Alternative Lengthening of Telomeres: Recombination Based Telomere Maintenance in Cancer Cell Lines

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I. Abstract

For cancer cells to develop a state of replicative immortality, they must avoid mechanisms that induce apoptosis. One of the main signals for a cell to undergo apoptosis comes from the telomeres. To avoid undergoing apoptosis, cancer cells remodel their telomeres thus suppressing signals for apoptosis such as DNA damage or the inappropriate configuration of proteins coating the telomere. The repetitive sequence structure of telomeric DNA poses a problem during DNA replication as it has the tendency to fold into unusual secondary structures. This results in incomplete replication and the subsequent shortening of telomeres after each division cycle. The majority of cells overcome progressive telomere shortening via a specialized DNA polymerase known as telomerase; however, a small subset of cells can successfully extend their telomeric DNA utilizing the alternative lengthening of telomeres (ALT) pathway. This pathway uses homologous recombination machinery to extend telomeres in the absence of telomerase. In this review, the structures and processes important to the ALT pathway are defined along with models for how these processes are believed to work. Major findings that serve as hallmarks in the field of ALT research and how these paved the way for future efforts to better characterize this pathway will be discussed.

Contents

II. Abbreviations

Structures and processes: ALT, alternative lengthening of telomeres; BIR, break induced replication; CRISPR, clustered regularly interspaced short palindromic repeat; DSB, double-strand breaks; DSBR, double-stranded break repair; HJ, Holliday junction; dHJ, double Holliday junction; NHEJ, non-homologous end joining; RCR, rolling circle replication; RI-1, RAD inhibitor 1; SCE, sister chromatid exchange; SDSA, synthesis-dependent strand annealing

Proteins: APBs, ALT-associated promyelocytic leukaemia nuclear bodies; APLF, aprataxin and PNK-like factor; ATM kinase, ataxiatelangiectasia mutated kinase; BCL11b, B-cell lymphoma/leukaemia 11B; BLM, bloom syndrome protein; BRCA2, breast cancer protein 2; BRIT, BRCT-repeat inhibitor of HTERT expression; COUP-TF2, chicken ovalbumin upstream promoter transcription factor 2 (aka NR2F2, nuclear receptor subfamily 2, group F, member 2); DNA-PKcs, DNA-dependent protein kinase catalytic subunit; FOG-1, friend of GATA protein 1 Hop2, homologous-pairing protein 2; HTERT, human telomerase reverse transcriptase; MDM2, mouse double minute 2 homolog; MMS21, methyl-methanesulfonate protein 21; MND1, meiotic nuclear division protein 1; MRE11, meiotic recombination 11; MRN, MRE11/RAD50/NBS1 complex; NBS1, Nijmegen breakage syndrome protein 1; NSE, non-smc element; NuRD, nucleosome-remodeling and histone-deacetylation complex; PML, promyelocytic leukaemia; POT1, protection of telomeres 1; RAD, radiation; RAP1, Ras-related protein 1; RECQL4, RECQ helicase-like 4; RTEL1, regulator of telomere elongation helicase; SALL1, sal-like 1; SMC, structural maintenance of chromosomes; SUMO, Small ubiquitin-like modifier; TIN2, TRF1 interacting protein 2; TPP1, tripeptidyl peptidase 1; TR4, testicular orphan nuclear receptor 4 TRF1, telomeric repeat factor 1; TRF2, telomeric repeat factor 2; WAPL, wings apart like protein; WRN, Werner syndrome ATP-dependent helicase; XLF, XRCC4-like factor; XRCC3,

X-ray repair cross-complementing protein 3; XRCC4, X-ray repair cross-complementing protein 4; ZNF827, zinc-finger protein 827

Genes: *DKC,* dyskeratosis congenital 1; *RAD52* epistasis gene group, DNA repair and recombination genes

III. Introduction

The incidence of cancer is continuing to rise. A better understanding of how cancer can overcome replicative mortality is essential in our fight against this deadly disease. Worldwide, it is estimated that cancer accounts for more deaths than HIV/AIDS, tuberculosis, and malaria combined, with 1 in every 7 deaths being attributed to cancer (American Cancer Society, 2016). Cancer cells are unique in their capacity to evade apoptosis, become self-sufficient in their growth signals, become insensitive to anti-growth signals, sustain angiogenesis to tumors, invade and metastasize tissue, and develop a limitless replicative potential (Hanahan and Weinberg, 2000). Specific cancer types accomplish some or all of these hallmarks through varying mechanistic strategies, the majority of which are still unknown. When it comes to characterizing the mechanisms involved in cancer's limitless replicative potential, the telomeres have become a target of interest (Muntoni and Reddel, 2005).

Telomeres are located at the end of chromosomes and play an important role in protecting the chromosome ends of linear DNA. A popular metaphor for the function of telomeres is comparing them to an aglet, which is the end of a shoelace (Blackburn, 2005). Telomeres help protect the ends of DNA from being degraded or eliciting a DNA damage response that could possibly result in apoptosis (O'Sullivan and Karlseder, 2010). Thus, cancer cells, with their limitless replicative potential, must develop mechanisms to maintain functional telomeres. Furthermore, without mechanisms in place to maintain telomere integrity, telomeres have the tendency to become shorter with each replication cycle as a result of incomplete replication at chromosome ends (Blackburn, 2005). Thus, cancer cells must also extend their telomeres after each replication cycle in order to continue replicating indefinitely.

Various mechanisms have been identified that help cancer cells overcome these processes, which have the potential to hinder successfully overcoming replicative mortality. The goal of these mechanisms is the same: maintain the structural integrity of the telomeres and extend them after each round of cell division. One method cancer cells have successfully been able to accomplish this is through the alternative lengthening of telomeres (ALT) pathway (Pickett and Reddel, 2015). This pathway uses homologous recombination based machinery to extend telomeres after each round of cell division, thus successfully avoiding replicative mortality. The differences between the ALT pathway and more canonical pathways that deal with telomere shortening can be used to identify potential therapeutic targets for treating ALT positive cancers. Better understanding the mechanisms involved in the ALT pathway can help solve various public health related issues, such as improving the accuracy of cancer screening techniques and developing more targeted therapeutic interventions.

IV. Background

About 55 years ago, Leonard Hayflick and Paul Moorhead published their discovery of the limited division potential of normal human cells when they were grown in culture (Hayflick and Moorhead, 1961). After cells reach this limit, they stop growing and enter into a replicative senescent state and eventually undergo apoptosis. In their experiment, Hayflick and Moorhead cultured an older male cell line and a younger female cell line both together and separately. When they observed the isolated older cell line stop proliferating, they also found that the mixed culture contained only the younger female cell line (Hayflick and Moorhead, 1961). This experiment demonstrated two things: somatic human cells have a finite replicative potential and the extracellular environment does not influence this potential. Thus, there seemed to be an endogenous mitotic clock that controlled when a cell was to enter into replicative senescence. When the mitotic clock of a somatic cell has expired and it has undergone its final cell division, it will enter into cellular senescence and is said to have reached its "Hayflick Limit" (Hayflick and Moorhead, 1961; Shay and Wright, 2011).

Today it is known that loss of telomeric DNA as a result of numerous mitotic divisions is responsible for controlling when cells reach their respective "Hayflick Limit" (Bodnar et al., 1998). Telomeres are stretches of G-rich tandemly repeated sequences of DNA (5'-TTAGGG-3' in vertebrates) that flank both ends of every chromosome (Pickett and Reddel, 2015). As cells proliferate and replicate their DNA, they must also replicate their telomeres to avoid continuous shortening of chromosomes. However, the repetitive nature of telomeric chromatin gives it the capacity to form various secondary structures such as G quadruplexes and T-loops, as well as other hypothetical structures inferred from *in vitro* studies such as triple helices, four-way junctions, and D-loops (Gilson and Geli, 2007). These structures pose a problem for the

canonical DNA replication machinery as they create a physical barrier that is difficult for the replication fork to pass through and complete replication. As a result, telomeres are often not fully replicated and subsequently shorten with each cycle of cell division.

