Identifying the amino acids of telomerase that are important for recruitment by TPP1

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Finally, I would like to show appreciation to my readers, Dr. Cadigan and Dr. Csankovszki, for being wonderful professors during my time here and for enthusiastically agreeing to read my work.

Abstract

Telomerase, a ribonucleoprotein enzyme, is important for maintaining telomere length at chromosome ends. Telomerase is recruited to telomere ends through its interaction with the shelterin protein TPP1. While the TPP1 interface of this interaction is well characterized, little is known about the telomerase surface. To identify important residues, a site-directed mutagenesis screen was designed based off sequence homology and the currently available structure of *Tetrahymena* telomerase. We performed telomerase recruitment to the telomeres using an approach that combines immunofluorescence (IF) and fluorescence *in situ* hybridization (FISH). Overall, eight novel amino acids in TERT were found to be important for telomerase recruitment to telomeres, providing critical insights into how telomerase is brought to the ends of chromosomes by TPP1.

Introduction

Telomeres are protein-DNA complexes that exist at the ends of chromosomes to serve a protective function for our cells (Palm and de Lange, 2008). Telomeres are characterized by a repetitive DNA sequence (GGTTAG in humans) that does not contain any genetic information, but is bound tightly by a six-membered protein complex known as shelterin in humans. Due to the end replication problem during the cellular process of DNA replication, telomeres become shorter. Once telomere length reaches a critically short length, regulatory mechanisms in the cells prevent further mitosis. This serves as a natural mechanism to thwart unregulated cell division that can result in cancer. However, certain cells, such as stem cells, need to keep dividing over time. Thus, telomeres in these cells need to be elongated as part of DNA replication during each cell cycle.

Telomerase, a reverse transcriptase, is responsible for solving this end-replication problem. As a ribonucleoprotein complex, telomerase contains the TR (telomerase RNA that contains the template for telomere DNA) subunit and TERT protein (catalytic enzyme subunit) to perform its function in the cell to extend telomeres ((Blackburn et al., 2015; Greider and Blackburn, 1989; Lingner et al., 1997). Normally, telomerase is only activated in cells that need to divide many times such as germline and somatic stem cells. It is therefore not surprising that mutations in telomerase or other genes that help with telomerase function result in diseases associated with severely short telomeres together known as telomeropathies. However, overexpression of telomerase provides cells of a large percentage of cancers (~90%) the ability to divide indefinitely, making telomerase a target for developing anti-cancer drugs (Kim et al., 1994).

The shelterin complex, which includes TPP1, TRF1, TRF2, Rap1, TIN2, and POT1 at chromosome ends serves to protect them from the DNA damage response complexes in the cell (Palm and de Lange, 2008). The shelterin component TPP1 specifically recruits telomerase to the telomere (Wang et al., 2007; Xin et al., 2007). The surface amino acids on TPP1 that are responsible for bringing telomerase to telomeres are well characterized. TPP1 has two identified regions of amino acids that are important for telomerase recruitment, the TEL patch and the NOB region (Figure 1B)(Grill et al., 2018; Nandakumar et al., 2012). Defects in these regions have been associated with telomeropathies. Two unrelated patients with aplastic anemia or Hoyeraal-Hreidarsson Syndrome suffer from a telomerase recruitment defect due to an in-frame deletion of K170, impacting the structure of the TEL patch (Bisht et al., 2016; Guo et al., 2014; Kocak et al., 2014). Some patients with dyskeratosis congenita-like features inhabit V94I and L95Q mutations in the NOB region of TPP1 (Tummala et al., 2018).

However, the interface on telomerase that interacts with TPP1 is not well characterized. There are three regions in the TERT protein that have been linked to telomerase recruitment (S). They are the telomerase essential N-terminal (TEN) domain, the insertion of fingers domain (IFD), and the C-terminal extension (CTE)(Figure 1A)(Chu et al., 2016; Schmidt et al., 2014; Sexton et al., 2012; Zaug et al., 2010; Zhong et al., 2012). One telomerase amino acid residue that was found to be important for recruitment through a charge swap experiment was K78 (in the TEN domain) and it interacts directly with E215 within the TEL patch (Schmidt et al., 2014; Zaug et al., 2010). Furthermore, structural data from *Tetrahymena* telomerase had indicated that the TEN domain and the IFD are positioned well to interact with TPP1 (Jiang et al., 2015). However recent studies suggest that the CTE is probably playing a role in catalysis and not

binding to TPP1 (Hoffman et al., 2017; Jiang et al., 2018). Therefore, in this study, we focused on the TEN domain and the IFD to determine the telomerase residues important for interaction with TPP1 based on sequence conservation between animal, ciliates, and yeast TERT homologs.

The scope of our experiments was to provide more information about the telomerase surface amino acids that are responsible for telomerase recruitment. We identify specific amino acids within the TEN domain and the IFD that are important for telomerase recruitment to telomeres. This data may help better resolve the structure of telomerase and further characterize the telomerase-TPP1 interaction directly.

Methods

Site-Directed Mutagenesis

The QuickChange® Site-Directed Mutagenesis Kit from Agilent Technologies was used along with complementary primers from Integrated DNA Technologies. Mutations in TERT were made in the p3X-FLAG-TERT-cDNA/myc-HIsC and the pTERT-cDNA6/myc-HisC vectors. To confirm the presence of the desired mutation and the lack of other errors made during the PCR reaction process, the obtained plasmids were sequenced.

Cell Culture

IF-FISH experiments used HeLA-EM2-11ht cells. For the experiments that used stably expressed FLAG-TPP1, a derived doxycycline-inducible strain was used. To induce FLAG-TPP1, 200ng/ml of doxycycline was used to active the tetracycline-inducible promoter located in the p6x-FLAG-B14 plasmid. Cells were grown in media that contained modified DMEM (Gibco; Dulbecco's Modified Eagle Medium; 11995-065), 100U/ml penicillin, 100 µg/mL of streptomycin, and 10% FBS. Cells were kept at 37°C in the presence of 5% CO2.

Immunoblots

Immunoblotting was performed with the following antibodies: mouse monoclonal anti-FLAG M2-HRP conjugate (Sigma; A8592; 1:10,000), Myc Antibody (1:5,000), mouse monoclonal anti-FLAG M2 (Sigma; F3165; 1:10,000). To reveal the primary antibodies by using chemiluminescence detection by ECL along with reagents (Pierce ECL Western Blotting Substrate; Thermo Scientific), secondary horseradish peroxidase-conjugated goat antibodies against mouse IG was used (Santa Cruz Biotechnology; 1:10,000). Previously published

protocols for the above antibodies were used. The data was visualized using a gel documentation system (ChemiDoc MP System; Bio-Rad or Odyssey Fc; LiCor).

