The Role of POT1a and POT1b in Telomere Replication of Mouse Tumor Cells

Abstract

Telomeres are the ends of chromosomes that protect genes during cellular replication. They have long been an interest in cancer research. However, the exact mechanisms in which they replicate are not yet fully understood. The replication process is important in understanding how various cancers, most notably chronic lymphocytic leukemias (CLL), familial melanomas (FM), breast cancer, and brain cancer, can be caused by telomere instability. In humans, the POT1 protein (protection of telomeres 1) play a role in regulating telomeres, and the two mouse homologs, POT1a and POT1b, have analogous functions. It is hypothesized that the deletion of POT1a, POT1b, or both, will result in replicative defects at telomeres in mouse tumor breast cells. Primary objectives are to characterize the phenotypes of telomeres during replication when POT1a, POT1b, or both are deleted, and to determine the effect of these protein deletions on the degree of replicative stress at telomeres. The analysis of fragile telomeres, replication forks, and telomeric proteins would enhance comprehension of the role POT1a and POT1b play in the telomere replication process.

Background and Significance

Telomeres are the protective ends of chromosomes, and proper functions at telomeres are important in preventing abnormal growth and atypical aging of cells. Dysfunctional telomeres are major characteristics of many diseases, such as Werner Syndrome and many forms of cancer, including but not limited to, chronic lymphocytic leukemias (CLL), familial melanomas (FM), breast cancer, and brain cancer. An understanding of telomeres 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. This results in a leading strand, where there is continuous replication, and a lagging strand, where DNA is replicated in fragments as the replication fork opens. This results in progressive shortening of telomeres after every cell cycle because the ends cannot be replicated. When telomeres have been eventually completely shortened, apoptosis or senescence is triggered in a cell to ensure that faulty DNA will not be replicated due to the shortening of the chromosome. The enzyme telomerase has the ability to elongate telomeres. It is normally inactive in somatic cells, but it is active in stem and germline cells. Active telomerase is one mechanism in which cancer cells can maintain telomere length and remain immortal (Shammas, 2011).

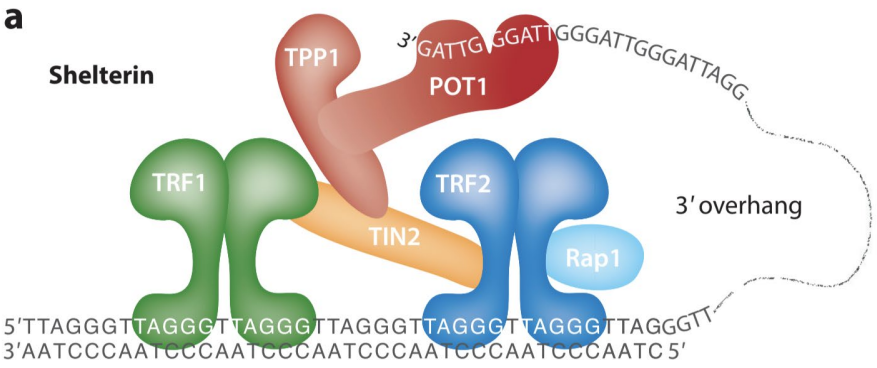

Figure 1: The shelterin complex (obtained from de Lange, 2018). The shelterin complex at telomeres consists of 6 proteins. Telomeres consist of TTAGGG repeats and a 3' overhang.

On the structural level, telomeres consist of the repetitive nucleotide sequence TTAGGG and a 3' overhang. They are regulated by a group of six proteins, known as the shelterin complex (Figure 1). The main role of shelterin is to prevent replicative stress and chromosomal abnormalities. 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 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 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 will go in between a piece of double stranded portion of telomeres, creating a loop and hiding the overhang (Wu et al, 2006).

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 plays a role in t-loop formation and maintenance. The primary function of Rap1 is to stabilize TRF2, and TIN2 stabilizes TRF1 and TRF2. TPP1 helps POT1 with its functions (de Lange, 2018).