Telomeres are usually associated with proteins that help stabilize its structure and protect them from exonuclease degradation. In mammals, telomeres are associated with the shelterin complex (Figure 1). The shelterin complex consists of six core proteins – telomeric-repeat binding factor 1 (TRF1), TRF2, TRF1 interacting protein 2 (TIN2), protection of telomeres 1 (POT1), the POT1 and TIN2 interacting protein (TPP1) and the transcriptional repressor/activator protein (RAP1) (Deng *et al.,* 2008). Many of the proteins involved in the shelterin complex help the 3' single-stranded overhang found at the end of the telomere invade into the telomeric DNA duplex creating a lariat structure known as the T-loop (Figure 1; Sishc *et al.*, 2015). The main functions of the shelterin complex and the T-loop are to prevent telomeres from nucleolytic degradation and protect the telomere from illegitimately eliciting a DNA damage response. These damage responses can lead to events such as erroneous non-homologous end joining, anaphase bridges, aneuploidy, and reactivation of enzymes involved in telomeric DNA replication (Shay and Wright, 2011).

The most common way cells replicate and therefore extend their telomeres is through utilizing an enzyme called telomerase. Telomerase is a specialized reverse transcriptase enzyme containing a catalytic subunit known as human telomerase reverse transcriptase (HTERT) that utilizes its telomerase RNA component (HTERC) to synthesize and extend telomeric DNA (Zhand *et al.,* 2012; Sishc *et al.,* 2015). Telomerase is generally only expressed in stem- and germ-line cells and transcriptionally suppressed in most somatic cells. Stem- and germ-line cells must avoid replicative mortality so they can continue to self-renew and maintain a pool of

progenitor cells. However, somatic cells eventually undergo apoptosis to balance the influx of cells from the stem cell niche and maintain tissue homeostasis. As a result, somatic cells usually lack telomerase and undergo programmed cell death when their telomeres shorten beyond a critical threshold.

After multiple cell divisions, somatic cells eventually activate a DNA damage response pathway as a result of impaired telomere function – the canonical pathway activates p53, inducing apoptosis or replicative senescence (Deng *et al.,* 2008). p53 is part of an extensive network of proteins involved in apoptosis and replicative senescence. One of the main proteins found to interact with p53 is MDM2, which regulates cellular concentrations of p53 by binding to it and facilitating proteolysis (Momand *et al.,* 1999). When the cell senses DNA damage, MDM2 is inhibited thus increasing concentrations of p53 in the cell. Increased p53 concentrations affects various cell signaling pathways involved in apoptosis and senescence. One way it does this is through its ability to act as a transcription factor capable of binding DNA in a sequence-specific fashion (Fridman and Lowe, 2003). Thus if p53, or a protein involved in its regulation, is rendered non-functional in some way, the cell may be able to bypass apoptosis and become tumorigenic. Likewise, if the cell finds a way to stop telomere shortening and never initiates a DNA damage response, p53 may never be activated and the cell may also avoid apoptosis and become a cancer.

It has been demonstrated that telomeres play an important role in aging disease. When somatic cells are tested for their expression of stathmin and EF-1a, which are considered biomarkers for DNA damage and telomeric dysfunction in a cell, there is an increase in expression of these proteins with increased age and age-related diseases in humans (Shammas, 2011). One age related disease that showcases the importance of maintaining proper telomere

length is Dyskeratosis Congenita (DC), which is the result of critically shortened telomeres due to insufficient telomere maintenance within cells. Approximately 50% of cases are due to an X-linked inherited mutation in the *DKC1* gene, which encodes a highly conserved nucleolar protein called dyskerin. Dyskerin associates with telomerase and it is thought that mutations in the *DKC1* gene are responsible for the reduction in functional telomerase levels observed in DC patients (Gu *et al.,* 2009; Calado and Young, 2012). The common clinical manifestations that result are characterized by abnormal skin pigmentation, nail dystrophy, and leukoplakia, as well as a predisposition to bone marrow failure, pulmonary fibrosis, and cancer (Pereboeva *et al.,* 2016).

In addition to genetically inherited mutations, other factors such as environmental and lifestyle stressors can also affect the structural integrity and length of telomeric DNA (Shammas, 2011). Things such as smoking, obesity, lack of exercise and consumption of unhealthy diet have the potential to accelerate telomere shortening. When telomere shortening is accelerated, individuals are at a higher risk for the early onset of many age-associated diseases such as coronary heart disease, heart failure, diabetes, increased cancer risk, and osteoporosis (Shammas, 2011). Thus, the telomeres are an important target of interest when considering physiologic responses to various public health problems. Analyzing the integrity of an individual's telomeric DNA could provide insight into whether they are at an especially high risk for disease when exposed to certain lifestyle stressors.

Approximately 85% to 90% of cancer cells deal with telomere shortening by up-regulating telomerase activity (Kim *et al.,* 1994; Sishc *et al.,* 2015). There are a number of ways this can be accomplished, and continued research is aimed to accurately characterize the biochemical processes involved. One of the most important targets of interest for explaining this

phenomenon is the catalytic subunit of telomerase, HTERT. One of the first cancers used to demonstrate the role of HTERT in up-regulation of telomerase activity was melanoma (Horn *et al.,* 2013). Horn et al. demonstrated that a germline mutation in the promoter region of the HTERT gene was responsible for an up-regulation of the HTERT gene and subsequent up-regulation of telomerase activity. This mutation was found to create a binding motif for transcription factors, which increased transcription of telomerase two-fold (Horn *et al.,* 2013).

The regulation of HTERT is very complicated and involves multiple transcription factors that either repress or activate the HTERT gene at the site of the promoter. In addition, other processes such as alternative mRNA splicing, phosphorylation, and direct modification of HTERT all help to regulate its cellular expression (Daniel *et al.,* 2012). Numerous transcription factors have been identified that act to regulate HTERT expression, many of them belonging to pathways involved in maintaining cellular homeostasis and cell cycle control (Daniel *et al.,* 2012). It is apparent that this extensive network of factors influencing HTERT leads to tighter control over its expression and therefore level of telomerase activity. It seems this could be both advantageous and dangerous to the cell since telomerase is such an essential enzyme. The extensive list of proteins directly involved in HTERT's expression increases the probability that one of these proteins may be mis-expressed and subsequently alter telomerase activity. Just as Horn et al. demonstrated; a mutation in the promoter region affects transcription levels of HTERT, it is also possible that mutations present in the various transcription factors involved in HTERT regulation have the potential to up-regulate telomerase activity.

