LIM Domain Proteins TRIP6 and LPP Associate with Shelterin to Mediate Telomere Protection

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Abstract

LIM DOMAIN PROTEINS TRIP6 AND LPP ASSOCIATE WITH SHELTERIN TO MEDIATE TELOMERE PROTECTION

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POT1 is the single stranded telomeric overhang binding protein of the shelterin complex, a group of six proteins essential for proper telomere function. The abrogation of POT1 DNA binding activity results in telomere elongation, or activation of the ATR DNA damage response at telomeres. Therefore, overhang binding represents the functionally relevant activity of POT1. Novel protein associations with the POT1 DNA binding domain are of great interest to explore and these possible interacting factors were sought using the yeast two-hybrid system. Bait containing the POT1 DNA binding domain was used leading to the isolation of LIM domain protein TRIP6 as a novel POT1 interacting factor. TRIP6 could co-immunoprecipitate with other shelterin components, arguing for association with the whole complex. Additionally, TRIP6 was detected at telomeres by Chromatin Immunoprecipitation and Immunofluorescence in Hela and HTC75 cells, which suggests association with telomeric DNA. TRIP6 depletion by siRNA led to the induction of telomere dysfunction induced foci, indicating a role in telomere protection. A closely related LIM protein, LPP, was also found at telomeres and was important for repressing the DNA damage response. A related LIM protein Zyxin was found not to associate with telomeres. We propose that TRIP6 and LPP represent a novel class of molecules at human telomeres involved in the repression of inappropriate DNA damage response at chromosome ends. All assays incorporate human cancer cell lines HTC75 and Hela 1.2.11. These results could advance our understanding on the repression of telomere-based senescence, an important tumor suppressor mechanism.

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Chapter 1 Introduction and Historical Overview

Mammalian Telomeres

The semi-conservative replication of linear eukaryote chromosomes results in the loss of small increments of DNA with each round of replication upon removal of the RNA primer. After several rounds of replication, a significant amount of DNA would be eroded from the chromosome ends. In order to circumvent the progressive DNA loss known as the end-replication problem, telomeres are present at the terminal ends of chromosomes. These protective nucleo-proteic structures found at the ends of linear chromosomes are essential chromosomal elements that ensure the proper replication and protection of chromosomes. The telomere DNA has a 3' end rich in guanosine and lacking cytosine with the complementary strand being rich in cytosine and lacking in guanosine. These properties result in the referring to these strands as G-rich and C-rich respectively. The length of telomeric DNA varies between organisms with human telomeres being comprised of 2-12 KB of double-stranded DNA with the human sequence being TTAGGG[1]. Adjacent to this duplex is a single stranded overhang at the 3' end that is between 50-150 nucleotides in length[4, 5] (Figure 1A). The G-rich overhang is the substrate for the enzyme telomerase as well as the binding for POT1, telomeric single-stranded DNA binding proteins which is critical to chromosome end protection[6]. The overhang is generated through a resectioning of the C-rich strand that predominantly generates an AATC-5' terminus. The overhang is known to be pivotal for the function of telomeres due to its involvement in the protective conformation referred to as t-loops that are thought to protect the telomeres from inappropriate processing possibly through the sequestration of the overhang[7] (Figure 1B).

The t-loop was revealed via electron microscopy in both mouse and human telomeres[7]. A t-loop occurs when the 3' overhang invades the duplex portion of the telomeres. The overhang will base pair with the C-rich strand and subsequently displace the G-rich strand. This site of displacement is referred to as the D loop. *In vivo*, telomeric proteins presumably maintain the t-loop configuration and the lariat is resolved once protein-free DNA is isolated. The actual size of the t-loop is not directly correlated to its functional capabilities but with the length of the telomeric repeat array[7]. The dynamics of the t-loop still requires further elucidation and it is unknown whether these t-loops maintain their structure all throughout the cell cycle or if there is a necessity of resolution during the DNA replication process.

The length of telomeres is of great importance with respect to genomic integrity. When telomeres reach a critical length they are detected by DNA-damage repair machinery. Short telomeres are known to induce senescence as well as deprotection of the telomeres through induction of ATM (repressed by TRF2) and ATR (repressed by POT1)[8]. This response leads to the formation of telomere dysfunction induced foci (TIF) formed by the accumulation of DNA repair proteins such as p53BP1, γH2AX and MCD1 for instance[9-11]. The minimal length at which telomeres maintain their functionality has not been clearly defined but a PCR mediated analysis of short telomeres in human fibroblasts showed that at least 13 TTAGGG repeats are required to prevent telomere fusions[12]. This programmed attrition of telomeres to yield senescence or apoptosis is seen as a tumor-suppressor mechanism serving as a mechanism to limit proliferation of

developing tumor cells. Constant telomere elongation leads to cellular immortalization, one of the steps required for cellular transformation.

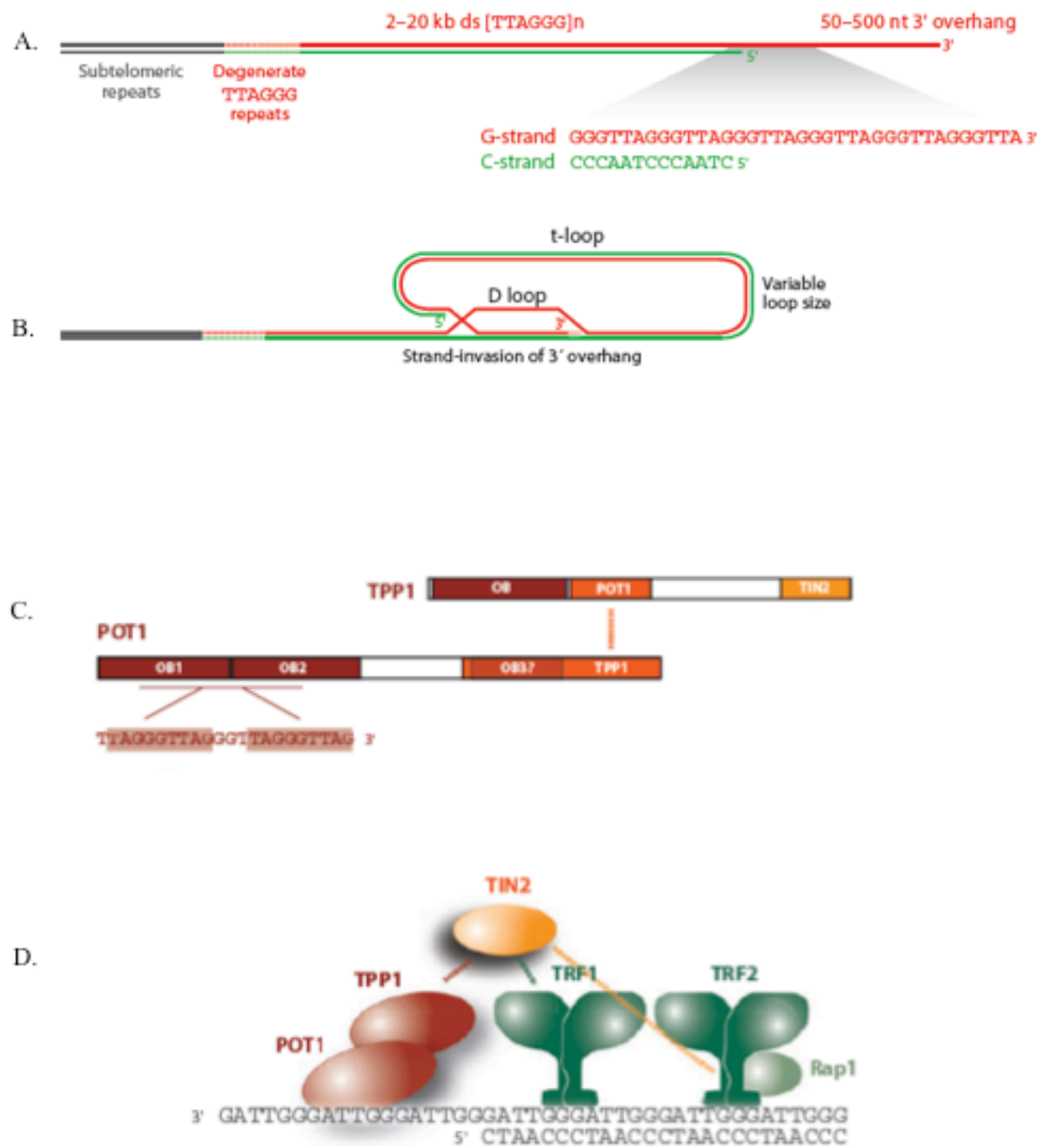


FIGURE 1. THE STRUCTURE OF HUMAN TELOMERES AND SHELTERIN

obtained from[2] A) Human chromosomes indicating the length and variability of the duplex and single-strand overhang. The display of nucleotide repeats underneath indicates that while the 3' is not precisely defined, there is an 80% chance of the C-strand ending with the sequence ATC-5'. B) Schematic of the t-loop C) Depiction of how POT1 and TPP1 interact and the representation of the oligosaccharide binding (OB) domains of POT1   D) A depiction of how shelterin may be positioned on telomeric DNA

Telomerase

The length of telomeres is regulated in eukaryotes. After approximately 50 to 70 doublings, the shortest telomeres are thought to induce an irreversible cell cycle arrest called senescence. The extension of the TTAGGG sequence through the 3' overhang is dictated by the telomerase enzyme and most telomerase positive cells use telomerase to compensate for the loss of sequences at chromosome ends[13]. The enzyme is a ribonucleoprotein that contains a catalytic subunit (TERT) and an essential RNA component (TERC). The RNA component has a region complementary to the telomeric repeat sequence and the specialized reverse transcriptase uses its RNA template to add G-rich telomeric repeats to the telomeric overhang[13-15]. The reverse-transcriptase component (TERT) of telomerase is conserved, expressed during early development and remains active in proliferative cells[16, 17].

Most human primary cells do not express telomerase leading to telomere length shortening with successive cell divisions due to semi conservative replication's inability to fully synthesize the terminus of the lagging strand. Attrition of these ends, in conjunction with a telomerase negative setting will eventually initiate senescence upon reaching a critically short length. In contrast, tumor cells have evaded senescence mostly through the re-expression of telomerase, bestowing upon them the characteristic of infinite replicative capability[18]. Over 80% of tumor cell lines express telomerase and can maintain their telomere length over time[19]. Telomerase therefore plays a pivotal role in the transformation of human cells in conjunction with Ras and SV40 early

region[20]. It is important to note that telomerase is not the only way in which termini of chromosomes are maintained. Another method is alternative lengthening of telomeres (ALT) and it has been seen in human cell lines that have circumvented the end replication problem without telomerase[21].

Shelterin

Telomere homeostasis is maintained through the relationship of the enzyme telomerase and telomere-associated proteins in telomerase positive cells. Since the enzyme telomerase specifically extends the 3' G-rich telomeric strand, the activity of the enzyme constitutes a positive factor in telomere length regulation. Factors are able to inhibit telomerase in cis leading to progressive telomere shortening over 60-80 population doublings. This argues for a set of activities binding the chromosome end and impacting the ability of telomerase to elongate the 3' end. A six telomere-specific protein complex, shelterin, is ubiquitously expressed and abundant at telomeres throughout the cell cycle[1]. Shelterin has currently been shown to determine the structure of the telomere ends, is involved in the formation of t-loops[1]. Shelterin collectively allows cells to distinguish their chromosome ends from DNA breaks and represses the initiation of DNA repair reactions.

Shelterin proteins are characterized by the specificity of their function at the telomeres as well as their abundance at the telomeres. The current shelterin model suggests that levels of this complex are directly correlated to the length of the telomere[22, 23]. The longer the telomere, the more shelterin is present at telomeres and consequently, the stronger the

cis-inhibition exerted on telomerase at that telomere. Conversely, a shorter telomere will have less shelterin present, leading to an increased probability of extension through the enzyme. The six components of shelterin can be found together in a single complex and also in smaller sub-complexes that lack one or two subunits[24]. The six proteins: TRF1, TRF2, POT1, TIN2, TPP1 and RAP1 and each play an integral role in preventing the induction of the DNA damage response pathway and in the regulation of telomere length.

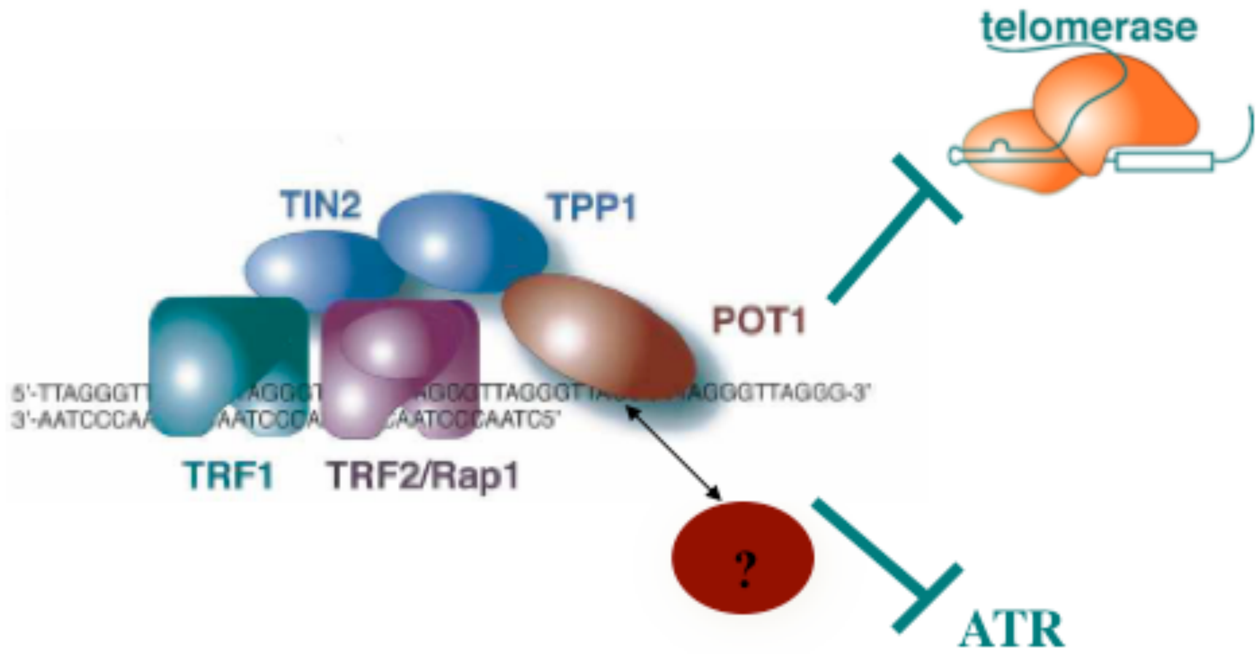


FIGURE 2 CENTRAL HYPOTHESES

adapted from[1] Depiction of shelterin domain structures and interactions and a novel protein that serves the purpose of modulating POT1 and may therefore be correlated to the ability of POT1 to repress the ATR pathway and negatively regulate telomere length

TRF1

TRF1 is the first component of shelterin to be identified and associates with the duplex TTAGGG repeats as a dimer[25, 26] (Figure 1D). It has an amino-terminal acidic region, a dimerization domain, and a carboxy-terminal SANT/Myb DNA binding domain, which confers specificity to the TTAGGG repeats[25, 27]. TRF1 is a dimer in solution and the TRF homology domain mediates this dimerization[25, 28]. TRF1 has been shown to modulate telomeric repeat arrays and this behavior may be correlated to the folding of telomere *in vivo*[7, 29]. TRF1 can be PARsylated, ubiquitylated, sumoylated and degraded by FBx4, an F-box protein[30-32]. In telomerase positive human cells, the overexpression of TRF1 acts as negative regulator of telomere length and its depletion results in telomere elongation but not in deprotection of the telomeres in human cells[2]. The deletion of TRF1 results in an increase in fragile telomeres which are characterized by multiple telomeric signals that spatially separated from the chromatid ends[33]. The deletion of TRF1 also impacts the replication of telomeric DNA through a general reduction in the efficiency of telomere replication[33].

TRF2

The identification of TRF2 is based primarily on its sequence similarity to the SANT/Myb domain of TRF1[34]. In TRF2 however there is a region rich in Glycine and Arginine that constitutes a basic domain in contrast to the acidic domain found in TRF1. TRF2 binds specifically through the Myb/SANT/Telobox domain. TRF2 is a sequence-specific DNA binding protein that binds the duplex at human telomeres as a dimer and

blocks the recognition of telomeres as ds-DNA breaks. The abrogation of TRF2 function results in apoptosis in a subset of mammalian cell types mediated through p53 and the ATM pathway and chromosomal abnormalities[35]. Conversely, the overexpression of TRF2 in primary human cells results in rapid telomere shortening and supports the role of TRF2 as a negative regulator of telomere length[36]. TRF2 has also been shown to promote the formation of t-loops and to stabilize them *in vitro*[29]. TRF2 can be phosphorylated, sumoylated and PARsylated[32, 37, 38]. Both TRF1 and TRF2 are found in large quantities at each telomere and are ubiquitously expressed.

Recently, the Arginine methylation of TRF2 in the GAR domain (the glycine and arginine rich region of TRF2) was implicated in the ability of TRF2 to prevent TIF formation. This is regulated by PRMT1, a major protein arginine methyltransferase that is present in mammalian cells[39].

