hTRF1 in various human cell lines did not affect cell viability, knockout of Trf1 in mice is embryonic lethal (Karlseder and Kachatrian et al. 2003). Recent studies using conditional Trf1 knockout mouse models revealed that deletion of murine TRF1 (mTRF1) also exposes telomere ends as fragile sits, and induces telomere instability in the forms of telomere fusion, concatenation, and multi-telomeric signals (Martinez and Thanasoula et al. 2009; Sfeir and Kosiyatrakul et al. 2009).

An important hTRF1-binding partner is human PinX1 (hPinX1), a 37-kDa protein with an N-terminal glycine-rich domain and C-terminal TRF1-binding motif (FXLXP). hPinX1 was originally identified through yeast two-hybrid screen using hTRF1 as a bait, but subsequently found to also inhibit telomerase in vitro and in vivo (Zhou and Lu 2001; Banik and Counter 2004). However, hPinX1 is also required to recruit telomerase to telomere during S phase (Cheung and Kung et al. 2012). The absence of murine PinX1 (mPinX1) in mice led to increased tumorigenesis, while silencing PinX1 in established telomerase-positive cancer cell lines resulted in growth inhibition (Zhou and Huang et al. 2011; Zhang and Bai et al. 2009). All these studies suggest that PinX1 has multiple roles in telomere length regulation, possibly depending on cellular context and cell cycle stages.

The interaction between hPinX1 and hTRF1 proved to enhance the telomeric DNA-binding ability of the latter (Yoo and Oh et al. 2009). hPinX1 is enriched at telomeres to during mitosis and can stabilize TRF1 at that phase of cell cycle (Yonekawa
and Yang et al. 2012). Based on these data, the interaction between hPinX1 and hTRF1 may be required for the stability of hTRF1 during mitosis.

One of the most important modifications on hTRF1 is poly-ADP ribosylation, mediated by TRF1-interacting, ankyrin-related ADP-ribose polymerase, tankyrase, (Smith and Giriat et al. 1998). Tankyrase belongs to the poly (adenosine diphosphate-ribose) polymerase (PARP) family that adds chains of ADP-ribose onto proteins, usually to inhibit target protein activity (Schreiber and Dantzer et al. 2006). This novel modification by tankyrase was demonstrated to disassociate hTRF1 from telomeres and lead to its degradation (Chang and Dynek et al. 2003; Cook and Dynek et al. 2002; Smith and de Lange 2000). Moreover, the TRF1-binding protein, hTIN2, was shown to inhibit this poly-ADP ribosylation of TRF1 mediated by tankyrase (Ye and de Lange 2004).

Knockdown of tankyrase by shRNA resulted in cell cycle arrest at mitosis, presumably due to unseparated sister telomere ends (Dynek and Smith 2004). Further, knockdown of hTRF1 or hTIN2 by shRNA rescued this mitotic arrest phenotype (Canudas and Houghtaling et al. 2007). It is therefore suggested that sister telomere ends are initially linked together after DNA replication through the interaction between hTRF1, hTIN2, and the cohesin complexes, which formed a ring-shape structure on each of the sister chromatids (Canudas and Houghtaling et al. 2007; Zhang and Kuznetsov et al. 2008). As such, modification and degradation of hTRF1 might be required for the
complete separation of sister chromatids at mitosis (Dynek and Smith 2004; McKerlie and Zhu 2011).

Other modifications on hTRF1 have also been identified, especially phosphorylation. Several studies have reported that hTRF1 is phosphorylated by various kinases during cell cycle. For example, the DNA-binding ability of hTRF1 was enhanced by phosphorylation of Cdk1/Plk1 (S435) and casein kinase 2 (T122) (Wu and Yang et al. 2008; Kim and Kang et al. 2008), Akt phosphorylated hTRF1 on T273, which was linked to telomere shortening (Chen and Teng et al. 2009), Ataxia telangiectasia mutated (ATM) kinase phosphorylation on T367 could promote the disassociation of hTRF1 from telomeric DNA (McKerlie and Lin et al. 2012), Cyclin B-Cdk1 phosphorylation on T371 of hTRF1 was essential for sister telomere resolution (McKerlie and Zhu 2011), and the mitotic defects caused by overexpression of Aurora A was dependent on S296 phosphorylation of hTRF1 (Ohishi and Hirota et al. 2010).

Collectively, these results indicate that hTRF1 is phosphorylated at multiple sites, resulting in different phenotypes. Notably, all these phosphorylation sites are located in different domains of hTRF1, suggesting that each event may control different functions of TRF1.

1.5 TRF2

Human TRF2 (hTRF2) is a 56-kDa protein, comprised of an N-terminal TRFH domain that promotes homodimerization and a C-terminal Myb domain that promotes
binding to telomeric dsDNA. One distinct difference between the two TRF proteins is that N-terminus of hTRF2 is composed of basic amino acids, rather than acidic amino acids found in hTRF1. (Broccoli and Smogorzewska et al. 1997)

Knock of Trf2 in mice is embryonic lethal (Ye and Donigian et al. 2004; Kim and Beausejour et al. 2004). One of the important roles of TRF2 is to prevent non-homologue end joining (NHEJ) at telomere ends. Overexpression of a dominate-negative mutant of hTRF2, or depletion of endogenous murine TRF2 (mTRF2) protein in mouse embryonic fibroblast (MEF), led to end-to-end fusion of telomeres, which was dependent on DNA ligase IV (van Steensel and Smogorzewska et al. 1998; Smogorzewska and Karlseder et al. 2002; Celli and de Lange 2005). The DNA damage responses caused by the absence or dominate-negative form of mTRF2 were primarily downstream of the ATM signaling pathway (Denchi and de Lange 2007). A recent study has shown that the dimerization domain of mTRF2 prevents the activation of ATM signaling, presumably through excluding sensor proteins from telomere ends. Further, mTRF2 could also inhibit the downstream amplification of ATM signaling by an ubiquitin-dependent mechanism. (Okamoto and Bartocci et al. 2013)

The binding partners of TRF2 also proved to be crucial for telomere protection and maintenance. hTRF2 is critical for preserving 3’ overhang of telomeres, in part by recruiting WRN, a helicase and exonuclease (Machwe and Xiao et al. 2004; Opresko and von Kobbe et al. 2002). Moreover, another nuclease interacting with TRF2, Snm1B
(Apollo) (Freibaum and Counter 2006; van Overbeek and de Lange 2006) was also important for protecting 3’ overhang length of telomeres (Akhter and Lam et al. 2010; Lam and Akhter et al. 2010) and resolving replication stress, possibly through interaction with replications fork complex (Mason and Das et al. 2013; Stringer and Counter 2012). Since telomere DNA repeats can be recognized as fragile sites, specific chromosome regions that challenge replication (Sfeir and Kosiyatrakul et al. 2009), these nucleases may be required during replication to ensure the normal progression of replication fork. Also, hTRF2 had been shown to interact with and inhibit activation of DNA damage signal transducer, the ATM kinase, as a mechanism to repress DNA damage signaling at telomeres (Karlseder and Hoke et al. 2004).

Various post-translational modifications have been identified on TRF2 that are important for its function in protecting and regulating the length of telomeres. ATM phosphorylation of T188 of hTRF2 plays a role in dsDNA break repair process (Huda and Tanaka et al. 2009; Tanaka and Mendonca et al. 2005). A p53-induced ubiquitination by E3 ligase Siah-1 on hTRF2 led to its degradation, suggesting a positive feedback between p53 activation and hTRF2 down-regulation during DNA damage signaling (Fujita and Horikawa et al. 2010). In contrast, acetylation on K293 of hTRF2 by histone acetyltransferase p300 can prevent hTRF2 degradation and thereby protect telomere integrity (Her and Chung 2013). Methylation of the basic domain of hTRF2 by protein
arginine methyltransferases (PRMTs) is also required for telomere stability and length maintenance (Mitchell and Glenfield et al. 2009).

Human RAP1 (hRAP1), a 44-kDa conservative member of telomere-binding protein complex, is another important binding partner of hTRF2 (Li and de Lange 2003), composed of four domains, a BRCT domain, a Myb HTH motif, a coiled region, and a C-terminal protein interaction domain (termed RCT), the last of which mediates its interaction with TRF2. hRAP1 has been recently shown to directly bind telomeric DNA and modulate hTRF2 localization on telomere (Arat and Griffith 2012). Initial studies revealed a role for hRAP1 in suppressing NEHJ by interacting with TRF2 (Sarthy and Bae et al. 2009; Bae and Baumann 2007; Bombarde and Boby et al. 2010). Conditional knockout of the Rap1 gene in mice further illustrated the role of mRAP1 in repressing homology-directed repair (HDR) (Sfeir and Kabir et al. 2010). These findings confirm the important role of RAP1 in telomere-binding protein complex. Surprisingly, further studies illustrated that RAP1 is also capable of regulating gene transcription in subtelomeric region (Martinez and Thanasoula et al. 2010) and the NF-κB signaling pathway (Teo and Ghosh et al. 2010). More recently, RAP1 was linked to cell metabolism, as Rap1 knockout mice exhibited dysregulation in adipose tissue and liver (Yeung and Ramirez et al. 2013), possibly through PPARα and PGC-1α signaling pathway (Martinez and Gomez-Lopez et al. 2013).
1.6 POT1

In the shelterin complex, POT1 is the only telomeric ssDNA-binding protein, which is critical for capping telomere ends and regulating telomere length (Loayza and De Lange 2003; Baumann and Cech 2001). Human POT1 (hPOT1) was originally discovered through a Basic Local Alignment Search Tool (BLAST) for homologues of fission yeast and ciliate telomeric ssDNA binding protein (Baumann and Cech 2001). hPOT1 has two oligonucleotide/oligosaccharide binding (OB) folds at N-terminal that specifically binds to ssDNA telomeric repeats (Lei and Podell et al. 2004). Several amino acids in these OB folds are critical to specifically distinguish telomeric DNA sequence (Wang and Podell et al. 2007). The C-terminal region of hPOT1 mediated its interactions with human TPP1 (hTPP1) (Ye and Hockemeyer et al. 2004; Liu and Safari et al. 2004) and human TRF2 (hTRF2) (Yang and Zheng et al. 2005).

Knockdown of hPOT1 in human cell lines resulted in reduced length of 3’ overhang, elevated levels of apoptosis, senescence, DNA damage response, and chromosome fusion (Hockemeyer and Sfeir et al. 2005; Veldman and Etheridge et al. 2004; Yang and Zhang et al. 2007). In mice there are two Pot1 genes, mPot1a and mPot1b. Knockout of mPot1a is embryonic lethal while mPot1b is dispensable for development. Further investigation of derived MEFs revealed that mPot1a was required for repressing DNA damage signaling at telomeres, while mPot1b regulated the length of 3’ overhang (Baumann 2006; Wu and Multani et al. 2006; Hockemeyer and Daniels et al. 2006).
DNA damage response caused by mPOT1a depletion was mediated by ataxia telangiectasia and Rad3-related protein (ATR) signaling pathway (Denchi and de Lange 2007). While mPOT1b is less relevant in preventing DNA damage signaling at telomeres, it has a unique role in regulating 3' overhang length. Several studies have reported that mPOT1b was essential for telomere overhang generation in conjunction with other telomeric accessory proteins (He and Multani et al. 2006; Wu and Takai et al. 2012).

When hPOT1 binds hTPP1, its specificity to telomeric DNA sequence is enhanced, and this interaction is required to exclude DNA damage sensor from telomere ssDNA and protect telomere ends from being detected as DNA damage sites (Barrientos and Kendellen et al. 2008; Nandakumar and Podell et al. 2010). Similarly, the interactions between either mPOT1a or mPOT1b with mTPP1 proved to be important for telomere protection (Hockemeyer and Palm et al. 2007). Another function of hPOT1 is to promote telomere elongation, presumably by recruiting telomerase through its interaction with hTPP1 (Xin and Liu et al. 2007; Latrick and Cech 2010; Wang and Podell et al. 2007).

Moreover, hPOT1 also co-immunoprecipitates with hTRF2, and hPOT1 mutants defective in this interaction fail to protect telomere ends from overhang loss and abnormal telomere elongation (Kendellen and Barrientos et al. 2009; Yang and Zheng et al. 2005). mTRF2 has also been shown to promote the formation of T-loops in vitro and
anchor mPOT1a and mPOT1b to telomere ends (Griffith and Comeau et al. 1999; Hockemeyer and Palm et al. 2007).

Limited information is known about the post-translational modifications on POT1, besides an ubiquitination site identified by genomic screen (Wagner and Beli et al. 2011). Nevertheless, the functions of POT1 on telomeres may still be regulated by the modifications of its binding partners, TPP1 and TRF2.

1.7 TPP1

hTPP1 is a 58-kDa protein containing an N-terminal oligonucleotide/oligosaccharide-binding (OB) fold, which participates in the telomerase recruitment, POT1-recruitment domain (RD), a Ser-rich region (S/T), and a TIN2-interacting domain (TID) (Xin and Liu et al. 2007).

Expression of truncated version of mTPP1, due to a point mutation in mouse mTpp1 allele, resulted in mild increased levels of Telomere-dysfunction Induced Foci (TIFs), chromosome fusions, as well as a mild reduction of mPOT1a, mPOT1b, and mTIN2 on telomeres, which were exacerbated by knockdown of residual mTPP1 protein by shRNA (Hockemeyer and Palm et al. 2007). Knockdown of hTPP1 in human cell lines also impaired the localization of hPOT1, recruitment of telomerase, and induced DNA damage response on telomeres (O’Connor and Safari et al. 2006; Abreu and Aritonovska et al. 2010). Knockout of the mTpp1 gene in mice was embryonic lethal, while mTpp1^{-/-} MEFs exhibited a loss of mPOT1a and mPOT1b on telomeres, elevated level of TIFs
mediated by ATR pathway, increased length of the telomeric ssDNA, and increased level of chromosome fusions (Kibe and Osawa et al. 2010). In summary, these results emphasized the important role of TPP1 in telomere protection and length regulation.

As discussed above, the interaction between TPP1 and POT1 is required for telomere protection and length regulation. Specifically, the hTPP1-hPOT1 interaction can exclude DNA damage sensors from 3’ overhang of telomere ends (Barrientos and Kendellen et al. 2008). Further, the TPP1-POT1 interaction promoted recruitment and processivity of telomerase through the OB fold of TPP1 (Latrick and Cech 2010; Wang and Podell et al. 2007; Zhong and Batista et al. 2012; Nandakumar and Bell et al. 2012). In addition, hTIN2 has also been shown to facilitate telomerase recruitment, possibly by anchoring hPOT1/hTPP1 on the telomere overhang (Abreu and Aritonovska et al. 2010).

