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Telomere dysfunction in human bone marrow failure syndromes

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Approximately 90% of all human cancers, in which some deregulation of cell cycle arrest or programmed cell death has occurred, express telomerase, a ribonucleoprotein whose activity is normally turned off in healthy somatic tissues. Additionally, small populations of self-renewing stem cells, such as hematopoietic stem cells, skin and hair follicle basal layer cells and intestinal basal crypt cells, have been shown to retain telomerase activity. Conversely, hereditary defects that result in shortened telomeres in humans have been shown to manifest most often as bone marrow failure or pulmonary fibrosis, along with a myriad of other symptoms, likely due to the loss of the stem and/or progenitor cells of affected tissues. The aim of this review is to highlight our knowledge of the mechanisms of telomere maintenance that contribute to the pathology of human disease caused by dysfunctional telomere homeostasis. Specifically, a new role for the *SNM1B/Apollo* nuclease in the pathologies of Hoyeraal-Hreidarsson syndrome will be discussed.

Key words: telomere, dyskeratosis congenita, telomerase, SNM1B, Apollo

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Introduction

The vitality of a cell lineage encompasses an intricate balance between forces that promote indefinite proliferative potential and forces that promote apoptosis or senescence. The length of a tract of G-rich double-stranded repetitive sequence found at the ends of linear chromosomes, termed telomeres, is a major determinant of the replicative limit of some human somatic cell types.[1] Telomeres erode with every cell division due to the inability of canonical DNA polymerases to completely replicate the 3′ end of each parental DNA strand. Most eukaryotes express the enzyme telomerase to combat telomere erosion. Telomerase is a ribonucleoprotein that adds a unique sequence onto the terminal 3′ overhang present at chromosome ends via its reverse transcriptase catalytic subunit, hTERT, with the aid of an RNA subunit, hTR, which provides the template for the telomere sequence.[2] Canonical telomere binding proteins TRF1, TRF2, POT1, TPP1, RAP1 and TIN2, collectively termed the shelterin complex in mammals, modulate telomere length by negatively and positively regulating telomerase activity at telomeres.[3] A multitude of other proteins, involved in processes such as DNA damage response, cell cycle regulation and RNA metabolism, play crucial roles in telomere maintenance by promoting the biogenesis or activity of telomerase or the integrity of the telomeric DNA itself. Telomeres are thought to evade a DNA damage response by adopting a conformation, termed T loop, where the 3′ overhang loops back to form a strand invasion intermediate within the double-stranded telomere duplex.[4] Uncapped or dysfunctional telomeres can be visualized through Telomere Dysfunction-Induced Foci (TIFs), which are comprised of several proteins and chromatin changes that coincide with telomeres upon the incitement of a DNA damage signal. Such foci may correspond to triggers of apoptosis or senescence.[5]