POT1 is an especially crucial protein in shelterin, which is bound to the protein TPP1 in the complex and the 3’ overhang. The protein prevents genome instability, including aberrant homologous recombination. In mice, there are there are two homologs of the human version of POT1: POT1a and POT1b. These homologs have an analogous role as in humans. The POT1 domains consist of 3 OB (oligonucleotide/oligosaccharide) folds and a Holliday junction resolvase-like junction. Mutations in the OB folds will result in the activation of a DDR and telomere instability (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, the replication fork is stalled 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 SMARCAL1 DNA translocase, that aid in repairing DNA and restarting replication at the replication forks (Poole et al., 2015). TRF1 is known to have a major role in telomere replication by recruiting the helicases BLM and RTEL1 to reduce fragile sites. It is thought that POT1 interacts with TRF1 during 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 slows down replication (Sfeir et al., 2009). More research is needed to determine the role of POT1 during telomere replication.

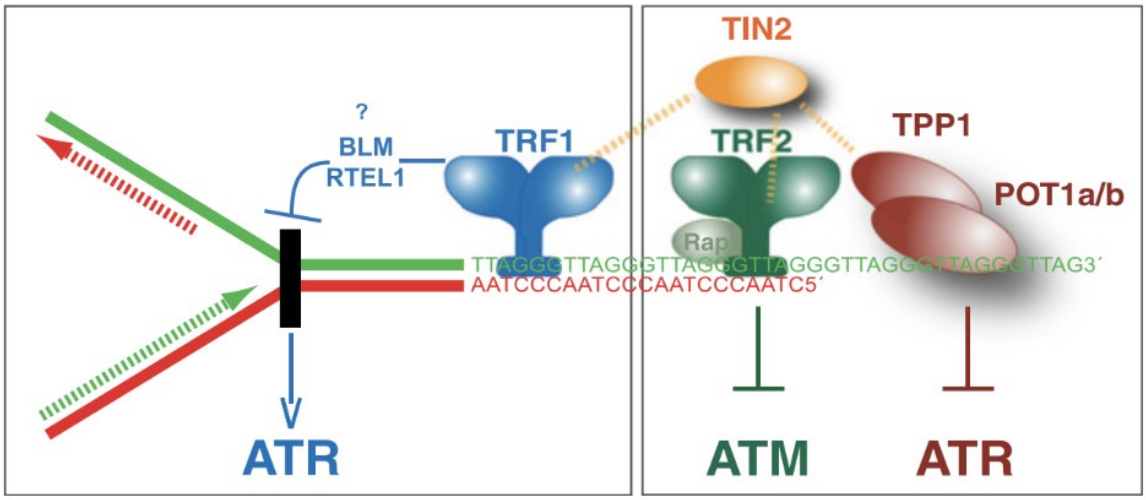

Figure 2: Replication fork at telomeres (obtained from Sfeir et al., 2009). The replication fork at telomeres is stalled because of the binding of shelterin to telomeres.

Hypothesis

I hypothesize that the loss 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 is currently unknown. Furthermore, studies have shown various similarities and differences between the POT1 proteins. Both are thought to work together to repress the NHEJ (non-homologous end joining) pathways (Wu et al., 2006). However, there are some differences, including 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. This would prevent forms of dysfunctional telomeres, including homologous recombination (HR), t-loop HR, chromosome fusions, and p53-dependent senescence (de Lange, 2018). Furthermore, POT1a is known to have a role in replication by

interacting with the WRN protein, which is a part of the RecQ helicase family. Together, the two proteins unwind G-quadruplexes during telomere replication (Wu et al., 2006).

The primary purpose of POT1b is the prevention of elongation of the 3' ssDNA in telomeres. The protein is involved with the formation of the 3' overhang. It recruits the heterotrimer CST complex, which contains the CTC1, STN1, and TEN1 proteins, to shelterin. The CST complex assists Polα/primase, an enzyme that can start DNA synthesis and replication. Together, the CST complex can proceed with DNA synthesis and determine the 3' overhang length. However, without POT1b, the 3' ssDNA will be abnormally elongated, and telomeres will quickly deteriorate. POT1b also has the ability to prevent the ATR pathway in the G1 phase (de Lange, 2018).