Recent studies have shown that post-translational modifications on TPP1 can also affect its function in telomere protection or telomerase recruitment. For example, TPP1 was phosphorylated on multiple sites by CDKs in a cell-cycle dependent manner, and mutation of one such phosphorylation site led to decreased telomerase activity and shorter telomeres (Zhang and Chen et al. 2013). The Akt signaling pathway participated in the homodimerization of TPP1, and its protection of telomere ends with POT1 (Han and Liu et al. 2013). Further, TPP1 was stabilized by an E3 ubiquitin ligase Rnf8 (Rai and Li et al. 2011). Collectively, these results indicate that the function of TPP1 is tightly regulated.
1.8 TIN2

hTIN2 is a 40-kDa telomere associate protein, composed of an N-terminal basic and acidic region that mediates the interaction with TPP1, a central TRF1-binding domain, and a C-terminal region frequently mutated in dyskeratosis congenita patients (Kim and Kaminker et al. 1999; Walne and Vulliamy et al. 2008).

hTIN2 plays a central role in shelterin complex, as it interacts with hTRF1, hTRF2, and hPOT1/hTPP1 to form different protein complexes on telomeres (O’Connor and Safari et al. 2006; Liu and O’Connor et al. 2004). Knockdown of hTIN2 by shRNA led to the reduction of hTRF1, hPOT1, hTRF2, hRAP1, and telomerase on telomeres, and dysregulation in cancer cell metabolism (Ye and Donigian et al. 2004; Abreu and Aritonovska et al. 2010; Takai and Kibe et al. 2011; Chen and Zhang et al. 2012). Knockout of mTin2 in mice was embryonic lethal, and deletion of mTin2 in MEFs led to telomere deprotection and instability, including loss of other telomere-associate proteins on telomeres, increased length of telomeric ssDNA, increased levels of DNA damage signaling at telomeres mediated by both ATM and ATR pathways, and increased levels of chromosome fusions, all of which were observed in mPot1a<sup>+</sup>, mTpp1<sup>+</sup>, and mTrf2<sup>+</sup> MEF (Takai and Kibe et al. 2011; Chiang and Kim et al. 2004). These results demonstrate that TIN2 is essential for the integrity and function of shelterin complex.

The interactions between TIN2 and other shelterin members proved to be crucial for telomere protection and length regulation. Specifically, the hTIN2-hTRF1 interaction
was important for the localization and stability of hTRF1 on telomeres (Ye and de Lange 2004), hTIN2 stabilizing of hTRF2 on telomeres can suppress a DNA damage responses (Ye and Donigian et al. 2004; Kim and Beausejour et al. 2004), and the hTIN2-hTPP1 interaction can promote telomerase recruitment to telomeres while inhibit mitochondrial localization of hTIN2 (Abreu and Aritonovska et al. 2010). Further, the mutant versions of TIN2 found in aging diseases, dyskeratosis congenita, lacked the ability to bind heterochromatin protein 1γ, resulting in cohesin defects and telomere shortening (Canudas and Houghtaling et al. 2011). Finally, an E3 ligase responsible for TIN2 degradation has been identified recently (Bhanot and Smith 2012). Collectively, these studies demonstrate the diverse roles of TIN2 in telomere maintenance and outside of telomere region.

1.9 Summary

The human shelterin complex is composed of the dsDNA-binding proteins, TRF1 and TRF2, the ssDNA-binding protein, POT1, and the associated proteins, RAP1, TPP1, and TIN2. This protein complex may help telomere ends to form T-loop, preventing chromosome ends from being detected as dsDNA breaks. The shelterin complex regulates the access of telomerase to telomere ends and cooperates with cohesin complex for sister chromatids separation during mitosis.

Protein-protein interactions among shelterin members are not only important for assembly at telomere ends, but are also critical for telomere protection and length
regulation. As mentioned above, TIN2 binds TRF1, TRF2, RAP1, TPP1, and POT1 and depletion of TIN2 leads to reduction of all these proteins on telomeres (Ye and Donigian et al. 2004; Abreu and Aritonovska et al. 2010; Takai and Kibe et al. 2011). POT1-TPP1 interaction was important for protecting the 3′ overhang of telomere ends and recruiting telomerase (Barrientos and Kendellen et al. 2008; Xin and Liu et al. 2007; Wang and Podell et al. 2007). The stability of TRF1, a telomere length negative regulator, and TRF2, a repressor of DNA damage response, relied on their interactions with TIN2 (Ye and Donigian et al. 2004; Ye and de Lange 2004). Further, interactions among TRF1, TIN2, and cohesin complex were important for sister chromatids separation (Canudas and Houghtaling et al. 2007). Thus, the protein-protein interaction among shelterin members and with other telomere accessory proteins proved to be required for telomere maintenance.

The functions of shelterin complex are coordinated within the cellular context as well, including cell proliferation, division, and DNA damage response signaling, by post-translational modifications. The stability of several shelterin members was regulated by ubiquitin-dependent degradation (Chang and Dynek et al. 2003; Rai and Li et al. 2011; Bhanot and Smith 2012). Phosphorylation of TPP1 by Akt, an important cell survival signaling pathway, promoted telomere protection (Han and Liu et al. 2013). ATM phosphorylation of TRF2 facilitated DNA damage repair on telomeres (Huda and Tanaka et al. 2009; Tanaka and Mendonca et al. 2005). Interestingly, multiple
phosphorylation sites were identified on TRF1 for different purposes, mediated by Akt, ATM, Cdk1/Plk1, and Aurora A (McKerlie and Zhu 2011; Wu and Yang et al. 2008; Kim and Kang et al. 2008; Chen and Teng et al. 2009; McKerlie and Lin et al. 2012; Ohishi and Hirota et al. 2010). As such, post-translational modifications of telomere associate proteins might lead to an integrated regulation of telomeres by various cellular signaling pathways.

In conclusion, protein-protein interaction and post-translational modification are critical methods of regulating the function of shelterin complex. Further studies in these two aspects are warranted to illuminate regulation of telomeres and its associate proteins.
2. Materials and Methods

2.1 Knockdown of PinX1 expression delays entry into mitosis

2.1.1 Retroviral infection and siRNA transfection

10^6 HeLa cells (catalogue # CCL-2, American Type Culture Collection) were stably infected with an amphotrophic retrovirus derived from pBabe-puro-GFP-histone-H2B (catalogue # 26790, Addgene) and selected for resistance to puromycin, as previously described (O’Hayer and Counter 2006).

For knockdown of hPinX1, HeLa cells were transiently transfected with 20 nM of a scramble control small interference RNA (siRNA) (5’-GUUUAUCAGCACUCGGUUGACUAGA-3’) or hPinX1 siRNA (5’-CCAGUAGAGAUAGCAGAGGACGCUA-3’) (Invitrogen) for 12 hours using Lipofectamine RNAiMAX reagents (catalogue # 13778075, Invitrogen) following manufacturer’s protocol. Cells were then expanded and 48 hours later lysates were collected for immunoblotting.

2.1.2 Immunoblot analysis

10^6 HeLa cells were lysed with RIPA lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% NaDoc) supplemented with 1 mM PMSF, 1 mM DTT, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 1 mM Na3VO4 and 1 mM NaF. Cell lysates were centrifuged at 4°C for 10 minutes. Protein concentrations of the supernatants were determined by Bradford assay. Equal amount of protein were mixed with 4x loading buffer (200 mM Tris-HCl pH 6.8, 400 mM DTT, 8% SDS, 0.4%
bromophenol blue, 40% glycerol) and boiled for 5 minutes. After electrophoresis, proteins were transferred onto PVDF membranes (catalogue # IPVH00010, EMD Millipore) at 4°C. The membranes were blocked with 5% non-fat milk in Tris Buffered Saline with Tween 20 (TBST) (50 mM Tris-HCl pH 7.6, 150 mM NaCl and 0.05% Tween 20), then incubated with first antibody at 4°C overnight. The membranes were washed twice with TBST at room temperature and then incubated with secondary antibody for 1 hour at room temperature. Finally, the membranes were incubated with ECL (catalogue # RPN2106, General Electric Healthcare) and exposed to hyper-film (catalogue # 28-9068-39, GE Healthcare).

2.1.3 Cell synchronization

For double thymidine block, HeLa cells were treated with 2 mM thymidine (catalogue # T9250, Sigma) for 19 hours and washed three times with phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4). Cells were then incubated fresh medium without addition of thymidine for 9 hours and treated with thymidine for another 16 hours. Cells were again washed three times with PBS, released into normal fresh medium and collected 0, 4, 8, 10, 12 or 15 hours later, denoted as G1/S, S, G2, M, Early G1 (EG1) and Middle G1 (MG1) phase of cell cycle (Kanda and Sullivan et al. 1998).
2.1.4 Live cell imaging

HeLa cells were stably infected with an amphotrophic retrovirus derived from pBabe-puro-GFP-histone-H2B (catalogue # 26790, Addgene) and a stable population was selected by puromycin as previously described (O'Hayer and Counter 2006). Resistant cells were transiently co-transfected with the aforementioned scramble siRNA or hPinX1 siRNA, and the BLOCK-iT Alexa Fluor Red fluorescent Oligo (catalogue # 14750-100, Invitrogen) to demark transfected cells. Cells were then arrested in G1/S phase by double thymidine block as described above, and released into G1/S phase. 7 hours and 30 minutes later, 23 transfected (BLOCK-iT positive) scramble siRNA or hPinX1 siRNA cells were monitored by live-cell imaging for a total of 17 hours using an Olympus VivaView FL incubator microscope. DIC (cells) and GFP (GFP-histone H2B) were monitored every 3 minutes, while RFP (BLOCK-iT Alexa Fluor Red fluorescent Oligo) was monitored every 30 minutes. Cells were considered to begin mitosis when GFP-histone-H2B labeled chromosomes began to aggregate, and considered to have completed mitosis when two daughter cells were completely separated (Kanda and Sullivan et al. 1998).

2.1.5 Analysis of apoptosis

HeLa cells were transiently transfected with the aforementioned scramble siRNA or hPinX1 siRNA and synchronized to G1/S phase by double thymidine block and released into cell cycle. 8 hours later, the cells were collected every hour for 5 hours, and
immunoblotted with α-tubulin (catalogue # T5201, Sigma), α-phospho-Histone-H3 (catalogue # 9701, Cell Signaling Technology), α-PinX1 (derived from affinity purified antibodies from rabbits immunized with the human PinX1 peptide encompassing amino acids 215-233), or the α-cleaved caspase-3 (Asp175) (5A1E) rabbit antibodies (catalogue # 9664, Cell Signaling Technology) at a 1:1000 dilution. Unsynchronized HeLa cells transiently transfected with PinX1 siRNA or scramble siRNA served as controls.

2.2 Identification of post-translational modification on TIN2

2.2.1 Plasmids and cloning

The pBabe-puro-Flag-TIN2WT, pBabe-puro-TIN2WT-HA, pMAL-c2x-Flag-TIN2WT and pEGFP-N1-TIN2WT were generated by introducing, in frame, an N-terminal Flag or a C-terminal HA epitope-tag in the human TINF2 cDNA (Barrientos and Kendellen et al. 2008) by PCR and subcloning the resultant cDNA into the EcoRI/HindIII sites of pBabe-puro (Morgenstern and Land 1990), the EcoRI/HindIII sites of pMAL-c2x (New England Lab) and the XhoI/HindIII sites of pEGFP-N1 (Clontech). The pBabe-puro-Flag, pMAL-c2x-Flag, and pEGFP-N1 TIN2S295A, TIN2S330A and the compound S295A/330A TIN2AA mutants were generated by introducing S295A, S330A or S295A/S330A mutations into the aforementioned Flag-TIN2WT cDNA and subcloning the resultant cDNAs into the EcoRI/HindIII sites of pBabe-puro, the EcoRI/HindIII sites of pMAL-c2x (New England Lab) and the XhoI/HindIII sites of pEGFP-N1 (Clontech). pBabe-puro-TIN2S295A-HA was generated by introducing the S295A mutation into the aforementioned TIN2WT-HA
cDNA, and subcloning the resultant cDNA into the EcoRI/HindIII sites of pBabe-puro. pQCXIP-Flag-TIN2WT was generated by subcloning the aforementioned Flag-TIN2WT cDNA into the NotI/AgeI sites of pQCXIP (catalogue # 6315, Clontech). pcDNA-Flag-RSK2Y707A was a kind gift from Dr. Sally Kornbluth. pCMV-myc-TRF1 and pEYFP-C1-TPP1 were previously described (Barrientos and Kendellen et al. 2008; Yonekawa and Yang et al. 2012). pSuper-retro-GFP-Neo-shTIN2-1 and -2 were generated by inserting the small hairpin sequence against TIN2, 5’-GGAGCACAUUCUUUGCCUG-3’ (Yang and He et al. 2011) and 5’-CCAACCCAGGUCAUAUCUAAG-3’, respectively, into pSuper-retro-GFP-Neo. All manipulated cDNAs were confirmed correct by sequencing.

2.2.2 Retroviral infection

For phospho-proteomic analysis of TIN2, 10⁵ HeLa cells were stably infected with an amphotrophic retrovirus derived from pQCXIP-Flag-TIN2 and selected for resistance to puromycin, as previously described (O’Hayer and Counter 2006). For analysis of TIN2 mutants, 10⁵ HeLa cells were infected with amphotrophic retroviruses derived from pBabe-puro encoding no transgene (vector), Flag-TIN2WT, -TIN2S295A, -TIN2S330A, or TIN2AA, or TIN2WT-HA or TIN2S295A-HA, and selected for stable resistance to puromycin, as described above.

2.2.3 Transient transfection

For analysis of RSK phosphorylation of TIN2, 10⁶ 293T cells (catalogue # CRL-11268, ATCC) were transiently transfected with pBabe-puro-Flag-TIN2WT and/or
pcDNA-Flag-RSK2\textsuperscript{Y707A} using the Fugene reagent (catalogue # E2691, Promega) according to the manufacture’s protocol.

2.2.4 Protein purification and mass spectrometry

10\textsuperscript{8} HeLa cells stably infected with pBabe-puro-Flag-TIN2\textsuperscript{WT} were lysed with one volume of high salt buffer (20 mM HEPES pH 8.0, 420 mM NaCl, 25% glycerol, 0.1 mM EDTA, 5 mM MgCl\textsubscript{2} and 0.2% NP-40) supplemented with a protease inhibitor cocktail (catalogue # 11836170001, Roche) and phosphatase inhibitor cocktails (catalogue # P5726 and P0044, Sigma). Cell lysates were diluted with three volume of dilution buffer (20 mM HEPES pH 8.0, 60 mM NaCl, 0.1 mM EDTA, 5 mM MgCl\textsubscript{2} and 0.07% NP-40) and incubated with M-2 resin (catalogue # A2220, Sigma) overnight at 4°C, then washed three times with lysis buffer at 4°C. Proteins were recovered by IgG elution buffer (catalogue #21004, Thermo Scientific) and resolved by SDS-PAGE on Novex\textsuperscript{®} 4-20% Tris-Glycine Mini Gels. The band corresponding to the molecular weight of TIN2 (40 kDa), identified after Coomassie Brilliant Blue staining, was excised for mass spectrometry analysis by Duke Proteomics Core Facility. In brief, the protein was subjected to in-gel tryptic digestion. Phosphor-peptides were then enriched by TiO\textsubscript{2} affinity chromatography and subjected to mass spectrometry analysis (Richardson and Soderblom et al. 2013).
2.2.5 Immunoblot analysis

10% of the described transiently transfected 293T cells or stably infected HeLa cells were lysed with RIPA lysis buffer supplemented with 1 mM PMSF, 1 mM DTT, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 mM Na$_3$VO$_4$ and 1 mM NaF. Cell lysates were centrifuged at 4°C for 10 minutes. Protein concentrations of the supernatants were determined by Bradford assay. Equal amounts of protein were mixed with 4x loading buffer and boiled for 5 minutes. SDS-PAGE gels were made either without or with 30 μM Phos-tag and 60 μM MnCl$_2$, according to manufacturer’s protocol (catalogue # Phos-tag AAL-107, Wako Pure Chemical Industries). After electrophoresis, proteins were transferred onto PVDF membranes (catalogue # IPVH00010, EMD Millipore) at 4°C. The membranes were blocked with 5% non-fat milk in Tris Buffered Saline with Tween 20 (TBST), and incubated with first antibody at 4°C overnight. The membranes were washed twice with TBST at room temperature, incubated with secondary antibody for 1 hour at room temperature. Finally, the membranes were incubated with ECL (catalogue # RPN2106, General Electric Healthcare) and exposed to hyper-film (catalogue # 28-9068-39, GE Healthcare). In some cases, the membranes were stripped with 7 M GuCl and 10 mM DTT, washed in ddH$_2$O at room temperature extensively and blocked again with 5% non-fat milk in TBST. Stripped membranes were then re-probed with another antibody. The following antibodies and concentrations were used: mouse anti-beta-tubulin at 1:1000 (catalogue #
T5201, Sigma), mouse anti-Flag at 1:1000 (catalogue # F1804, Sigma), mouse anti-HA at 1:1000 (catalogue # 2367, Cell Signaling Technology), rabbit anti-phospho-histone H3-Ser10 at 1:1000 (catalogue # 9701, CST), rabbit anti-Phospho-S6 Ribosomal Protein (Ser235/236) at 1:1000 (catalogue # 2211, CST), rabbit anti-TIN2 (Houghtaling and Cuttonaro et al. 2004) at 1:1000 (a kind gift from Dr. Susan Smith), rabbit anti-phospho-TIN2 Ser295, and rabbit anti-phospho-TIN2 Ser330 at 1:500 (Pierce customized antibodies derived from peptides C-PFRNLG [pS]PTQVISK-amide and C-STGKSK[pS]PCQTLG-amide, respectively).