TIN2

TIN2 was identified through its direct interaction with TRF1 by a yeast two-hybrid screen[40]. TIN2 is often referred to as the linchpin of the shelterin complex because it is capable of connecting three of the telomeric DNA-binding proteins, TRF1, TRF2 and POT1 through its interaction with TPP1 (Figure 1D). The ability of TIN2 to bind to TRF1 and TRF2 on telomeres has been shown to stabilize TRF2 at telomeres, supporting the idea that interactions between DNA-binding proteins increase their affinity for DNA when the binding sites are available[41]. TIN2 recruits TPP1 using an interaction domain at the N terminus and this site is distinct from that of TRF2. Mutations within TIN2 that

disrupt its ability to interact with these components lead to a deprotection phenotype. The interaction of TIN2 with TPP1 thereby mediates an important aspect of telomere protection through the recruitment of the TPP1-POT1 dimer. This close relationship with the TPP1-POT1 complex has been shown to be required for telomere protection. TIN2 deletion in the mouse resulted in an accumulation of the ssDNA binding RPA, lead to the activation of the ATR pathway response, and in other characteristics that are associated with POT1a/b deletions[42]. TIN2 also protects TRF1 from tankyrase and ultimately plays a role in the stabilization of the complex[41, 43].

Rap1

Rap1 forms a complex with TRF2 and is dependent upon TRF2 for localization to telomeres and overall stability[44]. Rap1 is composed of a BRCT domain, a single Myb domain, a coiled region, and a Rap1 carboxy-terminal domain[44]. Although the yeast ortholog of RAP1 is involved in telomere dynamics, its deletion in the mammalian system yields only a weak telomeric phenotype[45, 46]. Purified endogenous TRF2/Rap1 complexes suggest that the two proteins are present in a 1:1 stoichiometry and when TRF2 is absent, mammalian Rap1 does not bind the telomeres and its levels decline[47]. Rap1 is thought to have a negative role in telomere-length regulation and that the high presence of Rap1 binding sites prevents telomere elongation. Experiments have shown that when Rap1 is deleted, the telomere lengthens. Additionally, when telomeres shorten due to degradation or incomplete replication, the loss of Rap1 restores the ability of telomeres to be elongated by telomerase[48]. Rap1 is not essential in the mouse. Its removal leads to a significant increase in telomere recombination leading to sister

telomere exchanges but no fusions. Therefore Rap1 is a repressor of recombination through TRF2. Interestingly, Rap1 was recently found to have non-telomeric functions: it participates in the transcription of NFκB through association with IκB kinases. These are responsible for the phosphorylation and degradation of NFκB inhibitors[49].

TPP1

The carboxy-terminal of TPP1 binds to the amino-terminal half of TIN2 and its central 100-amino-acid region interacts with the carboxy-terminal portion of POT1 (Figure 1C). TPP1 is important for the recruitment of POT1 to the telomeres and its inhibition reflects this role. Knockdown of TPP1 results in telomere elongation, a phenotype analogous to POT1 depletion[50, 51].

POT1

POT1 is recruited to the shelterin complex through TPP1 to the Tin2-TRF1 and Tin2-TRF2 complexes[50, 52, 53]. The study of the mode of action of POT1 at telomeres is essential in the understanding of telomerase regulation in addition to the mechanism by which telomere protection is exerted by shelterin (Figure 2). For this reason the central hypothesis underlying the research described herein focuses on this particular component of the shelterin complex. Specifically, I sought to discover specific and novel protein interactions that are involved in the function of POT1 through its DNA binding domain. Therefore, my hypothesis is that there are specific POT1 partners that modulate the role of the POT1 DNA binding domains and thereby exert an influence on DNA damage response and/or telomere length regulation.

POT1 is a 634-amino-acid protein with two characterized OB folds that bind telomeric ssDNA and a C terminus that serves as a TPP1 binding domain. POT1 is the only protein within shelterin that can bind to the 3' single strand overhang and this is achieved through two OB folds found within the N terminus (Figure 1C). POT1 was identified based on its similarity to the protein that binds single strand telomeric DNA in *Oxytricha nova* and is highly conserved[54, 55]. POT1 binds the nonomer TAGGGTTAG with high affinity and strong sequence specificity [6, 56].

The OB folds of POT1 that bind the overhang are the regions that negatively regulate telomerase. This was demonstrated by expression of a N-terminal truncation of POT1 in telomerase-positive cells. This POT1 allele, termed POT1$^{\Delta OB}$, lacks DNA binding activity but is proficient for binding to TPP1 and is therefore unaffected in its localization to telomeres. The expression of POT1$^{\Delta OB}$ in telomerase-positive cells leads to extensive telomere elongation, implicating the overhang binding activity in mediating the negative regulation of telomerase at telomeres[23]. This regulation could impact the actual recruitment of the enzyme at the 3'end of chromosomes, the processivity of the enzyme once recruited, or both. The shRNA (which results in approximately 90 percent depletion) approach has also shown that the depletion of POT1 results in telomere elongation as well[57].

Knockdown of human POT1 expression also results in colocalization of DNA damage response proteins and increased premature senescence [55, 57]. An additional

consequence of POT1 depletion is a deregulation in the processing of the 5' end. Normally the 5' end of telomeres ends with the sequence ATC-5' and, upon depletion of POT1, the 5' end will terminate at a random position within the repeat[57]. POT1 is therefore pivotal for both telomere regulation and in the prevention of inappropriate DNA damage response at telomeres. This has been genetically confirmed in mouse embryonic fibroblasts (MEFs) via a double knockout[42]. In mice there are two POT1 proteins POT1a and POT1b. POT1a is responsible for the repression of the ATR associated DNA damage response while POT1b controls the resectioning of the 5' end.

The second domain, the TPP1 binding domain has the separate and essential function of recruiting POT1 to shelterin[50, 58]. The interaction through this domain underscores that the recruitment of POT1 to telomeres, and its DNA binding activity are distinct and separable functions. The study of how the DNA binding domain of POT1 at telomeres can be modulated can provide us with information on the regulation of telomerase as well as how the shelterin complex exerts its protective function. *In vitro,* POT1 and TPP1 affect accessibility of telomerase to the DNA as well as the repeat addition processivity of the enzyme[59].

Shelterin and DNA Damage Response

One very important aspect of telomeres and the shelterin complex is that it prevents the activation of DNA damage response to the ends of the chromosomes. The formation of telomeres with a single stranded overhang deeply resembles that of a double stranded break, so the protection of these ends from inappropriate processing is imperative to the fortification of genomic integrity. Mammalian cells have two phosphatidylinositol-3-

kinase related proteins ATM and ATR that stimulate pathways as a response to different kinds of DNA damage. The ATM pathway responds to double stranded breaks (DSBs) while the ATR pathway responds to lesions at single stranded DNA. Both kinases phosphorylate histone γH2AX resulting in the accumulation of additional DNA damage response factors at DSBs. This colocalization of these factors has been implicated in signal amplification and DNA repair. Both ATM and ATR can phosphorylate nucleoplasmic effector kinases Chk1 and Chk2 which can then impede cell cycle progression[2] .

The abrogation of certain shelterin proteins illustrates how important this complex is at preventing the activation of DNA damage response. The deletion or inhibition of TRF2 results in a large DNA damage response by the ATM pathway. This was evidenced by the colocalization of the DNA damage response factors with the telomeres referred to as telomere induced foci (TIFs). This DNA damage response when TRF2 is absent does not present itself when ATM is absent which supports ATM as the primary initiator of these pathway[8].

The ATR pathway is repressed by another shelterin protein POT1. The deletion of POT1 leads to TIFs. The presence of TIFs can be limited by the repression of the ATR pathway to support the relationship between POT1 and ATR. This property of POT1 in the repression of the ATR pathway is reliant on being associated with TPP1[8, 57]. TPP1 is responsible for recruiting POT1 to the complex and for stabilizing POT1's ability to bind the overhang. Therefore TPP1 is also relevant to the repression of ATR and its repression

also results in a DNA damage response akin to that of POT1[8, 60, 61].

Shelterin Accessory Factors

Shelterin represents a complex that assembles at telomeres and presents a number of interaction surfaces for other factors. Additionally, the variety of the potential outcomes due to modifications of shelterin proteins suggests that there are several factors that play a role in telomere protection be it permanent or transient in nature. Although shelterin is exclusive to telomeres, there are also a significant number of proteins that do not adhere to shelterin criteria but still have an impact on telomere function and regulation. These accessory factors make contributions to the regulation and maintenance of telomeres but are normally less abundant than shelterin and transiently associated[2].

Tankyrase is a prime example of a shelterin accessory factor. Tankyrase is a human telomeric poly (ADP-ribose) polymerase that can interact with the amino terminus of TRF1[31, 62]. Tankyrase can be found at telomeres through immunofluorescence and through ChIP with levels lower than that of actual shelterin proteins[23, 31]. Tankyrase1 is thought to be a positive regulator of extension of telomeres by telomerase. This interaction results in reduced affinity of TRF1 for telomeric DNA and subsequently plays a role in the positive regulation of telomere length[31]. Tankyrase has multiple targets and has recently been linked to Cherubism disease[63].

The recruitment or ability of additional proteins to interact with shelterin suggests that the six-protein complex may possibly receive modulation from essential proteins. Some

already characterized shelterin accessory factors play a role in telomere length regulation or in the repression of DNA repair. The transient association of these proteins with telomeres may also have roles in other contexts. Discovery of novel shelterin accessory factors and determination of how they modulate the shelterin complex may shed some light on when and how telomere length and protection are regulated.

The central hypothesis focuses on the shelterin protein POT1 and its ability to recruit additional factors to telomeres. These factors would serve to mediate the regulation of telomere length, telomere protection, and the onset of senescence. Due to POT1 binding the telomeric overhang, this shelterin protein ultimately mediates telomere length control that is exerted by telomerase. However, the method by which this is modulated is incompletely understood. It is possible that there are additional shelterin accessory factor like proteins that mediate functions of POT1 through their association with the DNA binding OB folds of said protein. This interaction may mediate POT1 binding the overhang and ultimately be a mediator of telomere function. My findings suggest that a previously uncharacterized interaction exists between members of the Zyxin LIM-domain family and shelterin, and that this relationship may confer stability by repressing the DNA damage response pathway. Furthermore, such a factor might mediate or repress the ATR response elicited by single stranded breaks particularly at the telomere overhang. I sought to determine factors that specifically interacted with the POT1 OB folds by 2-hybrid screen and from there characterize and ascribe a function to these factors.

LIM Domain Proteins

LIM domains are modular sequence elements that have been found in upwards of 60 gene products in the human proteome[3]. The LIM motif was initially identified in three transcription factors, *Caenorhabditis* elegans Lin-11, rat Isl-1, and *C.* Elegans mec-3[64-66]. LIM domains are made up of approximately 55 amino acids and are rich in cysteine, histidine, and aspartic acid residues. The structure of LIM domains has been verified through multidimensional NMR spectroscopy as well as X-ray crystallography. These assays have revealed that the LIM domain consists of two zinc fingers; each consisting of two orthogonally packed antiparallel beta-hairpins. Zinc fingers within a LIM domain contain two treble-clef fingers that are separated by an invariable linker region comprised of two amino acids. The linker region is thought to be essential for LIM-domain function as substitution within this dipeptide results in a significant reduction in binding activity.

LIM proteins are broadly categorized into four groups that reflect the similarity in organization and structure. There are nuclear only LIM proteins, LIM only, LIM actin associated and LIM catalytic groupings of these proteins that share a high level of similarity in the organization of these LIM domains. Group1 proteins contain LIM domains linked to a homeodomain and a potential transcription activation domain. Examples within this category include the three founder LIM proteins and have been categorized as involved in cell fat determination and differentiation. Group 2 proteins LMO proteins have one to five LIM domains without additional motifs and have the ability to be both nuclear, cytosolic or both. Group 3 proteins contain three to four tandem LIM domains at the C terminus in association with distinct N-terminal domains[3]. These members also contain a proline rich pre-LIM region. Proline-rich

regions of proteins can serve as molecular spacers or as surfaces for interaction with proteins that exhibit SH3 domains[67]. The LIM domain proteins of interest to my thesis are found within the group 3 LIM actin associated broad grouping. This grouping is characterized by the LIM protein Zyxin and includes Ajuba, Limd1, WTIP, Zyxin, LPP and TRIP6. LIM proteins that do not conform to the first three groups constitute their own group[3].

Human LIM proteins have been molecularly characterized as protein interacting domains as well as having scaffolding activities (primarily with tandem LIM domains)[68]. They can be comprised of solely LIM domains or they can be linked to other domains such as homeodomains, catalytic domains, cytoskeletal-binding domains or other protein-binding motifs. Information suggests that a LIM domain functions as a protein-binding interface to mediate protein-protein interactions[69]. A single LIM domain is thought to be sufficient to provide the specificity of interacting with a respective protein partner and has also been shown to bind several proteins simultaneously[3]. Based upon this behavior of LIM domains, it is believed that they are able to localize and modulate the activities of their binding partners. The four main categories that LIM domains can function is are as adaptors, competitors, auto inhibitors or localizers[3].

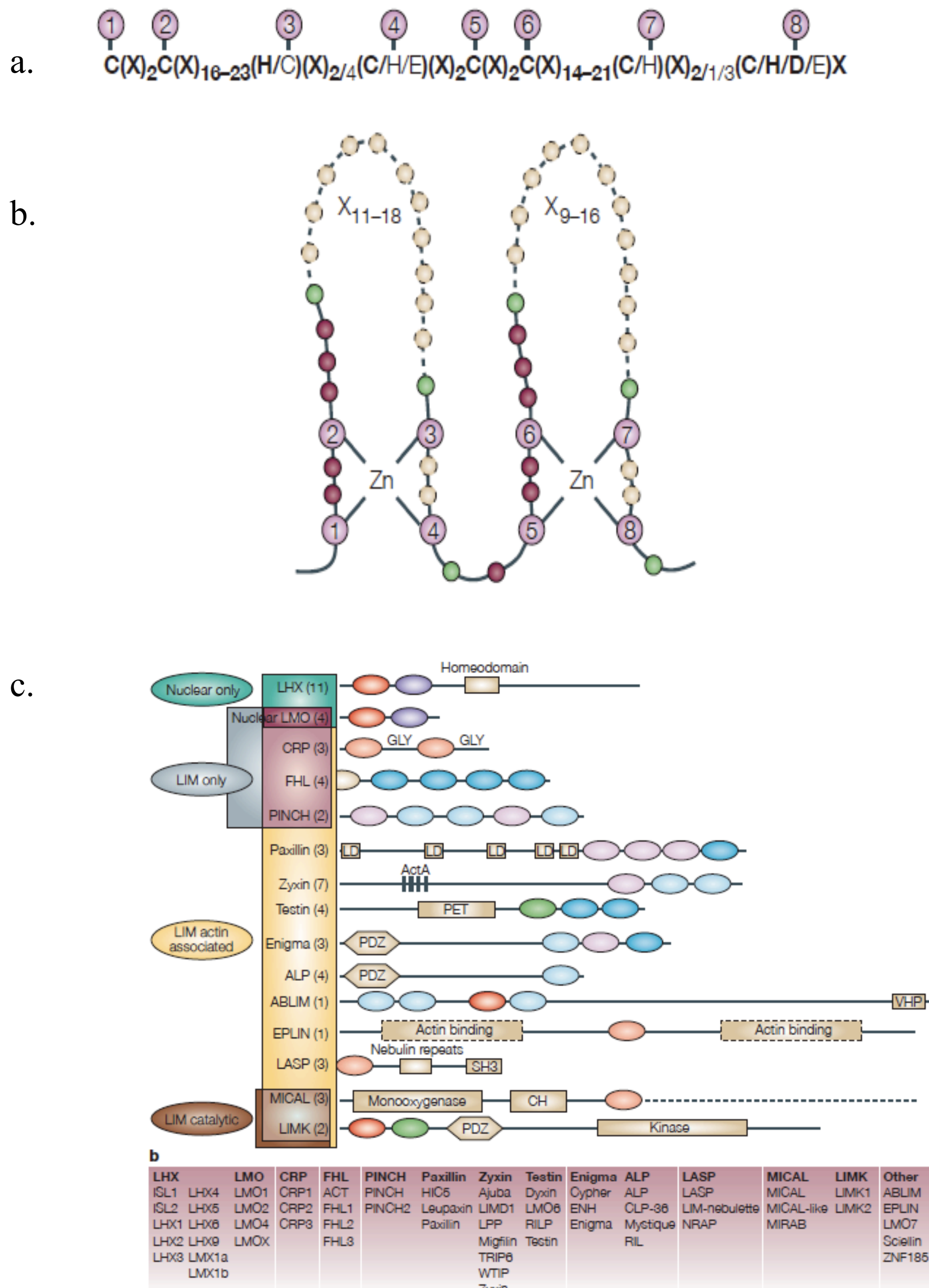


FIGURE 3 LIM DOMAINS SEQUENCE, TOPOLOGY AND FAMILY ORGANIZATION

as obtained from[3] a) The spacing and conserved sequence pattern of LIM domains b) Topology of zinc coordination with purple numbered circles indicating zinc-binding residues. Green circles represent aliphatic/bulky residues. Magenta spheres are non-conserved residues and dashed yellow circles indicate possible variable residues.

TRIP6

TRIP6, also known as Zyxin-related protein-1, was first identified as a binding partner for the thyroid hormone receptor via yeast two-hybrid screen. TRIP6 contains three tandem LIM domains in its C terminus and contains a proline-rich N-terminal region. Its molecular properties ascribe it to the same family as LIM domains Zyxin, LPP and Ajuba and it is most closely related to proteins Zyxin and LPP, a property that will be addressed regarding the associative property of this family with telomeres[3]. The TRIP6 human gene was assigned to a segment of chromosome 7q22 which is a region that has been shown to be deleted in malignant myeloid diseases and uterine leiomyoma[70].