IF-FISH

IF-FISH for telomerase recruitment was completed using a previously used protocol in the lab with Hela-EM2-11ht FLAG-Tpp1 stable cell lines (Bisht et al., 2016). IF-FISH for telomerase assembly used a slightly modified version of the Bisht et. al. protocol (change in antibodies used and cell line). Approximately 1.2 million cells of Hela-EM2-11ht cells (with no FLAG-TPP1) in a 6-well dish were transiently transfected with 1 µg of p3X-FLAG-TERT-cDNA6/myc-HisC and 3 µg of phTRmut-Bluescript II SK (+) plasmids using Lipofectamine 2000 (Fischer; 11668019), following the manufacture provided protocol. Twenty-four hours after transfection, cells were fixed onto coverslips at room temperature with PBS containing 4% formaldehyde for 10 minutes and then washed with PBS three times. Then cells were permeabilized with 0.3% Triton X-100 in PBS for five minutes. After this, cells were blocked in 0.1% Triton X-100 and 3% BSA in PBS for a half hour. To probe for FLAG signal, cells were incubated in blocking buffer (0.1% Triton X-100 and 3% BSA in PBS) containing mouse monoclonal anti-FLAG M2 (Sigma; F1804; 1:500). The cells were washed for five minutes in PBS three times. They were then incubated with Alexa Fluor 568-conguated anti-mouse IgG (Life Technologies). The cells were washed 3 times and fixed with 4% formaldehyde in PBS for ten minutes. Then, the cells were washed again three times with PBS. After, they were dehydrated with 70%, 95%, and 100% ethanol consecutively, for five minutes each. Cells were then air-dried for three minutes and rehydrated with 2X SSC 50% formamide for five minutes. To begin probing for the telomerase RNA, cells were pre-hybridized for one hour with hybridization

buffer. Hybridization buffer contains 100mg/ml dextran sulfate, 0.125mg.ml yeast tRNA, 1mg/ml BSA, 0.5 mg/ml salmon sperm DNA, 1mM vanadyl ribonuclesoide complexes (VRC), and 50% formamide in 2X SSC. The cells were then transferred to hybridization buffer containing three Cy5-conjugated probes against TR for sixteen hours at 37°C. Then, the incubated cells were washed twice for 30 minutes per wash with 2X SSC 50% formamide. The cover slides containing the cells were then mounted on microscope slides with ProLong Gold mounting medium with DAPI (Life Technologies). A laser scanning confocal microscope with a 100X oil objective (SP5; Leica, Germany) was used to image the cells. Images were then processed using ImageJ and Adobe.

To quantify the data from the IF-FISH experiments, individual spots of FLAG-tagged protein and individual spots of TR signal were counted for each sample. The number of overlapping spots within each cell was also counted. The percentage of TR foci that had overlapping FLAG signal was then calculated. Over 100 TR foci were counted for each sample in the recruitment IF-FISH assay and over 500 TR foci were counted for each sample in the assembly IF-FISH assay.

Co-Immunoprecipitation Assay

The Co-IP used HEK 293T cells grown at 37°C, with 5% CO2, and grown in modified DMEM (Gibco; Dulbecco's Modified Eagle Medium; 11995-065). Cells in 6-well plates were transiently transfected with 2 µg of DNA per sample, 1ug of each desired construct: Myc-TEN with an empty vector, Myc-TEN with FLAG-IFD, Myc-TPP1 with an empty control vector, Myc-TPP1 with FLAG-IFD, Myc-RAP1 with FLAG-IFD. Transfection was done using Lipofectamine 2000

(Fischer; 11668019), following the manufacture provided protocol. Twenty-four hours after transfection, cells were washed once with PBS. The cells were then incubated with Trypsin-EDTA (Gibco; 25300054) for five minutes at 37°C. Cells were then harvested with 500 µl of DMEM (Gibco; Dulbecco's Modified Eagle Medium; 11995-065) and 700 µl of FBS (Gibco; 26140079). Cells were then pelleted by centrifuging at 1000 rpm for ten minutes. The media/FBS mixture was decanted and the cells were washed with PBS. Cells were then again spun down as above. The PBS was decanted and cells were resuspended in 2X IP lysis buffer. The 2X IP lysis buffer contained 50mM Tris pH 7.4, 20% glycerol, 1mM EDTA, 150mM NaCl, 0.5% Triton X-100, 0.02% SDS, 1mM DTT, 100mM PMSF, and one protease inhibitor cocktail tablet (Sigma-Aldrich, cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail; 4693159001) and is kept on ice. Then 33 µl of ice-cold 4M NaCl was added to each sample. After inversion of the tubes, 433 ul of ice-cold water was added and then the sample was inverted again to mix. The samples were then centrifuged for ten minutes at 4°C at 13,000 g. The 2X IP lysis buffer was then diluted with water to make 1X IP lysis buffer. ANTI-FLAG beads (Sigma-Aldrich ANTI-FLAG M2 Affinity Gel; A2220) were used in the Co-IP. 30 µl of beads were used for each sample. The FLAG beads were washed three times with 1X IP lysis buffer and then resuspended in 100 µl of 1X IP lysis buffer for each sample. The tubes with the beads were then spun down and the supernatant was pulled off. The supernatant off the samples that were spun down were then separated into two tubes, one for the inputs (40 µl of sample) and one for the pull-down (760 µl of sample). An equal amount of 800 µl of supernatant was pulled off for each sample. The extract what was saved for the pull down was then incubated with the ANTI-FLAG beads. The samples were left nutating with the ANTI-FLAG beads in 4°C for about 16 hours. After this, the

cell samples were pelleted at 1000 rpm and the supernatant was discarded. The samples were then washed three times with 1X IP lysis buffer. 30 µl of 2X SDS dye with BME was then added to the sample and mixed. The cell extracts where then boiled for 10 minutes, spun down at 13,000 rpm for ten minutes, and then loaded and run on an SDS-PAGE gel for immunoblotting analysis. 20 µl of SDS dye with BME was also added to the input samples. Those samples were then boiled for ten minutes and then loaded onto an SDS-PAGE gel for immunoblotting analysis.

Results

A site-directed mutagenesis screen was designed to determine important amino acid residues on the hTERT surface. These residues were located in the TEN domain and the IFD region. Using sequence homology and the cryo-EM structure of *Tetrahymena* telomerase as a homology model of human TEN domain and IFD, we chose to mutate residues that were predicted to be on the surface of these domains (Figure 1C, 2A, 2B). In the screen, we selected twelve mutations in the IFD, three of which were single mutants and twelve of which were double mutants (Figure 2B). The TEN domain screen contained nineteen mutations, three of which were double mutations and sixteen of which were single mutations (Figure 2A). Throughout the experiments, we used the K78 TEN domain mutation as a positive control as it was previously shown to be defective in the telomerase-TPP1 interaction (Schmidt et al., 2014).