Telomerase Dysfunction in Human Disease

Dyskeratosis congenita (DC) was the first disease found to be caused by

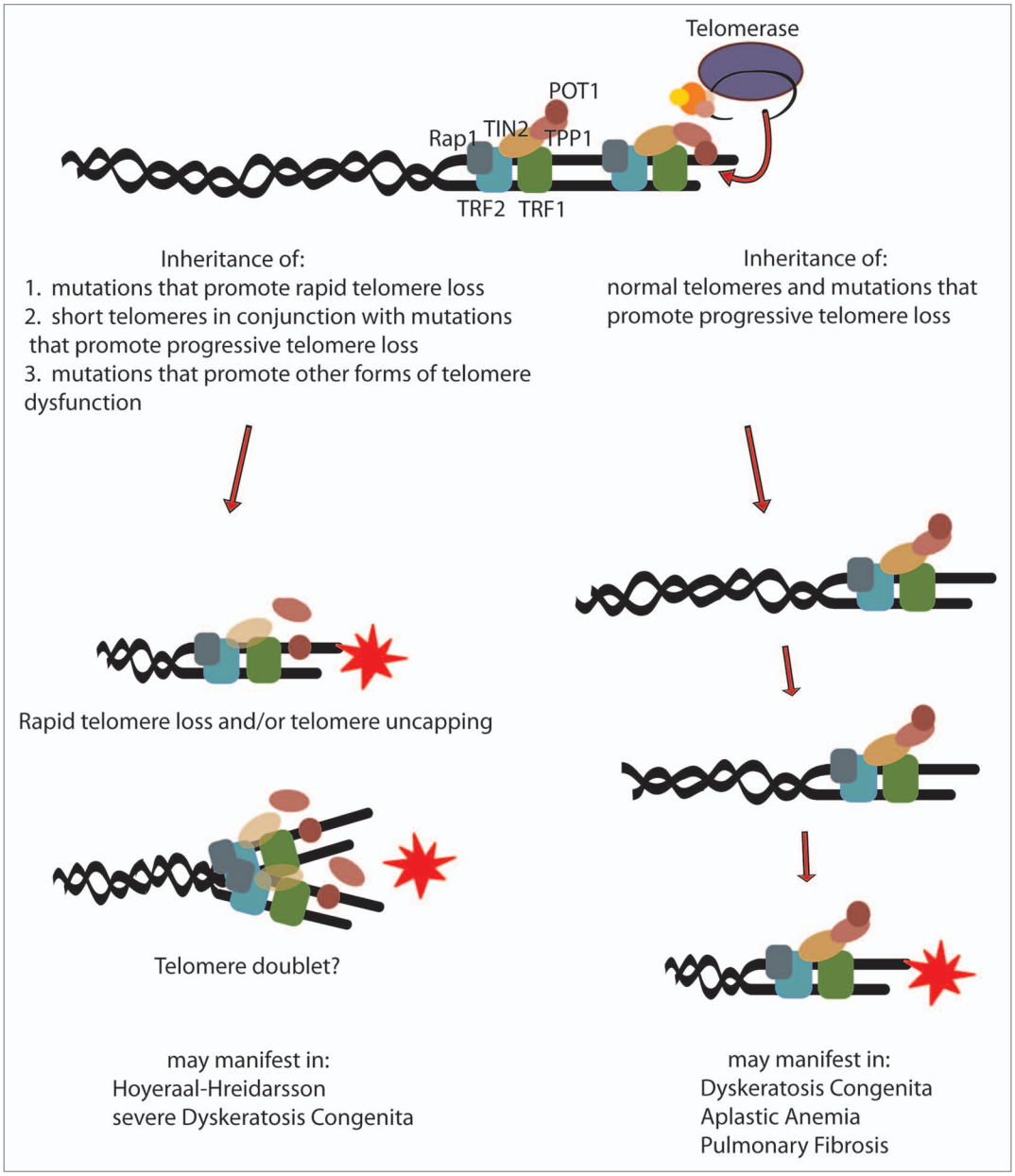

Figure 1. Several forms of telomere dysfunction—such as progressive or rapid telomere loss or hypothetical telomere doublets—manifest in varied severity of diseases of bone marrow failure in humans.

dysfunctional telomere maintenance in humans. Around 80% of DC patients exhibit the classic triad of diagnostic muco-cutaneous symptoms (abnormal skin pigmentation, nail dystrophy and oral leukoplakia) by an average of 10 years of age. They are over 90% likely to develop bone marrow failure, which causes almost 70% of patient deaths by 40 years of age. An additional 20% of patients succumb to either pulmonary complications or malignancy.[6] In 1998, mutations in *DKC1*, which encodes the protein dyskerin, first implicated aberrantly shortened telomeres as the causal defect in the X-linked syndrome.[7] Dyskerin, a protein that associates with small nucleolar RNAs (snoRNAs), interacts with the box H/ACA domain structure found at the 3' end of human telomerase RNA, hTR.[8,9] Aside from dyskerin, mutations in other snoRNA-associated proteins NOP10 and NHP2, which are required for telomerase biogenesis, have been identified in DC patients.[10,11] The box H/ACA RNA binding proteins dyskerin, NHP2, NOP10 and GAR1 promote stabilization and assembly of the entire telomerase complex, and biochemical purification of human telomerase has shown a close physical interaction with all four proteins.[12] Definitive support for telomerase complex dysfunction playing a causal role in DC pathology was provided by identification of disease-causing mutations in *hTERT* and *hTR* in DC patients.[13,14] Families with mutations in *hTR* or *hTERT* display anticipation, where successive generations inherit shorter telomeres and therefore exhibit initial DC