Figures

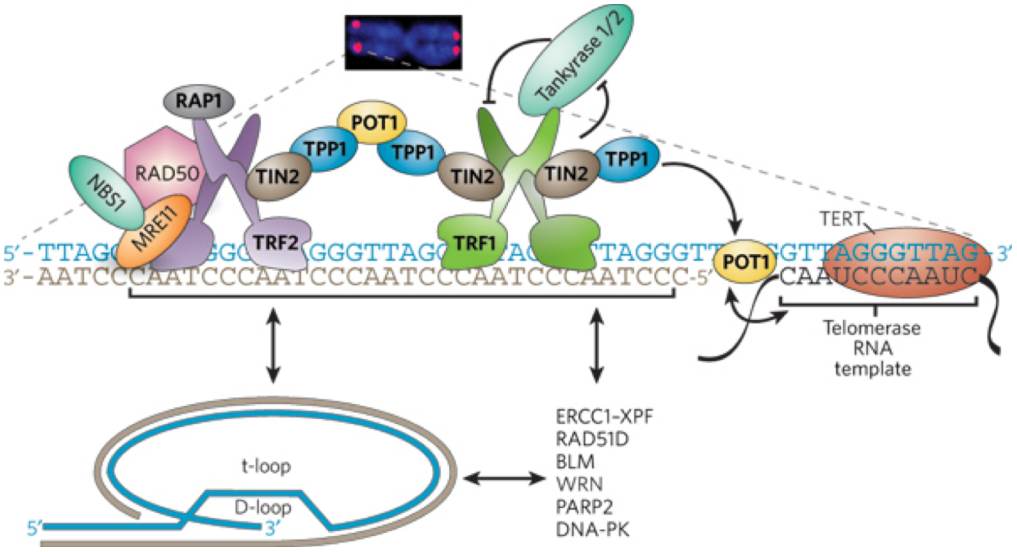

Figure 1. The fluorescence image at the top indicates the location of a telomere within a chromosome. There are a variety of proteins that bind to telomeric DNA, and components of the shelterin complex are represented in bold. The single-stranded DNA overhang is able to integrate into the double-stranded portion of the telomere, forming T-loops with displacement loops (D-loops). The shelterin protein complex also plays a role in regulating the telomere extension activity of telomerase.

(Figure adapted from Verdun and Karlseder, 2007).

V. Current State of the Field

In addition to up-regulation of telomerase, there is also a subset of cancer cell lines that utilize an alternative method of extending their telomeres to avoid replicative mortality. This mechanism is known as Alternative Lengthening of Telomeres (ALT), and evidence suggests that the homologous recombination machinery is responsible for the extension of telomeres in the absence of telomerase. About 10% to 15% of human cancers have been identified as ALT positive – the most common being subtypes of sarcomas and astrocytomas; however, it has also been identified in other tissues at a lower frequency, such as epithelial malignancies (Heaphy *et al.,* 2011). One of the more prevalent tumor types in which ALT is active is glioblastoma multiforme. This is the most common type of primary malignant brain tumor in adults, making ALT an attractive target for possible therapeutic interventions (Cesare and Reddel, 2010). Because the ALT mechanism utilizes machinery inherently different from other cells in the body, ALT research may have the potential to provide the basis for more accurate screening methods and more specific therapeutic treatments.

The ALT phenotype is commonly identified through assaying for large specialized ALT-associated promyelocytic leukaemia (PML) nuclear bodies (APBs) in which the PML nuclear bodies co-localize with telomeric DNA and associated proteins (Muntoni and Reddel, 2005). These large APBs contain telomeric DNA, TRF1, TRF2, and associated proteins making them distinct from other PML bodies in the same cell and other cell types (Royle *et al.,* 2008). In addition, APBs contain proteins involved in DNA recombination and repair indicating that these processes play a vital role in the ALT mechanism. The ALT phenotype can also be identified by an abundance of extrachromosomal telomeric DNA. The majority of this DNA can exist in alternative forms, including predominantly double-stranded telomeric circles (t-circles), partially

single-stranded circles (C- circles or G- Circles depending on if they are cytosine or guanine rich, respectively), linear double-stranded DNA, and "t-complex" DNA that has a very high molecular weight due to containing abnormal, highly branched structures (Cesare and Reddel, 2010). The heterogeneity found in ALT positive cell lines is indicative of the complexity involved in this mechanism. However, understanding the key phenotypic characteristics of ALT positive cells can help more accurately diagnose cancer cells that use this mechanism to avoid replicative mortality.

Homologous recombination is at the core of the ALT mechanisms, and it is through this action that ALT positive cancer cells are able to avoid replicative mortality and DNA damage responses triggered by corrupt telomeres. In 1911, biologist Thomas Hunt Morgan was studying the chromosome theory of heredity when he noticed that some traits considered to be linked together would separate while others would not. Morgan proposed that two paired homologous chromosomes could "cross over" to exchange information, and the proximity of two genes to one another on a chromosome arm was correlated with the probability that they would undergo recombination and become inherited together (Lobo and Shaw, 2008). Today, we know that homologous recombination serves many essential functions in both meiosis and mitosis. In meiosis, homologous recombination facilitates exchange of genetic information between maternal and paternal alleles generating more genetic diversity. In addition, it facilitates accurate segregation of homologous chromosomes during meiosis I by forming chiasmata and ensuring that aneuploidy does not occur. In mitosis, a specific type of homologous recombination called sister chromatid exchange (SCE) plays a major role in repairing various types of DNA damage such as double-strand breaks (DSBs), telomeres that have been incompletely replicated, DNA inter-strand crosslinks, and collapsed replication forks (Filippo *et al.,* 2008).

A large group of genes are needed for homologous recombination in both meiosis and mitosis. Collectively, these genes are known as the *RAD52* epistasis group, and these are highly conserved throughout most eukaryotic organisms. Protein products of the *RAD52* gene group in humans includes the MRN complex – consisting of MRE11, RAD50, and NBS1 – BRCA2, RAD52, RAD54, RAD54B, RAD51B-RAD51C complex, RAD51D-XRCC2 complex, RAD51C-XRCC3 complex, and HOP2-MND1 (Filippo *et al.,* 2008). All of these protein products have a specific function that is necessary for successful completion of homologous recombination. Many of these proteins are single-stranded DNA (ssDNA) binding proteins and mediator proteins that help stabilize intermediate structures throughout the homologous recombination process. Various models have been proposed to explain the role of the *RAD52* epistasis group in the biochemical mechanisms involved in recombination events. However, two models seem to be the most widely acknowledged: the double-strand break repair (DSBR) model and synthesis-dependent strand-annealing (SDSA) model (See Figure 2) (Symington, 2002).

Both the DSBR and SDSA models begin with a double stranded break followed by end resection and strand invasion on a homologous chromosome. In the DSBR model, the 3' end of the invading strand is extended via DNA synthesis after the single-stranded piece of DNA has invaded its homolog. This strand invasion forms a loop (known as the D-loop) that is able to pair with the adjacent side of the DSB. A double-Holliday-junction (dHJ) intermediate is formed when the 3' end of the non-invading strand is extended via DNA synthesis. At this point, the two Holliday junctions can be resolved in one of two ways: either creating a crossover or a non-crossover product. (Symington, 2002; Clancy, 2008; Filippo *et al.,* 2008). In the SDSA model, strand displacement occurs just as it does in the DSBR model; however, after DNA synthesis has occurred, the invading strand is displaced and is ligated with the other end of the break. Next, the

non-invading 3' end primes DNA synthesis in order to repair the DSB or gap. This always results in a non-crossover product and the genetic material on the undamaged homologue is not altered during the recombination event (Symington, 2002; Clancy, 2008; Filippo *et al.,* 2008).

Homologous recombination plays an important role in maintaining genome integrity directly; however, it is also utilized by other processes in the cell that require a genetic template to function. One of these is break induced replication (BIR, see figure 3), which is an important process in maintaining telomeric DNA. BIR is a recombination-dependent replication process used to repair broken chromosomes when a single-stranded overhang is present in DNA (Kraus *et al.,* 2001). Many times, this single-stranded overhang is the result of a disintegrated replication fork, which could be the case in telomeres. Abnormal structures found in telomeres hinder replication fork progression, and special proteins such as the helicases BLM, WRN, RECQL4 and RTEL1, the exonuclease Apollo and the scaffold protein SLX4 are required to dismantle these structures and facilitate replication fork progression through the telomere (Pickett and Reddel, 2015). However, when this is not successful BIR is often utilized to repair the incompletely replicated telomeric DNA.

