Understanding the Roles of POT1a and POT1b in Telomere Replication

Supervisor: Dean Sandy Chang

Abstract

Telomeres are protein-nucleic acid complexes that cap the ends of all eukaryotic chromosomes and are essential for the maintenance of genome stability. However, the mechanisms in which they are replicated are not yet fully understood. In humans, the POT1 (protection of telomeres 1) protein plays important roles in binding to the single-stranded overhang to prevent the activation of a DNA damage response and aberrant telomere replication. I hypothesize that the two mouse POT1 homologs, POT1a and POT1b, play analogous functions at telomeres. To test the hypothesis that deletion of POT1a, POT1b, or both proteins impact telomere replication in mouse tumor breast cells, I will examine for the presence of fragile telomeres and stalled replication forks at telomeres, both signs of telomere replication defects. Understanding how POT1a and POT1b function to maintain telomere replication will yield insights into how components of the shelterin complex evolved to maintain genome stability.

Background and Significance

Telomeres are the protective ends of chromosomes, and proper functions at telomeres are important in preventing abnormal growth and cellular aging. Dysfunctional telomeres are found in many diseases, such as the premature aging disease Werner Syndrome (Chang et al., 2004) and many forms of cancer, including but not limited to, chronic lymphocytic leukemias (CLL), familial melanomas (FM), breast cancer, and brain cancer (Gu et al., 2017). An understanding of how telomeres are maintained would help broaden insight into cancer mechanisms and direct the development of various cancer therapies.

Telomeres are major factors in determining the lifespan of a cell. During DNA replication, DNA polymerase can only add nucleotides in the 5' to 3' direction. DNA polymerase can replicate the leading TTAGGG G-strand, where there is a continuous replication in a 5' to 3' manner. However, since replication of the lagging C-strand requires initiation by the Okazaki RNA primer, there is always a loss of sequence information at the very end of the C-strand when the RNA primer is degraded. The inability of DNA polymerase to add nucleotides to the very end of the C-strand creates the "end-replication problem" and progressive telomere shortening with each round of cell division. Critically shortened telomeres are recognized as damaged DNA, triggering p53-dependent apoptosis and senescence (O'Connor, 2008).

The enzyme telomerase solves the end-replication problem. Telomerase is a reverse transcriptase, and it lengthens the 3' G-strand with DNA telomeric repeats copied with an internal RNA template. With the 3' G-strand overhang now elongated, DNA polymerase can now replicate the very end of the C-strand, preserving the length of the DNA and allowing for complete replication of telomeres (O'Connor, 2008). Telomerase expression is tightly regulated and is normally inactive in somatic cells, but it is active in stem and germline cells. Telomerase reactivation is the major mechanism in which cancer cells can maintain telomere length and remain immortal (Shammas, 2011).

On the structural level, telomeres are bound and regulated by a group of six proteins, known as the shelterin complex (Figure 1). The main role of shelterin is to solve the "end-protection problem" by preventing the activation of a DNA damage response at telomeres. Because the 3' overhang is a single-stranded piece of DNA, the cell may incorrectly identify the overhang as a damaged piece of DNA that needs repair, leading to the activation of DNA damage responses (DDR). There are multiple DNA enzymes and double-stranded break (DSB)

repair pathways that would attempt to repair the telomere. However, this will result in abnormalities, such as telomere sister chromatid exchanges (T-SCE) and end-to-end chromosome fusions (de Lange, 2018). One way in which telomeres hide the overhang to prevent DDR is with the formation of a t-loop. The single-stranded portion of the telomere gets tucked into the double stranded portion of telomeres, creating a loop and hiding the overhang (Wu et al., 2006).