The C-terminal LIM domains of human TRIP6 is present in all three cDNAs isolated via two-hybrid screening including the interaction between TRIP6 and POT1 and may therefore be important for mediating the array of interactions previously discovered. The consensus sequence of the LIM domain is defined as $CX_2CX_{16-23}HX_2CX_2CX_2CX_{16-21}CX2(C/H/D)$ where X represents any amino acid[71]. The number and spacing of the cysteine and histidine residues are highly conserved and known to represent segments that are metal binding domains. Each LIM domain binds two ions of zinc through a tetrahedral metal-coordinating center and the residues that bind the zinc are the most highly conserved[3]. A single LIM domain has two-tandem zinc finger like topology with a variable linker region to connect the two. Although these motifs are similar to GATA-type zinc fingers which in contrast are shown to bind DNA specifically, LIM domains are implicated in protein-protein interactions and not in DNA-protein interactions characteristic of zinc fingers[3, 69] .The structural function of LIM domains dictates their recognition as key components of the cells regulatory machinery. Protein-protein

interactions that occur through these domains have been shown to modulate activities of proteins or participate in the assembly of multi-component complexes.

TRIP6 contains a leucine rich nuclear export sequence (NES) in the pre LIM region that allows it to shuttle dynamically out of the nucleus and into the cytoplasm[72]. This movement is dependent on CRM1 and the treatment of TRIP6 with cytotoxin Leptomycin B can abrogate the proteins ability to leave the nucleus. TRIP6 possesses a YXXP motif from amino acid 55-58 that when phosphorylated at the Tyrosine residue by c-Src, can serve as a docking site for the SH2 domain of the adaptor protein Crk[73]. In addition to the LIM domain region in the carboxyl-terminal half of TRIP6, the last four residues of TRIP6 is a PDZ-binding motif that allows for an interaction between PDZ proteins. PDZ proteins function as scaffolds to facilitate multiprotein complexes or in directing a protein to anchor to the actin cytoskeleton[74].

There are many roles that TRIP6 has been shown to have with the majority of documented roles being within the cytoplasm. TRIP6 is enriched at focal adhesion and cell-cell contacts, and can regulate cellular adhesion and migration[75]. The knockdown of TRIP6 in human endothelial cells results in the disruption of cytoskeletal actin filaments. The ability of TRIP6 to be distributed along actin fibers is dependent on endoglin, a component of the TGF-β.

For instance TRIP6 has been demonstrated to play a role in actin cytoskeletal reorganization by associating with components of focal complexes such as paxillin, focal adhesion kinase and p130^{CAS}[76]. TRIP6 has also been shown to facilitate the activation of NF-κB through RIP2 and activates ERK through RIP2 and Nod1[74]. RIP2 (receptor-

interacting protein 2) is a member of the RIP kinase family and is involved in inflammation and immune response initiated by Nod1 (in addition to Toll-like receptors, TNF and IL-1)[77]. TRIP6 was shown to have an isoform that has a role in the nucleus, acting as a molecular platform so that target promoters can activate or repress signals[78]. These activities potentiate a role in the assembly of macromolecular complexes but how this interaction is achieved, whether directly or indirectly have yet to be determined.

LPP

LPP (LIM-containing lipoma-preferred partner) is an 80kDa LIM domain protein that is closely related to TRIP6 and is localized at sites of cell adhesion and transiently in the nucleus. It was originally discovered as a component of chromosome translocations found in benign tumors of human adipose tissue[79]. LPP also encodes a proline-rich pre-LIM region and three LIM domains at the carboxy-terminal end. All three LIM domains of LPP work together to target to focal adhesions and are required to target proteins to points of cell-cell and cell-matrix contacts[79, 80].

LPP is the most closely related LIM domain protein to TRIP6 and has been implicated in the development of human lipomas[70]. It has an extensive proline-rich N terminus and three C-terminal LIM domains with a highly similar sequence as that of TRIP6. LPP focalizes to focal adhesions and can be transiently translocated to the nucleus[81, 82]. At cell adhesions, LPP interacts with vasodilator-stimulated phosphoprotein through the proline-rich N terminus that contains VASP-binding motifs. LPP can also interact with

actinin through its actinin-binding site. Within the nucleus LPP has transcriptional activation capacity in the proline-rich region and also in the LIM domains. LPP is recruited to PEA3-dependent promoter regions and can enhance the transactivational potential of PEA3. PEA3 is a member of a family of transcriptional regulatory proteins and is regulated through receptor tyrosine kinases[83]. These sites have been identified as regulatory regions of genes associated with tumorigenesis and embryogenesis[80, 84]. LPP, akin to TRIP6, appears to employ a variety of functions that rely on the intracellular localization of the protein[67].

Zyxin

Zyxin is a low abundance phosphoprotein that is found to localize to focal adhesions and along actin filaments. It has previously been shown to play a role in cytoskeletal organization and nuclear-cytoplasmic communication through the interaction of intracellular signaling components. Zyxin contains a proline-rich N terminus and three cysteine and histidine rich LIM domain motif. One of the LIM domains in Zyxin has been shown to interact with proteins associated with myogenic potentiation[85]. The presence of LIM domains that are capable of mediating protein-protein interactions suggests that Zyxin may function through its ability to assembly multimeric complexes.

Biochemical studies of Zyxin indicate roles in cytoskeletal organization and nuclear-cytoplasmic communication through an interaction with multiple intracellular signaling components[86]. Zyxin is a low abundance phosphoprotein that is concentrated at adhesion plaques and along the actin filament bundles[87]. Subcellular localization of

Zyxin in human cells was demonstrated to show preference for patches at cell borders with a small level of nuclear staining. There is some level of nuclear staining that can be occasionally observed via indirect Immunofluorescence but these levels are variable[67]. The LIM motif of Zyxin has been shown to mediate protein-protein interactions and one of the LIM domains is implicated in myogenic potentiation[67].

Ajuba

Ajuba is a 60kD protein containing three LIM domains at the C-terminal portion. Ajuba plays a role in the formation and maintenance of cell-cell junctions[88, 89] and in cell migration[90, 91]. Ajuba is the most distantly related LIM domain protein studied with respect to the LIM proteins previously mentioned (TRIP6, LPP and Zyxin). Ajuba has been shown to participate in the assembly of many protein complexes. Ajuba can bind Grb2 and modulates serum-stimulated ERK activation[92] Ajuba has also been shown to contribute to adherin junctions by linking adhesive receptors to the actin cytoskeleton. In addition to cytosolic roles, Ajuba has also been ascribed a nuclear function. Ajuba was shown to function as a corepressor for the zinc-finger protein Snail and additionally in the negative regulation of retinoic acid[93].

The Dynamics and Relationship between LIM domain proteins

The similarity of proteins within each family subset suggests that a relationship may exist between highly homologous LIM domain proteins and the role that they play may have cooperative or antagonistic qualities. For example the Zyxin family member protein Ajuba has been shown to function as a modulator of retinoic acid signaling and its

depletion results in cell sensitivity to RA-stimulated transcription and differentiation[93]. Additionally, Ajuba can interact with RARα as well as other RAREs. This interaction can also be seen with Limd1 and WTIP in a manner reminiscent of Ajuba suggesting that these other LIM domain proteins may be potential corepressors for nuclear receptors. LIM domain proteins found within the same family such as TRIP6 and LPP prove negative in the ability to associate with RARα, which lends credence to a reorganization of where the similarity in these proteins lends itself to similar behavior within the cell.

My thesis represents analysis of the roles of TRIP6 and LPP in telomere function. I first show that TRIP6 can interact with POT1 by yeast two-hybrid screen (Chapter 3). After identification of a protein that interacts with the OB fold of POT1, I showed that TRIP6 can associate with telomeres (Chapter 4) and that this association is important for telomere function (Chapter 5). This was also proven to occur in LPP but not in Zyxin (Chapters 5 and 6). The analysis of LIM proteins at telomeres has revealed that these family members act in pairs both in the cytoplasm and nucleus. This could reflect redundancy or a partnership in their cellular roles.

Chapter 2 Materials and Methods

Two-hybrid screen/Isolation of TRIP6

A two-hybrid screen was carried out with the yeast reporter strain L40[94] using the human POT1ΔC C-terminal truncation (aa 1-379) fused to the LexA DNA binding domain as bait. The L-40 strain contains the HIS3 and LacZ reporter genes under the control of the LexA DNA binding site. The libraries used were the Hela S3 or human fetal liver hFL matchmaker cDNA library (Clonetech), containing random fusions to the GAL4 activation domain. The TRIP6 two-hybrid cDNA clones with three tandem LIM domains of the molecule were isolated and retransformed into L40 with the bait to confirm that the His+ and LacZ+ phenotypes were due to the library plasmid.

Two-hybrid assays

Two hybrid tests were performed in the L40 strain with the LexA-TRIP6 full length cloned in pBTM116 by PCR of the TRIP6 EST and tested against previously characterized and published fusions with the GAL4 activation domain: TRF1-GAD, TRF2-GAD, and POT1-GAD, all cloned into the pACT2 vector (Clonetech). The βGal liquid assays were performed as described in the Clonetech Mathmaker protocol and three independent colonies were essayed for each plasmid combination. The standard deviations reported are based on three separate experiments.

E. Coli GST-POT1

The cloning of POT1 and POT1$^{\Delta OB}$ have been described in [23]. The full-length POT1 cDNA was cloned as BamHI-XhoI fragments in FastBac HTb (Clonetech), adding a His6 tag to the N terminus and the transfections, virus amplifications and protein production were performed as described in the manufacturer's protocols. For protein purification from *E. Coli*, the POT1 and POT1$^{\Delta OB}$ cDNAs were cloned in the BAMHI and XhoI sites of pGEX-4T2, resulting in N-terminal GST fusions. GST fusion proteins were purified on glutathione beads as directed by the manufacturer. Post purification, protein was dialyzed against 20 mM Hepes, pH 7.9, 500 mM KCl (150 mM for GST fusions), and 20% glycerol. The binding affinity of POT1 declined 3-5 fold over a time frame of 2 weeks and stabilized afterward.

Band Shift Assays

The binding reactions were performed in 20ul of 20 mM glycine-NaOH, pH 9.0, 0.1 mM dithithreitol, 2% glycerol, 50ng of β-casein, 0.5ug of sonicated and denatured *E. coli* DNA and 0.25 nM probe. The protein was added last, and the binding reaction was incubated for 30 minutes at room temperature. For direct binding, a range of 0.1-5ug of protein was used. For competition assays, 05.-1ug of protein was added to the reactions containing up to 100 fold molar excess of unlabeled competitor oligonucleotide. Electrophoresis was performed in 0.6% agarose gels run in 0.1xTris borate EDTA. Gels were run for 40 minutes at 160 V, dried on Whatman DE81 paper at 80C and exposed on a phosphorimaging screen. Quantitation was performed using ImageQuant software.

Cell lines and antibodies

The HTC75 is a human fibrosarcoma cell line and a HT1080 derivative in which the pTet off control element has been stably transfected[95]. HTC75 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (BCS), 1% penicillin and 1% streptomycin. The retroviral transduction protocol is as follows. Phoenix packaging cells are plated and evenly distributed on Day 0. The next day cells are transfected with 20 μg DNA. Cell density is approximately 40-50% to ensure 90% confluency by Day 4. Media is changed 5-12 hours after transfection. The first supernatant is collected on Day 3 48 hours after transfection and 24 hours after changing medium. The virus is filtered and immediately used for infection. Total collections of 3 supernatants are done. For the infection cells are plated in order to be approximately 40% confluent on the day of infection (Day 3). The filtered virus receives 4μg/ml of Polybrene and 10% fetal bovine serum. After three rounds of infection, media is changed and a 24-48 hour waiting period is observed prior to the selection process.

The antibodies against TRIP6 and LPP were generated against a peptide conjugated to KLH and used for immunization into rabbits, as per the manufacturer's protocol (BioSynthesis, Lewisville, TX). The peptides were: NH2-GCPKKFAPVVAPKPKYNPYKQ-OH for LPP, and NH2-GCLNGGRGHASRRPDRQAYE-OH for TRIP6. TRF2 antibodies were 647 against the full-length protein made in Sf9 cells, or the anti-peptide 508. The TRF1 antibody was the anti-peptide 371; the POT1 antibodies were 1048 against the full-length protein made in Sf9 cells or the anti-peptide 978; and the Tin2 antibodies were the 864 made against the full-length protein in Sf9 cells. The p53Bp1 antibody was purchased from Novus

(NB100-304). Zyxin and PRMT1 antibodies were purchased from Abcam (71842 and 3768).

Plasmids

The TRIP6 and LPP cDNAs were purchased as full-length clones from the EST collection maintained by the ATCC or by Invitrogen respectively. The full-length cDNAs were amplified by PCR using primers with appropriate cloning sites (5' BglII and 3' XhoI) and cloned into pLPC-MYC to generate a MYC tagged version driven by the CMV promoter. The PCR oligonucleotides were obtained from Integrated DNA Technologies. :5' BglII AGATCTTCGGGGCCCACC TGGCTGCCCCCG and 5' XhoI CTCGAGTCAGCAGTCAGTGGTGACGGTGGC for TRIP6. LPP oligos are as follows: LPP 5' BglII 5' ATT AGA GAT CTC ACC CAT CTT GGC LPP 3' XhoI 5' TTA GAG CTC GAG CTA AAG GTC AGT.

The POT1$^{\Delta OB}$ construct is described [23] and the POT1$^{\Delta C}$ construct was cloned by PCR-cloning of amino acids 1-379 of POT1 into pLPC-MYC for expression into human cells, or pBTM116 to use as a bait for the two-hybrid screen. The POT1 and POT1$^{\Delta C}$ fusion with eGFP were performed using a vector constructed with the eGFP fragment with the pEGFPC1 vector (Clontech) subcloned into pBabe-Puro. A NLS was cloned as a BamHI fragment into the BglII site of the polylinker, and the full-length POT1 or POT1$^{\Delta C}$ fragments were cloned as BamHI-XhoI fragments downstream of the NLS, generating N-terminally GFP-tagged protein fusions actively transported into the nucleus.

RNA Interference

HTC75 cells were maintained in DMEM in the same way as aforementioned in the cell lines section. Dharmacon RNA Technologies synthesized TRIP6 and LPP specific siRNAs. For TRIP6 RNAi double-stranded siRNA were designed to target the following sequences: TRIP6 (6.1) siRNA 5'-AGGAGGAGACUGUGAGAAUUU-3' TRIP6 (6.2) siRNA 5'-CUGGAUAGGCUGACGAAGAUU-3' LPP siRNA (L.1) 5'CUCAUAAUGUGAAAUAUGA¬3' LPP siRNA (L.2)[96] 5'GCCAUUCUAUGCUGUGGAA-3' HTC75 cells were transfected using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Cells at a confluency of approximately 50-60% were plated into a 6-well plate 18-24 hours prior to transfection. Transfections were done once within a 24-hour interval and cells were processed 48 hours after the initial transfection. As a control, siRNA designed to target GFP (Dharmacon) was used.

Immunofluorescence

Immunostaining for TRF2 and 53BP1 proteins were performed on HTC75-Vector, MYC-TRIP6 or MYC-LPP cells plated onto glass coverslips. Cells were fixed with 2% formaldehyde in PBS (v/v) for 10 minutes at RT. Cells were permeabilized with 0.5% NP40 in PBS for 10 minutes at RT, washed two times in 1X PBS, and blocked with the PBG for 30 minutes. Coverslips were then incubated with the mouse anti-TRF2 antibody clone 4A794 (Millipore/Upstate Biotech) and a rabbit anti-p53BP1 antibody (Novus NB100-304A-1), both at a concentration of 1:1000 in PBG overnight.

Coverslips were then rinsed three times with PBG solution and incubated with secondary

TRITC-conjugated goat anti-rabbit antibody or FITC-conjugated donkey anti-mouse antibody (Jackson Immunoresearch) in PBG at a concentration of 1:1000 for 45 minutes at RT. Cover slips were rinsed two times with PBG. Coverslips were then incubated with PBG and 4,6-diamidino-2-phenylindone (DAPI) at 100 ng/ml to visualize the nuclei. Coverslips were mounted onto slides with embedding media. Images were collected with an Olympus BX61 fluorescence microscope using a 60X objective connected to a Hamamatsu ORCA-ER CCD camera, controlled by the SlideBook 5.1 image capture software.

Chromatin Immunoprecipitation (ChIP)

For ChIPs, cells were digested with trypsin, rinsed with PBS, and fixed with 37% formaldehyde in PBS for 60 minutes at room temperature, washed with PBS and lysed in 1% SDS, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA at a density of approximately 10^7 cells/ mL. Lysates were sonicated in order to obtain chromatin fragments < 1kb, and centrifuged for 10 minutes in a microfuge at 4°C. Two hundred microliters of lysates, diluted with 1.2 ml 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, and 150 mM NaCl was supplemented with antibody (typically 30 μl of crude serum or 5 μl of commercial antibody 9E10 and PRMT1) and rotated overnight at 4°C. Immunoprecipitated pellets were washed with 0.1% SDS, 1% Triton X-100, 2mM EDTA, pH 8.0, 20 mM Tris-HCl, pH 8.0, containing 150 mM NaCl in the first wash and 500 mM NaCl in the second wash. Additional washes were with 0.25M LiCl, 1% NP-40, 1% Na-deoxycholate, 1 mM EDTA, pH 8.0, 10 mM Tris-HCl, pH 8.0, and with 10mM

Tris-HCl, pH 8.0, 1mM EDTA. Chromatin was eluted from the beads with 500ul 1% SDS, 0.1M Na_2CO_3. After addition of 20 μL 5M NaCl, crosslinks were reversed for 4 hours at 65°C. Samples were supplemented with 20ul of 1M Tris-HCl, pH 6.3, 10ul 0.5M EDTA, and 20ug DNAse free RNase A, and incubated at 37°C for 30 minutes. Samples were digested with 40ug proteinase K for 60 minutes at 37°C, phenol extracted, and precipitated overnight at -20°C with 1ml ethanol. The precipitate was dissolved in 100ul of water, denatured in a sandbath at 95°C for 5 minutes, and dot blotted onto Hybond membranes in 2x SSC. 80% was loaded for the detection of telomeric sequences and 10% was loaded for nonspecific Alu sequences. Membranes were treated with 1.5M NaCl, 0.5N NaOH for 10 minutes, and 1M NaCl, 0.5M Tris-HCl, pH 7.0, for 10 minutes. Hybridization with a TTAGGG probe or an Alu prove was performed as described in [97]; membranes were washed three times in 2X SSC and exposed at varying times from 4 hours to overnight on a PhosphorImager screen. The quantification was performed using ImageQuant software. All lysates were normalized for cell number. For the total telomeric DNA samples, two 50ul aliquots (representing ¼ of the lysates used during immunoprecipitation) were processed along with the rest of the samples at the step of crosslink reversal. The average of the telomeric signal in the two totals were taken for the reference value of total DNA. The percentage of each Immunoprecipitation sample was calculated based on the signal relative to the corresponding total DNA signal. This results in the calculations accounting for changes in telomere length.