There are two major models (the second model has two sub-divisions) used to describe the mechanism of BIR (see figure 3). In both models, RAD51 mediates invasion of a homologous sequence by the the single-stranded piece of DNA forming a D-loop. In the first model, semi-conservative replication resolves the D-loop through the formation of a normal replication fork, resulting in synchronous leading and lagging strand synthesis (Kraus *et al.,* 2001; Malkova and Ira, 2013). In the second model, the D-loop migrates like a bubble which causes branch migration of an unresolved HJ. This results in synchronous synthesis of the leading and lagging strands, causing the new strands to be displaced and conservative inheritance

to occur. In the first subdivision of the second model, both newly synthesized strands are displaced from their templates. In the second subdivision of the second model, the leading strand is synthesized first while the lagging strand is not synthesized until the leading strand is displaced. After displacement, the lagging strand uses the leading strand as a template (Malkova and Ira, 2013).

Researching processes that account for BIR will likely help define the mechanisms required for ALT, as it has been suggested that BIR plays an integral role in ALT-mediated telomere lengthening. As previously mentioned, telomeric DNA consists of repeated sequences of TTAGGG. The 3' end containing this sequence is considered the G-strand, while the 5' end, containing a complementary sequence with more cytosines, is considered the C-strand (Nabetani and Ishikawa, 2010). This configuration allows the 3' single-stranded overhang to invade into the telomeric DNA duplex creating a lariat structure known as the T-loop (Figure 1; Sishc *et al.,* 2015). However, during BIR the 3' single-stranded G-strand invades the C-strand of another telomere with a homologous sequence. It is possible for this to be an adjacent telomere or one of the telomeres located on a sister chromosome; however, it is not fully known what proportion of ALT activity requires long range movement to accomplish successful recombination events. It could be that some telomeres are not able to successfully undergo recombination with adjacent telomeres due to sequence variation or incompatible protein arrangement. Thus they must develop other mechanisms to move to sister chromatids in order to successfully accomplish recombination events.

After invasion of the C-strand by the G-strand of another telomere, DNA synthesis is initiated at the 3'-end resulting in elongation of the G-strand up to the end of the template C-strand (Nabetani and Ishikawa, 2010). This process results in non-equivalent transfer of DNA

from the longer telomere to the shorter telomere causing the normal TTAGGG repeats to become interspersed with variable, non-canonical sequences. Telomere binding factors, such as the proteins involved in the shelterin complex, rely on the sequence specificity of telomeres to recognize and bind to the telomeric DNA. Therefore, when the BIR pathway induces sequence variation in telomeric DNA, many telomere binding factors may not be able to bind efficiently to telomeres, abrogating their function (Bechter *et al.,* 2004). As a result, telomeres may become more prone to nucleolytic degradation or eliciting a DNA damage response.

BIR induced variation of telomeric DNA can create nuclear receptor–binding TCAGGG variant repeat segments, which are high-affinity binding sites for a group of nuclear hormone receptors (Pickett and Reddel, 2015). Nuclear receptors such as TR4 and COUP-TF2 have both been demonstrated to play an important role in recruiting a zinc finger protein known as ZNF827 to telomeres in ALT positive cells (Conomos *et al.,* 2014). ZNF827, along with other similar proteins such as FOG-1, SALL1, and BCL11b help recruit the Nucleosome Remodeling Deacetylase complex (NuRD) to the telomere in a sequence specific manner (Lauberth and Rauchman, 2006; Conomos *et al.,* 2014). The NuRD complex harbors nucleosome-remodeling and histone-deacetylation components. Shelterin displacement can stimulated by the NuRD nucleosome remodeling function. In conjunction with ZNF827, the histone-deacetylation activity of the NuRD complex may play a role in compacting telomeric chromatin, thus countering histone demethylation. Increased histone acetylation causes a lower concentration of the shelterin complex being present on telomeres (Conomos *et al.,* 2014). Once the NuRD-ZNF827 complex has been established, it will then recruit proteins involved in DDR and homologous recombination, such as BRIT (BRCT-repeat inhibitor of HTERT expression). DDR recruitment

stimulates interactions between telomeres within APBs, successfully providing an environment conducive for HR to ensue (Conomos *et al.,* 2014; see figure 4).

The formation of APBs in ALT positive cells may rely on the accumulation of soluble PML protein at the telomere, and this accumulation may rely on sumoylation mediated by the SUMO E3 ligase MMS21 (also known as NSE2), which is a component of the SMC5/6 complex (Chung *et al.,* 2012). Interestingly, it has been speculated that one or more components of the NuRD-ZNF827 complex or the nuclear receptors responsible for its recruitment undergo sumoylation within the APBs (Conomos *et al.,* 2014). Thus, the SMC5/6 complex, or other proteins with sumoylation activity, may play a role in sumoylation events required for the formation and subsequent maintenance of APBs associated with telomeres in ALT positive cells. Furthermore, APBs in ALT positive cells can induce the assembly of filamentous telomeric bridges that may function in bringing long distance telomeres together to initiate HR events (Cesare and Reddel, 2010). Thus better characterizing the role of sumoylation in promoting or maintaining the ALT pathway could help identify what mechanisms are essential for ALT positive cells to successfully extend their telomeres using this pathway.

Another mechanism used to extend telomeric DNA that also relies on homologous recombination events is known as rolling-circle replication (RCR). RCR was originally described in yeast mitochondrial DNA as a way to deal with numerous replication cycles of linear DNA (Nosek *et al.,* 2005; Nabetani and Ishikawa, 2010). As previously mentioned, telomeric DNA can exist in various forms, including a lariat structure known as the T-loop (Cesare and Reddel, 2010). Recombination within these T-loops can either cause the telomere to rapidly shorten and form T-circles, which are extrachromasomal loops of DNA that have dissociated from the telomere (Nabetani and Ishikawa, 2010). It is thought that T-circles are formed when a t-loop

migrates and forms a Holliday junction, which is then resolved with the XRCC3 protein (Henson and Reddel, 2010). These T-circles can undergo RCR in order to rapidly elongate the telomeric DNA in a continuous and efficient process (Nabetani and Ishikawa, 2010). It is believed that RCR of T-circles produces long stretches of telomeric repeats that could be incised back into the chromosomal ends, and that T-circles use an excision-expansion-incision cycle to maintain telomeres in the absence of telomerase (Nosek *et al.,* 2005). It was discovered that the 3'-end of the G-tail can pair with t-circles in ALT cells. The G-strand can then be synthesized using the circular DNA as a template (Figure 5; Nabetani and Ishikawa, 2010).

T-loops and subsequent T-circles play an important role in extending telomeric DNA in ALT cells, but they are also essential for other functions such as maintaining structural integrity and protecting the telomere. As previously stated, many of the shelterin complex proteins help in the formation of a T-loop and therefore help protect the telomere (Figure 1; Sishc *et al.,* 2015). Two components of the shelterin complex have been identified as essential for the formation of T-loops, TRF1 and TRF2 (Baily *et al.,* 2001). When telomeres are depleted of TRF2, they become associated with DNA damage response factors and form telomere dysfunction induced foci (TIFs). When TRF2 is present on telomeres it can directly inhibit the ATM kinase, which plays a major role in activating p53 (Wang *et al.,* 2004). Thus, it seems that TRF2's ability to successfully facilitate the formation of a T-loop is essential to inhibiting proteins involved in DNA damage responses and apoptosis.