We first wished to determine if the hTERT mutants disrupted the ability of TPP1 to recruit telomerase to telomeres. To determine if the telomerase-TPP1 interaction was disrupted, we used immunofluorescence (IF) and fluorescence *in situ* hybridization (FISH) in the Hela-EM2-11ht derived cell line which stability expressed Flag-TPP1. Untagged WT or mutant hTERT was transiently transfected into our cell line, as well as telomerase RNA, hTR. We utilized IF to detect our FLAG TPP1 and the FISH to detect the hTR. If the signals for the two overlapped, then we assumed that telomerase was recruited to the telomere by TPP1. In our wildtype condition, we saw a high percentage (77%) of TR foci located to telomeres (Figure 3, Figure 5, Table 1, and Table 2). Our positive control, K78E, indicated a defect in recruitment as it showed only 10% of TR colocalizing with telomeres (Figure 3, Table 1). The mutants in the TEN domain that did show defects in recruitment were N125A, T128I, L139A, L140A, and R143A (Figure 3A,

Table 1). The mutants in the IFD that showed defects in recruitment were V790A/I792A, E793A/Q794A, L805A/F806A, and V818A/R819A (Figure 5A, Table 2). To note, the percentage of recruitment for all of our defective mutants were much lower than that of K78E. In summary, we identified five residues in the TEN domain and four double mutants in the IFD that all showed a reduced ability for telomerase to localize to telomeres.

It is known that when telomerase is not recruited to telomeres, it exists within Cajal bodies in cells (can be identified as the large non-recruited dots in our experiments) (Nandakumar and Cech, 2013). However, hTR without its hTERT component can locate to Cajal bodies as well. So, we next wanted to examine whether the defects we observed (through the visualization of Cajal bodies) in some of our mutants was due to a defect in the assembly of hTR with its protein hTERT unit or instead due to a true interruption of the TPP1-telomerase interaction. We used a Hela-EM2-11ht derived cell line (no FLAG TPP1) and transiently transfected in FLAG-TERT WT or FLAG-TERT mutants and hTR. Again, we utilized the IF to detect FLAG-TERT and the FISH to detect hTR signals and considered 50% co-localization between TERT and TR to be assembled. Our WT TERT showed high levels of assembly, along with our previous positive control K78E. Within the TEN domain, we saw that TERT colocalized with TR in Cajal bodies for N125A, T128I, L136A, and L140A, indicating that the ribonucleic protein was probably properly assembled (Figure 4, Table 1). We saw this occur within the following IFD mutants: V790A/I792A and E793A/Q794A (Figure 6, Table 2). These results suggest that the above-mentioned mutations have a true impact on the recruitment interaction of telomerase and TPP1. The remaining two mutations, R143A in the TEN domain and V818A/R819A, show a lack of co-localization between the protein subunit and RNA subunit of telomerase (Figure 4,

Figure 6, Table 1, and Table 2). This indicates that the defect in recruitment that was seen was not due to an interruption of the TPP1-telomease interaction but rather due to the upstream effect of poor protein assembly. While it is still possible that these residues are important for telomerase recruitment, that cannot be determined at this time.

To complement the above experiments, I decided to use co-immunoprecipitation experiments to directly assess binding between regions within TERT and binding with TPP1. Based off the cryo-EM structure of the *Tetrahymena* telomerase, the TEN and IFD domains interact closely with each other within the TERT subunit (Figure 1C). Some of our mutants appear to fall on TEN-IFD interface, so it is possible that the defects we observed were due to disruption of the TEN-IFD interaction. To tease this complexity out, I choose to use co-immunoprecipitation to test if the isolated TEN domain would precipitate with the IFD domain in the absence and presence of the identified mutations. However, I was not able to get the control WT TEN domain and WT IFD domain co-immunoprecipitation experiment to work. The domains were expressed in our Hela cell line, where the TEN domain expressed better than the IFD domain (Figure 7C, commonly seen in other experiments we have performed as well). I found that there was expression of all of our FLAG constructs in this framework (Figure 7A, right), as well as the Myc-tagged constructs (Figure 7B, left). The experiment also indicated that the FLAG-tagged constructs were being enriched in the immunoprecipitation (Figure 7A, left). However, it does not appear that the FLAG tagged IFD was able to pull down the MYC-tagged TEN domain in our immunoreaction system (Figure 7B). Surprisingly, FLAG-TEN domain was also not able to down TPP1, even though the TEN domain directly interacts with TPP1 (Schmidt et

al., 2014) (Figure 7B, left). This most likely indicates that a co-immunoprecipitation with the individual domains may not be a suitable avenue to assay this interaction.