The differences suggest that during telomere replication, POT1a and POT1b will have different functions. Deletion of either one of them and both are required to differentiate and quantify their effect during replication. I expect that deletion of POT1a will result in more common fragile sites and telomere dysfunction induced foci (TIFs) than deletion of POT1b because POT1a directly affects a potent DDR pathway and works to directly break apart G-quadruplexes. Deletion of both POT1a and POT1b will result in the greatest amount of aberrant telomere phenotypes.

I will use Cre-mediated deletion, a form of recombinant technology, to delete POT1a, POT1b, or both. With the edited gene, I will insert it into immortalized mouse embryonic fibroblasts (MEFs). From these MEFs, I can generate cell lines to examine (Hockemeyer et al., 2007). As a positive control, a cell line with wild type cells treated with aphidicolin will be generated because cells treated with aphidicolin resemble cells with common fragile sites. This cell line can serve as a comparison to cell lines generated from cells lacking the POT1 proteins (Sfeir et al, 2009). As a negative control, a cell line with wild type cells that are not treated with anything will be grown.

To determine the presence of fragile telomeres, I will use chromosome orientation fluorescence *in situ* hybridization (CO-FISH) to look at telomeres during metaphase. For a normal telomere, there will be a single fluorescent-colored signal from a chromatid end. This signal is approximately equal in intensity to its corresponding sister chromatid. Multiple telomere signals indicate the presence of fragile telomeres because the telomeres is likely broken apart or is no longer condensed. The number of multiple telomere signals in each of the different

scenarios can be counted to quantify the relative effects POT1a and POT1b have on telomere phenotype (Sfeir et al., 2009).

Additionally, I will test for the fragile telomere phenotype by performing a telomere dysfunction-induced foci (TIF) assay. I will use antibodies to the 53BP1 and γ-H2AX proteins because these proteins are indicators of double strand breaks and fragile telomeres. Then, I will look at the number of foci that formed because of the dysfunctional telomeres. The number of foci can be compared to determine the degree in which the POT1 proteins affect telomere replication (Mender and Shay, 2015).

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

Rationale: There are multiple indicators of replication stress, including the stalling of the replication fork at telomeres. The activation of the ATR or a p53-dependent pathway also suggests replication stress due to the increase in DNA damage responses. Because POT1a and POT1b involve different primary pathways, it is likely that with their deletions, different proteins will be involved. TRF1 may also behave differently as a result of POT1 deletions.

To analyze and track the replication fork, I will use DNA fiber analysis. This technique allows for the analysis of replication forks on the molecular level. For each group, 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 POT1a inducing more stress than the deletion of POT1b. Deletion of both proteins will result in the greatest amount of stress. The replication fork will be stalled, and the telomere replication rate will be the slowest in cells lacking both POT1 proteins.

Moreover, Immunofluorescence-Fluorescence *in situ* hybridization (IF-FISH) and chromatin immunoprecipitation (ChIP) analysis can be done to evaluate the localization of various proteins, including RPA, SMARCAL1, the CST complex, and TRF1 to telomeres. The

different levels of these proteins that bind at telomeres can give insight to the pathways, and thus, the replicative stress that results from the deletion of the POT1 proteins (Poole et al., 2015). With the deletion of POT1a, I predict that there will be an increase in association of the RPA protein to telomeres. I think there will be a decrease 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 from CO-FISH, which would affect my ability to differentiate the signals and identify fragile telomeres. A probable cause for this would be incomplete incorporation of 5-bromo-2′-deoxyuridine (BrdU) BrdU, which helps detect cell proliferation. If this occurs, I could allow for more time for replication of telomeres or inject BrdU in cells more frequently. Furthermore, to detect any potential problems beforehand, I could stain cells with anti-BrdU immunofluorescence and compare them with the cells tested with CO-FISH (Falconer et al., 2013).

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).

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