2.2.6 Immunoprecipitation and phosphatase treatment

10^6 of the described transiently transfected 293T cells or stably infected HeLa cells were lysed with RIPA lysis buffer as above. Equal amount of cell lysate were mixed with pre-washed Gamma beads (catalogue # 17-0885-01, GE Healthcare). The mixtures were rotated at 4°C for 2 hours. 4 µg anti-Flag, anti-HA, or anti-TIN2 antibodies were added and incubated at 4°C on an end-to-end rotator overnight. The beads were isolated by centrifugation and washed three times with RIPA lysis buffer at 4°C. Beads were boiled with 4x loading buffer for 5 minutes, and supernatants resolved by SDS-PAGE. In the case of phosphatase treatment, lysates were prepared in the absence of phosphatase inhibitors and subjected to immunoprecipitation as above. The protein-bound resins were washed as above and then either left untreated or treated with calf intestinal alkaline phosphatase (catalogue # M0290S, New England BioLabs).
for 1 hour at room temperature. The resins were boiled with 4x loading buffer for 5 minutes. The supernatants were then resolved by SDS-PAGE in the presence of Phos-tag reagent.

2.2.7 Cell synchronization by nocodazole arrest and double thymidine block

10% of the described stably infected HeLa cells were either left untreated, arrested in G2/M by overnight treatment with 0.6 µg/ml nocodazole (catalogue # M1404, Sigma), or synchronized at G1/S by the double thymidine block, as outlined below. In some cases, HeLa or 293T cell lines were treated with 10 µM H-89 (Sigma), 10 µM BI-D1870 (Enzo Life Science), 10 nM BI 2536 (Selleckbio) or 1 µM VX-680 (Selleckbio) for 30 minutes in the presence of nocodazole before harvesting. For double thymidine block, HeLa cells were treated with 2 mM thymidine (catalogue # T9250, Sigma) for 19 hours and washed three times with PBS. Cells were then incubated fresh medium without addition of thymidine for 9 hours and treated with thymidine for another 16 hours. Cells were again washed three times with PBS, released into normal fresh medium and collected at 0, 4, 8, 10, 12 or 15 hours, which were denoted as G1/S, S, G2, M, Early G1 (EG1) and Middle G1 (MG1) phase of cell cycle (Kanda and Sullivan et al. 1998). Untreated cells or cells at various states of cell cycle were collected and lysed for immunoprecipitation.
2.2.8 Fluorescence-activated cell sorting (FACS)

10^6 HeLa cells were harvested, washed with PBS, and fixed with methanol for 10 minutes at room temperature. Cells were then treated with 2 mg/ml RNase A solution (catalogue # R4642, Sigma) for 30 minutes at 37°C, stained with 0.1 mg/ml propidium iodide (catalogue # P4170, Sigma) for 30 minutes at 37°C. HeLa cells were then washed in PBS and sorted on a FACSCalibur flow cytometer (Becton Dickson) with CELLQUEST software.

2.2.9 In vitro RSK2 kinase assay

Maltose-binding protein (MBP), MBP-tagged TIN2^WT (MBP-TIN2^WT), MBP-TIN2^S295A, MBP-TIN2^S330A, and MBP-TIN2^AA recombinant protein were purified from BL21 competent bacteria. Specifically, bacteria transduced with the plasmids of pMAL-c2x, pMAL-c2x-Flag-TIN2^WT, pMAL-c2x-Flag-TIN2^S295A, pMAL-c2x-Flag-TIN2^S330A, and pMAL-c2x-Flag-TIN2^AA were lysed with lysis buffer (50 mM Tris-HCl pH8.0, 150 mM NaCl, 1% Triton X-100, 10% glycerol), immunoprecipitated with amylose resin for two hours at room temperature. The resins were then washed three times with lysis buffer at room temperature. MBP, MBP-TIN2^WT, MBP-TIN2^S295A, MBP-TIN2^S330A, and MBP-TIN2^AA recombinant protein were eluted with lysis buffer + 10 mM maltose. Purified proteins were dialyzed against 50 mM Tris-HCl pH8.0 and 150 mM NaCl for overnight at 4°C. 2 µg of purified MBP, MBP-TIN2^WT, MBP-TIN2^S295A, MBP-TIN2^S330A and MBP-TIN2^AA protein were incubated with 0.2 µg 6His-RSK2 (catalogue # ab60881, Abcam) in kinase
assay buffer (25 mM HEPES, pH 7.4, 10 mM MgCl₂, 1 mM EGTA, 0.2 mM EDTA, 1 mM DTT, 1 mM Na₃VO₄, 50 μM cold ATP and 5 μCi ATP³²) for 30 minutes at 30°C. The reactions were then mixed with 4x loading buffer, boiled for 5 minutes and resolved by SDS-PAGE. Acrylamide gels were stained by Coomassie Brilliant Blue and exposed to auto-radiographic film.

2.2.10 Protein stability analysis

293T cells transiently transfected with pBabe-puro-flag -TIN2WT, -TIN2S295A, -TIN2S330A or -TIN2AA were treated with 100 mg/ml cycloheximide (catalogue # C4859, Sigma) for 0, 1, 2, 4, 6 and 8 hours. Cells were harvested and lysed with RIPA buffer. Lysates were resolved by SDS-PAGE and immunoblotted with anti-Flag or anti-Tubulin antibody. Intensity of Flag-TIN2 bands were quantified by ImageJ, normalized to that of tubulin, and plotted against treatment time.

2.2.11 Immunofluorescence analysis

HeLa cells transiently transfected with pEGFP-N1- TIN2WT, -TIN2S295A, -TIN2S330A or -TIN2AA were seeded on coverslips. 24 hours later, cells were stained with MitoTracker Red CMXRos 1mM (catalogue # M-7512, Invitrogen) for 10 min at 37°C and then fixed, permeabilized, blocked, and mounted as previously described (Takai and Kibe et al. 2011). Colocalization of GFP fusion proteins and MitoTracker Red were assessed by Zeiss Axio Imager wide-field fluorescence microscope.


2.2.12 Telomere-dysfunction induced foci (TIF) assay

HeLa cells stably infected with pBabe-puro -TIN2<sup>WT</sup> or -TIN2<sup>AA</sup> were transiently transfected with pSuper-retro-GFP-Neo-shTIN2-1 and -2, and seeded on coverslips. 24 hours later, cells were fixed, permeabilized, and blocked as previously described (Takai and Kibe et al. 2011). The cells were then incubated with an anti-γH2AX antibody at 1:200 dilution for 1 hour at room temperature, followed by incubation with a donkey anti-mouse antibody conjugated with rhodamine RedX (Jackson Immuno-Research) at 1:200 dilution for 30 minutes at room temperature. After extensive washes with washing buffer (70% formamide, 10 mM Tris-HCl pH 7.2), cells were dehydrate in ethanol and denatured in pre-warmed 70% formamide (Catalogue #F7508, Sigma)/2X saline-sodium citrate (SSC) at 75°C for 10 minutes, followed by hybridization with 120nM Cy5-conjugated PNA Telomere C probe (Catalogue #F1003-5, PANAGENE) at 4°C overnight. Coverslips were then washed extensively with washing buffer and mounted in anti-fade solution (Catalogue #P-36931, Invitrogen). Co-localization of the telomere probe signal and the γH2AX signal in GFP-positive cells were blindly counted by overlaying the Cy5 and red channels. The difference of total Telomere-dysfunction Induced Foci (TIFs) in each genotype was assessed by student t-test.
2.3 Separation-of-function analysis of mPOT1a and mPOT1b

2.3.1 Plasmids cloning and mutagenesis

The pBabe-puro-Flag-mPot1aWT was generated by introducing, in frame, an N-terminal Flag epitope-tag in the murine Pot1a cDNA (a kind gift of Dr. Sandy Chang (Wu and Multani et al. 2006)) by PCR and subcloning the resultant cDNA into the EcoRI/HindIII sites of pBabe-puro. The pQCXIP-HA-mTPP1 was a kind gift of Dr. Sandy Chang. pcDNA3.1-HA-mTIN2 was generated by introducing an N-terminal HA in-frame HA epitope-tag in murine Tinf2 cDNA (Barrientos and Kendellen et al. 2008) by PCR and subcloning the cDNA into NheI/EcoRI sites of pcDNA3.1 (catalogue # V795-20, Life Technologies). For NAAIRS mutagenesis, a panel of 35 individual pBabe-puro-Flag-mPot1a mutants were generated using approaches previously described, in which the murine Pot1a cDNA sequence encoding six amino acids between 297aa and 616aa were mutated into the sequence of 5’-AATGCTGCTATACGATCG-3’ by PCR (Barrientos and Kendellen et al. 2008).

2.3.2 RNA extraction and RT-PCR

mRNAs were extracted from MEFs according to the manufacturer’s protocol (RNeasy Mini Kit, QIANGE, Cat # 74104) and reverse transcribed into cDNA using Omniscript RT kit (QIAGEN, Cat # 205110). The following primers were then used to PCR amplify the corresponding fragments, which were resolved by 1.5% agarose gel electrophoresis. Murine GAPDH forward 5’-GCACAGTCAAGGCGAGAAT-3’,
murine GAPDH reverse 5'–GCCTTCTCCATGGTGGTGA-3', mPot1a 5'UTR forward 5'–TATACCTTAAGAGGCTGGATTGTC-3', mPot1a 5'UTR reverse 5’- TGGGGAGGTC TTCATAGT-3', Flag forward 5’-GACTACAAAGACGATGACGAC-3’

2.3.3 Telomere-dysfunction induced foci (TIFs) assay

MEFs cells stably infected with pBabe-puro-TIN2WT, -TIN2N389 or -TIN2N497 were treated with Ad-Cre and then seeded on coverslips. 24 hours later, cells were fixed, permeabilized, blocked as previously described (Takai and Kibe et al. 2011). Blocked coverslips were incubated with an anti-γH2AX antibody at 1:200 dilution for 1 hour at room temperature, then a donkey anti-mouse antibody conjugated with rhodamine RedX (Jackson Immuno-Research) at 1:200 dilution for 30 minutes at room temperature. After extensive washes, cells were dehydrate in ethanol and denatured in pre-warmed 70% formamide (Catalogue #F7508, Sigma)/2X saline-sodium citrate (SSC) at 75°C for 10 minutes, followed by hybridization with 120nM Cy5-conjugated PNA Telomere C probe (Catalogue #F1003-5, PANAGENE) at 4°C overnight. Coverslips were then washed extensively and mounted in anti-fade solution (Catalogue #P-36931, Invitrogen). Co-localization of the telomere probe signal and the γH2AX signal were blindly counted by overlaying the Cy5 and red channels.
3. Knockdown of hPinX1 expression delays entry into mitosis

3.1 Introduction

hPinX1 is a 45-kDa protein, originally identified from a yeast two-hybrid screen using TRF1 as a bait. Indeed, ectopic expressed hPinX1 co-immunoprecipitates with hTRF1 (Zhou and Lu 2001). PinX1 also interacts with the RNA and protein subunits of telomerase, the enzyme that elongates telomere (Zhou and Lu 2001; Banik and Counter 2004) and can inhibit the activity of this enzyme in human cancer cell lines and murine embryonic fibroblasts (MEFs) (Zhou and Huang et al. 2011; Zhang and Bai et al. 2009). Therefore, PinX1 is a telomeric accessory protein that can negatively regulate telomerase.

Besides the interaction with telomerase, PinX1 appears play a role in chromosome segregation. Specifically, mPinX1+/- MEFs have elevated levels of anaphase bridges during mitosis (Zhou and Huang et al. 2011), which is associated with defects in chromosome separation. hPinX1 is also enriched on the outer plate of kinetochores and periphery of chromosomes during mitosis (Yuan and Li et al. 2009). As such, I explored the role of hPinX1 on telomeres during mitosis.
3.2 Results

3.2.1 hPinX1 depletion causes delayed mitotic entry

hPinX1 accumulates on chromosomes during mitosis, and knocking down hPinX1 expression leads to lagging chromosome segregation (Zhou and Huang et al. 2011; Yuan and Li et al. 2009). These results suggest a critical role for hPinX1 at mitosis. Indeed, in collaboration with Dr. Tohru Yonekawa in our lab, we determined that the absence of hPinX1 during mitosis led to reduced level of hTRF1 on telomeres (Yonekawa and Yang et al. 2012). Given this, I investigated the impact of knocking down hPinX1 expression during mitosis using live cell imaging on both the time taken for cells to enter mitosis and the length of this phase of the cell cycle.

HeLa cells were transiently co-transfected with either PinX1 siRNA or a control scramble siRNA and BLOCK-iT Alexa Fluor Red fluorescent Oligo to visualize transfected cells. Cells were then synchronized by double thymidine block and released into G1/S phase. Beginning just before entry into mitosis (7:30 hours post release), the BLOCK-iT-positive cells were visualized by live cell imaging. Control cells took, on average, 10 hours and 49 minutes to enter mitosis (Figure 2A), as defined as the time elapsed between release from the double thymidine block and the aggregation of chromosomes labeled with GFP-Histone-H2B. These cells took another 66 minutes to complete mitosis (Figure 2B), as defined as the time elapsed between the aggregation of chromosomes labeled with GFP-Histone-H2B and complete separation of two daughter
cells. In contrast, cells in which hPinX1 was knocked down by siRNA, as confirmed by immunoblot (Figure 2C), took on average 12 hours and 46 minutes to enter mitosis. However, once cells entered mitosis, the average length of mitosis was identical to scramble control cells, namely 61 minutes (Figure 2A, Figure 2B). Thus, knockdown of hPinX1 delays entry into mitosis.