symptoms earlier in life.[15,16] Patients also exhibit shorter telomeres at every age compared to healthy individuals.[17]

Most *hTR* and *hTERT* mutations that cause disease do so when heterozygous, indicating haplo-insufficent or dominant-negative effects on telomerase function.[18,19] Patients who are homozygous or multiply heterozygous for mutant alleles of *hTERT* or *hTR* display an exacerbated reduction of in vitro telomerase activity, although telomere length, as opposed to the presence of one or two mutant alleles in affected individuals, serves as the most reliable predictor of symptom severity.[20] In fact, 30 out of 72 families with individuals diagnosed with DC harbor the same *DKC1* mutation but exhibit a heterogeneous clinical presentation and severity of symptoms, likely due to variations in telomere length at individual chromosome ends or in polymorphisms in other genes that affect rates of telomere erosion during development.[21] Lymphoblastoid cell lines derived from DC patients exhibit accelerated telomere shortening that causes increased levels of cell death, which may represent an in vivo mechanism of dysfunction where stem cell depletion manifests in the pathologies seen in these patients.[22]

The high prevalence of aplastic anemia (AA) and pulmonary failure in DC patients led to an investigation of whether dysfunctional telomere replication plays a more general role in these pathologies. Indeed, mutations in *hTR* were found in several AA patients, whose telomeres were shorter than healthy controls.[23] Patients suffering from other bone marrow disorders such as paroxysmal nocturnal hemoglobinuria (PNH) and myelodysplasia (MDS) also were found to harbor *hTR* mutations.[24] More recently, mutations in *hTR* and *hTERT* were identified in patients diagnosed with idiopathic pulmonary fibrosis (IFP), along with reports suggesting that liver cirrhosis may also occur as a consequence of telomerase deficiency.[25-27]

Note that telomere dysfunction may result in pathologies that are distinct from those observed in bone marrow failure and related disorders caused by telomerase deficiency. For example, the rare accelerated aging disease Werner syndrome results from mutations in the WRN helicase, which has been shown to cooperate with POT1, TRF1 and TRF2 to perform various tasks at human telomeres.[28,29] In contrast to telomerase deficiency, WRN dysfunction affects different tissues or cellular compartments, eliciting loss of hair, cataracts, diabetes mellitus and osteoporosis accompanied by premature death at a median age of 54 due to cardiovascular disease.[30] Thus, WRN deficiency may result in telomeric stress that affects a unique subset of cell types.

Dysfunctional Telomere Capping Proteins in Human Disease

Deleterious mutations in single alleles of the gene *TINF2*, which encodes one of the shelterin complex proteins, TIN2, were identified in several patients diagnosed with DC and ataxia-pancytopenia.[31,32] TIN2 serves as a scaffold for shelterin proteins TPP1 and POT1 by tethering them to telomeric double-stranded DNA via interactions with TRF1 and TRF2.[33,34] DC patients with *TINF2* mutations exhibit an earlier onset and a higher severity of symptoms than most patients with *hTR* or *hTERT* mutations.[35] The dramatically short telomeres seen in cells from patients carrying mutated *TINF2* could be symptomatic of rapid telomere dysfunction due to telomere uncapping. This is unlike the progressive telomere shortening seen over generations in patients with dysfunctional telomerase, because *TINF2* mutations arise de novo in the germ cells of parents of DC patients. Thus, accelerated telomere shortening due to telomere deprotection is the likely mechanism at fault since these patients inherit telomeres of normal length. Even though a dramatic reduction of in vitro telomerase activity has been reported for one ataxia-pancytopenia patient, unperturbed hTR levels in several DC patients with *TINF2* mutations supports a telomere uncapping/deprotection pathology in most cases.[31,32] Inconsistent with what is seen in the former patients, TIN2 has been shown to act as a negative regulator of telomerase in vitro, where siRNA-mediated knock-down of TIN2 in human cells elicits progressive telomere elongation.[36] The basis of this discrepancy remains unclear and requires further analysis of the effect of TIN2 mutations on protein function in patients. For example, overexpression of TIN2 lacking its carboxy-terminus leads to telomere attrition in human cells, whereas overexpression of TIN2 lacking its amino-terminus leads to telomere extension.[37] Thus, analysis of mutant TIN2 proteins that elicit human telomere dysfunction may reveal variants that exhibit effects similar to that of in vitro C-terminal truncation of this protein.