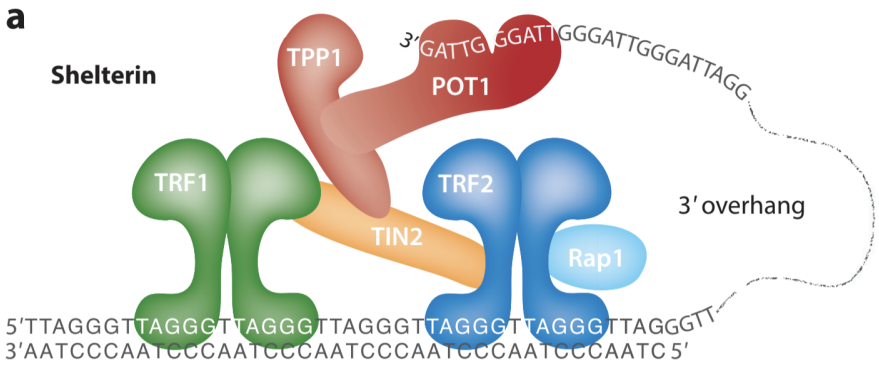

Figure 1: The shelterin complex (from de Lange, 2018). The shelterin complex at telomeres consists of 6 proteins. Telomeres consist of TTAGGG repeats and a 3' overhang.

The six proteins in the shelterin complex are TRF1 (telomere repeat factor 1), TRF2 (telomere repeat factor 2), Rap1 (Repressor / Activator Protein 1), TIN2 (TRF1-interacting nuclear protein 2), TPP1 (TIN2- and POT1-organizing protein), and POT1 (protection of telomeres 1). TRF1, TRF2, TIN2, and Rap1 form the core of the complex. TRF1 prevents replication fork stalling, and TRF2 prevents the activation of aberrant telomere repair. Rap1 binds to TRF2 to enhance its functions, and TIN2 stabilizes TRF1 and TRF2. TPP1 forms a heterodimer with POT1 and functions to recruit telomerase to telomeres (de Lange, 2018).

POT1-TPP1 are especially crucial shelterin components and bind to the 3' overhang. The heterodimer prevents genome instability, including aberrant homologous recombination. In mice, there are there are two POT1 homologs: POT1a and POT1b. These homologs play analogous roles as in humans. All POT1 proteins contain conserved domains consisting of 3 OB (oligonucleotide/oligosaccharide) folds and a Holliday junction resolvase-like junction that interacts with the 3' single-stranded telomere overhang. Mutations in the OB folds results in the activation of a DDR and telomere instability, suggesting that POT1 is required to maintain genome stability (Gu et al., 2017).

While shelterin helps to prevent the activation of a DDR, a problem arises during telomere replication. Because shelterin binds to telomeres, replication forks stall because of the physical barrier presented by shelterin (Figure 2). This results in incomplete replication and generation of fragile telomeres, which are locations where gaps and breaks are likely to form, inducing replicative stress. There are enzymes, such as the SMARCAL1 DNA translocase, that bind to stalled replication forks and aid in repairing DNA and restarting replication at these forks (Poole et al., 2015). TRF1 is known to play a major role in telomere replication by recruiting the helicases BLM and RTEL1 to reduce the formation of fragile telomeres. It is thought that POT1 interacts with TRF1 during telomere replication. Furthermore, POT1 competes with RPA (replication protein A), a protein that is recruited in the ATR DNA damage response pathway, to bind to telomeres. Preventing RPA binding to the single-stranded overhangs by POT1 is thought to be important for proper telomere replication (Sfeir et al., 2009). However, additional research is needed to determine the role that POT1 plays during telomere replication.