Figures and Tables

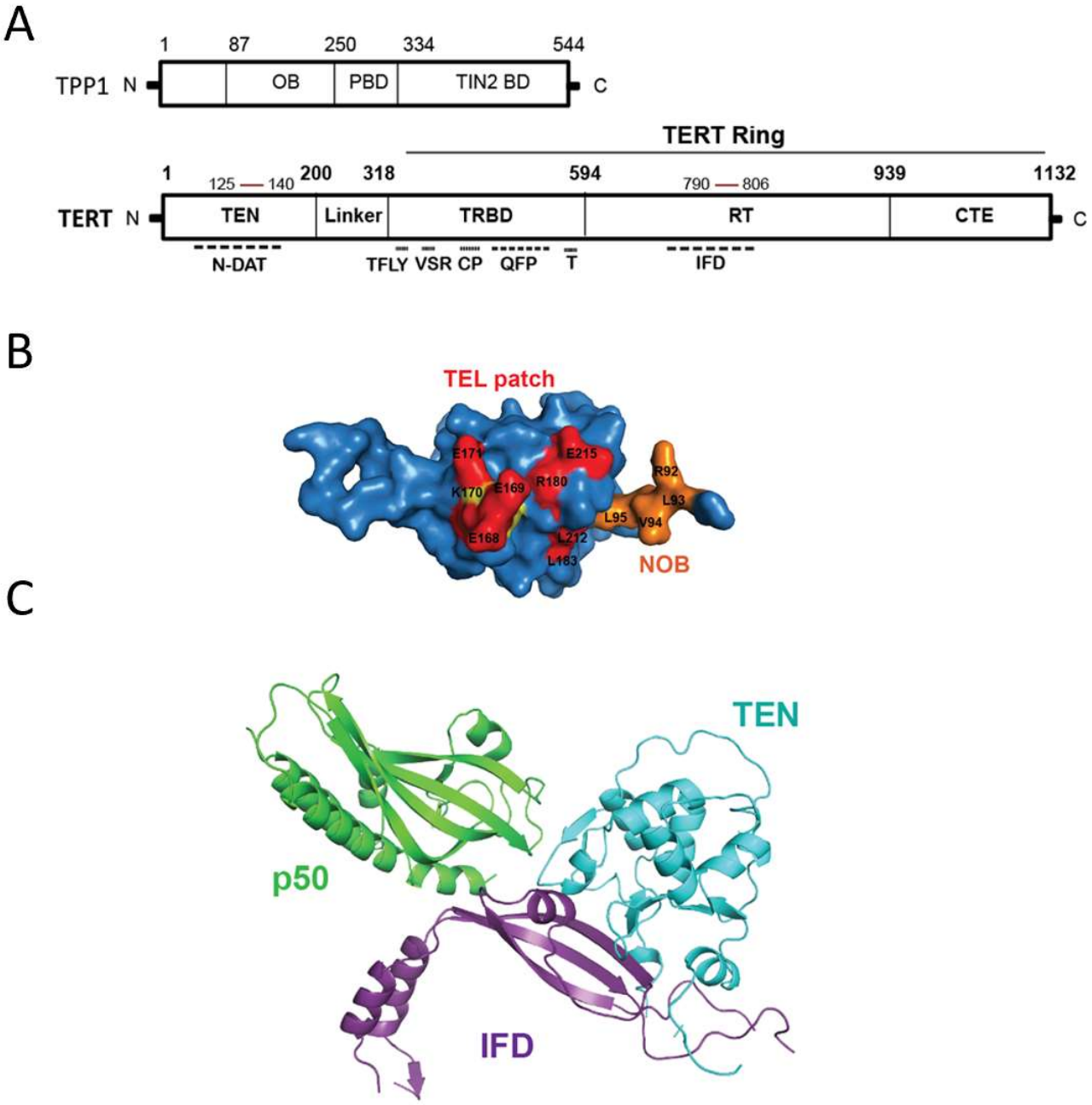


Figure 1: Important domains in the telomerase-TPP1 interaction

(A) Domain diagram of TPP1 and TERT. (B) A visual representation of the solved crystal structure of the OB domain of TPP1 (PDB 2146), illustrating the TEL Patch in red and NOB in orange. K170 is in yellow. (C) A visual representation of the solved Cryo-EM structure of the *Tetrahymena* telomerase (PDB ID: 6D6V) indicating the TEN domain in teal, the IFD in purple and p50 (a TPP1 homolog) in green. *Figures A, B, and C were adapted from Eric M. Smith, 2018.*

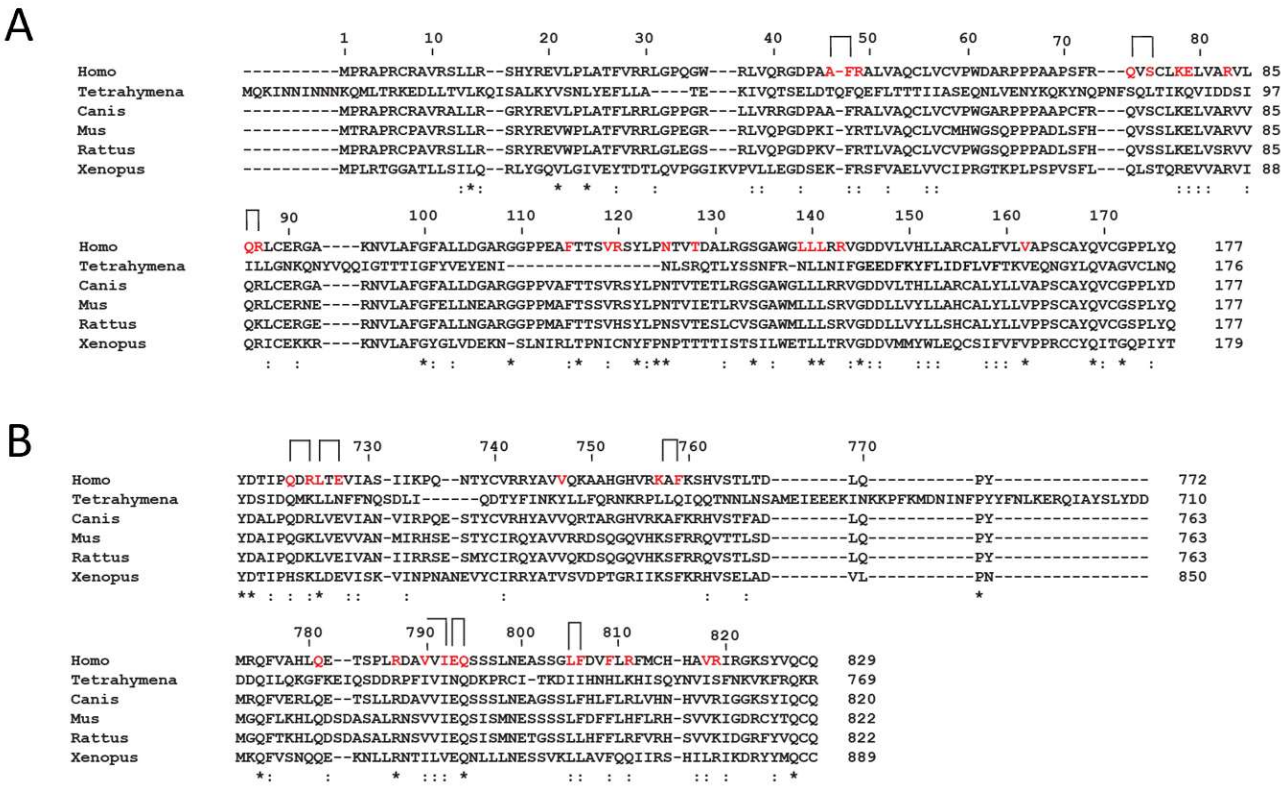


Figure 2: Sequence Homology Mutagenesis Screen

(A) A sequence alignment of the TEN domain with eukaryotic TERT homologs. Alignment wa completed by ClustalW and further refined. Red indicated mutated residues and brackets indicate double mutants. (B) A sequence alignment of the IFD domain with eukaryotic TERT homologs. Alignment was completed by MUSCLE and further refined. Red indicated mutated residues and brackets indicate double mutants. *Figures A and B were adapted from Eric M. Smith, 2018.*