3.2.2 PinX1 depletion causes elevated apoptosis during mitosis

Since mitotic delay has been associated with apoptosis (Vakifahmetoglu and Olsson et al. 2008), I explored whether the delayed entrance into mitosis upon knockdown of hPinX1 resulted in apoptosis. Specifically, scramble and hPinX1-knockdown cells were released from a double thymidine block, and beginning 8 hours later (just prior to mitotic entry), extracts were collected every hour and immunoblotted to detect tubulin (as a loading control), phospho-histone H3 (as a marker of mitosis), hPinX1 (to validate knockdown), and finally, cleaved Caspase 3 (as a measure of apoptosis). Scramble control cells entered mitosis, as defined by an increase in phosphorylated histone H3, 10 hours post release, and during the course of the experiment, exhibited little evidence of cleaved Caspase 3. As already noted, PinX1-knockdown cells exhibited a delay in entry into mitosis, with elevated phosphorylated histone H3 at 11 hours post release, but also elevated Caspase 3 cleavage beginning at the earliest time point and extending throughout the period of analysis, but not in
asynchronous cells (Figure 2C). Thus, knockdown of hPinX1 increases the amount of Caspase 3 cleavage.
Figure 2: Knockdown of hPinX1 delays entry into mitosis

Mean length of time +/- SD to enter (A) or to complete (B) mitosis beginning 8 hours after release from a double thymidine block, as assessed in 23 HeLa cells stably expressing GFP-histone H2B and transfected (as verified by BLOCK-iT Alexa Fluor Red oligonucleotide fluorescence) with either a scrambled control siRNA (●) or a hPinX1 siRNA (■). **, P <= 0.01; N.S., not significant.

(C) HeLa cells transfected with a scrambled control siRNA or a hPinX1 siRNA were synchronized by double thymidine block, released, collected at the time points indicated above the gels, and immunoblotted to detect endogenous PinX1, phosphorylated histone H3 (P-HH3), and cleaved Caspase 3. Data are representative of two replicate experiments. A, asynchronous cells.
3.3 Discussion

Endogenous hPinX1 is primarily localized to telomeres during mitosis (Yonekawa and Yang et al. 2012), and knockdown of hPinX1 resulted in lagging chromosome segregation (Yuan and Li et al. 2009). This prompted me to study the role of hPinX1 in cell cycle progression, especially during mitosis. In this study, I evaluated the effects of knocking down hPinX1 expression in HeLa cell lines via live cell imaging and apoptosis analysis. These experiments revealed that knockdown of hPinX1 led to delayed entry into metaphase and increased levels of apoptosis.

hPinX1 binds to hTRF1 specifically between G2/M and early M phase, which appears to stabilize hTRF1, as knockdown of hPinX1 leads to a reduction of hTRF1 during mitosis, possibly by blocking the association of the E3 ligase Fbx4 to hTRF1 (Yonekawa and Yang et al. 2012; Zeng and Wang et al. 2010). However, another telomere-associate protein, hTIN2, binds with a stronger affinity to the same site of hTRF1, suggesting hPinX1 binding to hTRF1 may be dictated by the residency of hTIN2 (Chen and Yang et al. 2008). Although the level of hTIN2 on telomeres remains stable during cell cycle (data not shown), it is possible that the hTIN2-hTRF1 interaction is disrupted during early mitosis, during which hPinX1-hTRF1 interaction is required for normal mitotic entry.

Previous research has shown that the hTIN2-hTRF1 interaction is essential to prevent poly ADP-ribosylation modification by tankyrase on hTRF1, which leads to the
disassociation of hTRF1 from telomere and its degradation (Smith and Giriat et al. 1998; Chang and Dynek et al. 2003; Ye and de Lange 2004). Further, this modification and degradation of hTRF1 by tankyrase is required for sister telomere separation, possibly through the degradation of cohesin complex on sister chromatids (Canudas and Houghtaling et al. 2007; Dynek and Smith 2004). Based on these data, it is tempting to speculate that the protective roles of hPinX1 and hTIN2 on hTRF1 are required at different stages of cell cycle to ensure telomere protection and sister chromatids separation. During interphase, hTIN2 binds to hTRF1 to ensure its stability on telomere. When cells start to enter mitosis, the interaction between hTRF1 and hTIN2 is disrupted, resulting in the degradation of hTRF1 via tankyrase modification. After the separation of sister chromatids, hPinX1 binds to and escorts newly synthesized hTRF1 onto telomeres. Apparently, this reloading of hTRF1 is required to protect telomere ends in daughter cells and failure at this step resulted in delayed entry into mitosis.

In agreement with this hypothesis, the knockdown or knockout of PinX1 results in elevated levels of anaphase bridges, a phenotype characteristic of abnormal chromosome separation (Zhou and Huang et al. 2011; Yuan and Li et al. 2009). Further, phosphorylation of TRF1 by Cdk1 resulted in disassociation of TRF1 from telomere during the early stage of mitosis, coinciding with the timing of PinX1-TRF1 interaction. Taken together, these observations suggest that this phosphorylation event could facilitate the interaction between PinX1 and TRF1 (McKerlie and Zhu 2011). Lastly, as
TRF1 is important for telomere protection, knockdown of PinX1 led to increased level of DNA damage signaling at telomere (Zhang and Bai et al. 2009). Thus the elevated level of apoptosis observed in my study may stem from the persistent DNA damage responses in cells.

In summary, my research has shed light on the function of PinX1 during mitosis, as knockdown of hPinX1 delayed entry into mitosis and induced apoptosis. Although other functions of PinX1 may play a role in these phenotypes, such as localization to kinetochores during mitosis, my hypothesis is that PinX1-TRF1 interaction is important for reloading TRF1 onto the telomeres of sister chromatids. The molecular mechanisms behind these events still remain to be determined.
4. Identification of post-translational modification on TIN2

4.1 Introduction

The TRF1-interacting nuclear factor 2 (TINF2, hereafter referred to as TIN2) is a 40-kDa protein integral to telomere function. TIN2 has no known enzymatic activity, but interacts with double-stranded (ds) and single-stranded (ss) telomeric DNA-binding proteins (Loayza and De Lange 2003; Kim and Kaminker et al. 1999). Specifically, TIN2 directly binds to the telomeric dsDNA-binding proteins TRF1 and TRF2, and to TPP1, which forms a heterodimer with the telomeric ssDNA-binding protein POT1 (Ye and Donigian et al. 2004; Kim and Beausjour et al. 2004; Kim and Kaminker et al. 1999; Houghtaling and Cuttonaro et al. 2004). Knockdown of TIN2 in human cells results in moderate telomere elongation, as well as destabilization of TRF1 and TRF2 (Ye and Donigian et al. 2004; Ye and de Lange 2004). Knockout of TIN2 in murine embryonic fibroblasts results in severe telomere dysfunction phenotypes such as high levels of telomere-dysfunction induced foci (TIFs), telomere fusions, as well as telomere sister chromatid exchange (T-SCE) (Takai and Kibe et al. 2011).

Circumstantial evidence suggests that TIN2 might be phosphorylated in a cell cycle dependent fashion. First, the increase in T-SCE upon loss of TIN2 suggests a role for TIN2 in mitosis (Takai and Kibe et al. 2011), and many proteins are regulated at mitosis by phosphorylation (Fisher and Krasinska et al. 2012). Second, TIN2 is found in a complex with TRF1 and cohesin (Canudas and Houghtaling et al. 2007), the latter of
which are functionally regulated by phosphorylation during mitosis (Hauf and Roitinger et al. 2005; Hauf and Waizenegger et al. 2001; Gorr and Boos et al. 2005).

Third, at least two other telomere proteins have been reported to be phosphorylated. Specifically, TRF1 is phosphorylated at mitosis to allow sister telomere resolution (McKerlie and Zhu 2011), and the phosphorylation of TPP1 during G2/M phase is related to higher telomerase activity (Zhang and Chen et al. 2013). Given these observations, I explored the possibility that TIN2 may be phosphorylated, perhaps at mitosis.

4.2 Results

4.2.1 Mass spectrometry identifies two phosphorylated residues in TIN2

To investigate whether TIN2 is phosphorylated, I ectopically expressed a Flag epitope-tagged version of the more commonly studied shorter cDNA version of TIN2 (Flag-TIN2) in HeLa cells (Kaminker and Kim et al. 2009). This protein was then immunoprecipitated by virtue of the Flag epitope and resolved by SDS-PAGE, revealing primarily one band at 40 kDa, the estimated molecular weight of TIN2 (Figure 3, left). The band was excised and subjected to in-gel tryptic digestion, after which the phosphopeptides were enriched by TiO2 affinity chromatography and subjected to mass spectrometry analysis (Richardson and Soderblom et al. 2013). This analysis revealed two phosphorylation sites on TIN2, namely serines (S) 295 and 330 (Figure 3, right). Encouragingly, phosphorylation of TIN2 on S295/T297 and/or S330 were detected in
unbiased whole phospho-proteome analysis of HeLa cells throughout the cell cycle (Olsen and Vermeulen et al. 2010), HeLa cells treated with the drug rapamycin (Chen and Yang et al. 2009), and in nocodazole-arrested HeLa cells (Dephoure and Zhou et al. 2008). Given our identification of these two sites, and their detection in unbiased phospho-proteomic screens, we reasoned that S295 and S330 might be bona fide phosphorylation sites in TIN2.
Identification of phosphorylation sites on TIN2 by mass spectrometry. Left, A lysate from HeLa cells stably infected with a retrovirus encoding N-terminal Flag epitope-tagged TIN2 (Flag-TIN2) was subjected to immunoprecipitation (IP) with an αFlag antibody, resolved by SDS-PAGE and detected by Coomassie Brilliant Blue staining. M: marker lane. Right, The purified protein was then recovered and digested by trypsin, followed by TiO2 enrichment and mass spectrometry analysis, revealing two peptides with phosphorylated serine residues (denoted with an *). Representative of one experiment.
4.2.2 Validation of TIN2 phosphorylation on S295 and S330 by Phos-tag analysis

To determine whether S295 and S330 of TIN2 are indeed phosphorylated, as suggested by mass spectrometry analysis, Flag-TIN2 cDNA was mutated to encode either a S295 to alanine (A) mutation (S295A) or a S330 to A mutation (S330A). These two mutants, as well as a control wild-type version of Flag-TIN2, were stably expressed in HeLa cells. All three proteins were immunoprecipitated by virtue of the Flag tag and resolved by SDS-PAGE containing the dinuclear metal complex Phos-tag reagent, which can specifically bind to phospho groups on proteins and impede their migration (Yang and Xue et al. 2010). TIN2 was then detected by immunoblot with an anti-TIN2 antibody. This analysis revealed four major bands from lysates derived from HeLa cells expressing wild-type TIN2: one band residing at the molecular weight of TIN2, corresponding to the unphosphorylated protein, and three supershifted bands. The lowest of these supershifted bands was absent in cells stably expressing the S330A TIN2 mutant, indicating that this band corresponds to S330 phosphorylation. Interestingly, this lower supershifted band appeared as either a singlet or doublet (Figure 4A, Figure 7A, Figure 7B, Figure 9A). As phosphorylation of S2448 of mTOR similarly yields more than one band using the Phos-tag reagent (Kinoshita and Kinoshita-Kikuta et al. 2009), the doublet may represent altered migration of TIN2 when phosphorylated on S330, although other possibilities cannot be excluded. The second supershifted band was absent in cells stably expressing S295A mutant, indicating that this band corresponds to
phosphorylation at S295. The highest supershifted band was absent in cells expressing either of the S295A or S330A TIN2 mutants, indicating that this band corresponds to the doubly phosphorylated protein (Figure 4A, left). Finally, the intensity of these supershifted bands were either abolished or greatly reduced by phosphatase treatment, whereas there was no change in the intensity of the band corresponding to the unphosphorylated protein (Figure 4, right), arguing that the three supershifted bands represent the indicated phosphorylation events. As no further bands were detected, and the intensity of all three supershifted bands was diminished by phosphatase treatment, these results support the contention that TIN2 is phosphorylated principally on S295 and S330.

4.2.3 Validation of TIN2 phosphorylation on S295 and S330 by phosphorylation-specific antibodies

To independently validate the observation that S295 and S330 in TIN2 are phosphorylated, phosphorylation-specific antibodies against these two sites were generated. Specifically, serum was collected from rabbits inoculated with one of the phospho-mimicking peptides C-PFRNLGSPPTQISK-amide or C-STGKSKSPCQTLG-amide. Lysates derived from HeLa cells expressing a C-terminal HA epitope-tagged TIN2 (TIN2-HA) in either the wild-type or S295A mutant configuration were immunoprecipitated with an anti-HA antibody, resolved by SDS-PAGE and immunoblotted with the antibody against the aforementioned S295 phosphorylated TIN2 peptide, termed anti-Phos-S295 for ease of description. The antibody detected a
band corresponding to the molecular weight of TIN2 in cells expressing wild type, but not S295A mutant TIN2 (Figure 4B, top). Moreover, when ectopic Flag-TIN2 was immunoprecipitated from HeLa cells with an anti-Flag antibody followed by treatment of calf intestinal phosphatase and resolved by SDS-PAGE, the band detected by the anti-Phos-295 antibody was lost (Figure 5A). Similarly, lysates derived from HeLa cells expressing Flag-TIN2 in either the wild-type or S330A mutant configuration were immunoprecipitated with an anti-Flag antibody, resolved by SDS-PAGE and immunoblotted with the antibody against the aforementioned S330 phosphorylated TIN2 peptide, termed anti-Phos-S330 for ease of description. This antibody detected a band corresponding to the molecular weight of TIN2 in cells expressing wild type, but not S330A mutant TIN2 (Figure 4B, bottom). Moreover, treatment of the Flag immunoprecipitates with calf intestinal phosphatase reduced the intensity of the Flag-TIN2 band detected by the anti-Phos-330 antibody by roughly half (Figure 5B), although admittedly this was not as large a difference as that seen with the anti-Phos-295 antibody. Thus, the use of phosphorylation-specific antibodies supports the conclusion from the Phos-tag analysis that TIN2 is phosphorylated on S295 and S330.
(A) Detection of phosphorylation of TIN2 at S295 and S330 by the Phos-tag reagent. Lysates from HeLa cells stably infected with a retrovirus encoding Flag-TIN2 in the wild-type (WT), S330A, or S295A configuration were subjected to immunoprecipitation (IP) with αFlag, and then either left untreated or treated with calf intestine phosphatase (CIP), followed by SDS-PAGE either in the presence (top) or the absence (bottom) of the Phos-tag reagent and immunoblotted (IB) with an αTIN2 antibody. The supershifted bands corresponding to S295, S330, or S295 and S330 phosphorylation, as well as the unphosphorylated (UP) TIN2 protein are denoted on the left. Representative of three experiments.

(B) Detection of S295 and S330 phosphorylation of TIN2 with phospho-specific antibodies. Lysates from HeLa cells stably infected with a retrovirus encoding C-terminal HA epitope-tagged TIN2 (TIN2-HA) or Flag-TIN2 in wild type, S295A or S330A configuration were subjected to immunoprecipitation (IP) with either an αHA or αFlag antibody, resolved by SDS-PAGE and immunoblotted (IB) with either an αPhos-S295 or αPhos-S330 antibody to detected the phosphorylated TIN2, and either an αHA or αFlag antibody to detect total ectopic TIN2 as a loading control. Representative of two experiments.