Co-Immunoprecipitation from 293T cells

Human 293T cells were plated and transfected 24-32 hours later using calcium chloride

coprecipitation method and 20-40 μg of plasmid DNA per dish. Medium was changed after 12 hours and cells were harvested 24-30-post transfection. Cells were removed from dish using cold PBS, collected by centrifugation and lysed in cold buffer composed of 50mM Tris-HCl pH 7.4, 20% glycerol, 1mM EDTA, 150mM NaCl, 05% Triton X-100, 0.02% SDS, 1mM DTT, 2mM PMSF, 1ug/ml aprotinin, 10 μg/ml pepstatin, and 1μg/ml leupeptin. After, 5M NaCl was added to bring NaCl concentration to 400mM. After 20 minutes on ice water at a volume equal to that of the lysis buffer was added and thoroughly mixed before 10-minute centrifugation at top speed. Supernatants were collected and used directly for IP. Lysates prepared from 4-5 dishes were mixed with antibodies at 30ul for crude serum and 5ul for commercial antibodies and gently rotated for 4 hours. During the final half hour, 30 μL of protein G-Sepharose beads were added to each tube. G-Sepharose beads were preblocked o/n with 10% BSA in PBS. Beads were washed three times with PBS and eluted with Laemmli loading buffer at equal volumes to the original lysates and analyzed by SDS-PAGE and immunoblotting.

Chapter 3 Characterization of TRIP6, a POT1 Interacting Factor in Human Cells

Introduction

The processes that result in the regulation of telomere length have a significant impact on genomic integrity and in two major problems within human cancer biology. One problem is that the efficient maintenance of telomeres is necessary, in conjunction with other genetic elements, for full oncogenic transformation of human cells[20]. Secondly, the gradual shortening of telomeres, which happens during the replication process, is correlated to a finite replicative life span for cells in culture. The termination of this lifespan results in cellular senescence, which is significant for tumor suppression *in vivo*[98]. The enzyme telomerase catalyzes the addition of TTAGGG repeats and is negatively regulated by shelterin. Collectively, the negative regulation of shelterin results in a stable average telomere length over time in cells that are telomerase positive. A complete understanding of the method behind the regulation of telomere length has not been currently elucidated.

Of the shelterin proteins at telomeres, POT1 is the only overhang-binding component of shelterin and it has been shown to mediate telomere length regulation and protect telomeres from inappropriate processing[1]. The location at the 3’ overhang puts POT1 in a critical position with respect to the accessibility of the telomeres for extension and the molecular effectors of POT1 function are incompletely understood.

The DNA binding activity of POT1 has been shown via mouse conditional knock out to repress ATR kinase, a component of the DNA damage response pathway[8, 42]. The removal of POT1 in mouse cells leads to an increase of accumulation of DNA damage proteins at telomeres. Examples of these proteins include p53BP1, γH2AX[9] and MDC1[11]. These proteins are indicative of a loss of protective properties at telomeres and this ultimately yields end-to-end telomere fusions and the formation of telomere dysfunction induced foci. In addition to protection, POT1 plays an important role in the cis-inhibition of telomerase. In telomerase positive cells, depletion of POT1 by siRNA results in the elongation of telomere length[57].

In contrast to the negative regulatory role of POT1, *in vitro* systems have indicated a positive role for POT1 and the POT1-TPP1 heterodimer in telomerase activity on a model telomere seed[99]. POT1 by itself can lead to increased recruitment of telomerase and this is dictated by distance between the binding site and the 3' end of the DNA. In conjunction with TPP1, the dimer is shown to increase repeat addition processivity of the enzyme through a domain in TPP1.

The primary interest of my research is in the possible factors associated with the protein POT1. The central hypothesis of this proposal is that proteins mediate the function of POT1 through association with the DNA binding OB folds and that this DNA binding of POT1 to the 3' single strand imparts its significance through its regulation of telomerase in *cis* (Figure 2). It is possible that undefined proteins interact with the POT1 DNA binding domain and thereby mediate the function of POT1. The assay employed to find

additional protein-protein interactions is the yeast two-hybrid screen. I describe below the POT1 bait used for this screen as well as the isolation of a POT1 interacting protein and its preliminary characterization. Results from this screen may shed light concerning proteins found at telomeres that do not fit shelterin criteria, and increase our understanding of POT1 regulation and, as such, of chromosome integrity.

Results

POT1 Constructs

POT1 is made up two separable moieties. The first domain is made up of the OB folds, required for single stranded DNA binding. Current sequence analysis suggests a third OB fold that to date has not been ascribed with a specific function[2]. OB folds 1 and 2 are required for binding the telomeric overhang with a high degree of affinity and specificity[56]. The second domain is the TPP1 binding domain, which allows POT1 to be recruited by TPP1 and ultimately incorporated into the shelterin complex.

Several POT1 deletion constructs were cloned into the pLPC-MYC retroviral vector and transduced into HT1080 cells. A C-terminal truncation was constructed lacking the TPP1-interacting domain (herein referred to as POT1$^{\Delta C)}$. The POT1 allele we constructed, termed POT1$^{\Delta C}$, contains the first 379 amino acids of the protein, with a full DNA-binding domain as described in [18] with an additional 79 amino acids, but not the TPP1-binding domain, located in the C-terminal region[55]. Analysis of this constructs provides information concerning whether the intact OB folds in this construct were sufficient for localization at telomeres.

The expression of POT1$^{\Delta C}$ was confirmed by Western blotting in HTC75 and HeLa cells. It was found to be at a lower molecular weight than full-length POT1 but detectable at a predicted weight of 43kD (Figure 4A). A gel-shift assay (EMSA) comparing GST fusions of hPOT1 and hPOT1$^{\Delta C}$ revealed that hPOT$^{\Delta C}$ could bind DNA *in vitro* as well as the

full-length protein (Figure 4B). In order to allow for the intracellular localization of this construct an NLS-GFP fusion containing hPOT$^{\Delta C}$ in fusion with a pBabe vector was used. In comparison to the full length POT1, the telomeric association of GFP-POT1 was readily detectable using epifluorescence and co-staining with TRF1 (Figure 4C). In contrast, GFP-POT1$^{\Delta C}$ did not exhibit telomeric association. This construct could not efficiently target to telomeres and exhibited a diffuse overall nuclear staining pattern. This inability to colocalize to the shelterin protein TRF1 in conjunction with the EMSA data concludes that the DNA binding to the telomeres overhang is not the primary targeting activity in POT1. POT1 is recruited to telomeres through its interaction with TPP1. Additionally, DNA binding by itself is insufficient for the accumulation of POT1 at telomeres. This information provides us with a viable bait candidate for our two-hybrid screen. This bait has the ability to identify proteins that associate with the DNA binding domain of POT1 and will not result in the re-isolation of TPP1.

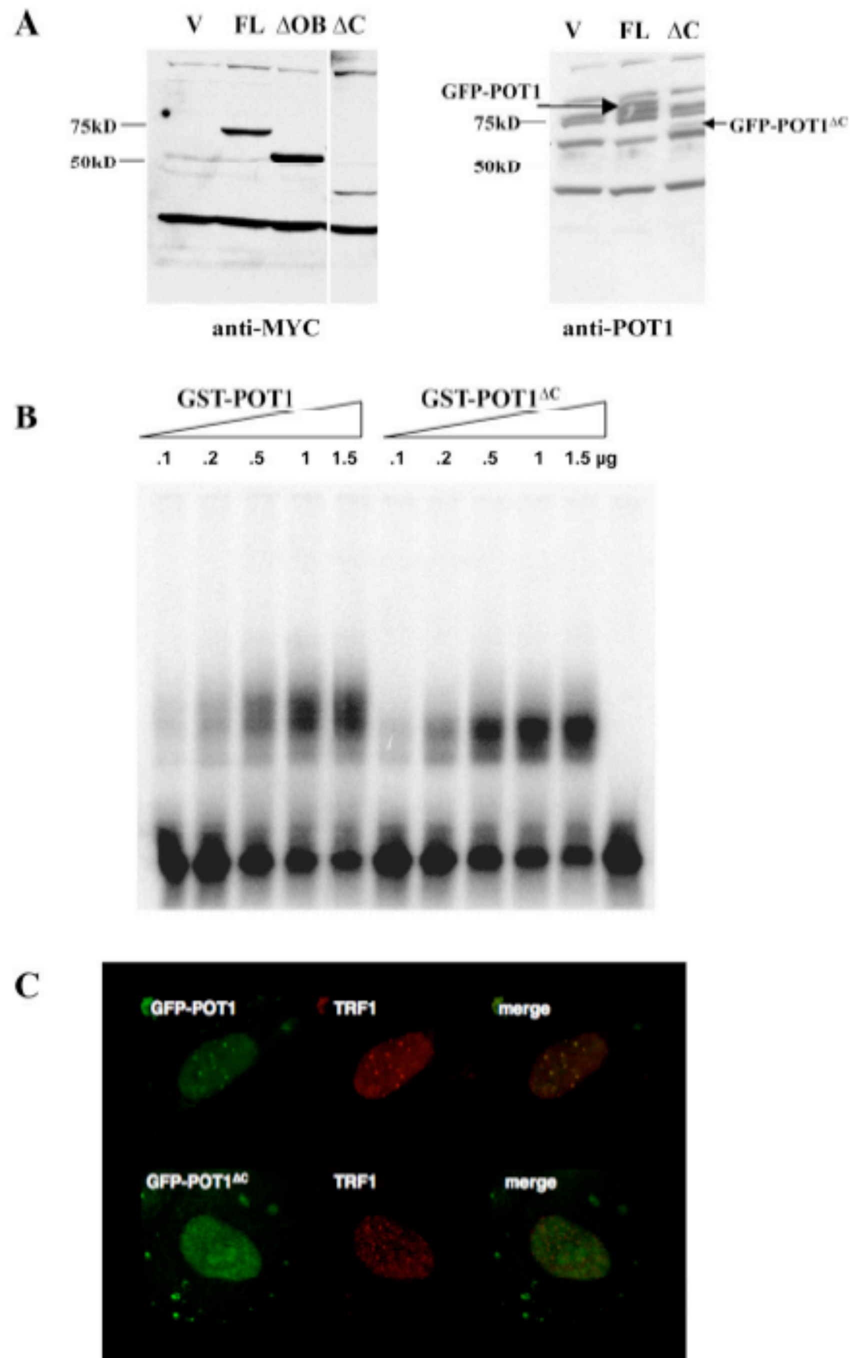


FIGURE 4. LOCALIZATION AND DNA BINDING ACTIVITY OF THE POT1$^{\Delta C}$ ALLELE.

A. Expression levels of MYC- or GFP- tagged alleles in HTC75 cells. The full-length (FL, 71kD), POT1$^{\Delta OB}$ (ΔOB, MW 57kD) and POT1$^{\Delta C}$ (ΔC, MW 43kD) are shown along a vector-only control. Blots proved with the 9E10 (anti-MYC) (left) or 978 (anti-POT1) antibodies (right) are shown. B. Gel shift assay for GST-POT1 and GST-POT1$^{\Delta C}$. A ^{32}P-labelled oligonucleotide containing the POT1 minimal binding site was incubated with the amounts of GST fusion protein shown on top. The free probe is visible at the bottom of the autoradiogram. C. Intranuclear localization of GFP-NLS-POT1 and GFP-NLS-POT1$^{\Delta C.}$ The GFP-tagged protein is detected in the FITC channel (left), and telomeres are stained with an anti-TRF1 antibody (371, middle panels). The overlay is shown in the right panels.

Yeast two-hybrid screen reveals POT1 interacting protein

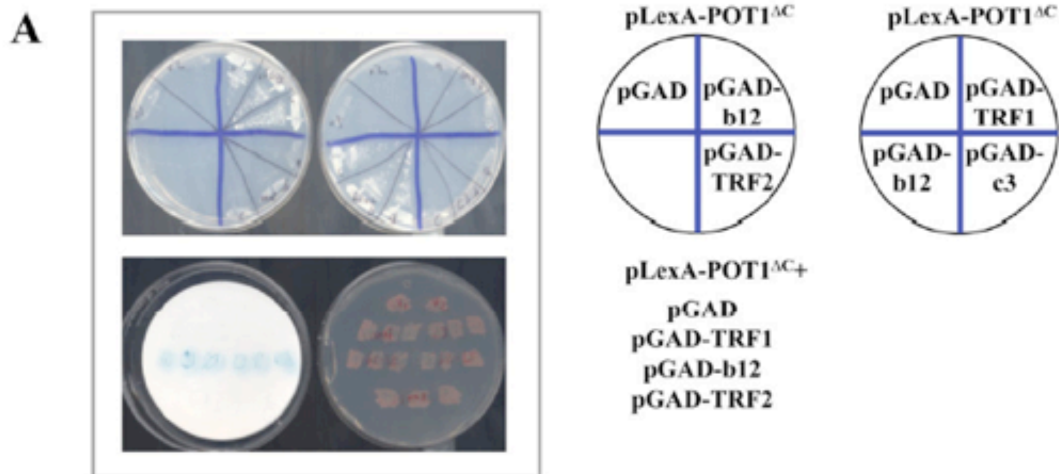
In order to find proteins that bind to POT1, the construct $POT^{\Delta C}$ was used as bait in a yeast two-hybrid screen. The yeast two-hybrid screen was performed by Diego Loayza at Rockefeller University and was designed for β-Gal activity. The goal of this screen was to identify human proteins interacting with the two N-terminal OB folds of POT1. The absence of the TPP1 interaction domain prevents the re-isolation of TPP1-containing plasmids.

A control used for this two-hybrid screen was the full-length POT1 in order to ensure that binding is not due to an aberrant C-terminus. The L40 yeast strain was used for the screen and it contains integrated copies of the GAL4 UAS upstream of the HIS3 and LacZ genes.

The primary screen isolated phenotypically His3+ transformants on 2mM 3-AT, and the positives were tested for β-Gal activity. Out of 1.2×10^6 transformants that were screened, three transformants consistently showed HIS+ and LacZ+ phenotypes and had a significant level of activation compared to control bait-prey fusions (Figure 5). The library plasmids were then isolated, re-tested, and the insert was sequenced. The sequence revealed that all three inserts corresponded to the 3' half of the human TRIP6 cDNA. This segment of human TRIP6 cDNA starts at amino acid 220 and ends with the final residue at position 476 and contains all three of the C-terminal LIM domains. Test performed in yeast confirmed that the clones interacted with full-length POT1, supporting

that the interaction detected in yeast was not an artifact of the truncated C terminus (Figure 5B).

TRIP6 is a 476 amino acid protein with a predicted molecular weight of 50kD. The TRIP6 gene is located at human chromosome 7q22, a region with chromosomal rearrangements that are associated with several human diseases such as myleodysplasia[70, 100]. It was previously identified via yeast two-hybrid screen as a thyroid hormone receptor interacting protein that is documented as being involved in cytoskeletal rearrangements[101]. Some laboratories also ascribe a nuclear function to TRIP6 but at the time of the yeast two-hybrid screen, no role had been associated to this protein with telomere protection. A nuclear involvement for this protein is supported through the presence of the nuclear export sequence (NES) and its mutation results in nuclear accumulation.