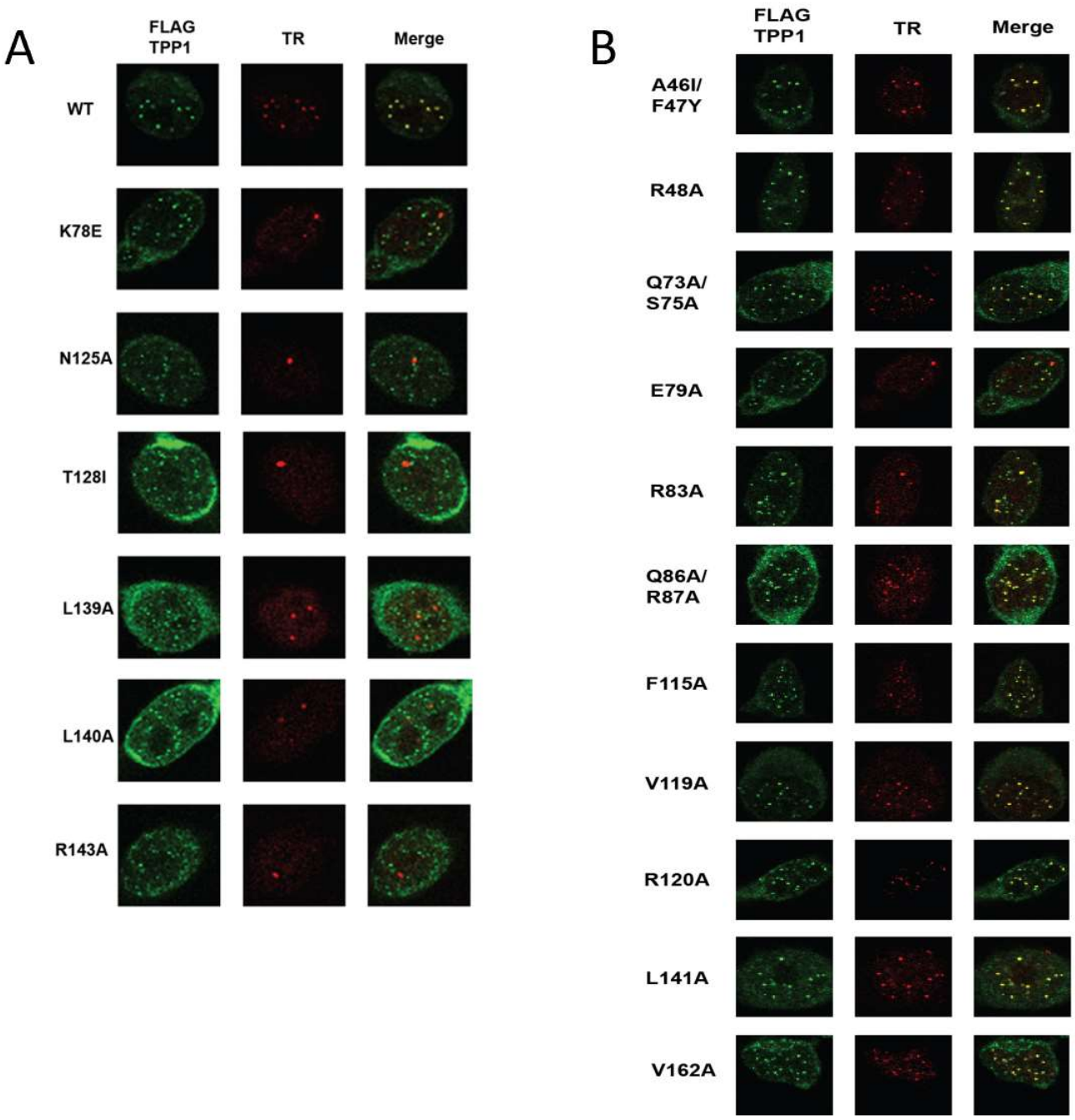


Figure 3: IF-FISH used to determine telomerase recruitment in the TEN Domain mutants

Telomerase recruitment to telomeres was analyzed using immunofluorescence-fluorescence *in situ* hybridization using stable HeLa cells overexpressing FLAG TPP1. Untagged TERT mutants and telomerase RNA (TR) was transiently transfected as indicated. The green spots indicated

FLAG-TPP1 and the red indicated TR foci. Yellow foci in the merge signify recruitment of TR to the telomeres. Quantification is found in Table 1. (A) These TEN domain mutants indicated recruitment defects. (B) These TEN domain mutants indicated wild type recruitment. *Figure was adapted from Eric M. Smith, 2018.*

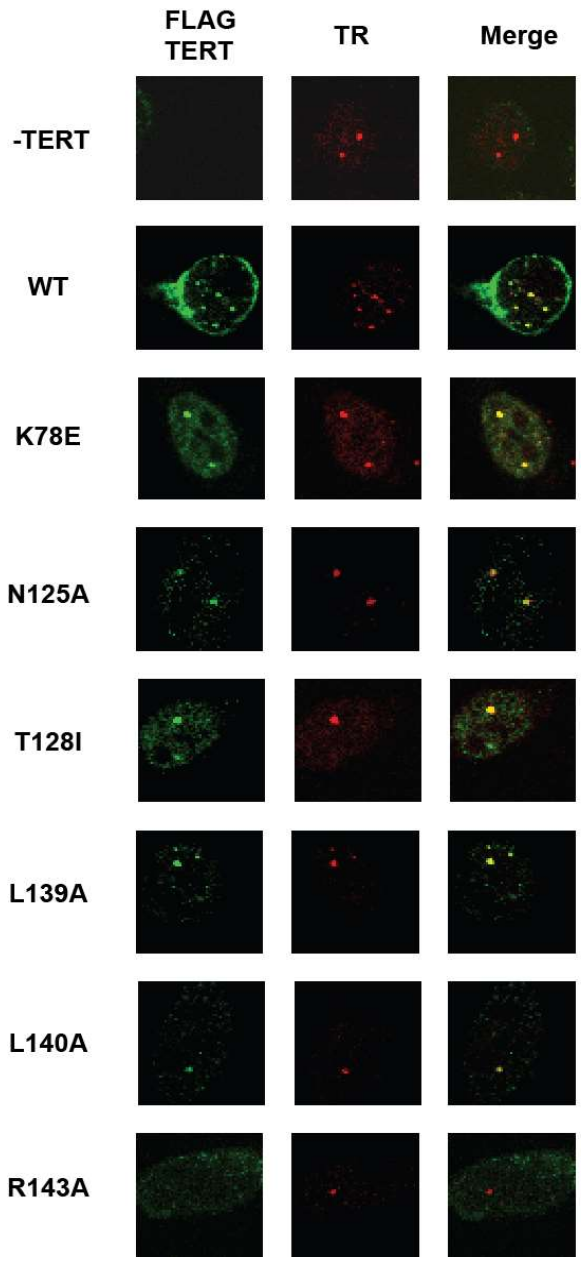


Figure 4: IF-FISH to determine telomerase assembly in TEN domain mutants that are defective in recruitment