Figure 4: TIN2 is phosphorylated on Serine 295 and Serine 330 ( Detected by Phos-tag SDS-PAGE and phosphorylation-specific antibody)
Figure 5: Immunoblot with TIN2 phosphorylation-specific antibodies after phosphatase treatment

Lysates from HeLa cells stably infected with a retrovirus encoding Flag-TIN2 were subjected to immunoprecipitation (IP) with an αFlag antibody and then either treated with vehicle (DMSO) or calf intestine phosphatase (CIP), followed by SDS-PAGE and immunoblot (IB) with an αTIN2 antibody in addition to an (A) αPhos-S295 or (B) αPhos-S330 antibody. Bar graphs depict the relative level of the αPhos-S295 or αPhos-S330 signal normalized to total TIN2. Representative of one experiment.
4.2.4 TIN2 is preferentially phosphorylated on S295 and S330 during mitosis

As phosphorylation events are often tightly regulated throughout the cell cycle (Fisher and Krasinska et al. 2012) and an unbiased phospho-proteomic analysis revealed that TIN2 was preferentially phosphorylated on S295 in nocodazole-treated HeLa cells (Dephoure and Zhou et al. 2008), I tested whether phosphorylation of TIN2 on S295 and/or S330 varied during the cell cycle.

Specifically, HeLa cells stably expressing Flag-TIN2 in the wild type, S295A, or S330A mutant configuration were arrested at G1/S phase by double thymidine block and then released to progress through cell cycle. Cell cycle phases were assigned based on previous FACS analysis (Zhang and Bai et al. 2009) and confirmed by immunoblot detection of serine 10 phosphorylated histone H3, which is elevated during mitosis (Hendzel and Wei et al. 1997). No obvious differences in the cell cycle profile, as determined by FACS, were noted between HeLa cells stably infected with a vector encoding no transgene, wild type Flag-TIN2, or when both S295 and S330 were mutated to alanine (AA) in Flag-TIN2 (Figure 6). Flag-TIN2 protein was then immunoprecipitated with an anti-Flag antibody from lysates derived from these three cell lines when untreated (and hence asynchronous), arrested at G1/S by double thymidine block, or at intervals after release from this block that corresponded to S, G2, M and either early G1 (EG1) or middle G1 (MG1) phases of the cell cycle. Immunoprecipitated Flag-TIN2 was then resolved by Phos-tag integrated SDS-PAGE and
detected with an anti-TIN2 antibody. Equal loading was verified by immunoblot detection of tubulin (Figure 7A).
Figure 6: FACS analysis of HeLa cells expressing wild-type or AA mutant TIN2

HeLa cells stably infected with retroviruses encoding no transgene (vector only), Flag-TIN2\textsuperscript{WT} or Flag-TIN2\textsuperscript{AA} were harvested, stained with propidium iodide and subjected to fluorescence-activated cell sorting (FACS) analysis. Representative of two experiments.
Immunoblot of the Phos-tag integrated SDS-PAGE gel revealed four bands in lysates derived from asynchronous cells expressing wild-type TIN2. Namely those corresponding to unphosphorylated, S295, S330, and to a smaller degree, S295/S330 phosphorylated TIN2. In the immunoprecipitates from extracts of cells enriched in G1/S, S or G2 phases of the cell cycle, only the bottom two supershifted bands were readily detectable, suggesting that TIN2 is normally mono-phosphorylated during these phases of the cell cycle. However, the intensity of these bands increased, and the band corresponding to doubly phosphorylated TIN2 was clearly evident in immune-precipitates from extracts isolated from cells in M and early G1 (Figure 7A). These results suggest that TIN2 is principally phosphorylated at both S295 and S330 during mitosis.

To determine if one site is preferentially phosphorylated at mitosis over the other, we compared the phosphorylation status of TIN2 as above throughout the cell cycle in HeLa cells stably expressing either S295A or S330A mutant Flag-TIN2. As expected, only two bands were detected in asynchronous populations of cells expressing either of these mutant proteins, corresponding to the unphosphorylated protein and TIN2 phosphorylated on S295 or S330. The level of singly phosphorylated TIN2 increased at M and middle G1 phases in HeLa cells expressing either of the mutants, pointing towards a coordinated phosphorylation of TIN2 at mitosis. The S295A mutant variably appeared more strongly phosphorylated, suggesting a possible bias towards
phosphorylation of S330 (Figure 7A). In conclusion, TIN2 is phosphorylated on S295 and S330, and phosphorylation of both these sites coordinately increases during mitosis.

4.2.5 Validation of TIN2 phosphorylation on S295 and S330 during mitosis.

To independently validate phosphorylation of TIN2 at mitosis, HeLa cells expressing wild type, S295A, or S330A Flag-TIN2 were either left untreated (asynchronous population) or arrested at mitosis by treatment with nocodazole, as confirmed by elevated levels of phosphorylated histone H3. Flag-TIN2 was then immunoprecipitated by virtue of the Flag-epitope tag followed by resolution using Phos-tag integrated SDS-PAGE and detected by immunoblot with an anti-TIN2 antibody. Again, tubulin served as a loading control (Figure 7B). Consistent with the previous results, cells expressing wild-type TIN2 that were treated with nocodazole exhibited three supershifted bands that were nearly undetectable in the untreated asynchronous population. The increase in the intensity of these bands could be specifically ascribed to phosphorylation of S295 and S330, as nocodazole treatment similarly increased the level of the bands corresponding to S295 and S330 phosphorylated TIN2 in cells expressing the S330A and S295A mutants, respectively. Finally, as an added control, we demonstrate that all three supershifted bands were lost in cells expressing Flag-TIN2-AA mutant in which both S295 and S330 were mutated to alanine (Figure 7B). Thus, TIN2 is phosphorylated on S295 and S330 during mitosis, as assessed by two independent methods of cell synchronization.
Figure 7: TIN2 is phosphorylated on S295 and S330 during mitosis

(A) Detection of S295 and S330 phosphorylation of TIN2 during mitosis by the Phos-tag reagent after release from a double thymidine block. HeLa cells stably infected with a retrovirus encoding Flag-TIN2 in the WT, S330A, or S295A configuration were collected from asynchronous populations (A), populations arrested with a double thymidine block corresponding to the G1/S phase of the cell cycle, or populations at the points corresponding to S, G2, M and early or middle G1 (EG1 or MG1) after release from the double thymidine block. Derived lysates were then either subjected to (top)
immunoprecipitation (IP) with an αFlag antibody and resolved by SDS-PAGE in the presence of the Phos-tag reagent and immunoblotted (IB) with an αTIN2 antibody or (bottom) resolved by normal SDS-PAGE and immunoblotted with either an αPhos-HH3 antibody to monitor cell cycle progression or an αTubulin antibody as a loading control. The supershifted bands corresponding to S295, S330, or S295 and S330 phosphorylation, as well as the unphosphorylated (UP) TIN2 protein, are denoted on the left of the upper panels. Left and right panels are different exposures. Representative of two experiments.

(B) Detection of S295 and S330 phosphorylation of TIN2 by the Phos-tag reagent in cells arrested with nocodazole. HeLa cells stably infected with a retrovirus encoding Flag-TIN2 in the WT, S330A, S295A or AA configuration, as well as pBabe-puro empty vector only control (V), were collected from asynchronous populations (Asyn) or populations arrested in G2/M by treatment with nocodazole (Noc). Derived lysates were then subjected to either (top) immunoprecipitation (IP) with αFlag and resolved by SDS-PAGE in the presence of the Phos-tag reagent and immunoblotted (IB) with an αTIN2 antibody, or (bottom) resolved by normal SDS-PAGE and immunoblotted with either an αPhos-HH3 antibody to monitor cell cycle progression or an αTubulin antibody as a loading control. The supershifted bands corresponding to S295, S330, or S295 and S330 phosphorylation, as well as the unphosphorylated (UP) TIN2 protein, are denoted on the left of the upper panel. Representative of three experiments.

(C) Detection of S295 phosphorylation of endogenous TIN2 with a phosphorylation-specific antibody in cells arrested with nocodazole. Lysates from HeLa cells were collected from asynchronous populations (Asyn) or populations arrested in G2/M by treatment with nocodazole (Noc), resolved by SDS-PAGE and immunoblotted (IB) with an αPhos-S295, αTIN2, αPhos-HH3 or, as a loading control, αHH3 antibodies. Representative of two experiments.

(D) Detection of S330 phosphorylation of endogenous TIN2 with a phosphorylation-specific antibody in cells arrested with nocodazole. HeLa cells were collected from asynchronous populations (Asyn) or populations arrested in G2/M by treatment with nocodazole (Noc). Derived lysates were then either subjected to (top) immunoprecipitation (IP) with an αTIN2 antibody, resolved by SDS-PAGE in the presence of the Phos-tag reagent and immunoblotted (IB) with either an αPhos-S330 or αTIN2 antibody, or (bottom) resolved by normal SDS-PAGE and immunoblotted with αPhos-HH3, to monitor cell cycle progression, or αTubulin, as a loading control, antibodies. Representative of one experiment.
4.2.6 Endogenous TIN2 is phosphorylated on S295 and S330 at mitosis.

To determine whether endogenous TIN2 is preferentially phosphorylated on S295 and S330 at mitosis, HeLa cells were either left untreated (asynchronous population) or treated with nocodazole to arrest cells in mitosis, as validated by the appropriate absence or presence of phosphorylated histone H3, respectively. Lysates from the two cell populations were then immunoblotted with the anti-Phos-S295 antibody, revealing a clear increase in the band corresponding to S295-phosphorylated TIN2 in cells treated with nocodazole (Figure 7C). On the other hand, detecting endogenous S330-phosphorylated TIN2 with the anti-Phos-S330 antibody provide to be more challenging. Specifically, endogenous TIN2 first had to be immunoprecipitated with an anti-TIN2 antibody, followed by detection with the anti-Phos-S330 antibody. Moreover, because of cross-reactivity with unphosphorylated TIN2, which was only manifested at the endogenous level (and not observed with ectopic TIN2, Figure 4B), immunoprecipitated TIN2 needed to be further resolved by Phos-tag integrated SDS-PAGE to separate the phosphorylated species prior to immunoblot with the anti-Phos-S330 antibody. Finally, as both the TIN2 and Phos-S330 antibodies were derived from rabbits, we were forced to use a light chain specific secondary antibody, which only detected the doubly phosphorylated species. Nevertheless, this analysis revealed a single band corresponding to S295/S330 phosphorylated TIN2 in HeLa cells treated with
nocodazole, but not in asynchronous HeLa cells (Figure 7D). We thus conclude that endogenous TIN2 is phosphorylated on S295 and S330 at mitosis.

4.2.7 The RSK inhibitor BI-D1870 reduces TIN2 phosphorylation.

To identify possible kinases responsible for the mitotic phosphorylation of TIN2, unsynchronized or nocodazole-arrested HeLa cells expressing wild-type Flag-TIN2 were treated with either vehicle alone (DMSO) as a control, or small molecular compounds known to inhibit common mitotic kinases. Specifically, cells were treated with 10µM BI-D1870, which is reported to inhibit the family of p90 ribosome S6 kinases (RSK) (Sapkota and Cummings et al. 2007; Bain and Plater et al. 2007), 10nM BI 2536, which is reported to inhibit Polo-like kinase 1 (Steegmaier and Hoffmann et al. 2007), 10µM VX-680, which is reported to inhibit the family of Aurora kinases (Bain and Plater et al. 2007; Tyler and Shpiro et al. 2007), and lastly, 10µM H-89, which is reported to inhibit protein kinase A (Bain and Plater et al. 2007). Derived lysates were then subjected to immunoprecipitation with an anti-Flag antibody followed by immunoblot with either the anti-Phos-S330 antibody to detect phosphorylated TIN2 or an anti-TIN2 antibody to detect total TIN2 protein as a loading control. As expected, S330 phosphorylation was higher in nocodazole-arrested compared to asynchronous HeLa cells. More importantly, S330 phosphorylation was essentially undetectable in cells treated with BI-D1870. The other three inhibitors also reduced the level of phosphorylated TIN2, but much less so compared to BI-D1870 (Figure 8A). Interestingly H-89 and VX-680 are known to inhibit
RSK kinases (Bain and Plater et al. 2007), although these results do not exclude the possibility that other mitotic kinases aside from the RSK family may yet phosphorylate TIN2. To rule out the possibility that this loss of TIN2 phosphorylation arose because BI-D1870 permitted cells to exit mitosis, DMSO, nocodazole, and nocodazole+BI-D1870 treated HeLa cells were stained with propidium iodide and the DNA content determined by FACS analysis. As expected, nocodazole treatment increased the number of cells arresting with a 4N content compared to DMSO-treated cells, and this did not change when cells were co-treated with nocodazole and BI-D1870 (Figure 8B). Taken together, these data suggest that the RSK family of kinases may phosphorylate TIN2 during mitosis.
Figure 8: Kinase inhibitors screen for TIN2 phosphorylation

(A) Detection of S330 phosphorylation of TIN2 with a phosphorylation-specific antibody in cells arrested with nocodazole and treated with kinase inhibitors. HeLa cells stably expressing wild-type Flag-TIN2 were treated with DMSO, H-89, BI-D1870, BI 2536 or VX-680 in the presence of either nocodazole (Noc) or vehicle (DMSO). Derived lysates were immunoprecipitated (IP) with an αFlag antibody, resolved by SDS-PAGE and immunoblotted (IB) with αPhos-S330 or, as a loading control, αTIN2 antibodies. Representative of two experiments.

(B) DNA profiles of HeLa cells treated with BI-D1870. HeLa cells treated with DMSO, nocodazole (Noc) or nocodazole+ BI-D1870 were harvested, stained with propidium iodide and subjected to fluorescence-activated cell sorting (FACS) analysis. Representative of two experiments.
4.2.8 Cellular TIN2 is phosphorylated by the mitotic kinase RSK2.

To evaluate whether RSK kinases can phosphorylate TIN2, 293T cells were engineered to transiently express Flag-TIN2. Transfected cells were then untreated, treated with nocodazole to arrest cells in mitosis, treated with BI-D1870 to inhibit RSK kinases, or treated with both compounds. Thereafter, Flag-TIN2 was immunoprecipitated with an anti-Flag antibody, resolved by Phos-tag SDS-PAGE and detected with an anti-TIN2 antibody. As expected, treatment with nocodazole increased all three phosphorylated forms of TIN2 compared to untreated cells, and this increase in mitotic phosphorylation was inhibited by BI-D1870 (Figure 9A). Further, 293T cells were transiently transfected with a vector expressing Flag-TIN2 and RSK2\textsuperscript{Y707A}, a constitutively active version (Poteet-Smith and Smith et al. 1999) of the RSK2 member of the RSK family of kinases, comprised of RSK1, RSK2, RSK3 and RSK4. The cells were then either left untreated or treated with BI-D1870. The derived lysates were split into two portions. The first portion was resolved by SDS-PAGE and immunoblotted with an antibody detecting S235/S236 phosphorylated S6 ribosomal protein, a known substrate of RSK2 (Roux and Shahbazian et al. 2007), or tubulin as a loading control. In the second portion, Flag-TIN2 was immunoprecipitated with an anti-Flag antibody, resolved by Phos-tag SDS-PAGE and detected with an anti-TIN2 antibody. Both positive-control S6 ribosomal protein and TIN2 were phosphorylated in the presence of RSK2\textsuperscript{Y707A}, and this
phosphorylation was decreased by BI-D1870 treatment (Figure 9B). These results argue that TIN2 is phosphorylated on both S295 and S330 by RSK2 during mitosis.