As previously discussed, one of the main proteins that interacts with p53 is MDM2, which can bind to p53 and facilitating proteolysis (Momand *et al.,* 1999). Because ATM kinase activates p53, it would be interesting to see how this affects the MDM2 protein activity in the cell. For example, when cells are depleted of TRF2, telomeres become associated with DNA

damage response elements. Some of these elements may directly or indirectly inhibited the activity of MDM2, thus successfully reducing the proteolysis of p53. Furthermore, the ability for TRF2 to successfully form a T-loop could recruit proteins that interact with MDM2 and thus inhibit its inhibition. The cross-regulation of p53 by the ATM kinase and MDM2 could provide a better understanding of how other pathways are affected by the successful induction of a T-loop in the telomere.

In addition to TRF2 facilitating the formation of T-loops to protect telomeres from eliciting DNA damage responses, T-loop products also help keep the telomeres from undergoing a process called non-homologous end joining (NHEJ) (Baily *et al.,* 2001). When a DSB is present in DNA, NHEJ may be used to ligate the DSB back together. In the case of telomeres, the ligation can occur between telomeres on two different chromosomes, leading to various problems such as chromosomal abnormalities and aneuploidy (O'Sullivan and Karlseder, 2010). When the telomere is not folded into a T-loop conformation, it has free ends that can be recognized as a DSB. The first step of NHEJ is the binding of a protein complex called Ku70-Ku80 to the perceived DSB. This protein acts as a scaffold to recruit other proteins necessary for NHEJ, such as DNA-PKcs, XRCC4, XLF, DNA Ligase IV, and APLF (Davis and Chen, 2013). These proteins work together to bring the two ends of a DSB into close proximity with each other (Figure 6). The DNA-PKcs complex is then either autophosphorylated or phosphorylated by the ATM kinase, thus inducing a conformational change causing its release from the DNA. DNA Ligase IV can then ligate the DSB together before all proteins dissociate from the DNA, except for Ku. Ku is the last protein to dissociate, and is subsequently ubiquitinated and degraded in the proteasome (Davis and Chen, 2013).

Figures

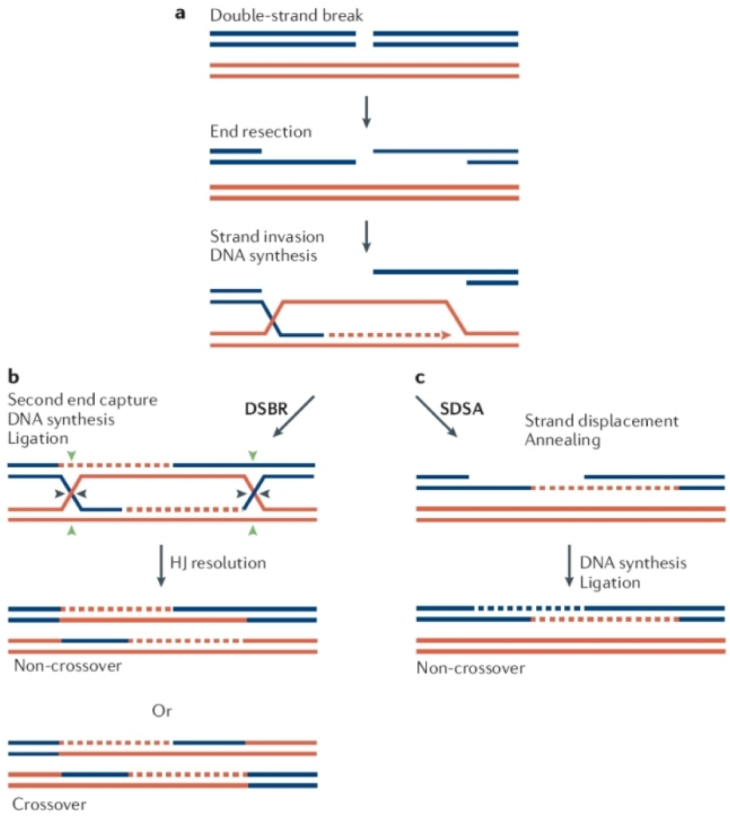

Figure 2. Demonstration of both double-strand break repair (DSBR) and synthesis-dependent strand annealing (SDSA) models:

A) In both models, a double-strand break initiates the process followed by resection to provide 3' single-stranded DNA (ssDNA) overhangs. These strands then invade a homologous sequence before it is synthesized at the invading end.

B) In the DSBR model, the second DSB end is captured to form an intermediate with two Holliday junctions (HJs). The structure is resolved at the HJ in a non-crossover or crossover manner after second end capture, DNA synthesis, and ligation.

C) In the SDSA model, the strand is displaced then annealed to the ssDNA on the other break end. The gap is then filled by DNA synthesis and ligation occurs. The SDSA model always results in a non-crossover product.

(Figure adapted from Clancy, 2008)

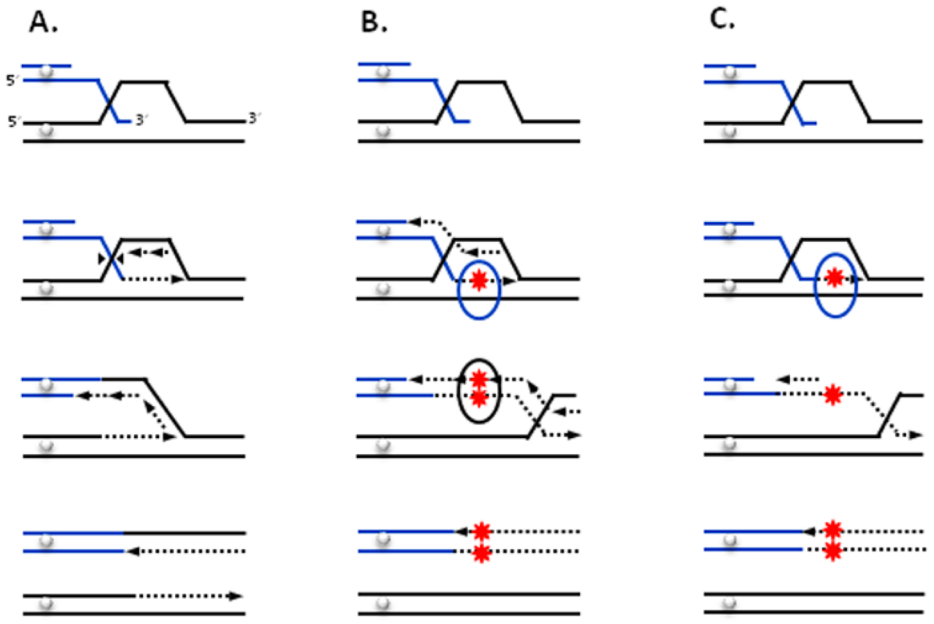

Figure 3. Proposed Models for BIR

A) After the single-stranded piece of DNA invades the homologous chromosome, a HJ is formed. This is resolved by unidirectional progression of a replication fork resulting in semi-conservative DNA synthesis.

B) This is the first sub-division of the second model. A D-loop is formed and migrates as a bubble while both the leading and lagging strands are synthesized. Both of these strands are displaced leading to conservative inheritance.

C) This is the second sub-division of the second model. A D-loop is formed and migrates as a bubble while the leading strand is synthesized first. After the leading strand has been displaced, the lagging strand uses it as a template.

Note: The blue ovals indicate positions where a quick dissociation of newly synthesized DNA occurs. One star indicates a position of replication error, such as nucleotide mis-incorporation, that results from this quick dissociation (B, C). The black oval indicates the inability of mismatch repair to decipher between two newly synthesized strands resulting in replication error being present in both strands (two stars).