Telomerase assembly was analyzed in for telomerase TEN domain recruitment mutants using immunofluorescence-fluorescence *in situ* hybridization in HeLa cells. FLAG-TERT mutants and telomerase RNA (TR) were transiently transfected as indicated. The green spots indicate FLAG-

TERT and the red indicate TR foci. Yellow foci in the merge signify the colocalization of the protein and RNA subunit of telomerase. *Figure was adapted from Eric M. Smith, 2018.*

Mutation	% Recruited	%Assembled
Wild Type	77	91
A46I/F47Y	91	N/A
R48A	80	N/A
Q73A/S75A	75	N/A
K78E	10	84
E79A	41	N/A
R83A	66	N/A
Q86A/R87A	70	N/A
F115A	80	N/A
V119A	72	N/A
R120A	75	N/A
N125A	1	73
T128I	2	75
L139A	3	61
L140A	2	71
L141A	47	N/A
R143A	0.4	0
V162A	36	N/A

Table 1: Summary of telomerase recruitment and assembly quantification in the TEN domain.

This table presents quantification of the data represented by the images in Figures 3 and 4.

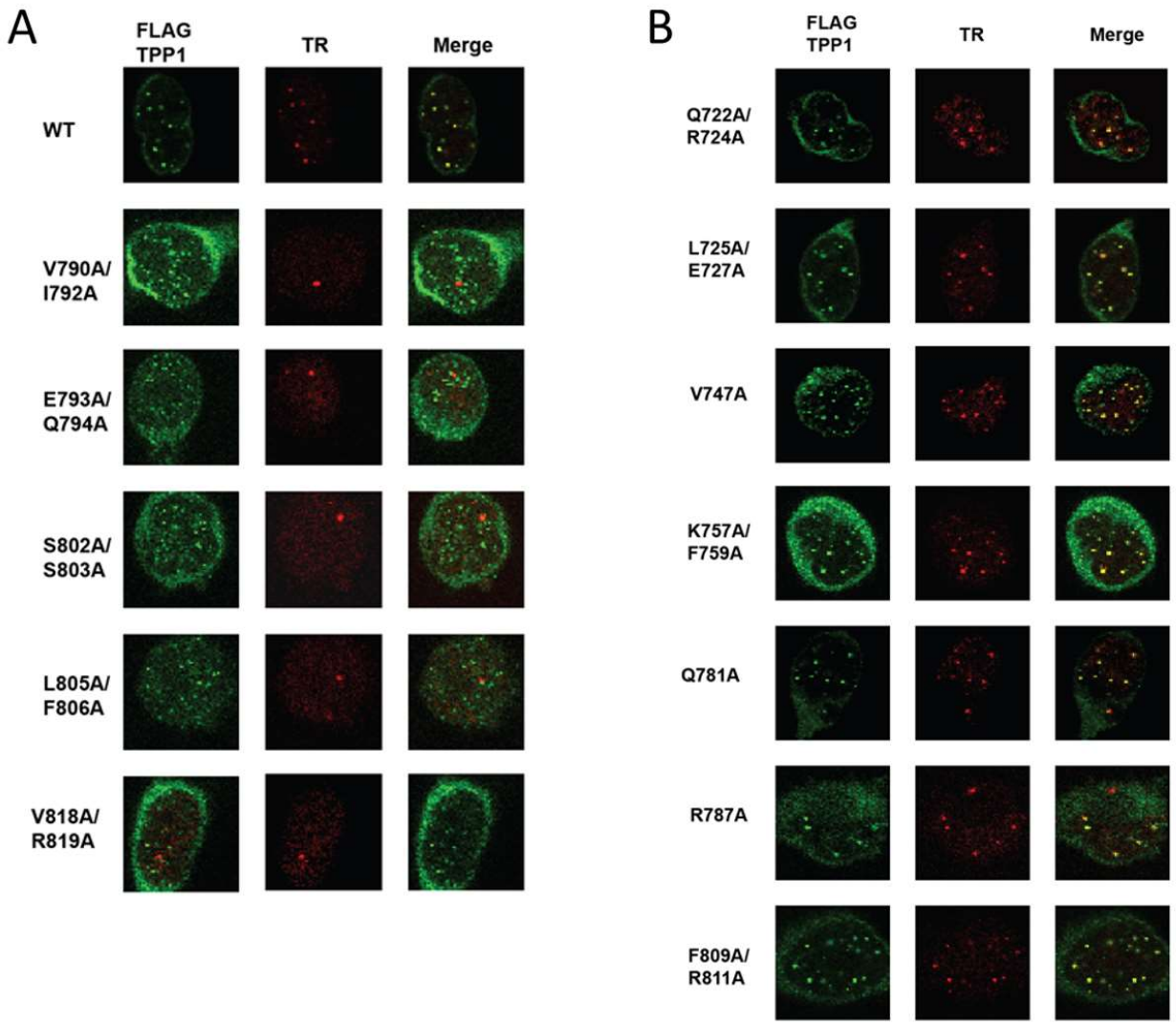


Figure 5: IF-FISH used to determine telomerase recruitment in the IFD mutants

Telomerase recruitment to telomeres was analyzed using immunofluorescence-fluorescence *in situ* hybridization using stable HeLa cells overexpressing FLAG TPP1. Untagged TERT mutants and telomerase RNA (TR) was transiently transfected as indicated. The green spots indicated FLAG-TPP1 and the red indicated TR foci. Yellow foci in the merge signify recruitment of TR to the telomeres. Quantification is found in Table 1. (A) These IFD domain mutants indicated

recruitment defects. (B) These IFD domain mutants indicated wild type recruitment. *Figure was adapted from Eric M. Smith, 2018.*