**4.2.9 TIN2 is phosphorylated by RSK2 in vitro.**

To assess whether RSK2 can directly phosphorylate TIN2, a recombinant 6-His epitope-tagged version of wild-type RSK2 was incubated with recombinant and purified maltose-binding protein (MBP)-tagged wild type, S295A, S330A, and AA mutant TIN2 protein in the presence of P32-labeled ATP. Reaction products were resolved by SDS-PAGE and exposed to auto-radiographic film to visualize phosphorylated protein. Equal amounts of recombinant MBP or MBP-TIN2 protein in each reaction were confirmed by Coomassie Brilliant Blue staining. Consistent with previous reports (Poteet-Smith and Smith et al. 1999; Vik and Ryder 1997), 6His-RSK2 exhibited some auto-phosphorylation. However, recombinant MBP-TIN2 was phosphorylated in the presence of 6His-RSK2, and this phosphorylation was reduced if S295, S330, or especially if both S295 and S330 were mutated (Figure 9C). Further, the phosphorylation level of MBP-TIN2 was increased upon incubated with 6His-RSK2, and this increase was reduced with BI-D1870 (Figure 10). Admittedly, not all phosphorylation was lost when both S295 and S330 were mutated. However, since BI-D1870 was quite effective at reducing TIN2 phosphorylation (Figure 8A, Figure 9A, Figure 9B), no other phosphorylated residues were identified in TIN2 by mass spectrometry (Figure 3), and mutating both S295 and S330 blocked all detectable phosphorylation of TIN2 in cells
(Figure 7B), we suspect that this residual phosphorylation may be spurious, although other possibilities cannot be discounted. In summary, these data support the conclusion that RSK2 can phosphorylate TIN2.
Figure 9: TIN2 is phosphorylated by the mitotic kinase RSK2 in vivo and in vitro

(A) Detection of S295 and S330 phosphorylation of TIN2 by the Phos-tag reagent in asynchronous or nocodazole arrested cells with or without the RSK2 inhibitor BI-D1870. 293T cells were either untreated or treated with nocodazole (Noc), BI-D1870, or both compounds. Derived lysates were then subjected to immunoprecipitation (IP) with an αFlag antibody and resolved by SDS-PAGE in the presence of the Phos-tag reagent and immunoblotted (IB) with an αTIN2 antibody. The supershifted bands corresponding to S295, S330, or S295 and S330 phosphorylation, as well as the unphosphorylated (UP) TIN2 protein, are denoted on the left. Representative of two experiments.

(B) Detection of S295 and S330 phosphorylation of TIN2 by the Phos-tag reagent in asynchronous cells with ectopic RSK2 and/or the RSK2 inhibitor BI-D1870. 293T cells transiently transfected with Flag-TIN2 and the Y707A constitutively active mutant form of RSK2 (Flag-RSK2<sup>Y707A</sup>) were either left untreated or treated with RSK kinase inhibitor BI-D1870. Derived lysates were split into two portions. The first portion of the lysates was subjected to immunoprecipitation (IP) with an αFlag antibody, resolved by SDS-PAGE in the presence of the Phos-tag reagent and immunoblotted (IB) with an αTIN2 antibody. The supershifted bands corresponding to S295, S330, or S295 and S330 phosphorylation, as well as the unphosphorylated (UP) TIN2 protein, are denoted on the left (top). The second portion of the lysates were resolved by normal SDS-PAGE and immunoblotted with either an αPhospho-S6 antibody to monitor RSK2 kinase activity, or an αTubulin antibody as a loading control (bottom). Representative of two experiments.
(C) Detection of TIN2 phosphorylation by RSK2 in vitro. Recombinant maltose-binding protein (MBP) or N-terminal MBP-tagged TIN2 (MBP-TIN2) in the WT, S295A, S330A or AA mutant configuration were captured with amylose resin and eluted with maltose. No protein (-) or equal amounts of the aforementioned purified MBP-TIN2 proteins were incubated with recombinant N-terminal 6His-tagged RSK2 (6His-RSK2) in the presence of ATP$^{32}$, after which the reaction products were resolved by SDS-PAGE and either (top) exposed to autographic film or (bottom) stained with Coomassie Brilliant Blue (CBB staining). Phosphorylated (P$^{32}$) MBP-TIN2 and a non-specific band (*) are denoted on the left top panel. MBP-TIN2 and MBP are denoted on the left bottom panel. Representative of two experiments.
Figure 10: In vitro phosphorylation of TIN2 by RSK2

Recombinant N-terminal MBP-tagged TIN2 (MBP-TIN2) in the absence or presence of recombinant N-terminal 6His-tagged RSK2 (6His-RSK2) and/or BID-1870 were incubated with ATP$^{32}$. Reaction products were resolved by SDS-PAGE and either (top) exposed to autographic film or (bottom) stained with Coomassie Brilliant Blue (CBB staining). Arrow: Phosphorylated (top) or purified (bottom) MBP-TIN2. Representative of one experiment.
4.3 Discussion

We identified two, and only two phosphorylation sites on TIN2 by mass spectrometry analysis, namely S295 and S330. Mutational analysis of these two residues, coupled with two independent phosphorylation detection assays, namely Phos-Tag separation of phosphorylated species and phosphorylation-specific antibodies, confirmed that TIN2 can indeed be phosphorylated on both these sites. The phosphorylation was also detected on endogenous TIN2. Furthermore, both sites were preferentially phosphorylated in mitosis, either when cells entered mitosis after release from a double thymidine block or when cells were arrested with nocodazole. Lastly, the mitotic kinase RSK2 was found to phosphorylate TIN2 on S295 and S330 both in vitro and in vivo. Thus, we identify phosphorylation of S295 and S330 as a new regulated post-translational modification of TIN2.

The consequence of TIN2 phosphorylation during mitosis remains to be determined. Preliminary analysis has failed to uncover any overt difference between the wild type and AA mutant. Specifically, the wild type and AA mutants of TIN2 both co-immunoprecipitates with the known TIN2-interacting proteins TRF1 and TPP1 (Figure 11). Although TIN2 can be ubiquitinated (Bhanot and Smith 2012), the levels of Flag-TIN2 wild-type and AA mutant protein after cells were treated with cycloheximide were nevertheless similar (Figure 12). Even though TIN2 can localize to mitochondria (Chen and Zhang et al. 2012), the wild type as well as the S295A, S330A, and AA mutant
versions of GFP-tagged TIN2 exhibited similar co-localization with the mitochondrial marker Mito-Tracker Red (Figure 13). Finally, although telomere-induced foci (TIF) are observed in Tin2−/− murine cells (Takai and Kibe et al. 2011), both the wild type and phosphorylation mutants of TIN2 suppressed the number of TIFs induced in HeLa cells by TIN2 shRNA (Figure 14). However, as telomere sister chromatid exchanges are elevated in Tin2−/− murine cells (Takai and Kibe et al. 2011), perhaps phosphorylation is related to this aspect of TIN2 function. Alternatively, S295 and S330 reside close to mutation sites found in dyskeratosis congenital patients (Walne and Vulliamy et al. 2008) that affect binding to heterochromatin protein 1γ and telomere length (Canudas and Houghtaling et al. 2011). Thus, perhaps mitotic phosphorylation of TIN2 is instead involved in telomere length regulation. Finally, as RSK phosphorylated TIN2, and inhibiting this kinase in mitotic cells reduced TIN2 phosphorylation, TIN2 phosphorylation may be linked with functions of RSK2. In this regard, RSK2 promotes G2/M transition (Cude and Wang et al. 2007) and maintains spindle assembly checkpoint (Vigneron and Brioudes et al. 2010).
Figure 11: Co-immunoprecipitation of wild-type and AA mutant TIN2 with TPP1 and TRF1

Cell lysates from 293T cells transiently co-transfected with a pBabe-puro vector (V), pBabe-puro-Flag-TIN2^{WT} (WT) or pBabe-puro-Flag-TIN2^{AA} (AA) and either (A) pEGFP-TPP1 or (B) pCMV-myc-TRF1 were subjected to immunoprecipitation (IP) with an αFlag antibody, resolved by SDS-PAGE and immunoblotted (IB) with αFlag, αGFP or αMyc antibodies. Representative of two experiments.
Figure 12: Normalized protein level of wild-type and AA mutant TIN2 after treatment of cells with cycloheximide

293T cells transiently transfected with pBabe-puro-Flag-TIN2\textsuperscript{WT} (●) or pBabe-puro-Flag-TIN2\textsuperscript{AA} (○) were untreated (0 time point) or treated with 100 µg/ml cycloheximide for 1, 2, 4, 6 or 8 hours. Lysates were resolved by SDS-PAGE and immunoblotted (IB) with αFlag and αTubulin antibodies. The intensity of Flag-TIN2 bands were quantified by imageJ, normalized to that of tubulin, and plotted against time. Representative of two experiments.
Figure 13: Co-localization analysis of wild-type and phosphorylation mutant versions of TIN2 with Mito-Tracker Red

Confocal microscope imaging of HeLa cells transiently transfected with pEGFP-N1-TIN2 in the WT, 295A, 330A, or AA configurations. DNA was visualized by staining with DAPI, ectopic TIN2 proteins were visualized by virtue of GFP, mitochondria were visualized by staining with MitoTracker Red, and co-localization between TIN2 and mitochondria were visualized by overlaying the latter two images. Each panel represents one of six cells imaged.
HeLa cells stably infected with pBabe-puro encoding no transgene (vector) or Flag-TIN2 in the wild-type (WT) or AA configuration were transiently transfected with pSuper-retro-GFP-Neo with no insert (vector) or TIN2 shRNA sequences 1 and 2 (shRNA1, 2) verified by (A) immunoblot analysis (IB) to reduce endogenous TIN2 levels (immunoblot with αTubulin antibody serves as a loading control). Cells were then (B) hybridized in situ with a Cy5-labelled PNA telomere C probe and an α-γH2AX antibody, and the number of co-localization of the two probes representing telomere-dysfunction induced foci (TIF) per cell was quantitated.

Figure 14: Analysis of telomere-dysfunction induced foci in TIN2 knockdown cells rescued with wild-type or phosphorylation mutant versions of TIN2
5. Separation-of-function analysis of mPOT1a

5.1 Introduction

Previous screening of a panel of NAAIRS substitution mutants revealed two classes of separation-of-function mutants of human POT1 (hPOT1). First, a NAAIRS substitution at 317aa, termed hPOT1ΔTPP1, had reduced affinity for human TPP1 (hTPP1) but neither human TRF2 (hTRF2) nor telomeric DNA. Expression of this mutant in human cells elicited a DNA damage response on telomere ends (Barrientos and Kendellen et al. 2008). Second, a NAAIRS mutant at 545aa, termed hPOT1ΔTRF2, had reduced the affinity for hTRF2, but not hTPP1 or telomeric DNA. Expression of this mutant in human cells could induce telomere elongation (Kendellen and Barrientos et al. 2009). Based on these data, hPOT1 binding to hTPP1 and hTRF2 are required to protect telomere ends and regulate telomere length, respectively.

One significant difference between human and mice is that there are two copies of the Pot1 gene in the mouse genome. Pot1a is located on chromosome 6 while Pot1b is located on chromosome 17 (Hockemeyer and Daniels et al. 2006). Both of the encoded proteins are ubiquitously expressed throughout the embryo development. Murine POT1a (mPOT1a) and murine POT1b (mPOT1b) share 75% similarity in protein sequence, whereas they seem to have different roles in telomere protection (Hockemeyer and Daniels et al. 2006). Specifically, mPot1a is an essential gene, and mPot1−/− MEFs have an increased level of T-SCE, anaphase bridges, chromosome fusions, and DNA breaks.
In contrast, \textit{mPot1b} knockout mice are viable and fertile with no apparent increase in DNA damage responses, although there is an increase in the length of the telomere overhang (Wu and Multani et al. 2006; Hockemeyer and Daniels et al. 2006). Loss of \textit{mPot1a} evidently induces TIFs, and double knockout of \textit{mPot1a} and \textit{mPot1b} further exacerbates the telomere deprotection, as evidenced by an elevated level of TIFs. Interestingly, overexpressing mPOT1a can fully compensate this defect, whereas mPOT1b can only weakly rescue this defect, indicating that mPOT1a plays a major role in suppressing DNA damage response at telomeres, whereas POT1b may have limited function in this regard.

The phenotypes of two \textit{mPot1} knockout mice suggest that these genes are both required for telomere protection (Baumann 2006). Based on the highly conserved sequences, mPOT1a and mPOT1b may protect telomere ends through a similar mechanism as observed with hPOT1. Indeed, the interactions between mPOT1a and mPOT1b with murine TPP1 (mTPP1) proved to be important for telomere protection (Hockemeyer and Palm et al. 2007). More recently, \textit{mTpp1} knockout mice further suggested that mPOT1a and mPOT1b require mTPP1 in preventing DNA damage responses and chromosome instability (Kibe and Osawa et al. 2010). However, there is no evidence that mTRF2 binds mPOT1a or mPOT1b, although mTRF2 has been shown to form T-loops \textit{in vitro} and anchor mPOT1a and mPOT1b to telomere ends (Griffith and Comeau et al. 1999; Hockemeyer and Palm et al. 2007). To explore the effect of
substitution mutants on the function of mPOT1a, I created a panel of NAAIRS substitution mutants of mPOT1a.

5.2 Results

5.2.1 Identification of mTPP1-binding mutants of mPOT1a by NAAIRS mutagenesis

Previous studies identified two separation-of-function mutants of hPOT1, one of which could not bind to hTPP1 but still interacted with hTRF2 (hPOT1ΔTPP1) and the other of which could not bind to hTRF2 but still interacted with hTPP1 (hPOT1ΔTRF2). To determine if similar mutants altered mPOT1a function, I introduced NAAIRS substitution mutant into mPOT1a corresponding to the ΔTPP1 mutant of human POT1 (Figure 15A). Wild type (WT) and ΔTPP1 mutant versions of mPOT1a were then co-transfected with pQCXIP-HA-mTPP1 into 293T cells, which were lysed and immunoprecipitated with anti-Flag antibody. Elutes were resolved by SDS-PAGE and immunoblotted with anti-Flag, anti-HA, and anti-Tubulin antibodies (Figure 15B). Equal loading levels were confirmed by Tubulin immunoblot. Despite the homology between hPOT1 and mPOT1a in the region mutated in the ΔTPP1 mutant, I found that amount of mTPP1 immunoprecipitated by WT and ΔTPP1 mPOT1a were nevertheless comparable.

Given the aforementioned results, I created a panel of 35 individual NAAIRS mutants, in which the sequence encoding six amino acids between 297aa and 616aa of mPOT1a was systematically mutated to the sequence encoding NAAIRS. In order to identify separation-of-function mutants, these NAAIRS mutants were subjected to
immunoprecipitation with mTPP1. Among these NAAIRS mutants, mPOT1a^{N389} and mPOT1a^{N497} had significantly reduced affinity to mTPP1, comparing to mPOT1a^{WT}.