(Figure adapted from Malkova and Ira, 2013)

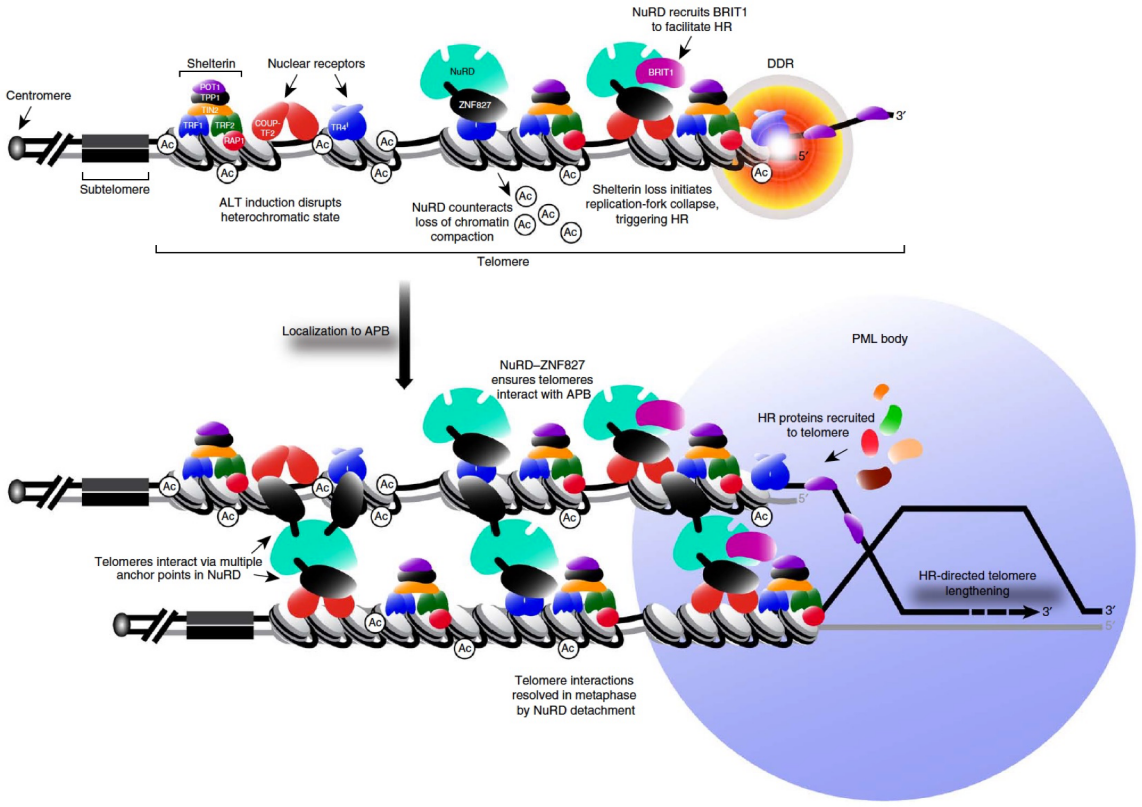

Figure 4. Model for NuRD-ZNF827 activity on the telomere

Nuclear receptors such as COUP-TF2 and TR4 bind to telomere at nuclear receptor–binding TCAGGG variant repeat segments. This causes recruitment of ZNF827 which recruits NuRD to form a NuRD-ZNF827 complex. This complex helps recruit the BRIT1 protein to facilitate HR through increasing telomere interactions with other telomeres and PML bodies.

(Figure adapted from Conomos *et al.,* 2014).

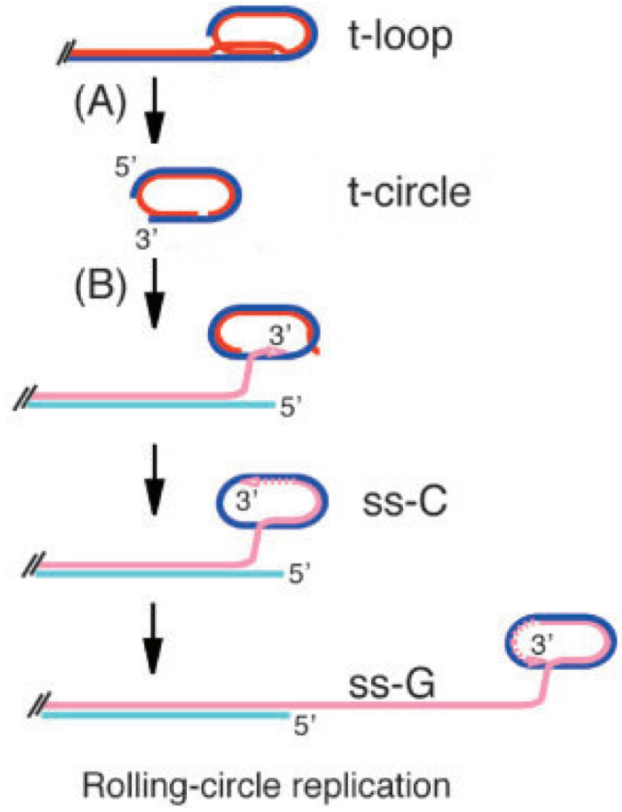

Figure 5. Rolling-circle replication

A) Homologous recombination within the t-loop causes formation of an extrachromasomal t-circle.
B) The G-tail of the telomere can then invade the t-circle, thus initiating rolling-circle replication.

(Figure adapted from Nabetani and Ishikawa, 2010).

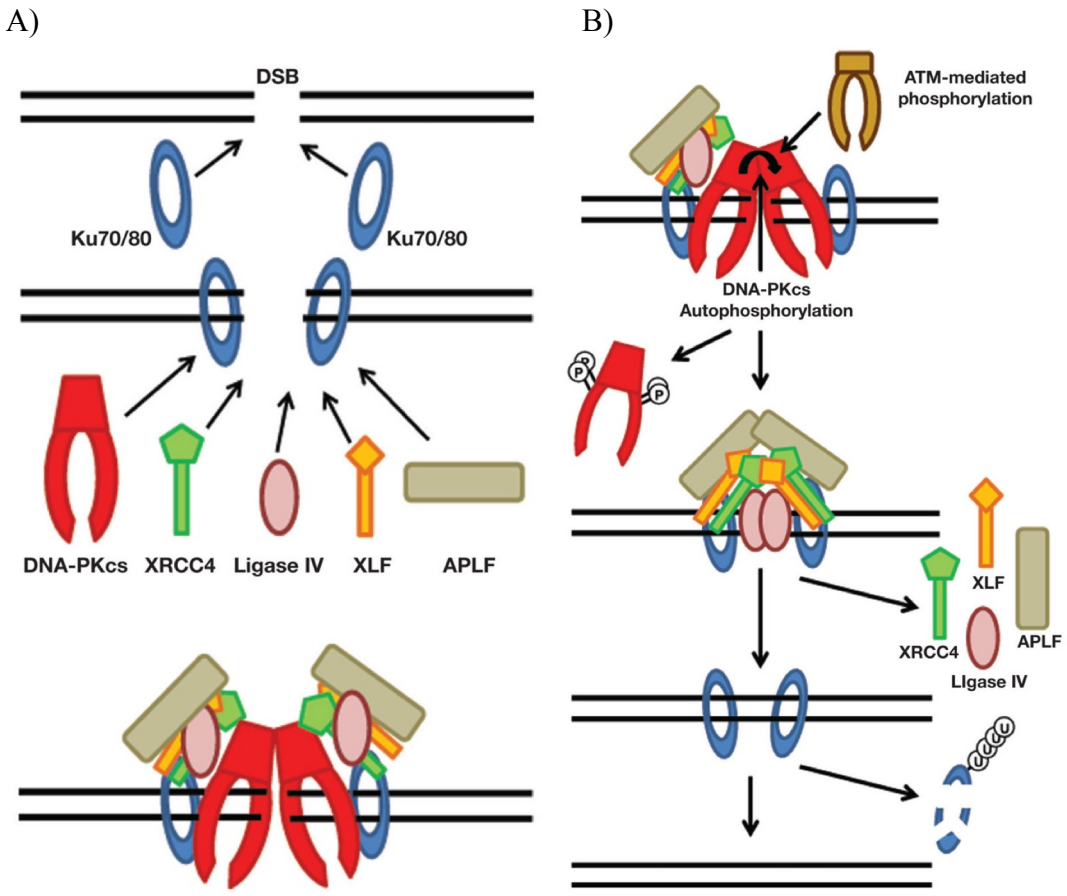

Figure 6. Mechanism of Non-Homologous End Joining

A) Ku binds to the ends of the broken DNA molecule (the end of the telomere). This then functions as a scaffold to recruit the other proteins involved in the NHEJ machinery. These then interact with each other to form a stable complex at the DSB

B) The DNA-PKcs protein is either phosphorylated by ATM or is autophosphorylated, resulting in its release. DNA-Ligase IV then facilitates end joining before the proteins involved in the NHEJ machinery dissociate from the DNA. Ku is the last to dissociate and is ubiquinated and subsequently degraded.