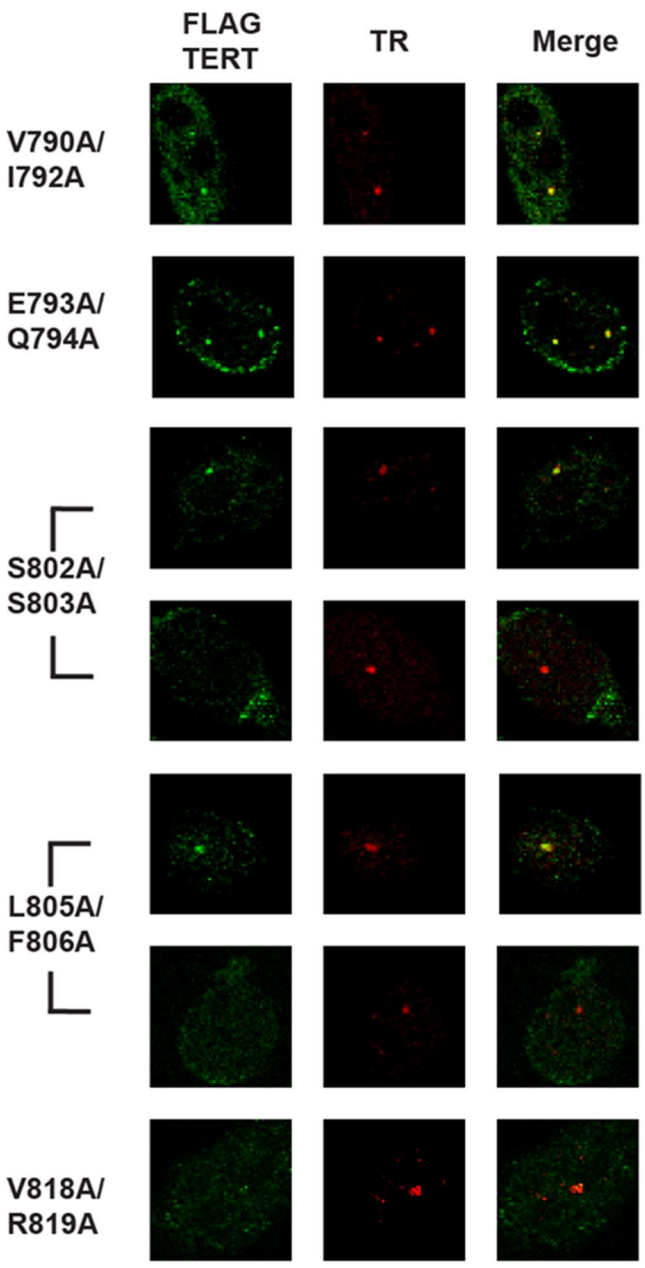


Figure 6: IF-FISH to determine telomerase assembly in IFD mutants that are defective in recruitment

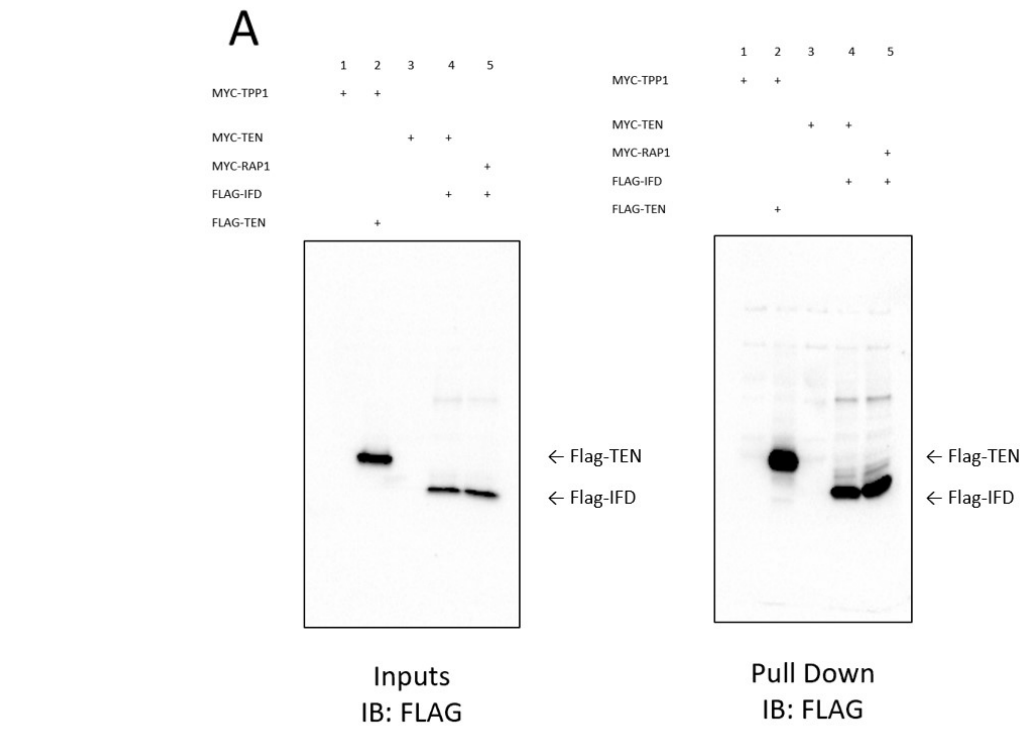
Telomerase assembly was analyzed in for telomerase TEN domain recruitment mutants using immunofluorescence-fluorescence *in situ* hybridization in HeLa cells. FLAG-TERT mutants and

telomerase RNA (TR) were transiently transfected as indicated. The green spots indicate FLAG-TERT and the red indicate TR foci. Yellow foci in the merge signify the colocalization of the protein and RNA subunit of telomerase. Two panels were shown for S802A/S803A and L805A/F806A to illustrate how the mutant demonstrated both proper assembly and an assembly defect. *Figure was adapted from Eric M. Smith, 2018.*

Mutation	% Recruited	%Assembled
WT	77	91
Q722A/R724A	52	N/A
L725A/E727A	74	N/A
V747A	42	N/A
L757A	69	N/A
Q781A	58	N/A
R787A	40	N/A
V790A/I792A	0.7	71
E793AQ794A	1.5	85
L805A/F806A	1.5	49
F809A/R811A	78	56
V818A/R819A	7	34

Table 2: Summary of telomerase recruitment and assembly quantification in the IFD domain.

This table presents quantification of the data represented by the images in Figure 5 and 6.