Specifically, 293T cells were co-transfected with the pBabe-puro vector encoding no transgene or Flag-mPOT1a-WT, -N389, and -N497 and pQCXIP-HA-hTPP1. The lysates derived from these cells were subjected to immunoprecipitation with anti-Flag antibody. Elutes were then resolved by SDS-PAGE and immunoblotted with anti-Flag, anti-HA, and anti-Tubulin antibodies (Figure 16). Equal loading was confirmed by Tubulin immunoblot. While mPOT1a^{WT} readily co-immunoprecipitated with mTPP1, both mPOT1a^{N389} and mPOT1a^{N497} mutants bound significant less mTPP1, indicating a defect in mTPP1 binding ability. Therefore, mPOT1a^{N389} and mPOT1a^{N497} are mTPP1-binding mutants based on the immunoprecipitation experiment. Nevertheless, the telomere-related functions of these mutants remain to be determined.
Figure 15: Co-immunoprecipitation analysis of wild-type and mutant of mPOT1a with mTPP1

(A) DNA sequence alignment between hPOT1 and mPOT1a. The mutated region of hPOT1 ΔhTPP1 was highlighted.

(B) Cell lysates from 293T cells transiently co-transfected with a pBabe-puro-Flag-mPOT1aWT or pBabe-puro-Flag-mPOT1aΔhTPP1 and pQCXIP-HA-mTPP1 were subjected to immunoprecipitation (IP) with an αFlag antibody, resolved by SDS-PAGE and immunoblotted (IB) with αFlag, αHA or αTubulin antibodies.
Figure 16: NAAIRS mutants of mPOT1a have defects in binding to mTPP1

Cell lysates from 293T cells transiently co-transfected with a pBabe-puro vector, pBabe-puro-Flag-mPOT1a<sup>WT</sup>, pBabe-puro-Flag-mPOT1a<sup>N389</sup>, pBabe-puro-Flag-mPOT1a<sup>N497</sup>, and pQCXIP-HA-mTPP1 were subjected to immunoprecipitation (IP) with an αFlag antibody, resolved by SDS-PAGE and immunoblotted (IB) with αFlag, αHA or αTubulin antibodies.
5.2.2 Characterization of mTPP1-binding mutants of mPOT1a

To further explore the potential defects in these two NAAIRS mutants of mPOT1a, the binding affinity to another telomere-associate protein, mTIN2, was investigated. Since mTIN2 is known to interact with mPOT1 through mTPP1 (Takai and Kibe et al. 2011), mTPP1-binding mutants of mPOT1a should similarly have reduced affinity for mTIN2.

The pBabe-puro vector encoding no transgene or Flag-mPOT1a-WT, -N389 or -N497 mutant versions of mPOT1a, or pBabe-puro-Flag-mPOT1b, were co-transfected with pcDNA-HA-mTIN2 into 293T cells. The cells were then lysed and immunoprecipitated with anti-Flag antibody. Elutes were resolved by SDS-PAGE and immunoblotted with anti-Flag, anti-HA, or anti-Tubulin antibodies (Figure 17). Both mPOT1a and mPOT1b readily co-immunoprecipitated with mTIN2, although mPOT1b had less affinity for mTIN2 compared to mPOT1a. Further, both N389 and N497 mutants of mPOT1a had significantly lower affinity for mTIN2.

To investigate whether these two mutants of mPOT1a had defects in telomere protection, telomere-dysfunction induced foci (TIFs) were evaluated in mPot1a\textsuperscript{+/−} MEFs, in which mPOT1a WT and two mTPP1-binding mutants were stably expressed via retroviral infection. Specifically, mPot1a\textsuperscript{−/−} MEFs were infected with retrovirus derived from pBabe-puro encoding no transgene (vector) or Flag-mPOT1a-WT, -N389, or -N497. The MEFs were then either left untreated or treated with Ad-Cre for 48h to convert
mPot1a<sup>F/F</sup> to an mPot1<sup>a</sup><sup>−/−</sup> genotype. The efficacy of Ad-Cre and expression of Flag-tagged mPOT1a proteins were confirmed by immunoblot, although N389 mutant expressed at a lower level compared to N497 mutant and wild type mPOT1a (Figure 18). MEFs were then permeabilized, fixed, blocked, and hybridized in situ with a Cy5-labelled PNA telomere C probe and an anti-γH2AX antibody to assess the level of TIFs. The co-localization of PNA probe and γH2AX signals was assessed blindly and the results in each group were plotted (Figure 19). The following results were observed: first, stable expression of WT, N389, and N497 mutant versions of mPOT1a protein in mPot1a<sup>F/F</sup> MEF did not induce DNA damage signaling on telomere ends comparing to vector only control, suggesting that the two mTPP1-binding mutants of mPOT1a do not have dominate-negative effect. Second, deletion of endogenous mPOT1a protein resulted in elevated DNA damaging signaling, but not to the same extend reported previously (Wu and Multani et al. 2006; Hockemeyer and Daniels et al. 2006). Third, exogenously expressed wild type or N389 mutant of mPOT1a neither rescued nor exacerbated the increased levels of TIFs in the absence of endogenous mPOT1a protein. However, because expression of WT mPOT1a did not suppress the elevated level of TIFs upon Ad-Cre mediated conversion of MEFs, no conclusions can be drawn with regards to the ability of the mPOT1a mutants in telomere protection.
Figure 17: Interaction between mPOT1 WT/mutants and mTIN2

Cell lysates from 293T cells transiently co-transfected with a pBabe-puro with no transgene or with Flag-mPOT1a-WT, mPOT1a-N389, or mPOT1a-N497 and pcDNA-HA-mTIN2 were subjected to immunoprecipitation (IP) with an αFlag antibody, resolved by SDS-PAGE and immunoblotted (IB) with αFlag, αHA, or αTubulin antibodies.
Mouse embryonic fibroblasts (MEF) from \textit{mPot1a^{f/f}} genetic engineered mice stably infected with retrovirus derived from pBabe-puro encoding with no transgene (empty) or with Flag-mPOT1a-WT, -N389, or -N497 mutants were either left untreated or treated with Ad-Cre.

(A) The RNA was purified from these MEFs, reverse transcribed and PCR amplified with primers targeted to 5’UTR of \textit{mPot1a}, Flag epitope, and GAPDH. The amplified DNA fragments were then resolved by 1.5% agarose gel.

(B) Cell lysates derived from these MEFs were subjected to immunoprecipitation (IP) with an \(\alpha\)Flag antibody, resolved by SDS-PAGE, and immunoblotted (IB) with \(\alpha\)Flag or \(\alpha\)Tubulin antibodies.
Mouse embryonic fibroblasts (MEF) from *mPot1a* knockout mice stably infected with retrovirus derived from pBabe-puro with no transgene (vector) or Flag-mPot1a-WT, -N389, or -N497 mutants were either untreated or treated with Ad-Cre. Cells were then hybridized *in situ* with a Cy5-labelled PNA telomere C probe and an α-γH2AX antibody, and the number of co-localization of the two probes representing telomere-dysfunction induced foci (TIF) per cell was calculated and plotted. At least 40 cells from each group were analyzed.
5.3 Discussion

To study the function of POT1 proteins in mice, I created a panel of C-terminal NAAIRS mutants of mPOT1a that I screened for reduced affinity to mTPP1. Among these NAAIRS mutants, N389 and N497 of mPOT1a have significantly reduced affinity to mTPP1 (Figure 16).

To further characterize the defects of these two mutants, the binding affinity to other telomere-associate proteins were evaluated. Although hPOT1 binds to hTRF2 (Barrientos and Kendellen et al. 2008; Kendellen and Barrientos et al. 2009), the interaction between mPOT1a, mPOT1b and mTRF2 was not detected either in vivo or in vitro (Data not shown). Nevertheless, it is possible that other murine telomere-binding proteins or accessory proteins are needed to facilitate this interaction. As such, mTIN2 was chosen as a surrogate to mTRF2, since previous studies have shown that hTIN2 formed different protein complex with hTRF1, hTRF2 and hTPP1 (Ye and Donigian et al. 2004; O’Connor and Safari et al. 2006; Houghtaling and Cuttonaro et al. 2004). However, both the mPOT1a NAAIRS mutants N389 and N497 failed to immunoprecipitate with mTIN2, although a strong interaction was captured with wild-type mPOT1a and mPOT1b (Figure 17). Thus, these two mPOT1a NAAIRS mutants have reduced binding to both mTPP1 and mTIN2.

In order to assess the telomere-protecting function of these two mutants, I analyzed TIFs level in mPot1aF/F MEFs expressing WT and mutant mPOT1a after Ad-Cre
treatment. I confirmed that Ad-Cre treatment resulted in the recombination between the two loxP sites in mPot1a gene, successfully converting mPot1a^{+/−} to mPot1a^{−/−} genotype (Figure 18). In this setting I found that the N389 and N497 mutant versions of mPOT1a did not elicit large amount of DNA damage signals at telomere ends, comparing to WT mPOT1a, in the presence or absence of endogenous mPOT1a. However, because introducing WT mPOT1a into mPot1a^{−/−} MEFs failed to rescue the phenotype of elevated levels of TIFs, no conclusions can be drawn. The difference of TIFs levels between mPot1a^{+/−} and mPot1a^{−/−} MEF in our setting was not as significant as observed by others (Hockemeyer and Daniels et al. 2006). These discrepancies raised the possibility that genetic disruption of mPot1a gene was not complete, so that the residual endogenous mPOT1 escaped from deletion is still able to partially fulfill its functions on telomeres. Indeed, it has been proposed that the genetic deletion within exon 1 and 2 could potentially result in the production of truncated mPOT1a protein (Hockemeyer and Daniels et al. 2006), which complicates the interpretation of our data. Thus further analysis of mPOT1a mutants is required.
6. Conclusion and future directions

6.1 Summary of studies in telomere-associate protein regulation

Previous studies revealed the importance of telomere-associate proteins of the shelterin complex in protecting telomere ends from being detected as damaged DNA and controlling telomere length during cell proliferation. Specifically, the telomeric dsDNA-binding protein TRF2 and the telomeric ssDNA-binding protein POT1 inhibit ATM- and ATR-mediated DNA damage detection pathways, respectively (Denchi and de Lange 2007). RAP1 is also capable of inhibiting homologous recombination (Sfeir and Kabir et al. 2010). Further, the POT1-TPP1 heterodimer is both important to protect 3’ overhang of telomere and recruit telomerase to telomere ends (Ye and Hockemeyer et al. 2004; Barrientos and Kendellen et al. 2008; Xin and Liu et al. 2007; Hockemeyer and Palm et al. 2007). Another telomeric dsDNA-binding protein, TRF1, is a negative regulator of telomere length, and interacts with PinX1, a known telomerase inhibitor (Loayza and De Lange 2003; Yonekawa and Yang et al. 2012; Chen and Yang et al. 2008). TIN2, as a central member of this protein-DNA complex, interacts with each member directly or indirectly to form different sub-complex on telomere (Ye and Donigian et al. 2004; O’Connor and Safari et al. 2006; Takai and Kibe et al. 2011).

Although the protective roles of telomere-associate proteins have been described, whether other signaling pathways or protein-protein interactions modulate these functions is not well understood. For example, multiple post-translational modifications
of TRF1 have been found at mitosis, suggesting the function, localization, or stability of TRF1 may be regulated during mitosis by such modifications (summarized in Chapter 1.3.1). Similarly, the TRF1-binding protein, PinX1, was recently found to localize to telomeres during mitosis and knockdown of PinX1 led to instability of TRF1 during mitosis (Yonekawa and Yang et al. 2012). These observations prompted me to further study the roles of PinX1 during mitosis. Indeed, the absence of PinX1 during mitosis resulted in delayed entry into mitosis with unchanged length of mitosis comparing to control cells, as evident by live cell imaging analysis (Figure 2A, Figure 2B). Moreover, knockdown of PinX1 induced apoptosis during mitosis, at least as measured by an elevated level of Caspase 3 (Figure 2C). Finally, in a collaboration efforts with Dr. Yonekawa, I found that PinX1 is critical for reloading of TRF1 during mitosis, when TRF1 levels on telomere is first reduced then restored to normal (Yonekawa and Yang et al. 2012). Collectively, these studies suggest that PinX1 is an essential chaperon of TRF1 for its stability on telomeres during mitosis and required for mitosis progression.

TIN2 has also been shown to protect TRF1 from modification by tankyrase (Ye and de Lange 2004) and inhibit binding of PinX1 to TRF1 (Yonekawa and Yang et al. 2012). Further, it is know that TIN2 has a higher affinity to TRF1 comparing with PinX1 (Chen and Yang et al. 2008). Therefore, I hypothesize that TIN2-TRF1 interaction is disrupted when cells enter mitosis, so that PinX1 could bind with TRF1 and help its relocation to telomeres. To test this hypothesis, I purified TIN2 protein from mitotic cell
populations, and subjected the protein to mass spectrometry analysis. This analysis revealed two phosphorylation sites on TIN2, serine 295 and serine 330 (Figure 3). These two serines were also reported by previous whole genomic phospho-sites screen, but further characterization was not available. To independently validate these results, Phos-tag integrated SDS-PAGE and phosphorylation-specific antibodies were employed (Figure 4). Notably, multiple super-shifted bands were observed above major TIN2 band after immunoprecipitation, which correlate to single or double phosphorylated TIN2, distinguished by serine-to-alanine mutants of TIN2. Further, these super-shifted bands almost completely disappeared after phosphatase treatment, indicating they were phosphorylated TIN2. Similar results were observed using phospho-specific antibodies (Figure 5).

Next, I asked whether these two phosphorylation events were cell cycle dependent. To answer this question, I synchronized cells and released them into different stages of cell cycle. The TIN2 protein immunoprecipitated from these different cell populations were then analyzed by Phos-tag integrated SDS-PAGE and phospho-specific antibodies (Figure 7). Based on the intensity of phosphorylated TIN2 bands, I concluded that both phosphorylation events on TIN2 mainly occurred during mitosis, which was also confirmed by nocodazole treatment, another method to synchronized cell at mitosis.
I then carried out a small-scale mitotic kinase inhibitors screen (Figure 8) to identify mitotic kinases responsible for the phosphorylation on TIN2, which revealed RSK kinase family as potential candidates. Moreover, RSK kinase proved to phosphorylate TIN2 both in vitro and in vivo (Figure 9, Figure 10). In addition, I have tried various approaches to identify the function of these two phosphorylation events on TIN2, including protein-protein interaction (Figure 11), stability (Figure 12), localization (Figure 13) and TIFs assay (Figure 14), although no distinct differences were observed between wild-type and unphosphorylatable mutants groups were observed. In conclusion, I have identified two serine sites on TIN2 that are specifically phosphorylated during mitosis. RSK kinase family potentially mediates these phosphorylation events on TIN2. However, the effect of these modifications on TIN2 function remains to be determined.

In addition, I have also studied murine POT1a and mPOT1b proteins via NAAIRS mutagenesis. Previously, our lab characterized the separation-of-function mutants of human POT1, which revealed that the diverse functions of POT1 depend on different protein-protein interactions (Barrientos and Kendellen et al. 2008; Kendellen and Barrientos et al. 2009). In order to investigate these mutants in vivo, I attempted to generate corresponding separation-of-function mutants of mPOT1a and mPOT1b via NAAIRS mutagenesis, ultimately to create mice encoding these mutant proteins. The two mPOT1 proteins bound mTPP1. However, neither mPOT1a nor mPOT1b bound
mTRF2. Nevertheless, I did identify two mutants of mPOT1a that exhibited reduced binding to mTPP1 and its binding partner mTIN2, although the effect of these mutations on mPOT1a function remains to be identified.