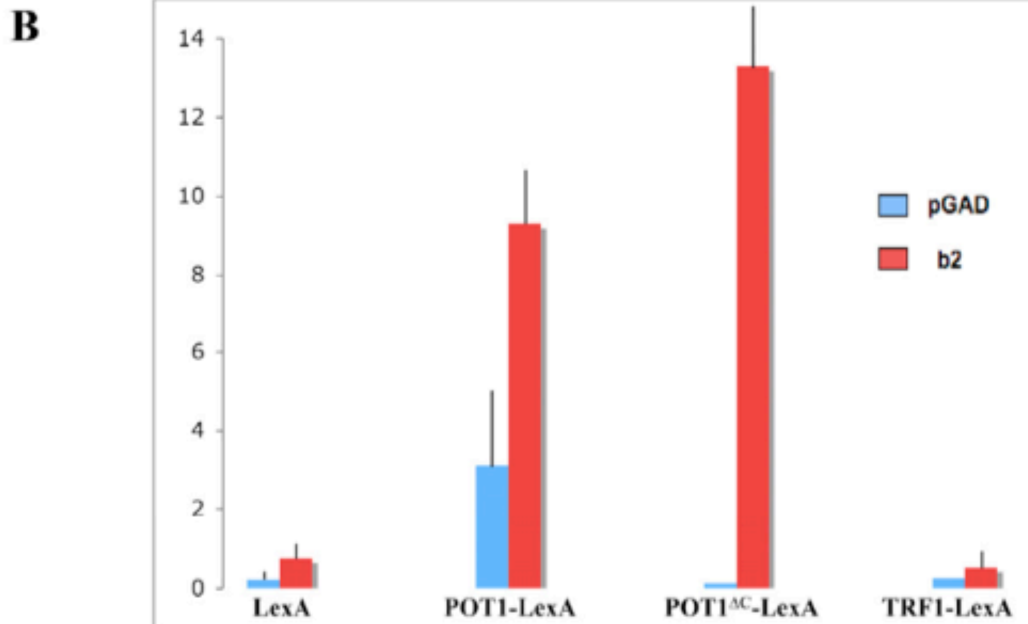


FIGURE 5. THE LIM DOMAINS OF TRIP6 INTERACT WITH POT1 $^{\Delta C}$ BY YEAST-2-HYBRID

A. Top: His phenotypes of the B40 strain carrying the pLexA-POT1$^{\Delta C}$ bait plasmid and the plasmids shown on the right side: the b12 positive clone and clone, c3 proved negative upon retransformation. Bottom: Patch LacZ assay of the same strains, showing that the b12 clone activates the LacZ reporter gene. B. Liquid β-Gal assays using the B40 yeast strain and the bait plasmids shown at the bottom, with the GAD vector or GAD-b2 clone. The b12 clone activates LacZ with LexA-POT1 or LexA-POT1$^{\Delta C}$, but not with LexA-TRF1. Std. deviations were calculated on three independent yeast colonies, each assayed three times.

Cloning and expression of the full-length human TRIP6 cDNA

In order to study the POT1-TRIP6 interaction in human cells, the full-length TRIP6 cDNA was obtained as an EST obtained from the ATCC and cloned through PCR. Full length TRIP6 cDNA was then introduced into a retroviral mammalian expression vector (pLPC) in fusion with a FLAG or MYC epitope tag, in order to express the tagged full-length cDNA in human cells. We first set out to confirm the interaction between POT1 and TRIP6 detected in yeast and to also confirm if that interaction occurs through the OB folds of POT1. Transient co-transfection in 293T cells with full-length POT1, POT1$^{\Delta C}$ or POT1$^{\Delta OB}$ was performed to ask whether MYC-POT1 could coimmunoprecipitate specifically with FLAG-TRIP6. Because the library plasmid recovered contained all three LIM domains, it is likely that the interaction takes place between the LIM domains of TRIP6 and the OB folds of POT1. If that is true, the interaction should be detectable in a co-Immunoprecipitation assay with the full length POT1, POT1$^{\Delta C}$, but perhaps not with that of POT1$^{\Delta OB}$.

The 293T transient co-transfections followed by immunoprecipitation revealed that MYC-POT1 could precipitate FLAG-TRIP6 confirming the yeast two-hybrid screen results. Also observed was an increase in the amount of POT1 in the presence of exogenous TRIP6, suggestive of stabilization that is often seen upon co-expression of interacting partners (Figure 6A, lane 6). POT1$^{\Delta C}$ also showed an increase in protein levels, although in this construct no interaction was readily detected. It is possible that POT1$^{\Delta C}$ is not expressed at levels high enough to detect an interaction in this particular experiment. In contrast, hPOT1$^{\Delta OB}$ did not show an interaction with TRIP6 despite a

robust expression of the transfected protein and there was no change in that level of expression upon the introduction of TRIP6.

We have also confirmed the interaction of POT1 and TRIP6 through immunoprecipitation using FLAG TRIP6 with rabbit anti-POT1 sera; in this method we are detecting an interaction between exogenous TRIP6 and endogenous POT1 (data not shown). Although POT1$^{\Delta OB}$ displays a consistent robust expression level, no interaction or stabilization was detected with POT1$^{\Delta OB}$ and TRIP6 (Figure 6B), suggesting, in conformity with the setup of the yeast two-hybrid screen, that the N-terminal OB folds are important for the POT1-TRIP6 interaction.

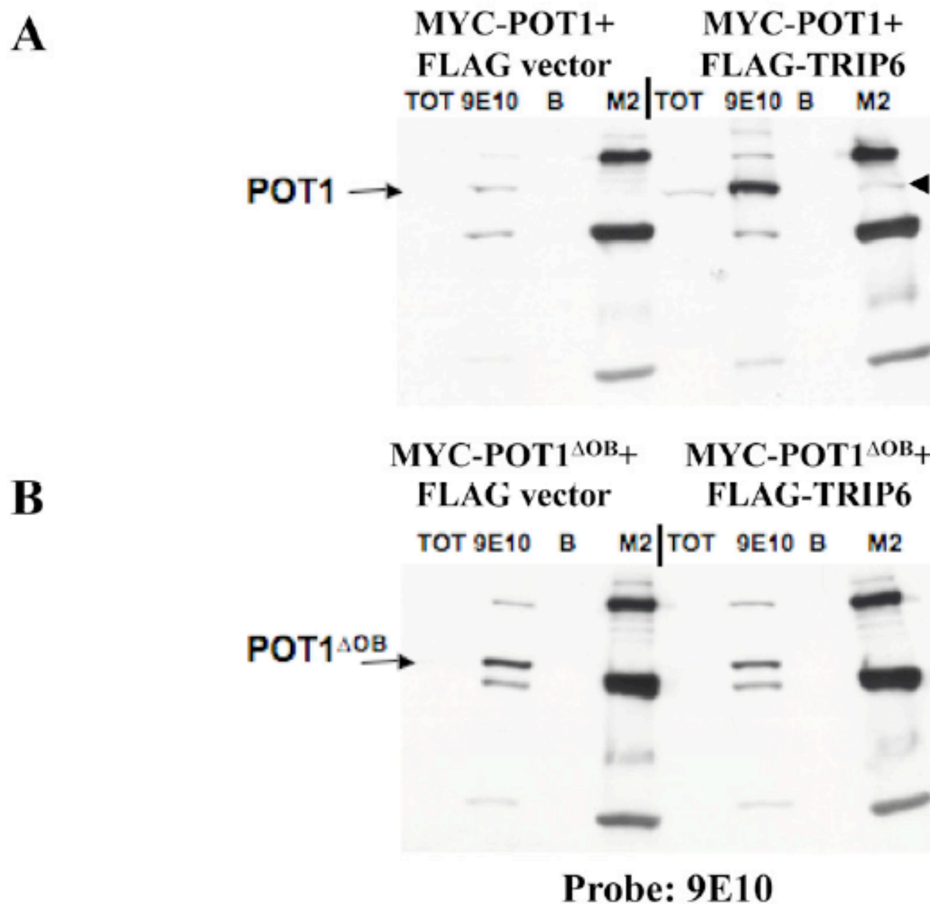


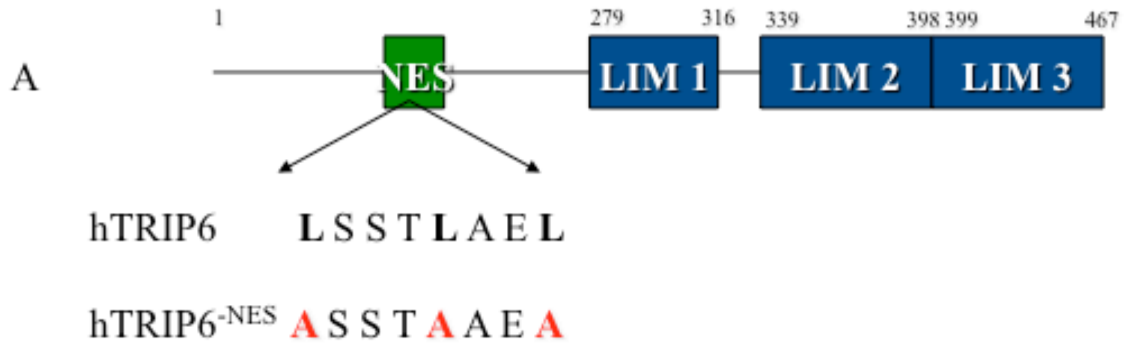
FIGURE 6. POT1 INTERACTS WITH TRIP6 IN TRANSIENT TRANSFECTIONS.

A) IP-Western blots on lysates made from transiently transfected 203T cells to detect co-immunoprecipitation of the transfected proteins. MYC-POT1 was co-transfected with FLAG-TRIP6 or with FLAG-Vector, and the lysates were used for Immunoprecipitation with the anti-MYC (9E10) or anti-FLAG (M2), as shown on top. A total fraction (TOT) and beads only control (B) were run alongside. The blot was proved with the 9E10 antibody and the arrow indicates the position of MYC-POT1. The immunoprecipitated MYC-POT1 by the FLAG antibody is shown with the black triangle. **(B)** Same as indicated in A, except the cell line MYC0POT1$^{\Delta OB}$ is used in the co-transfection.

After validating this protein interaction, full-length cDNA of TRIP6 was cloned into a retroviral expression vector in frame with a MYC tag. These constructs were then transduced into the stable cell lines HTC75, HCT116 and Hela1.2.11 (Figure 7B). Over subsequent population doublings, this expression was lost in Hela1.2.11. TRIP6 contains a leucine rich nuclear export sequence that is located within the N-terminal region. Treatments with leptomycin B, an inhibitor of Crm-1 dependent nuclear export in addition to deletion or mutations of the NES are enough to abolish shuttling activity[68]. A mutation in the nuclear export sequence of TRIP6 was also cloned (herein referred to as TRIP6^{-NES}) and was retrovirally transduced into HTC75 and HCT116 cell lines. These lines contain alanines in the location of proline at residue 100, 104 and 107 (Figure 7A). Western blots reveal strong expression of TRIP6 and TRIP^{-NES} is the HTC75 and HCT116 cell lines but not in Hela 1.2.11 cells (Figure 7B, data not shown). This mutant should result in the retention of TRIP6 to the nucleus and ultimately potentiate nuclear overexpression.

TRIP6 is found primarily in focal adhesion plaques so both the TRIP6 and TRIP6^{-NES} construct were fixed on coverslips to ascertain their localization pattern. Immunofluorescence confirms that TRIP6 is naturally found in the cytoplasm to a significant degree (Figure 8). The TRIP6^{-NES} construct however was confined to the nucleus as expected[72]. In order to verify that this construct localization of TRIP6 is due to blocking the canonical NES pathway, TRIP6 was treated with leptomycin B. Leptomycin B is a cytotoxin that is shown to bind the CRM1 protein and its

administration has been shown to block the CRM1 pathway[102]. Treatments with leptomycin B, an inhibitor of the Crm-1 dependent nuclear export for four hours showed that TRIP6 is now capable of accumulating in the nucleus (Figure 8). This information indicates that TRIP6 is capable of shuttling dynamically between the nucleus and the cytoplasm.



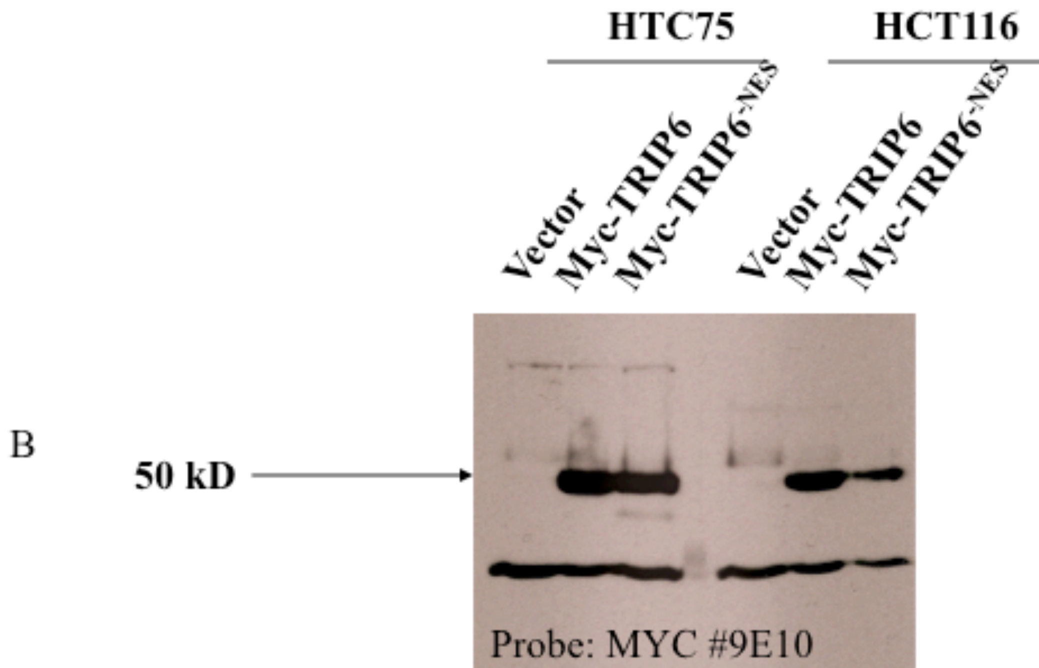


FIGURE 7 STABLE RETROVIRAL TRANSDUCTION OF TRIP6

(A) (TOP) Schematic of TRIP6 outlining location of the nuclear export sequence (NES) and the tandem LIM domains (LIM1-3). Numbers indicate residue locations. (LOWER) Denotation of residues that comprise the leucine rich NES. Red letters indicate site of mutation to create a cell line with a comprised NES. (B) Western Blot showing expression levels for Myc-TRIP6 and MYC-TRIP6^{-NES}. Blot was probed with anti-MYC antibody (9E10).

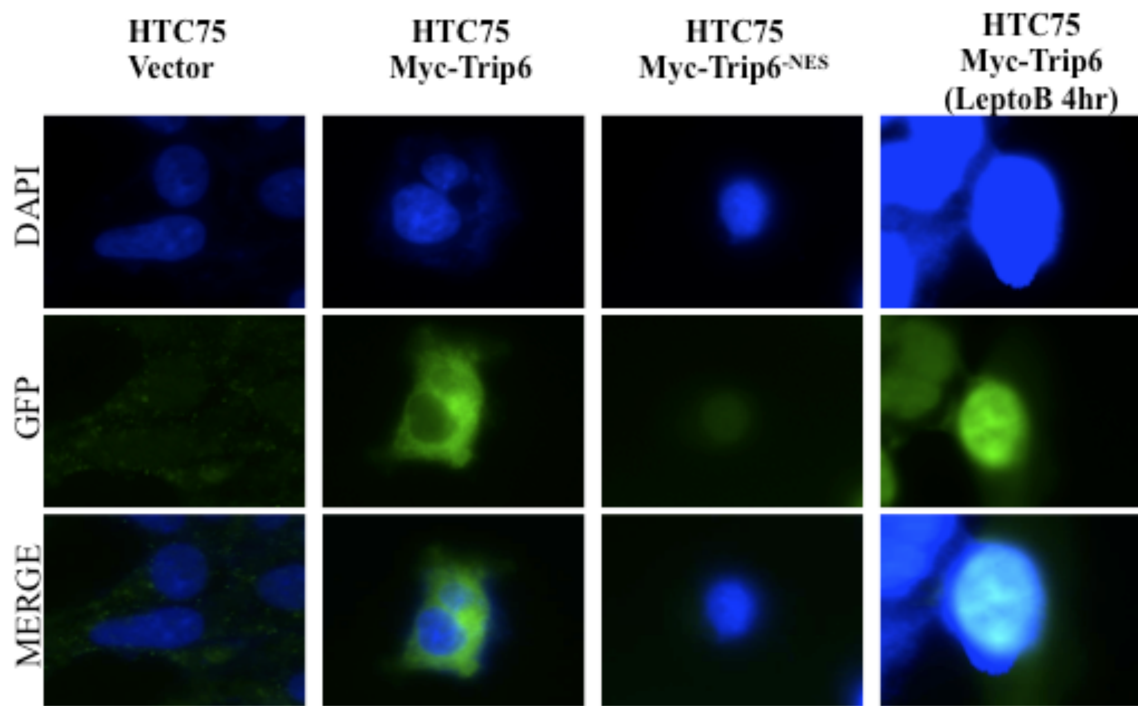


FIGURE 8 IMMUNOFLUORESCENCE IN HTC75 TRIP6 CELL LINES.

DAPI stain for nucleus detection and the GFP channel indicates localization of TRIP6 and TRIP6-NES construct.