(Figure adapted from Davis and Chen, 2013)

VI. Future Direction

Many of the future developments in the field of ALT research will require a deeper understanding of how processes involved in the ALT pathway interact with each other to provide an environment conducive to telomere lengthening. Proteins involved in chromatin remodeling have been demonstrated in telomere maintenance and their loss of function has been linked with ALT activation (Heaphy *et al.,* 2011). It seems that the process of telomeric chromatin remodeling is essential for providing an environment conducive to recruiting proteins that subsequently help activate the ALT pathway. Two proteins involved in chromatin remodeling, the ATP-dependent helicase protein ATRX and its H3.3-specific histone chaperone DAXX, are both constituents of PML bodies, and some ALT positive cancer cells in humans have been shown to be correlated with a mutated, non-functional ATRX-DAXX protein complex (Heaphy *et al.,* 2011; Pickett and Reddel, 2015). Furthermore, the histone-deacetylation activity of NuRD-ZNF827 in chromatin remodeling could result in the recruitment of proteins involved in increasing the interactions of telomeres with other telomeres and APBs (Conomos *et al.,* 2014). Better characterization of how processes like these interact with each other to remodel chromatin in a way that provides a platform for events involved in the ALT pathway will be an important avenue to explore in the future.

Just as the activation of some proteins is essential to induce the ALT pathway, there are also proteins that must be suppressed. Mechanisms involved in the balance of activating and suppressing proteins involved in the ALT pathway are important to identify how this pathway is controlled. The structural maintenance of chromosomes (SMC) family of proteins has been identified as playing a role in maintaining this balance. The SMC family of proteins consists of 6 variations that bind together in different combinations to form three possible multi-subunit

protein complexes: cohesin, condensin, or the SMC5/6 protein (Potts and Yu, 2007). Cohesin and the SMC5/6 complex both play a pivotal role in the ALT pathway. The cohesin complex consists of the SMC1/3 heterodimer and its primary function is to maintain cohesion between sister chromatids in S phase until the metaphase-anaphase transition; however, cohesin has also been demonstrated as playing an important role in repairing DSBs by HR (Wu *et al.,* 2012). The SMC5/6 complex has been shown to play a role in maintaining cohesin association with DSBs through its sumoylation activity (Wu *et al.,* 2012). The exact functions of the SMC5/6 heterodimer in the cell are not fully understood, but it seems to be involved in DNA repair by homologous recombination, restart of collapsed replication forks, maintenance of telomere homeostasis, and ribosomal DNA (rDNA) stability (Roy *et al.,* 2015). The Smc5/6 protein complex also consists of 4 accessory proteins that are necessary for DNA binding and DNA damage repair (NSE1 through NSE4). The accessory protein NSE2, also known as MMS21, is responsible for the sumoylation activity of the SMC5/6 complex.

An important protein involved in the process of SMC5/6 regulation of cohesin maintenance is WAPL (Wu *et al.,* 2012). WAPL has been demonstrated as playing a crucial role in facilitating sister chromatid resolution during mitosis by acting as a negative cohesin regulator (Gandhi *et al.,* 2006). Therefore, it seems that in order for cohesin to be maintained at a DSB on the telomere, WAPL must be inhibited. Wu *et al.* (2012) demonstrated that the SCC1/RAD21 subunit of cohesin is sumoylated by MMS21/NSE1, resulting in successful negative regulation of WAPL and subsequent maintenance of cohesin at DSBs. Because it has been proposed that the ALT pathway uses DSBs and HR as a possible means to extend telomeres, the sumoylation activity of the SMC5/6 complex could play an important role in facilitating HR by maintaining cohesin that is associated with telomeres.

In addition to SMC5/6's role in sumoylating cohesin, it has also been found to play an important role in sumoylating other proteins involved in the ALT pathway, such as those that constitute the shelterin complex (Potts and Yu, 2007). In their experiment, Potts and Yu (2007) used RNAi to knock-down the MMS21 sumoylating subunit of the SMC5/6 complex and subsequently observed a decrease in APB formation. Furthermore, they demonstrated that the formation of APBs relied of sumoylation of protein constituents of the shelterin complex. After various other experiments, Potts and Yu (2007) concluded that sumoylation of the shelterin complex by the SMC5/6 heterodimer was essential for the formation of APBs. However, it should be noted that the authors later suggested that the RNAi used in this experiment was subject to off target effects (Wu *et al.,* 2012). It is known that components of the shelterin complex inhibit initiation of a DNA damage response at the telomere. For example, exogenous expression of the protein TRF2 can suppress the induction of a telomere-specific DNA damage response (Cesare *et al.,* 2009). As previously discussed, the shelterin complex can help facilitate the formation of a T-loop, which is necessary to protect the telomeric DNA from nucleolytic degredation (Baily *et al.,* 2001). Therefore, loss of shelterin function or inhibiting its telomere binding properties are likely to be linked with ALT establishment. Sumoylation events mediated by the SMC5/6 complex could contribute to the functionality of the shelterin complex.

It appears that many of the proteins involved in suppressing a telomeric specific DNA damage response either have a reduced binding affinity to the telomere or lose their ability to bind all together. In addition to sequence variation playing a role in the decreased binding affinity, there could also be a decreased expression of these proteins due to mutations in the promoter regions that control their expression. Exogenous expression of some of these proteins, such as TRF2, has been found to be sufficient in suppressing induction of a telomere specific

DNA damage response (Cesare *et al.,* 2009). Thus, these proteins may have an adequate binding affinity but just be expressed in lower concentrations. Future research may aim to investigate not only sufficient binding interactions with telomeres, but also the adequate regulation of these proteins in the cell. In addition, the concentration of proteins involved in the ALT pathway may be regulated by posttranslational modification or degradation. Peuscher and Jacobs (2012) demonstrated that the shelterin protein TIN2 can protect TRF1 from ubiquitylation-induced proteasomal degradation. Therefore, the concentrations of proteins involved in the shelterin complex are contingent upon their ability to successfully elude proteosomal degradation. It is possible that there are other mechanisms involved in mediating degradation of proteins involved in the ALT pathway. Fluctuations in the balance of functional protein turnover could either inhibit or activate the ALT pathway. Thus, it would be advantageous to more thoroughly identify mechanisms involved in regulating both expression and post-translational modification of proteins involved in the ALT pathway.