6.2 Future directions

Future studies can be divided into two parts: 1) To analyze the regulation and function of multiple phosphorylation events on TRF1; 2) To further explore the impact of TIN2 phosphorylation in the absence of endogenous TIN2 and whether these modifications relate to non-telomeric function.

6.2.1 Roles of multiple phosphorylation events on TRF1

Since phosphorylation of TIN2 proved to be cell-cycle dependent, modifications of other telomere-associate proteins might also be controlled during cell cycle. Based on the multiple phosphorylation sites found on TRF1 previously, it is possible that TRF1 can also be regulated by phosphorylation in a cell-cycle-dependent fashion. In fact, these phosphorylation events on TRF1 proved to have diverse, and sometimes even opposite effects. For example, phosphorylation on S435 and T122 of TRF1 by Cdk1/plk1 and casein kinase 2 respectively could enhance its DNA binding ability (Wu and Yang et al. 2008; Kim and Kang et al. 2008), while ATM mediated T367 phosphorylation on TRF1 led to its disassociation from telomeres (McKerlie and Lin et al. 2012).
It is conceivable that these modifications could occur at different stages of cell cycle or under different cellular context, such as growth or stress signaling. The consequences of these modifications may include altered protein configuration, protein-protein interaction, stability and localization. For instance, transient TRF1 degradation is critical for sister chromatids separation (Dynek and Smith 2004; McKerlie and Zhu 2011), while reloading of TRF1 onto chromosome ends is required for telomere protection at the end of mitosis (McKerlie and Zhu 2011). Therefore, TRF1 and/or the telomere structure need these different modifications to react properly to cell-cycle progression and extracellular signaling. It is also possible that these phosphorylation events happened at approximately same time. As such, the final effect on TRF1 may depend on the most prevalent modification, presumably the strongest signal. However, this is less likely since most of the kinases responsible for these modifications on TRF1 are activated at different stages of cell cycle, or through different signaling pathways. Further, even though some modifications are mediated by mitotic kinases, these phosphorylation events on TRF1 might still have to occur in a specific order during mitosis.

In order to investigate these phosphorylation events on TRF1, the Phos-tag integrated SDS-PAGE could be used. The Phos-tag reagent can specifically bind to phospho-groups on proteins and slow their migration during SDS-PAGE (Yang and Xue et al. 2010; Kinoshita and Kinoshita-Kikuta et al. 2009). Thus, either exogenous or endogenous TRF1 could be immunoprecipitated from cells arrested in different stages of
cell cycle by double thymidine block and resolved by Phos-tag integrated SDS-PAGE. If specific phosphorylation events occur in a cell-cycle-dependent fashion, they would be identified by this approach. Given that a number of phosphorylation events occur on TRF1, it may be hard to distinguish multiple super-shifted bands at the same time due to limited separation ability of SDS-PAGE. To avoid this problem, unphosphorylatable mutants containing specific serines/threonines-to-alanines mutations could be used to help to determine the identity of each super-shifted bands of TRF1. Further, these results could be confirmed by different types of cell synchronization methods and phospho-specific antibodies. Since the development of phospho-specific antibodies can be time-consuming, using Phos-tag integrated SDS-PAGE would certainly help to identify the phosphorylation events that are worth pursuing, and guide the development of phospho-specific antibodies.

Next, the function of phosphorylation-of-interest could be investigated by phospho-specific antibodies. The localization of phosphorylated TRF1 could be assessed by immunofluorescence using phospho-specific antibodies. It is likely most of the phosphorylation of TRF1 occurs while the protein is bound to telomeres. However, other groups have shown that phosphorylation could result in the disassociation of TRF1 from telomeres (McKerlie and Lin et al. 2012). The fate of unbound TRF1 needs to be studied. Although tankyrase can readily modify unprotected TRF1, resulting in degradation of TRF1 (Smith and de Lange 2000), it is possible that TRF1 is degraded immediately after
released from telomeres, or unbound TRF1 is trafficked to other subcellular locations for reuse. These questions could be answered by assessing the colocalization of the signal from TRF1 phospho-specific antibodies with telomeric signal.

The phosphorylation of telomere-associate proteins may also be important for sub-complex formation and protein-protein interactions. TRF1 is known to bind to TIN2, PinX1, and Tankyrase (Ye and Donigian et al. 2004; Zhou and Lu 2001; Ye and de Lange 2004). Since the ADP-ribosylation by tankyrase leads to degradation of TRF1, their interaction may be transient and hard to capture. Interestingly, both TIN2 and PinX1 are able to protect TRF1 from tankyrase, as they have overlapped binding sites on TRF1 (Chen and Yang et al. 2008). TIN2 has a higher affinity than PinX1 for TRF1 (Chen and Yang et al. 2008), which raises a question that how PinX1 competes with TIN2 to interact with TRF1. Further, TIN2 has been shown to form sub-complexes on telomeres, such as TIN2-TRF1, TIN2-TPP1-POT1, TRF1-TIN2-TPP1-POT1 or TRF1-TIN2-TRF2 (Ye and Donigian et al. 2004; O'Connor and Safari et al. 2006). It is possible that phosphorylation of TRF1 could impact the formation of these sub-complexes and facilitate the structural change of telomere-associate protein complex.

To investigate the role of TRF1 phosphorylation on its protein-protein interactions, the binding partners of TRF1 could be immunoprecipitated. Anti-TRF1 and phospho-specific antibodies could then be used to detect the levels of total and phosphorylated TRF1 pulled down by these proteins. For example, phosphorylation of
TRF1 could favor its interaction with PinX1 over TIN2, which would be more frequently found in PinX1-TRF1 interaction, rather than TIN2-TRF1 interaction. On the other hand, fractionation of telomere-associated proteins has demonstrated the existence of different subcomplexes within shelterin complex (O'Connor and Safari et al. 2006). The phosphorylation status of TRF1 could be analyzed by phospho-specific antibodies after fractionation to reveal the relative abundance of different phosphorylation events on TRF1 within different subcomplexes. In addition, these methods can be used in adjunction with cell synchronization to further demonstrate cell-cycle-dependent regulation of TRF1 and other telomere-associate proteins.

To summarize, multiple modifications on TRF1 are the evidence of multi-layer regulation of its function. With Phos-tag integrated SDS-PAGE and phospho-specific antibodies, the timing and function of these phosphorylation events on TRF1 could be elucidated, which enables an integrated view of the diverse roles of TRF1 on telomeres.

6.2.2 Continue to explore the impact of TIN2 phosphorylation

6.2.2.1 Characterize TIN2 phosphorylation in other systems

Although I have identified the two phosphorylation sites on TIN2 and demonstrated that they are regulated in a cell-cycle-dependent fashion, the function of these phosphorylation events is still unclear. Based on my research, no obvious telomere dysfunction was observed when unphosphorylatable mutants of TIN2 were expressed in human cell lines. It is possible that endogenous TIN2 protein was capable of blocking
the adverse effect of these mutants. Knocking down endogenous TIN2 protein by
shRNA has been employed to minimize this caveat. In TIFs assay, the knockdown of
endogenous TIN2 elicited significant amounts of DNA damage responses at telomeres,
consistent with the phenotypes observed in \( mTIN2^{+/−} \) MEFs. However, the knockdown of
endogenous TIN2 protein in HeLa cells did not significantly increase the frequency of
telomere sister-chromatid exchange, which was reported in \( mTIN2^{+/−} \) MEF (Takai and
Kibe et al. 2011). This unchanged level of T-SCE after knocking down TIN2 in HeLa cells
could mean that the residual endogenous TIN2 still protect the integrity of telomeres,
and any defects caused by unphosphorylatable mutants of TIN2 were undetected.
Therefore, it is still possible that phosphorylation on TIN2 is essential to prevent
abnormal telomere sister-chromatid exchange, however, a more robust reduction of
endogenous TIN2 protein is needed to test this possibility.

TIN2 phosphorylation could also be important for sister-chromatid separation
during mitosis. The cohesin complexes on each sister chromatid were linked together
after DNA replication (Zhang and Kuznetsov et al. 2008). In order for sister chromatids
to be separated normally during mitosis, the linkage between these cohesin complexes
need to be disrupted (Canudas and Houghtaling et al. 2007). Interestingly, both TRF1
and TIN2 were shown to interact with a member of telomeric cohesin complex (Canudas
and Houghtaling et al. 2007). Further, the knockdown of either TRF1 or TIN2 was able to
rescue the mitotic arrest phenotype induced by unseparated sister chromatids (Canudas
and Houghtaling et al. 2007). Therefore, the phosphorylation of TIN2 could yet play a role in the process of sister-chromatid separation.

Despite that the defects in telomere sister-chromatid separation and exchange were not observed in HeLa cell line upon overexpressing unphosphorylatable mutants of TIN2, further studies in other systems could still shed light on the function of TIN2 phosphorylation. First, residual endogenous TIN2 still exist in HeLa cells after shRNA knockdown. A more robust depletion may be required for analysis of sister-chromatid separation phenotypes, such as nuclease-based genome editing system TALEN (Bedell and Wang et al. 2012). Second, HeLa is an established cancer cell line, its genomic instability might block the effect of TIN2 phosphorylation on sister chromatid exchange (Macville and Schrock et al. 1999). In addition, the HeLa cell line likely has partially deactivated cell-cycle checkpoint system (Harris and Lamb et al. 2002), and therefore the mitotic arrest caused by unseparated sister chromatids could be difficult to detect during cell division.

Due to these problems, a primary human fibroblast line might be used instead of cancer cell lines for exploring the function of TIN2 phosphorylation. However, disrupting endogenous TIN2 in primary cell lines might be challenging, because the telomere deprotection and genomic instability caused by TIN2 depletion could result in reduced cell viability (Takai and Kibe et al. 2011). In this case, wild-type and unphosphorylatable mutants of TIN2 would need to be exogenously expressed in the
fibroblast before the disruption of endogenous TIN2. Similarly, \( \text{Tin2}^{f/f} \) MEF could also be used, despite only one of two phosphorylation sites, S330, identified in human TIN2 is conserved in murine TIN2 protein. By using this approach, the function of TIN2 phosphorylation in telomere sister chromatid exchange and sister chromatids separation could be assessed by chromosome-oriented fluorescence \( \text{in situ} \) hybridization in human fibroblasts, which have a lower background of sister chromatid exchange and intact cell-cycle checkpoint system.

6.2.2.2 Explore the potential non-telomeric roles of TIN2 phosphorylation

Besides the telomere-related phenotypes, these two phosphorylation events of TIN2 could also play a role outside of telomeres. It has recently been reported that TIN2 localizes to mitochondria and modulates metabolic processes (Chen and Zhang et al. 2012). RAP1, another member of telomere-associate protein complex, was also recently shown to be a transcriptional factor for metabolic process (Yeung and Ramirez et al. 2013; Martinez and Gomez-Lopez et al. 2013). Further, telomere dysfunction induced by telomerase depletion in mice resulted in impaired mitochondria function/biogenesis and gluconeogenesis, possibly through p53-dependent repression on PGC1\( \alpha \) and PGC1\( \beta \) pathway (Sahin and Colla et al. 2011). These observations suggest a link between telomere-associate proteins and cellular regulation of metabolism. Thus, the impact of TIN2 phosphorylation in mitochondrial function could be investigated. Although no obvious differences were observed in mitochondrial localization between wild-type and
unphosphorylatable mutants of TIN2, it is still possible that phosphorylated TIN2 could regulate the metabolic function of mitochondria. First, the relative ratio of phosphorylated TIN2 on mitochondria and telomeres/nuclear could be assessed by fractionation, followed by immunoblotting against phospo-specific and TIN2 antibodies. This result would quantitatively illustrate whether these phosphorylation on TIN2 can facilitate its translocation to mitochondria. In fact, one of the substrates of RSK kinase family, Nur77, is translocated to mitochondria after being phosphorylated (Wang and Rud et al. 2009). Second, the impact of TIN2 phosphorylation on mitochondria could be assessed by overexpressing wild-type and unphosphorylatable mutants of TIN2 in the absence of endogenous TIN2. As noticed previously, the localization of TIN2 to mitochondria could change mitochondrial morphology (Chen and Zhang et al. 2012), suggesting a relationship between TIN2 and fusion-fission cycles of mitochondria. The morphology and metabolic process of the mitochondria from these cells could be assessed by fluorescence microscopy and various metabolic assays. Further, the status of TIN2 phosphorylation on mitochondria can be assessed when known pathways regulating mitochondria morphology such as Mfn1/2 or Drp1 are disrupted (Chan 2012). In summary, these approaches would help to clarify the relationship between TIN2 phosphorylation and mitochondrial function.

While the function of TIN2 phosphorylation in mitochondrial metabolism is intriguing, it is also possible that TIN2 phosphorylation could have indirect non-
telomeric function through other telomere-associate proteins. As mentioned above, RAP1 was discovered as an active transcriptional factor for gene regulation outside of telomeres. Since the stability of RAP1 on telomeres depends on its binding to TRF2, the phosphorylation of TIN2 could potentially change the stability of TRF2 on telomeres, and therefore modulate unbound RAP1 level and indirectly regulate its downstream gene transcription (Ye and Donigian et al. 2004; Kim and Beausejour et al. 2004). To explore this possibility, the abundance of TRF2 and RAP1 on telomeres needs to be assessed with either exogenously expressed wild-type or unphosphorylatable mutants of TIN2 in the absence of endogenous TIN2. The known downstream signaling pathways regulated by RAP1 could also be monitored. Last but not least, a longer version of TIN2 based on alternative splicing has been reported, which still contains the two serines identified by my study (Kaminker and Kim et al. 2009). The only known difference observed between two types of TIN2 is that the longer version of TIN2 is able to associate with nuclear matrix, suggesting its role in tethering telomeres to nuclear matrix. It would be interesting to investigate whether phosphorylation of TIN2 could alter the nuclear matrix binding ability of the longer version of this protein. Again, the abundance of wild-type or unphosphorylatable mutants of longer version of TIN2 in nuclear matrix could be assessed by fractionation, followed by immunoblotting against phospho-specific or TIN2 antibodies.
In summary, although the phosphorylation of TIN2 did not have an obvious role in telomere protection, it is still possible that these modifications of TIN2 are essential for non-telomeric function of telomere-associate proteins and future studies would shed light on the role of TIN2 phosphorylation outside telomere region.

6.3 Conclusions

In order to investigate the regulation of telomere-associate proteins, I have characterized the importance of PinX1 in mitosis progression, identified two serine sites on TIN2 that are phosphorylated by RSK kinase family during mitosis, and studied the NAAIRS mutants of murine POT1a and POT1b. The results of my studies demonstrate that both protein-protein interaction and post-translational modification can regulate the diverse functions of telomere-associate proteins, based on the cell-cycle status and their localization. Future studies are required to generate an integrated view on the regulation of telomere-associate proteins. The comprehensive understanding of these protein-protein interactions and post-translational modifications will help not only to dissect the diverse roles of telomere-associate proteins on telomere protection, length regulation, and non-telomeric functions, but perhaps even guide the development of novel therapies against aging diseases and cancer.
References


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PAPERS