A
1 2 3 4 5
MYC-TPP1 + +
MYC-TEN + +
MYC-RAP1 +
FLAG-IFD + +
FLAG-TEN +
← Flag-TEN
← Flag-IFD
Inputs
IB: FLAG
1 2 3 4 5
MYC-TPP1 + +
MYC-TEN + +
MYC-RAP1 +
FLAG-IFD + +
FLAG-TEN +
← Flag-TEN
← Flag-IFD
Pull Down
IB: FLAG

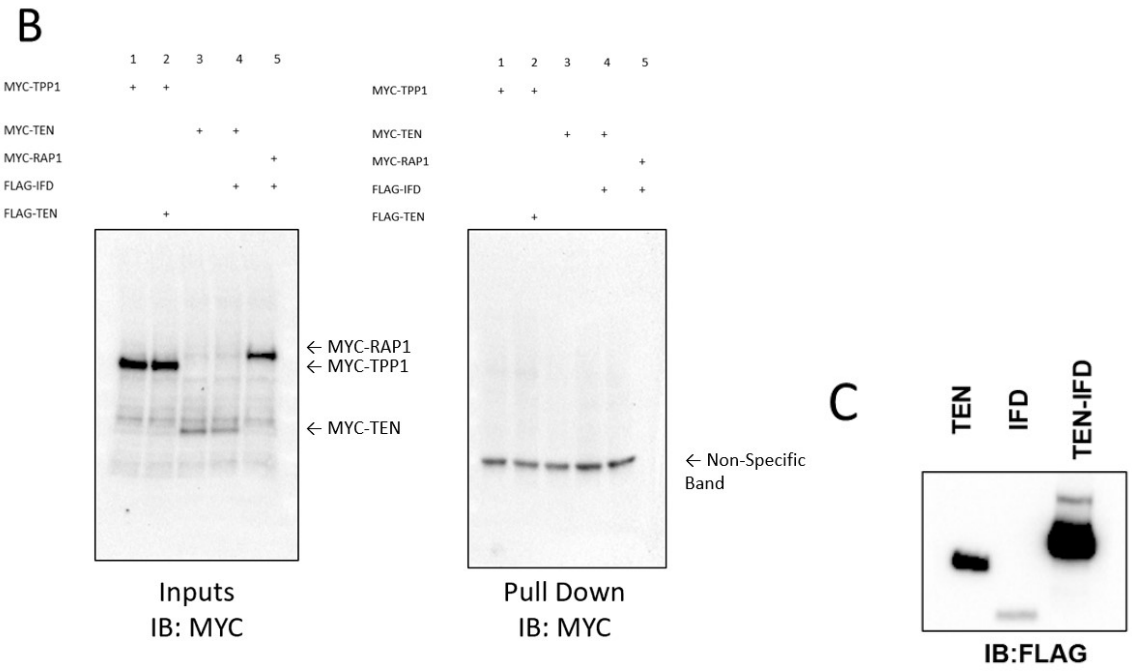

B
1 2 3 4 5
MYC-TPP1 + +
MYC-TEN + +
MYC-RAP1 +
FLAG-IFD + +
FLAG-TEN +
← MYC-RAP1
← MYC-TPP1
← MYC-TEN
Inputs
IB: MYC
1 2 3 4 5
MYC-TPP1 + +
MYC-TEN + +
MYC-RAP1 +
FLAG-IFD + +
FLAG-TEN +
← Non-Specific Band
Pull Down
IB: MYC
C
TEN
IFD
TEN-IFD
IB:FLAG

Figure 7: Co-Immunoprecipitation of the TEN domain and IFD in telomerase

(A) The inputs and output of the Co-IP probed with Anti-Flag antibodies. The data indicates that all FLAG-tagged constructs were expressed and then enriched on the anti-FLAG beads. (B) The inputs and outputs of the Co-IP probed with Anti-Myc antibodies. The data indicates that all Myc tagged constructs expressed but were not co-immunoprecipitated with the FLAG constructs. A non-specific band is seen in the Myc blot, potentially due to non-specific binding of the antibody. (C) Expression levels of the TEN domain, IFD, and the TEN-IFD constructs.

Figure C was adapted from Eric M. Smith, 2018

Discussion and Future Directions

Our study identifies important residues on telomerase that are important for recruitment to telomeres. We mapped these residues through the use of a site-directed mutagenesis screen based off sequence homology and the structure of the *Tetrahymena* telomerase. We used IF-FISH assays to first determine which of our mutations led to defects in the recruitment of telomerase to telomeres by measuring if our TR foci localized with TPP1 in Hela cells. To further examine the recruitment defects, IF-FISH was again used to determine if telomerase was properly assembled. With these two assays, nine resides were found to be important in the recruitment of telomerase to telomeres. Due to the loss of recruitment to TPP1, it seems that these residues on telomerase are important for the telomerase-TPP1 interaction. It may even be possible that they are interacting with the NOB or TEL patch of TPP1.

However, these experiments alone do not determine if the amino acids residues are important for a direct interaction with TPP1 or rather instead a stabilizing interaction between the TEN domain and the IFD within telomerase. To better tease out if the defects were due to interactions between the TEN domain and the IFD or the interaction with TPP1, it may still be useful to optimize the use co-immunoprecipitation experiments. One reason this experiment may have failed is due to the hydrophobicity of the IFD. This may lead to misfolding of the domain, making it difficult to see the interaction. I had used RAP1 protein as a control in this experiment as it does not directly interact with telomerase and could therefore serve to detect any non-specific interactions. So, if the IFD had been misfolded in a way that made it bind non-specifically to other proteins via hydrophobic interactions, then that would be seen through the

immunoprecipitation of RAP1 with the IFD. Based on the results, it does not seem that the IFD is misfolded to the point of being grossly non-specific in its interactions as it does not immune-precipitate any protein we tested (Figure 7B, left, lanes 4 and 5). So, if the IFD is misfolded, this misfolding does not make it more likely to bind other cognate or non-cognate protein partners. Interestingly, when the IFD is linked to the TEN domain, there is strong expression of this construct (Figure 7C). It is possible that the IFD needs to be in close proximity to the TEN to fold correctly but this relationship is not necessary reciprocally (data not shown).

While the co-immunoprecipitation experiment has not worked yet, it still might be possible to optimize the conditions in the future. One potential approach may be to change the boundaries of the TEN and IFD domains and see if this impacts the expression and stability of the constructs. Another potential approach would be to link the TEN domain and IFD with a linker that is cleavable. This way, the expression of both domains would be higher due to the increased stability we saw but the domains could potentially be separated from each other, and purified individually to be used in a pull-down experiment.

In addition, many of the important residues found in the IFD were double mutants we created. I would be especially be interested in separating these double mutants that were found to be defective in recruitment, such as V790A and I792A, even if they had an assembly defect to look at this interaction between the TEN domain and the IFD. It may be possible (though potentially unlikely due to the structure of the IFD and TEN domain) that one of the residues may be more important than the other or even that only one of the residues is truly responsible for the defect. Currently, I am in the process of separating the E793A/Q794A mutant.

Overall, this project identified important residues for telomerase recruitment on the surface of telomerase. This provides more insight into the surface of the TERT protein, as well as how it may be interacting with the NOB and TEL patch of TPP1. This information may be useful in the future, when looking for a way to inhibit overzealous telomerase activation in cancer cells, for example.

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