Sequence alignment for LIM domain proteins reveals very similar protein LPP

In our initial analysis of TRIP6, we found that the human genome encodes a highly related protein called Lipoma Preferred Partner (LPP). LPP is another member of the Zyxin family and a level of homology exists between the three C-terminal LIM domains. Sequence identity confirms a 60%, 77%, and 75% similarity between each respective LIM domain[70]. LPP also contains a nuclear export sequence. This argues for the ability of the protein to shuttle dynamically between the nucleus and the cytoplasm like TRIP6. The next most closely related LIM domain proteins are Zyxin and Ajuba within this family and further analysis of LIM domain protein behavior at telomeres is of great interest to pursue (Figure 9). Upon assessment of homology we found TRIP6 and LPP to have an e value at or above $6e^{-104}$ and the relationship between Zyxin with TRIP6 or LPP is at or below $3e^{-68}$ as acquired from the NCBI blast engine.

```
TRIP6    1 --------------MSGPTWLPPKQPEPARAPQGRAIPRGTPGPPPAHGAALQ-------
LPP      1 --------------MSHPSWLPPKSTGEPLGHVPARMETTHSFGNPSISVSTQ-------
Zyxin    1 MAAPRPSPAISVSVSAPAFYAPQKKFGPVVAPKPKVNPFRPGDSEPPPAPGAQRAQMGRV

TRIP6   40 ----PHPRVNFCPLPSEQCYQAPGGPEDRGPAWVGSHGVLQHTQGLPADRGGLRP-----
LPP     40 ----QPPKKFAPVVAPKPKYNPYKQPGGEGDFLPPPPPPLDDSSALPSISGNFPP--PPP
Zyxin   61 GEIPPPPPEDFPLPPPPLAGDGDDAEGALGGAFPPPPPPIEESFPPAPLEEEIFPSPPPP

TRIP6   91 ---------------------GSLDAEIDLLSSTLAELNGGRGHASRRPDR---------
LPP     94 LDEEAFKVQGNPGGKTLEERRSSLDAEIDSLTSILADLECSSPYKPRPPQSSTGSTASPP
Zyxin  121 PEEEGGPEAPIPPPPQPREKVSSIDLEIDSLSSLLDDMTKNDPFKARVSSG---------

TRIP6  121 -----------QAYEPPPPPAYRTGSLKPNPASPLPASPYG---------GPTPASYTTA
LPP    154 VSTPVTGHKRMVIPNQPPLTATKKSTLKPQPAPQAGPIPVAPIGTLKPQPQPVPASYTTA
Zyxin  172 -----------YVPPPVATPFSSKSSTKPAAGGTAPLPPWKSPSSSQPLPQVPAPAQSQT

TRIP6  161 STPAGPAFPVQVKVAQP-VRGCGPPRRGASQASGPLPGPHFPLPGRGEVWGPGYRS----
LPP    214 STSSRPTFNVQVKSAQPSPHYMAAPSSGQIYGSGPQGYNTQPVPVSGQCPPPSTRGGMDY
Zyxin  221 QFHVQPQPQPKPQVQLHVQSQTQPVSLANTQPRGPPASSPAPAPKFSPVTPKFTPVASKF

TRIP6  216 --------------QREPGPGAKEEAAGVSGPAGRGRGGEHGPQVPLSQP----------
LPP    274 AYIPPPGLQPEPGYGYAPNQGRYYEGYYAAGPGYGGRNDSDPTYGQQGHPNTWKREPGYT
Zyxin  281 SPGAPG--------GSGSQPNQKLGHPEALSAGTGSPQPPSFTYAQQREKPRVQEKQHPV

TRIP6  252 -------------------------------------------------------PEDEL
LPP    334 PPGAGNQNPPGMYPVTGPKKTYITDPVSAPCAPPLQPKGGHSGQLGPSSVAPSFRPEDEL
Zyxin  333 PPPAQNQNQVRSPGAPGP---------------------------------LTLKEVEEL

TRIP6  257 DRLTKKLVHDMNHPPSGEYFG--QCGGCGEDVVGDGAGVVALDRVFHVGCFVCSTCRAQL
LPP    394 EHLTKKMLYDMENPPADEYFG--RCARCGENVVGEGTGCTAMDQVFHVDCFTCIICNNKL
Zyxin  360 EQLTQQLMQDMEHPQRQNVAVNELCGRCHQPLARAQPAVRALGQLFHIACFTCHQCAQQL

TRIP6  315 RGQHFYAVERRAYCEGCYVATLEKCATCSQPILDRILRAMGKAYHPGCFTCVVCHRGLDG
LPP    452 RGQPFYAVEKKAYCEPCYINTLEQCNVCSKPIMERILRATGKAYHPHCFTCVMCHRSLDG
Zyxin  420 QGQQFYSLEGAPYCEGCYTDTLEKCNTCGEPITDRMLRATGKAYHPHCFTCVVCARPLEG

TRIP6  375 IPFTVDATSQIHCIEDFHRKFAPRCSVCGGAIMPEPGQEETVRIVALDRSFHIGCYKCEE
LPP    512 IPFTVDAGGLIHCIEDFHKKFAPRCSVCKEPIMPAPGQEETVRIVALDRDFHVHCYRCED
Zyxin  480 TSFIVDQANRPHCVPDYHKQYAPRCSVCSEPIMPEPGRDETVRVVALDKNFHMKCYKCED

TRIP6  435 CGLLLSSEGECQGCYPLDGHILCKACSAWRIQELSATVTTDC
LPP    572 CGGLLS-EGDNQGCYPLDGHILCKTCNSARIRVLTAKASTDL
Zyxin  540 CGKPLSIEADDNGCFPLDGHVLCRKCHTARAQT---------
```

FIGURE 9 BOXSHADE 3.21 SHADING OF MULTIPLE-ALIGNMENT FOR TRIP6, LPP AND ZYXIN .

Black boxes indicate sequence homology between proteins

Discussion

TRIP6 is a member of a subfamily of LIM domain proteins that include Zyxin, LPP, and Ajuba. Characterization of these proteins shows primary localization to focal adhesion plaques. Members of this subfamily contain three tandem LIM domains in the C terminus. LIM domains are protein motifs derived from the homeodomains of three *C. Elegans* proteins Lin11, Isl-1 and Mec-3 and have a highly conserved cysteine-rich sequence. LIM proteins are present in a diverse amount of eukaryotic proteins and have varying biological functions.

The construct of TRIP6 that came out of the yeast two-hybrid screen was composed of amino acid residue 220 to the C terminus, which is predominantly the LIM domain region of TRIP6. The transient co-transfection validates the interaction between TRIP6 and POT1 in human cells but also reveals an interaction that is present within the OB fold region of POT1. The POT1$^{\Delta OB}$ construct lacking the DNA binding domain of POT1 fails to interact with TRIP6. This data reveals a partner that is capable of interacting with the region of POT1 responsible for maintaining telomere length. Although the exact position of the TRIP6-POT1 interaction has not been evaluated based upon this data, the LIM domain appears to be the important portion of TRIP6.

The behavior of this interaction is not surprising due to literature that illustrates how the LIM domains of TRIP6 and its family members is capable of protein-protein interactions. The LIM domain is a pivotal protein structure for the biological function of proteins

within this category and supports the import of their ability to function in the assembly of multiprotein complexes[68].

TRIP6 in particular has been shown to interact with a variety of both cytoplasmic and nuclear proteins and has the ability to shuttle in and out of the nucleus under appropriate conditions. This behavior is of particular interest because it supports a possible dynamic relationship of this subfamily at telomeres. The second highly homologous protein LPP has introduced the possibility that a pair of LIM domain proteins play a role at the telomeres. TRIP6 and LPP may have a role in independent or dependent pathways.

Chapter 4 Characterization of TRIP6 and LPP as novel telomeric proteins

Introduction

The yeast two-hybrid and transient co-transfection in 293T cells reveals that TRIP6 can interact with POT1 most likely through its OB folds. TRIP6 is a LIM domain protein that was first identified through a two-hybrid screen and was shown to interact with the thyroid hormone receptor in a hormone-dependent manner[101]. TRIP6 belongs to a class of LIM domain proteins that includes Zyxin, Trip6 and LPP. The function of members of this subfamily has not been completely understood and members of this family are found to primarily localize to focal adhesion plaques. TRIP6 has been identified as interacting with a variety of nuclear proteins such as thyroid hormone receptor and v-Rel; TRIP6 has also been shown to interact with cytoplasmic proteins protein phosphatase PTP1E and PTPBL [72]. The localization of TRIP6 is primarily cytosolic due to the presence of a nuclear export signal near the N-terminus of the protein [72]. This leucine-rich NES is found between residues 100 and 107. Movement of TRIP6 from the nucleus to the cytoplasm is dependent on Crm1 and treatment with the Crm1 inhibitor leptomycin B can block this movement[72]. In addition to having a signal for nuclear export, TRIP6 aa 280-480, also contains a sequence capable of targeting a heterologous protein to the nucleus. This C-terminus portion of TRIP6 contains the three tandem LIM domains that have been outlined in the introduction chapter. The stable transduction of TRIP6 or LPP cDNA into telomerase positive cell lines allows for subsequent long-term analysis of LIM domain protein behavior at telomeres.

Results

Co-Immunoprecipitation

Co-Immunoprecipitation assays were done in order to further support the interaction of TRIP6 and POT1 in stable cell lines. Analyses in cell lines overexpressing Myc-TRIP6 indicate a weak but reproducible signal using various forms of anti-POT1 sera (Figure 10). In addition, two anti-TRIP6 peptide antibodies were raised in rabbits to confer if cell lines overexpressing POT1 could be precipitated using TRIP6. The Western blot did not reveal a co-immunoprecipitation in exogenously expressed POT1 with TRIP6 antibodies (5923, 5924). It is of import to note that Western blots and IPs using cell lines with overexpressed POT1 have yielded poor results in prior assays employed within the lab and in literature, which suggests that the inability to confirm the interaction in the reverse orientation may be due to poor expression levels of this protein in our stable cell lines.

We next sought to ascertain if an interaction existed between TRIP6 and the other shelterin complex proteins. Co-immunoprecipitation assays revealed a very robust precipitation of TRIP6 was seen using anti-TRF2 (647) antibody (Figure 10). Additionally TRIP6 can successfully be pulled down with TIN2 and TRF1 antibodies (Figure 10A). In order to verify that the interaction between TRF2 and TRIP6 was specific, another co-IP involving MYC-POLA2 retrovirally transduced into HTC75 cells was performed. POLA2 is a nuclear protein involved in DNA replication and has been previously shown not to interact with shelterin. MYC-POLA2 was not pulled down using the TRF2 antibody, arguing for a specific pull down for MYC-TRIP6 (Figure 11). Co-Immunoprecipitation indicate that TRIP6 is in complex with shelterin but we cannot rule out a direct interaction between TRIP6 and other shelterin components that were not

detected using the yeast two-hybrid tests.

LPP, like TRIP6 localizes in focal adhesions, membrane attachment sites of cells to the extracellular matrix. LPP is also capable of being translocated to the nucleus and its LIM domains are thought to be indispensable to this intranuclear localization[81, 82]. As the yeast two-hybrid data suggests that the interaction between POT1 and TRIP6 is mediated through the LIM domains, it became necessary to assess whether other LIM domains, particularly LPP, could associate with the same shelterin components. In order to test another protein within the Zyxin LIM domain family, LPP was retrovirally transduced into HCT116 and HTC75 cell lines. LPP provided similar results to TRIP6 upon co-IP, a weak but reproducible association with POT1 and a robust signal with the TRF2 antibody. The similarity of results observed with TRIP6 and LPP suggest some redundancy in their roles as partners of POT1 and TRF2 (Figure 10B). Additionally, by IP-Western, MYC-LPP was found to co-precipitate with POT1, TRF2, TRF1 and TIN2 antibodies (Figure 10B).

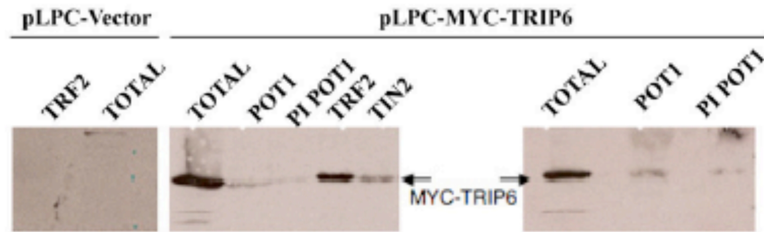
Chromatin Immunoprecipitation

In order to address whether TRIP6 or LPP associate with shelterin at telomeres we applied the Chromatin Immunoprecipitation (ChIP) technique in HTC75 cell lines. This assay has been extensively used in order to study the presence of shelterin components or other proteins on telomeric DNA. Anti-peptide rabbit sera against TRIP6 or LPP were used for this analysis, which were raised against epitopes in the N-terminus, which were significantly divergent between the two proteins. We confirmed that the TRIP6 and LPP

sera were competent for Immunoprecipitation and not cross-reacting bands. In asynchronous HTC75 cells, TRIP6 was found to associate with telomeres with a yield of about 5% of total TTAGGG DNA (Figure 12B), which is about half of the yield which is seen for POT1 in this assay and comparable to the yield obtained for TPP1. TRF1 antibodies were used as a positive control and were able to pull down 20% of total telomeric sequences in keeping with previously published results[23]. LPP is also capable of being detected at telomeres by ChIP with a yield of 3.5% of total DNA. The yields for Alu sequences are used as internal control sequences were between 0.5 and 1% for all samples.

We also probed the telomeric association of TRIP6 and LPP by ChIP in cells expressing POT1$^{\Delta OB}$. These cells have truncated OB folds, the region of POT1 that binds the single stranded overhang. The abrogation of POT1's DNA binding ability in telomerase positive cells leaves the enzyme free to elongate these ends. As a result these cell lines have highly elongated telomeres, concomitant with a lower expression of endogenous full-length POT1. The overall increase in telomere length is directly proportional to the presence of shelterin which supports that shelterin coats the telomere throughout and not only at the distal ends. This quantitative behavior was observed for both TRIP6 and LPP with an increase in the percent yield when looking at the POT1$^{\Delta OB}$ line. A significantly stronger signal on the dot blot (Figure 12) was seen and correlated to the significantly longer telomeres in POT1$^{\Delta OB}$ cells.

A



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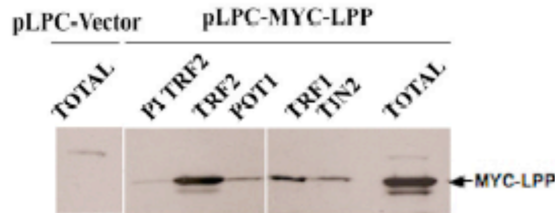


FIGURE 10. TRIP6 AND LPP CO-IMMUNOPRECIPITATE WITH SEVERAL SHELTERIN COMPONENTS.

(A) IP-Western blots on lysates made from HTC75 cells obtained through retroviral transduction, stably expressing MYC-TRIP6 (50kD). The vector only control is shown on the left. The lysates were used for immunoprecipitations with the antibodies listed on top, and analyzed for the amounts of MYC-TRIP6 by Western blot with the MYC 9E10 antibody. The total fraction was run alongside as indicated. The POT1 sera were the anti-epitope #4955 (left panel) and the anti-baculovirus POT1 #1048 (right panel). (B) Same as A, using a stably expressing MYC-LPP (66kD) HTC75 cells.

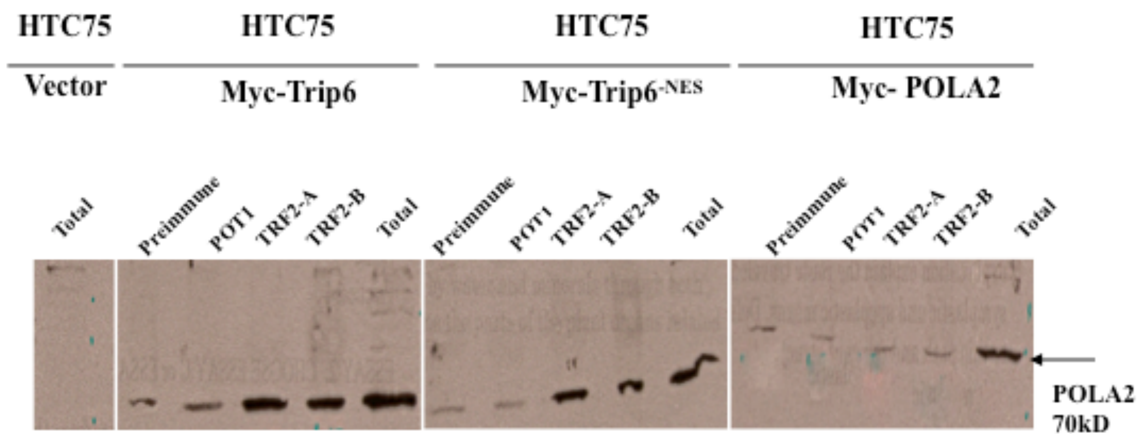
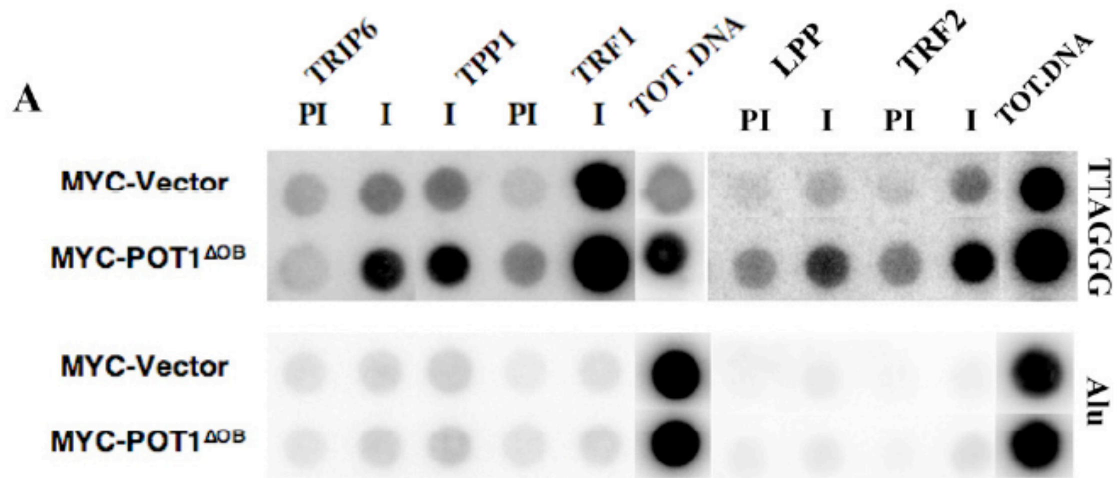


FIGURE 11. LIM PROTEINS TRIP6 AND LPP CO-IMMUNOPRECIPITATE WITH TRF2.

IP-western blots on lysates made from HTC75 cells stably expressing MYC-TRIP6, MYC-TRIP6^{-NES} or MYC-POLA2 as indicated on the top. Lysates were used for immunoprecipitations with anti-TRF2 rabbit antibodies, and analyzed for the amounts of each respective protein by western blot with the 9E10 antibody. The total fraction was run alongside as indicated. TRIP6: 50kD; TRIP6^{-NES} : 50kD; POLA2: 70kD.