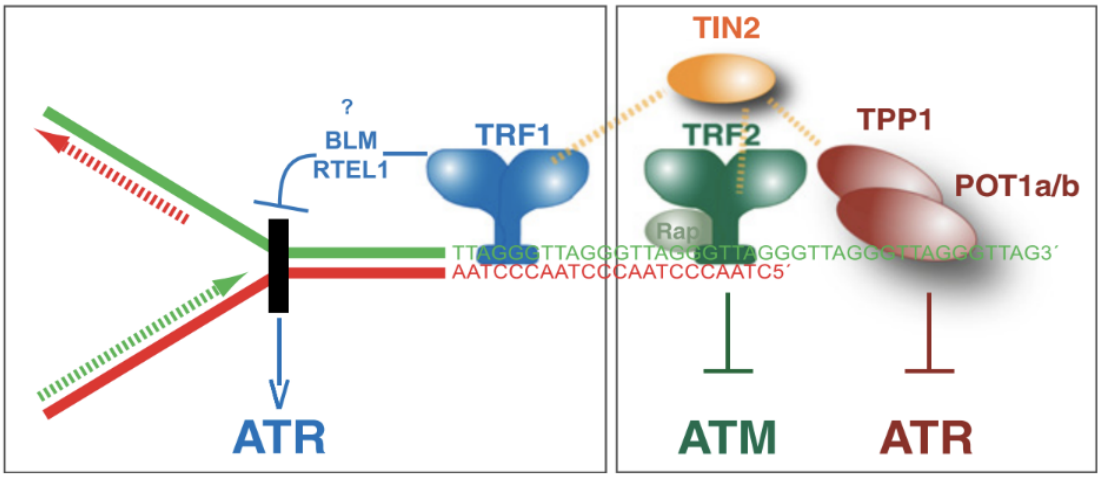

Figure 2: Replication fork at telomeres (from Sfeir et al., 2009). The replication fork at telomeres is stalled because of the binding of shelterin to telomeres.

Hypothesis

I hypothesize that the deletion of POT1a, POT1b, or both will result in telomeric replication defects in mouse tumor breast cells.

Specific Aim 1: Characterization of telomere replication phenotypes when POT1a, POT1b, or both are deleted from telomeres.

Rationale: While the role of TRF1 in telomere replication has already been studied, the role of POT1a and POT1b in telomere replication is currently unknown. Furthermore, studies from the Chang lab have shown that various similarities and differences exist between the POT1 proteins. While both are thought to work together to repress the NHEJ (non-homologous end joining) DNA repair pathways (Wu et al., 2006), there are some differences in their primary functions.

POT1a mainly prevents DNA damage response to maintain telomere stability. Throughout the entire cell cycle, POT1a represses the ATR pathway by preventing RPA from binding to telomeres (de Lange, 2018), preventing the activation of aberrant homologous recombination (HR), t-loop HR, end-to-end chromosome fusions, and p53-dependent senescence (de Lange, 2018). Furthermore, POT1a is known to have a role in telomere replication by interacting with the WRN protein, which is a part of the RecQ helicase family. WRN is thought to be required to unwind G-quadruplexes during telomere replication (Wu et al., 2006).

The primary purpose of POT1b is to regulate the elongation of the 3' single-stranded telomere DNA. POT1b recruits the heterotrimer CST complex, which contains the CTC1, STN1, and TEN1 proteins, to telomeres. The CST complex assists Polα/primase, an enzyme that can start DNA synthesis and replication, to help synthesize the telomere 5' C-strand. Without POT1b, the C-strand will not be elongated, resulting in an abnormally elongated 3' single-stranded G-strand, and eventually total telomere length shortening (de Lange, 2018).

These differences suggest that POT1a and POT1b might play unique roles during telomere replication. Deletion of either one of them or both are required to differentiate and quantify their individual effects during replication. I expect that deletion of POT1a will result in more telomere fragile sites and telomere dysfunction induced foci (TIFs) than deletion of POT1b because POT1a directly affects a potent DDR pathway and works to directly prevent the formation of secondary structures called G-quadruplexes that might break the G-strand. Since POT1b is needed to replicate the C-strand, replication forks might stall in the absence of POT1b. I anticipate that deletion of both POT1a and POT1b will result in the greatest amount of aberrant telomere phenotypes.