In addition to mitotic specific proteins, some meiotic specific proteins have also been identified as playing a role in the ALT pathway. One of these protein complexes is HOP2-MND1, which ensures that recombination between homologous chromosomes is favored over recombination between sister chromatids during meiosis (Cho *et al.,* 2014). HOP2-MND1 simulates D-loop formation through interacting with two recombinases RAD51 and DMC1, which are involved in bringing homologous DNA molecules together so HR can occur (Filippo *et al.,* 2008). One reason this process could be advantageous is because it increases the possible templates available for the ALT pathway to work through HR. Arnoult and Karlseder (2014) suggest that telomeric chromatin in ALT positive cells may contain features that allow interaction between RAD51, HOP2, and MND1 that cells not expressing the ALT pathway do

not have. Thus, long-range movement to homologous chromosomes mediated by HOP2-MND1 and RAD51 could increase sequence variation in telomeric DNA producing sequences conducive to the ALT pathway. Sequence variation in the telomeric DNA of ALT positive cells has been shown to be important for the proteins involved in the ALT pathway (Lee *et al.,* 2014). In the ALT pathway, HOP2-MND1 has been demonstrated as localizing to the telomeres, and knocking down either HOP2 or MND1 results in significant reduction in APB formation (Cho *et al.,* 2014). As previously mentioned, APB formation is a phenotypic indicator of ALT positive cells. Thus, it appears that HOP2-MND1 is important for the formation of APBs and the successful functioning of the ALT pathway. Because HOP2-MND1 is meiosis specific, it could be a potential therapeutic target to treat ALT positive cancer cells as it is not thought to be expressed by somatic cells during mitotic homologous recombination. As a result, ALT positive cancer cells could theoretically be targeted specifically as somatic cells would not be affected by inhibiting this protein.

One way the HOP2-MND1 protein complex could be targeted specifically is by using a Crispr-Cas9 genome editing strategy. The Crispr-Cas9 machinery can be used to introduce a DSB into the genome at a specific location in order to insert a sequence of interest (Sander and Joung, 2014). This machinery could be used to inhibit the HOP2-MND1 protein complex by disrupting the promoter region of the *HOP2* or *MND1* gene just upstream of the protein coding region. The Crispr-Cas9 machinery uses a specific RNA known as crRNA to direct Cas9 to cleave complementary target DNA sequences adjacent to sequences known as protospacer adjacent motifs (PAMs). Following this, NHEJ can be used to produce a small insertion or a small deletion in the DNA (Sander and Joung, 2014). In addition to introducing a DSB in the promoter region, it could also be possible to introduce a premature stop codon in the protein

coding region of either the *HOP2* or *MND1* gene. This would result in a truncated protein that could be rendered non-functional. To specifically target and transfect cancer cells with the Crispr-Cas9 machinery, researchers could utilize a viral capsid that contains a binding site specific for a cell surface receptor found on ALT positive cells. It has been demonstrated that in telomerase positive cells, peptides generated by degradation of HTERT, the catalytic subunit of telomerase, can be presented on the cell surface via the major histocompatibility complex (MHC) (Reddel, 2014). If it is discovered that ALT specific protein degradation products are presented in a similar way on ALT positive cells, they could be used as specific targets for the Crispr-Cas9 containing viral capsids.

In addition to utilizing the Crispr-Cas9 machinery to facilitate knockout of the HOP2-MND1 protein in ALT positive cells, small-molecule inhibitors could also be developed that specifically target the HOP2-MND1 to disrupt its activity. These small-molecule inhibitors could potentially disrupt the DNA binding activity or the assembly of the HOP2-MND1 protein. It has been suggested that knockdown of HOP2 or MND1reduces telomere chromatid exchanges by 50% or greater in ALT positive cells (Cho *et al.,* 2014). Thus, the HOP2-MND1 protein complex could be a potential attractive target for therapeutic interventions in the future of ALT research. Proteins involved in meditating the activity of the HOP2-MND1 protein complex could also be potential targets to develop small-molecule inhibitors against. The RAD51 protein plays an integral part in HOP2-MND1 mediated HR events, and small-molecule inhibitors have been developed that inhibit the activity of RAD51. The small molecule inhibitor (E)-3-benzyl-2-(2-(pyridin-3-yl) vinyl) quinazolin-4(3H)-one, also known as B02, can efficiently and specifically inhibit the DNA strand exchange activity of the RAD51 protein (Huang and Mazin, 2014). Thus

it could be possible to use a small-molecule inhibitor in ALT positive cells lines to successfully inhibit the activity of RAD51 required for HOP2-MND1 mediated HR events.

Other small-molecule inhibitors of RAD51 have also been discovered and affect the function of RAD51 in different ways. One of these molecules, 3-chloro-1-(3,4-dichlo rophenyl)-4-(4-morpholinyl)-1H-pyrrole-2,5-dione, also known as RI-1 (RAD Inhibitor 1), inhibits the formation of RAD51 foci in human cells (Budke *et al.,* 2012). The ability of small-molecule inhibitors of RAD51 to disrupt the proper function of this protein can also result in other effects, such as an increase sensitivity to other chemotherapeutic drugs. Many common chemotherapeutic drugs cause interstrand cross-links in DNA, thus successfully inhibiting replication of DNA during S-phase, which subsequently may result in apoptosis. It has been shown that the small-molecule inhibitor RI-1 could increase the sensitivity of cancer cells to these sorts of chemotherapeutic drugs (Budke *et al.,* 2012). As ALT research continues and potential targets to inhibit this pathway are identified, small-molecule inhibitors will be important to consider. The ability of these small-molecule inhibitors to directly inhibit the mechanisms that facilitate replicative immortality in ALT positive cells, as well as increase the efficiency of other chemotherapeutic drugs, makes them useful tools when applying mechanisms identified in ALT research to a clinical setting.

VII. Conclusion

The ALT pathway is a complicated yet efficient way for cancer cells to avoid replicative mortality and progress through multiple cell divisions without initiating a DNA damage response potentially resulting in apoptosis. In somatic cells, the telomeres serve as a "mitotic clock" that helps determine when a cell has undergone a sufficient number of cell cycles and is ready to become senescent. This regulation is important to maintain a sufficient equilibrium between dividing and dying cells in the human body. Cells become cancerous when they are able to avoid cellular checkpoints that signal the cell to undergo apoptosis if its DNA has become damaged. One way cells are able to do this is by up-regulating mechanisms used to extend telomeres and shield telomeric DNA from eliciting a DNA damage response. Many cancer cells do this by up-regulating telomerase, but there is a subset of 10 to 15% of cancers that utilize the ALT mechanism to accomplish this goal.

Homologous recombination is at the heart of the ALT mechanism, and it is through its action that ALT positive cancer cells are able to acquire a template for telomeric DNA extension. There are many mechanisms involved in HR, and most of them have been shown to play some role in the ALT pathway. Various proteins used in the canonical HR pathways are also used in the ALT pathway. Thus, these proteins have been of interest to researchers as they try to understand both what causes a cell to initiate the ALT pathway and what signals are appropriate to maintain its utilization as a cancer cell continues to undergo subsequent cell divisions. The secondary structures found in telomeres allows for HR mediated events to occur in less than perfect ways, resulting in telomeric heterogeneity further convoluting what causes the ALT phenotype to be observed.

As the field of ALT research continues to expand, new mechanistic steps essential to the ALT pathway are continuing to be discovered. Proteins involved in inducing telomeric DNA associations with APBs has provided important insight into what environment is conducive for telomeres to initiate and maintain the ALT pathway. Proteins in the structural maintenance of chromosomes family have been demonstrated as important mediators of other events necessary for the ALT pathway to be successfully implemented. The sumoylation activity of proteins will be an important avenue to research as we begin to better characterize the effects this has on protein organization at the telomeres. A more thorough investigation of proteins specifically involved in meiosis that also play a part in the ALT pathway will be essential for future therapeutic applications of ALT research. Time and time again, ALT research has proven to be an important avenue to explore when characterizing how cancer cells successfully avoid replicative mortality.

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