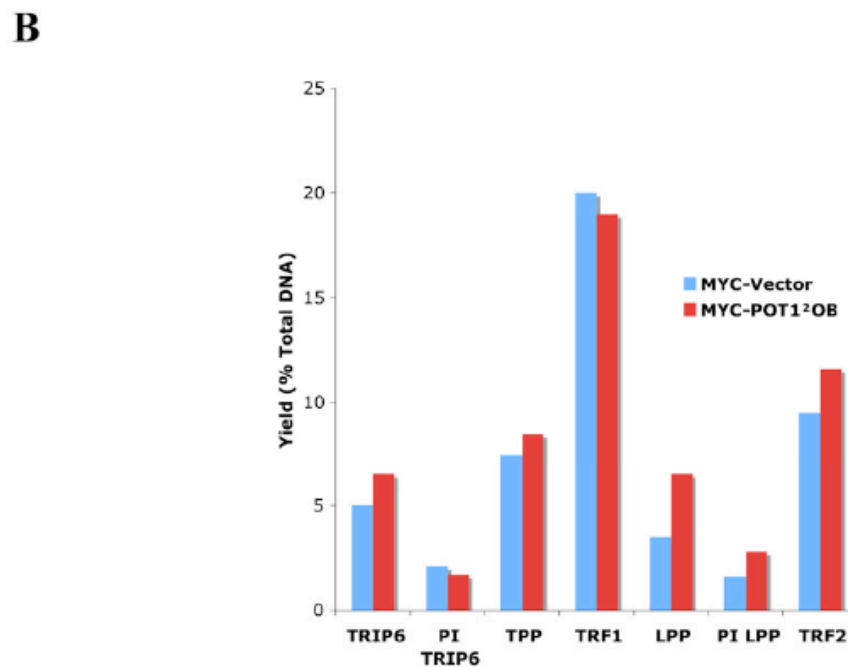


FIGURE 12. TRIP6 AND LPP ARE DETECTED AT TELOMERES BY CHIP.

A) Chromatin immunoprecipitations with fixed lysates prepared from HTC75 cell lines indicated on the left. The antibodies used are listed on top (I = Immune, PI = Preimmune), and the total DNA fraction is on the right side of each blot as indicated. Extracted DNA samples were dot-blotted on Nitrocellulose, and probed with a TTAGGG probe (TOP) or with an Alu prove (BOTTOM) as a control. The yields calculated for the samples proved with Alu were all below 0.5%. (B) Histogram of the values for the yields as % of total DNA of the samples shown in A.

Discussion

TRIP6 and LPP can associate specifically with the telomeric repeats as evidenced by the ChIP. This interaction may be mediated through the highly similar C-terminal LIM domains, although the specificity of the interaction requires elucidation. The correlation between a stronger signal on the dot-blot for TRIP6 and LPP may be correlated to the significantly longer telomeres in POT1 $^{\Delta OB}$ cells and this pattern is seen among telomeric or telomere-associated proteins such as TRF1, RAP1 or MRE11[23]. From the CoIP and ChIP data we can postulate that not only do TRIP6 and LPP associate with shelterin, they associate with telomeric DNA and as the telomere length increases, so does the presence of these proteins along the telomere. This suggests that these LIM proteins are found to associate with shelterin as it coats the telomere.

It is of import to note that by overexpressing POT1 with a truncated OB fold we are increasing the ratio between POT1 with and without an intact OB fold. The fact that this overexpression yields higher levels of TRIP6 and LPP bound to the telomere modifies that previous hypothesis as gleaned through the yeast two-hybrid screen. It seems that the OB folds are not responsible for the direct recruitment of these proteins but may instead play a role in the function of TRIP6 and LPP. It is possible that TRIP6 and LPP could associate for the purpose of recruitment with another shelterin protein such as TRF2. More work is required to verify this as a valid model for the presence of TRIP6 and LPP at the telomeres.

Chapter 5 TRIP6 and LPP Play a Protective Role at the Telomeres

Introduction

At this juncture we have been able to isolate a novel interaction between the LIM domain proteins TRIP6 and LPP with shelterin components as well as the telomeric DNA. With their ability to dynamically shuttle between the cytoplasm and nucleus, it is of interest to determine what impact they impart to telomeres. To analyze the possible roles of TRIP6 and LPP in telomere function we first examined telomere length in HTC75 cells over 60 population doublings in cells overexpressing either TRIP6 or LPP. The impact of shelterin components depletion or overexpression is usually visible if normally detected within this span, but no effect was observed for TRIP6 or LPP. We then turned to the analysis of TRIP6 and LPP siRNA depletion on telomere protection in HTC75 cells.

Telomere dysfunction has been implicated in tumorigenesis and aging. This occurs through the deprotection of telomeres through reaching a critically short length or through the impairment of telomere protective proteins such as shelterin. This loss of telomere function leads to senescence, apoptosis, end-to-end fusions, and cell cycle arrest. Inhibition of the shelterin protein TRF2 can result in an ATM pathway dependent response, and POT1 inhibition yields an ATR pathway response. Signs of telomere dysfunction can be illustrated through telomere dysfunction induced foci (TIFs) which results in an accumulation of DNA damage response proteins such as 53BP1 and γH2AX found to colocalize with the shelterin protein TRF1[9]. The binding of DNA damage

response proteins is indicative of cells perceiving unprotected telomeres as sites of DNA damage.

Results

siRNA of TRIP6 and LPP leads to DNA damage response

Analysis of the possible short-term effects 24-48 hours post transfection of TRIP6 or LPP depletion on the induction of a DNA damage response at telomeres were conducted. The DNA damage response can be monitored by the induction of p53BP1 foci and their localization with telomeres. This association is indicative of telomere de-protection [9, 10]. For the siRNA assay we used target sites as mentioned in the RNA interference materials and methods. This led to partial depletion of exogenous MYC-TRIP6 or MYC-LPP as observed by Western blot (Figure 13).

The depletion of TRIP6 using the appropriate siRNA construct led to a significant increase of p53BP1 foci in the nuclei, which suggests the induction of DNA damage response. The number of p53BP1 nuclear foci increased from an average of 1 to 2.65 per nucleus. The quantitation of this DNA includes all cells and may indicate a slightly lower value due to the inclusion of untransfected cells.

In particular, we observed the induction of TIFs as observed by the colocalization of p53BP1 with shelterin component TRF2 (Figure 14A). Depletion of TRIP6 yields 40% of the nuclei observed showing 3 or more TIFs, which indicates that this increase in levels of DNA damage response is also occurring at the telomeres. This value is a 2.7 fold

increase over the background that was detected using GFP as a negative control (Figure 14B).

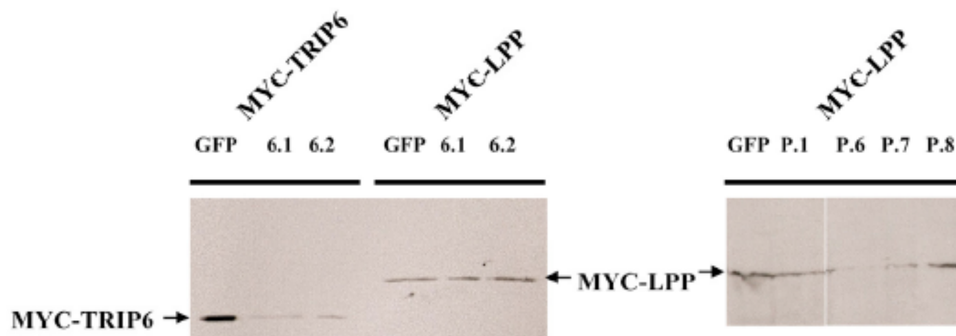
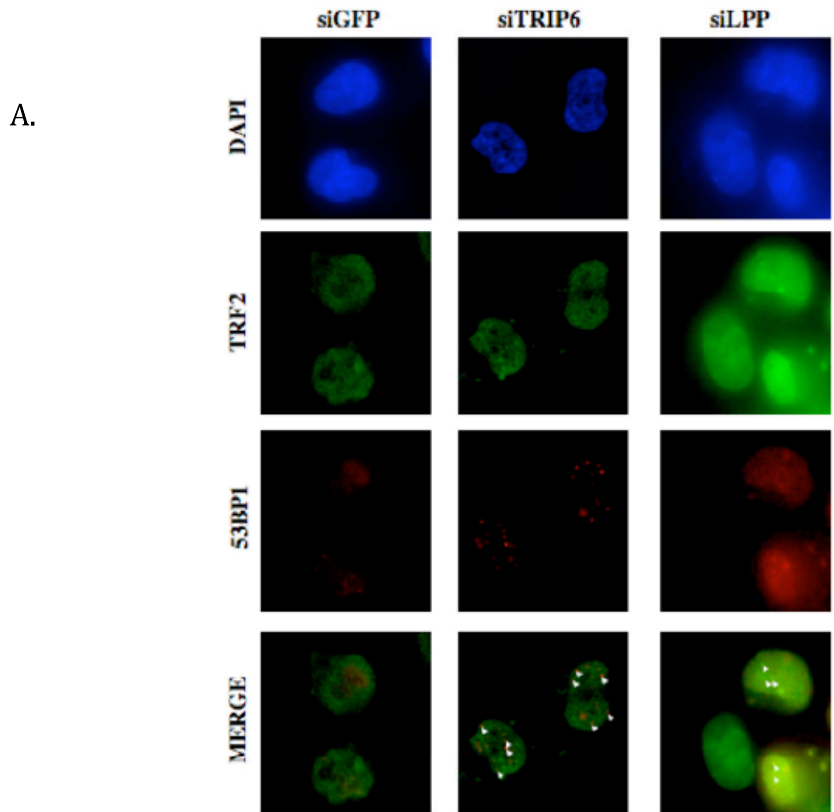


FIGURE 13. DEPLETION OF TRIP6 OR LPP BY SIRNA.

Western blot of lysate prepared from MYC-TRIP6 or MYC-LPP expressing cells transfected with the siRNA constructs indicated on top. The anti-MYC 9E10 antibody is used as a probe. All further siRNA treatments of TRIP6 and LPP were done using the 6.2 siRNA for TRIP6 and P.1 for LPP depletion.

A.



B.

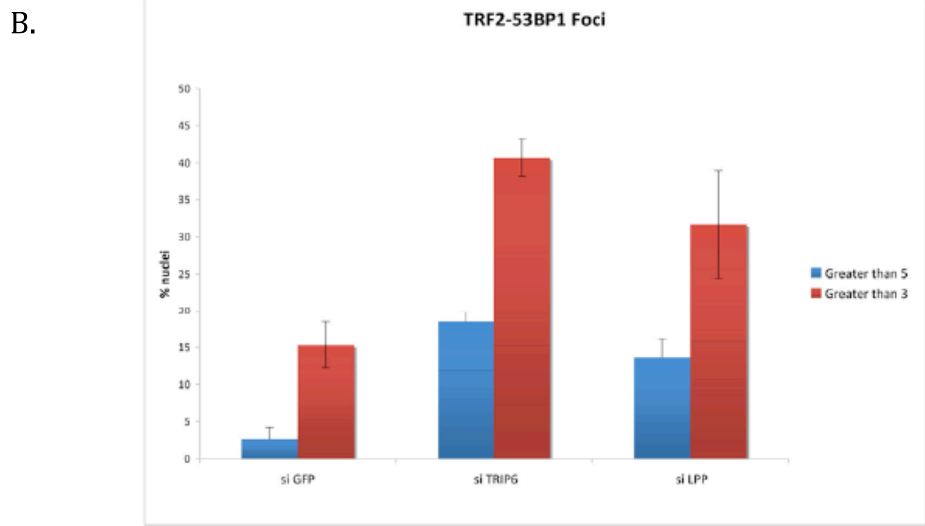


FIGURE 14. DEPLETION OF TRIP6 OR LPP LEADS TO TIF FORMATION.

A. Immunofluorescence showing the intranuclear localization of p53BP1 in TRIP6-depleted HTC75 cells (middle panels), LPP-depleted cells (right panels) or control siRNA GFP (left panels). The detected fluorescence (DAPI, FITC for TRF2, TRITC for p53BP1) is indicated on the left and white triangle points indicate the co-localized TRF2-p53BP1 foci. B. Histogram of the values for co-localized p53BP1 and TRF2 foci (left, greater than 3 in red, right greater than 5 in blue per nuclei) as a perfect of the total nuclei counted. 100 nuclei were counted for each set, and the standard deviation was calculated on three separate experiments.

Similar results were found with the siRNA depletion of LPP (Figure 14). An overall increase of p53BP1 was evident, from 1 to 1.8 foci per nucleus. The amount of TIF formation was 32% of cells with 3 or more foci co-localizing with TRF2. This represents a two-fold increase over the background (Figure 14B).

After the independent depletion of TRIP6 and LPP a double siRNA depletion was done in order to ascertain if this protective quality of LIM domain proteins operated along distinct pathways. Double depletion did not yield a significant change in DNA damage response levels but does not necessarily support that these proteins are within the same pathway.

Discussion

Depletion of either TRIP6 or LPP alone was sufficient to yield a DNA damage response at telomeres. From this we can conclude that both are necessary to fully protect telomeres through a possible cooperation with POT1. Our results suggest that TRIP6 and LPP both contribute to the protection of telomeres through the prevention of the DNA damage response pathway that is elicited through the activation of ATM or ATR. The high sequence similarity between TRIP6 and LPP likely accounts for their mutual recruitment to telomeres and although our data argues that each is independently important, it is possible that they modulate activities through a common pathway.

Chapter 6

TRIP6 and LPP, but not Zyxin or Ajuba are present at a subset of telomeres in human cells

Introduction

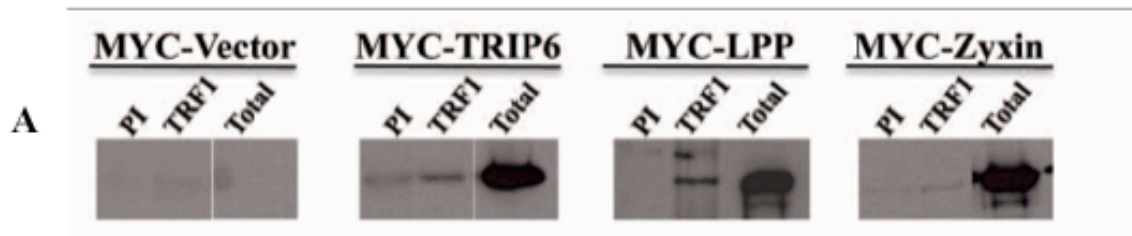
The LIM domain similarity between TRIP6 and LPP warranted further investigation regarding the specificity of these LIM proteins. The next most closely related LIM protein was Zyxin followed by Ajuba both of which were tested regarding their association with telomeres. The e value obtained from the NCBI blast engine between TRIP6 and LPP is at or above 6e -104, and the e value between Zyxin with TRIP6 or LPP is at or below 3e-68. Although the degree of homology is significantly high, it is still much lower than that observed between TRIP6 and LPP.

Results

Co-Immunoprecipitation

In order to verify the specificity of the telomere association observed for TRIP6 and LPP, Zyxin was cloned into a retroviral vector in frame with the MYC epitope (Figure 15B). Attempts to do the same with Ajuba cDNA resulted in cell death upon selection. A co-immunoprecipitation assay was done in order to determine if Zyxin was in complex with TRF1. Whole lysates for the three lines, in addition to control cells were used for the immunoprecipitation with TRF1, TRF2, TIN2, or POT1 antibodies. As previously shown, TRIP6 and LPP are capable of being precipitated by TRF1 antibodies as well as additional shelterin antibodies. Zyxin was incapable of being pulled down using TRF1 or any other shelterin antibodies tested (Figure 15A).

Additionally, a coimmunoprecipitation utilizing MYC tagged overexpressed POT1 and $POT1^{\Delta OB}$ were done. This not only provides the ability to test Zyxin overexpressed in addition to commercial antibodies, but it also allows it to incorporate Ajuba using a commercial antibody due to the inability to create a stable retrovirally transduced cell line. These results corroborated the previous figure in that cell lines overexpressing shelterin protein POT1 could not be found in complex with either Zyxin or Ajuba.



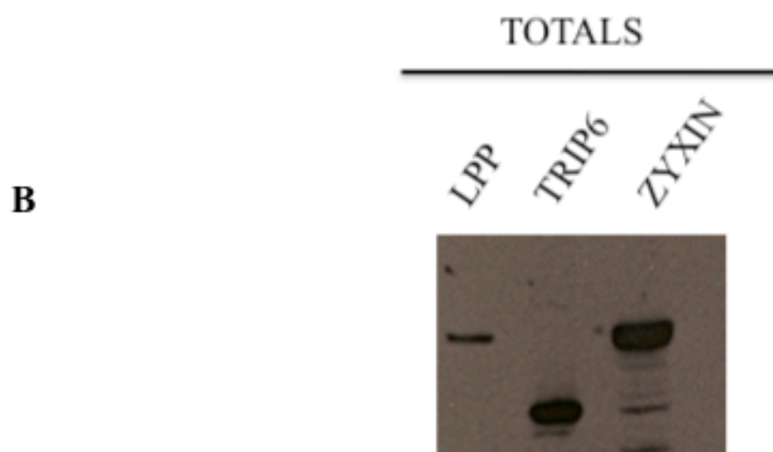


FIGURE 15. LIM PROTEINS TRIP6 AND LPP CO-IMMUNOPRECIPITATE WITH SHELTERIN COMPONENTS, BUT ZYXIN DOES NOT

A. IP-western blots on lysates made from HTC75 cells stably expressing MYC-TRIP6, MYC-LPP or MYC-Zyxin. Lysates were used for immunoprecipitations with anti-TRF1 rabbit antibodies and analyzed for amounts of each respective protein using the 9E10 antibody. B. Western blots for HTC75 LPP, TRIP6 and Zyxin. Western blot of lysate prepared from MYC-TRIP6, MYC-LPP and MYC-Zyxin expressing cells to test presence of exogenous protein. The anti-MYC 9E10 antibody is used as a probe.