To test these notions, I will generate $POT1a^{-/-}$ and $POT1b^{-/-}$ mouse embryo fibroblasts (MEFs) from POT1a and POT1b null mice previously generated in the Chang lab (Wu et al.,

2006). As a positive control, wild type (WT) MEFs treated with aphidicolin will be generated (cells treated with aphidicolin are defective in replication and resemble cells with common fragile sites) (Sfeir et al., 2009). This cell line can serve as a comparison to MEFs lacking the POT1 proteins (Wu et al., 2006). As a negative control, a cell line with wild type cells that are not treated with anything will be analyzed.

To determine the presence of fragile telomeres, I will use chromosome orientation fluorescence *in situ* hybridization (CO-FISH) to look at telomeres in WT, POT1a$^{-/-}$, POT1b$^{-/-}$, and in WT cells treated with aphidicolin during metaphase. If telomeres are replicated properly, there will be a single fluorescent-colored signal from a chromatid end. This signal should be approximately equal in intensity to its corresponding sister chromatid. If telomeres are not replicated normally, as I would expect in POT1a$^{-/-}$, POT1b$^{-/-}$, and in WT cells treated with aphidicolin, multiple telomere signals would be present, indicating the presence of fragile telomeres because these telomeres are likely broken apart or is no longer condensed. The number of multiple telomere signals in each of the different scenarios will be counted to quantify the relative effects POT1a and POT1b have on telomere phenotype (Sfeir et al., 2009).

In a second experiment, I will test for the fragile telomere phenotype by performing a telomere dysfunction-induced foci (TIF) assay. I will use antibodies to the DNA damage marker proteins 53BP1 and γ-H2AX to detect their localization to telomeres, because these proteins are indicators of double strand breaks. Finding 53BP1 and γ-H2AX at telomeres would suggest the presence of fragile telomeres. The number of TIFs will be compared to determine the degree in which the POT1 proteins affect telomere replication (Mender and Shay, 2015).

Specific Aim 2: To determine whether deletion of POT1a or POT1b increase replication stress at telomeres.

Rationale: An important indicator of the stalling of the replication fork at telomeres is the activation of the ATR DNA damage responses. Because POT1a is involved in repressing the ATR DDR and POT1b is involved in regulation of the 3' single-stranded G-overhang, these two proteins likely play different roles in ATR-dependent telomere replication.

To analyze how POT1a and POT1b function in telomere replication, I will perform DNA fiber analysis. This technique allows for the analysis of replication forks on the molecular level. For WT MEFs (plus or minus aphidicolin), POT1a$^{-/-}$, POT1b$^{-/-}$, and POT1a$^{-/-}$; POT1b$^{-/-}$ double-

deletion MEFs, I will label telomeres with two thymidine analogs: 5-iodo-2′-deoxyuridine (IdU), which is red, and 5-chloro-2′-deoxyuridine (CldU), which is green. Different pulses will be sent to instigate the restart of telomere replication at the forks. With a fluorescent microscope, the change in the fluorescent-colored patterns can be tracked and examined. The replication fork progression, origin of replication, and nucleolytic degradation can be determined from the red and green patterns after each pulse (Quinet et al., 2017). I expect that deletion of either POT1 proteins will greatly amplify replicative stress with the deletion of POT1b inducing more stress than the deletion of POT1a. Deletion of both proteins will result in the greatest amount of stress, leading to increased fork stalling, and the telomere replication rate will be the slowest in cells lacking both POT1 proteins.

Second, I will use the anti-SMARCAL1 antibody and use immunofluorescence to examine and quantitate the amount of stalled replication forks present in these cells (Poole et al., 2015). I will also examine the localization of other proteins, including RPA, the CST complex, and TRF1 and correlate their localization with SMARCAL1 foci. With the deletion of POT1a, I predict that there will be an increase in association of the RPA protein to telomeres, while I anticipate that there will be a complete absence in the recruitment of CST complex with the deletion of POT1b.