Chromatin Immunoprecipitation

Next, we determined whether Zyxin could associate with the telomeres through the ChIP assay. This assay has previously allowed us to demonstrate the telomere association for TRIP6 and LPP. This was done using HTC75 POT1 and HTC75 POT1ΔOB in order to see if a correlation also existed with the average length of the telomere and the levels of Zyxin present. Results show that neither Zyxin nor Ajuba was found at telomeres in significant levels (Figure 16A).

Within the ChIP we included antibodies for PRMT1, a methyl transferase in an effort to shed light on the possible mechanism by which these LIM proteins function at telomeres. Our results led to the hypothesis that TRIP6 and LPP recruit a methyl transferase to telomeres in the same way that Ajuba does at RARE promoters[93]. This hypothesis is supported by findings that TRF2 is methylated at specific Arginine residues by the methyl transferase PRMT1, a modification important to mediate telomere protection[39]. Quantitation of the dot blot reveals that PRMT1 is present at telomeres in levels comparable to TRIP6 and LPP (Figure 16B).

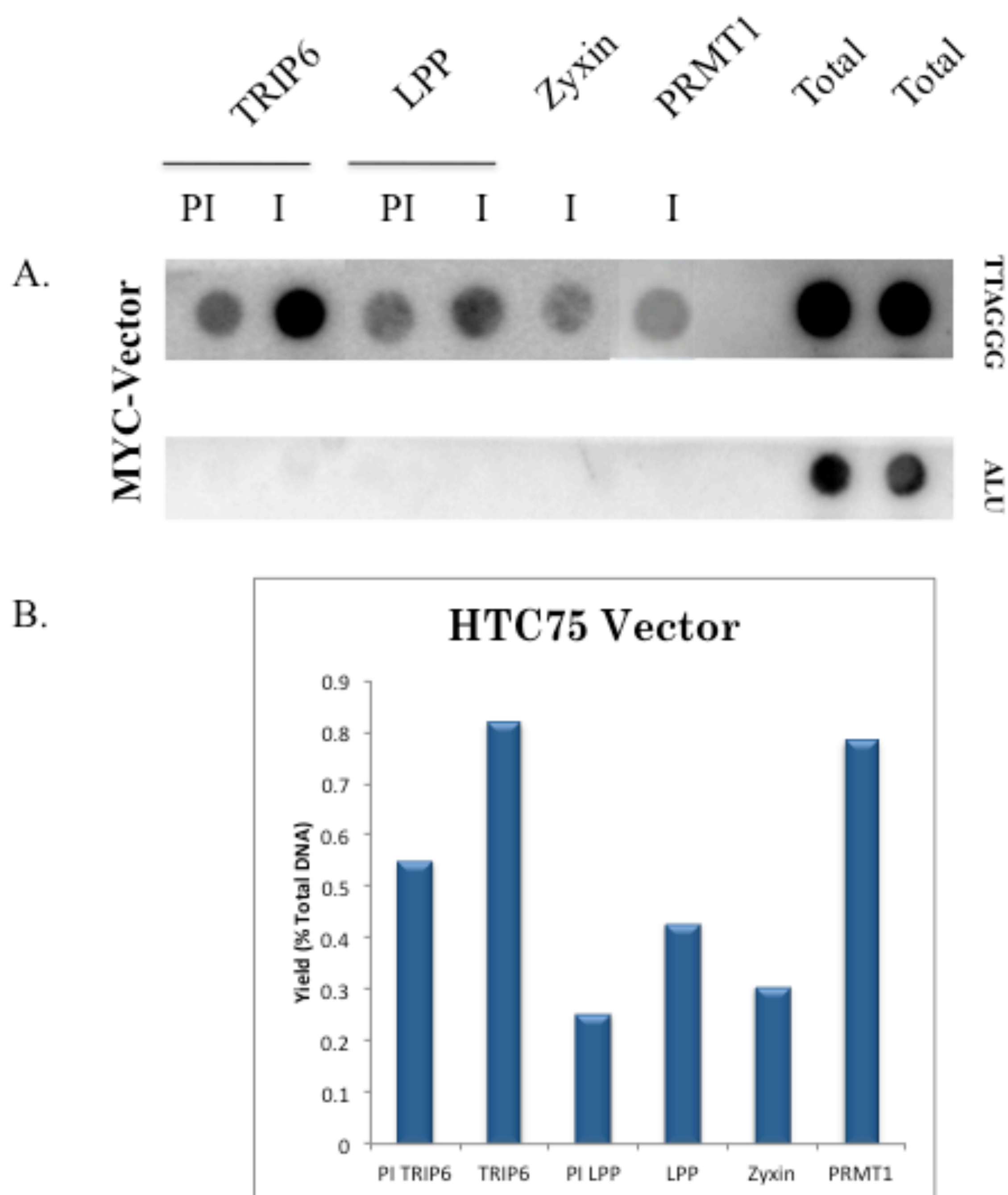


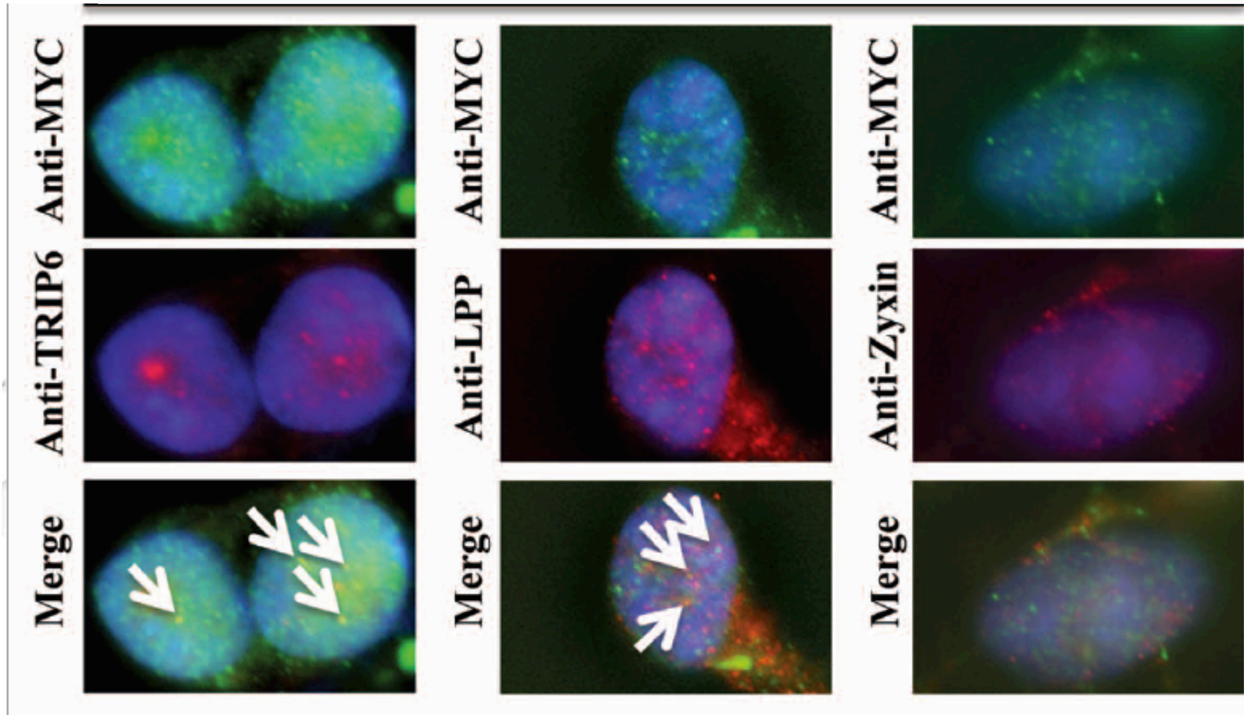
FIGURE 16 TRIP6, LPP AND PRMT1 ARE DETECTED BY TELOMERES BY CHIP.

(A) Dot blot of ChIP. Chromatin Immunoprecipitation with fixed lysates prepared from HTC75 cell lines indicated on the left. The antibodies used are listed on top (I=Immune, PI=PreImmune), and the Total DNA fraction is on the right side of each blot as indicated. Extracted DNA samples were dot-blotted on nitrocellulose and probed with a TTAGGG probe (TOP) or with an Alu probe (BOTTOM) as a control. (B) Histogram of the values for the yields as % of total DNA of the samples shown in (A). Signals were quantified by ImageQuant analysis. The percentage of precipitated DNA was calculated relative to the input signal.

Immunofluorescence

Lastly, immunofluorescence analysis of group 3 LIM domain proteins demonstrates that they have the potential to actively shuttle between the cytoplasm and the nucleus with predominant staining at steady state within the cytoplasm[72]. This ability to move in and out of the nucleus is mediated by the nuclear export sequence found in the N-terminus. With the specificity of TRIP6 and LPP at telomeres being demonstrated, we wanted to understand whether some of the nuclear pool could be detected at telomeres by immunofluorescence. To this end, Hela 1.2.11 cells stably expressing MYC-TRF1 were fixed and incubated with rabbit-LIM domain proteins TRIP6, LPP and Zyxin in conjunction with the anti-MYC antibody. The MYC antibody is used to detect levels of TRF1 at telomeres.

In addition to cytoplasmic staining of the LIM proteins, there was also punctate staining present within the nucleus (Figure 17A). For TRIP6 we found 32% of nuclei with 2 or more foci co-localizing with telomeres. For LPP we found 31% of nuclei with 2 or more co-localized foci. For Zyxin, although a degree of punctate staining was visualized and 0% of the foci indicated 2 or more foci present. 19% of the cells had one focus co-localizing with TRF1 and 83% of the cells had no colocalization present (Figure 17B).



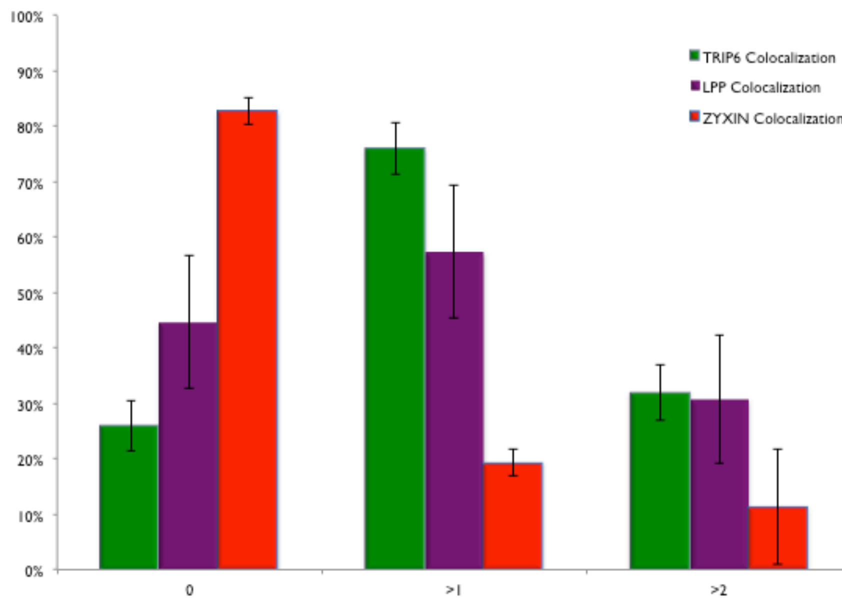


FIGURE 17 INTRANUCLEAR LOCALIZATION OF TRIP6, LPP AND ZYXIN.

A. TRF1 was detected with the MYC 9E10 antibody in the FITC channel. TRIP6, LPP and Zyxin with rabbit antibodies TRIP6 50kD; LPP 70kD; Zyxin 60kD were detected in the TRITC channel. Colocalization between shelterin protein TRF1 and appropriate rabbit antibodies result in yellow foci upon merge. Colocalization within the merge panels is also indicated using the arrows. B. Quantitation of colocalization levels in TRIP6, LPP and Zyxin of varying stringencies.

Discussion

This data suggests that TRIP6 and LPP are robustly and consistently detected at a subset of telomeres whereas Zyxin and Ajuba do not display an interaction with telomeres or the shelterin complex. This assessment is based upon the fact that TRIP6 is not found within all nuclei in the immunofluorescence assay. One possible interpretation that requires experimental corroboration is that TRIP6 and LPP associate with a subset of telomeres that are critically short. This association with telomeres could also be occurring in a highly regulated manner such as during S phase, a time at which telomerase needs to act, possibly leaving telomeres in a vulnerable state and requiring the presence of stabilizing or protecting factors such as TRIP6 and LPP.

Chapter 7 Concluding Remarks

Ultimately I have revealed that there is a new group of proteins that play a role at the telomeres and that this function is thought to confer resistance to DNA damage response factors. I have explored the specificity that is conferred with respect to telomere association for group 3 LIM proteins. TRIP6 and LPP can be found at telomeres through various assays, but the next closest family member, Zyxin, was negative. The behavior of these proteins in the prevention of eliciting a DNA damage response suggest a oncogenic role at the telomere level, which is ultimately supported by its cytoplasmic roles[74].

It would be of great interest to explore which pathway is engaged through the repression of TRIP6 and LPP, the ATM pathway or the ATR pathway. TRIP6 was shown to interact directly with POT1, the shelterin protein associated with ATR. Through subsequent we also see a strong interaction of TRIP6 with TRF2, the shelterin protein associated with repression of the ATM pathway. We can initially look at Chk1 and Chk2 phosphorylation after the depletion of TRIP6/LPP as Chk1 and Chk2 phosphorylation are downstream of ATR and ATM pathway respectively.

One question that arises from our current understanding of how TRIP6 and LPP behave at telomeres is as follows: why are TRIP6 and LPP recruited only to specific telomeres and not all telomeres? The IF and ChIP data previously mentioned allows us to speculate that based upon the quantitative levels of these LIM proteins at telomeres, TRIP6 and LPP may participate in the protection of only a subset of telomeres. We speculate that the

presence of these LIM proteins occurs in a regulated manner such as during S phase. Recent advances have increased our knowledge regarding the relationship between overhang binding factors in the regulation of telomere replication and in the prevention of DNA damage response in S phase.

It has been demonstrated that RPA, a non-specific single stranded DNA binding protein, essential for DNA replication, recombination and repair, is an effective competitor for POT1 on the telomeric overhang[103]. In vitro, the affinity of RPA for single stranded TTAGGG sequences is in fact higher than that of POT1. In early S phase, at a time of active telomere replication, RPA can outcompete POT1 for binding of the overhang. As S phase progresses, RPA is selectively removed from the overhang by hnRNPA1, allowing POT1 to bind and ultimately inhibit ATR pathway signaling. As telomeres shorten, the relationship between RPA and POT1 becomes decidedly more favorable to the presence of RPA. This is possibly due in part to the lower amounts of shelterin and therefore of POT1 in cis. The direct impact of this fluctuation may be seen through the induction of senescence. We speculate that the ratio of POT1 to RPA is important and distinct at each telomere. A low level of POT1 versus RPA, which would be experienced on short telomeres, would result in the activation of ATR due to the presence of RPA and not POT1 at the single stranded overhang (Figure 18).

An interesting speculation regarding this relationship is that POT1 and RPA are under the control of pathways with the capability to either delay the senescence point through the inhibition of ATR activation, or by influencing the binding affinity of POT1 on short

telomeres. As TRIP6 interacts with POT1 specifically at the OB folds,[104] the region that binds the overhang, *in vitro* studies on the interactions of these players on the overhang might be informative.

FIGURE 18 MODEL FOR POSSIBLE ROLES OF TRIP6 AND LPP IN TELOMERE PROTECTION

Another avenue to consider is that TRIP6 and LPP may have an affinity for short telomeres and may lower the critical point at which telomeres signal into senescence. The signal that results from critically short telomeres may in turn recruit TRIP6 and LPP and possibly PRMT1 for protection (Figure 18). Factoring in the presence of PRMT1, this protection would require the Arginine methylase activity of PRMT1 on TRF2 which has been shown to be important *in vivo*[39], or on other telomere associated proteins. The inclusion of a PRMT1 pathway is further supported by the induction of senescence that occurs upon the depletion of PRMT1 by shRNA in primary cells. This would position TRIP6, LPP and PRMT1 in a category of import in the determination of senescence in primary cells. The possible regulatory effect of group 3 LIM proteins on methyltransferases leads to testable models on the induction of senescence and in the repression of DNA damage response, principally in human primary cells.

Another hypothesis for the critically short telomere theory is that TRIP6 and LPP are recruited through interactions with POT1 or TRF2, which we have been able to detect,[104] but may not occur on t-looped telomeres. This would prevent TRIP6 and LPP from being effectively recruited at long telomeres but make the association more stable at shorter telomeres with an exposed overhang. The overexpression of each shelterin protein for the purpose of studying the ability to study TRIP6 and LPP association at telomeres of variable length may shed light on the veracity of this theory.

Our current model suggests a role for LIM proteins TRIP6 and LPP at telomeres through

the influence exerted upon shelterin proteins POT1 and TRF2. Both shelterin proteins are associated with DNA damage response pathways (ATR and ATM respectively). Within this study, specific aims were set forth and elucidated regarding a role of a previously uncharacterized relationship between LIM proteins and telomeres. Further biochemical assays regarding the specificity of these interactions would not only shed light on the behavior and regulation of these proteins at telomeres, but would also help to characterize the behavior of LIM domains in a broader sense.

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