Potential Pitfalls and Alternate Strategies

One possible challenge is if there is a presence of overlapping signals detected in my CO-FISH images, which would affect my ability to differentiate the immunofluorescent signals and identify fragile telomeres. A possible cause for this is incomplete incorporation of 5-bromo-2′-deoxyuridine (BrdU) into newly replicated telomeres. If this occurs, I will allow for more time for telomere replication in the presence of BrdU. Furthermore, to detect any potential problems before CO-FISH analysis, I will stain cells with anti-BrdU antibody and compare them with the cells processed for CO-FISH, and not use CO-FISH to analyze cells that improperly incorporated BrdU (Falconer et al., 2010).

Another potential pitfall involves DNA fiber analysis. After labeling the DNA with IdU and CldU, the DNA is spread onto glass by dynamic molecular combing. The DNA combing machine can accurately measure the speed of replication fork progression, but the force of the machine can cause DNA breaks because the telomeres are fragile. As a result, replication rate

and possible stalling of the replication fork cannot be seen. To resolve this problem, the strength of the DNA backbone could be increased by adding Na^+ ions in the buffer because the ions would be attracted to DNA, forming a protective coat and preventing breakage (Quinet et al., 2017).

Personal Statement

Ever since I took ninth grade biology, I was intrigued by cells and how they worked together. My interest in cancer biology began later that year when I saw my biology teacher miraculously recover from cancer without conventional treatments. My interest in research continually grew when I took an introductory science research class my sophomore year in high school, where I had the opportunity conduct an experiment with bacterial soaps. I designed, researched, and constructed a model of an efficient passive solar house, and I also represented my school in the Molloy College Science Fair for my project, The Effect of Visual Factors on Memory. This class sparked my desire to conduct research because I discovered that I loved being creative to design analytical and ingenious solutions to problems. In the summers of 2016 and 2017, I attended Camp Cardiac and Camp Neuro at the NYU Langone Center and New York Medical College, respectively. I had the opportunity to talk to many doctors and medical students about the medical field while studying the basics of cardiology and neurology.

My fascination with medicine and cancer continued throughout senior year when I took a medical technology class and was able to conduct urinalysis and dissect a fetal pig. I also participated in the Spark Challenge sponsored by Northwell Health with my class. We toured the Monter Cancer Center in North New Hyde Park, NY, and I was able to listen to doctors, nurses, and pharmacologists talk about their daily lives helping cancer patients. Through this experience, I was able to fully see the work of clinicians in the cancer field behind the scenes. Their passion, calmness, and care for cancer patients inspire me to enter the cancer field.

I am currently taking the first-year seminar MB&B 050 Topics in Cancer Biology. So far, I have learned how to read primary literature documenting landmark cancer discoveries. While reading articles published in Nature, Cell, and the New England Journal of Medicine is challenging, I am excited to learn about the molecular mechanisms of cancer and the many laboratory techniques needed to do ground breaking research. I hope to apply this information to the more specific area of research I will be conducting this summer. From my many interactions

with my mentor Dr. Chang, I am learning how to ask good experimental questions and apply research concepts to my projects this summer. I am very excited to have the opportunity to do research in the Chang lab, especially in the field of telomeres and their role in cancer. I will not only have the chance to apply the biology I have learned in class, but also, I will be able to immerse myself in the cancer field, a topic I have always wanted to learn more about because of the complexity of this disease. By gaining exposure to bench research, I will learn important laboratory techniques and obtain a deeper understanding of cancer biology. More importantly, I will develop problem solving skills and learn critical thinking skills needed to solve scientific problems. Studying the roles that POT1 play in telomere replication will prepare me for my aspirations to major in Molecular Biophysics and Biochemistry. Since my mentor is a physician-scientist, I will also be able to gain invaluable insights into his career, which will help me to decide if I want to be an oncologist or a physician-scientist. Obtaining funding through the First-Year Summer Fellowship will thus give me an invaluable beginning to my research career.

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