The former studies of *TINF2* deficiency suggest that other shelterin components, *TRF2*, *TRF1*, *RAP1*, *TPP1* and *hPOT1*, may be plausible candidates for the dysfunction seen in patients suffering from inherited bone marrow failure disorders. For example, several lines of evidence allude to the possible involvement of hPOT1 in the pathologies of these diseases. *mPot1b*$^{-/-}$ mice, which lack one of two mouse POT1 genes, display a massive increase in apoptosis in highly proliferative cells of the testes and intestine.[38] In the context of a telomerase haploinsufficiency, *mPot1b*$^{-/-}$ phenotypes are exacerbated and manifest in reduced body size as well as dyskeratosis congenita-like symptoms of hyperpigmentation of the extremities and nail dystrophy.[38] These mice also have a severely reduced lifespan accompanied by symptoms suggestive of bone marrow failure towards the end of their life. While total telomere length is unaffected in the liver tissue of *mPot1b*$^{-/-}$, *mTR*$^{+/-}$ mice, immortalized *mPot1b*$^{-/-}$, *mTR*$^{+/-}$ mouse embryonic fibroblasts (MEFs) exhibit a loss of overall telomere length.[38] An independent study has shown that mouse POT1 proteins protect telomeres from initiating DNA damage response signaling and from nuclease-mediated erosion of the 5' end of the chromosome terminus.[39]

hPOT1 binds to single-stranded telomeric DNA and is tethered to the double-stranded portion of the telomere through its shelterin binding partners.[40-42] hPOT1 has been shown to positively and negatively regulate telomere length, and its knock-down consistently led to DNA damage response signaling at telomeres, affirming a role in telomere protection.[43-45] While hPOT1 binding to the terminal single-stranded 3' end of a telomeric substrate precludes telomerase extension, interaction of hPOT1 with TPP1 can stimulate telomerase processivity.[46,47] TPP1 itself has been

shown to possess dual roles in telomere length regulation, where cells transformed with TPP1 lacking its OB-fold exhibited telomere shortening while cells transformed with TPP1 lacking the first 86 amino acids of the N-terminus exhibited extensive telomere elongation.[48] Two Pot1 proteins in *Arabidopsis thaliana* take on different roles in telomere length regulation, where AtPot1a associates with the telomerase complex and is essential for telomerase activity in vivo, while AtPot1b functions in chromosome end protection.[49,50]

Recently, a protein with POT1 homology in *C. elegans*, MRT-1, was shown to be essential for telomere maintenance in vivo.[51] MRT-1 is a dual-domain protein with homology to the second OB-fold of hPOT1 and to the nuclease domain of the SNM1 family of proteins, which function in DNA damage repair and cell cycle checkpoint response.[52,53] A mutation in the OB-fold of *mrt-1(yp2)* not only abolishes the ability of MRT-1 to bind single-stranded DNA in vitro and abrogates telomere replication in vivo, but also results in increased susceptibility to ICL-induced damage. The mild deficiency in ICL repair seen in this mutant might be caused by a defect in interacting with substrates, due to a mutant OB-fold. While the role of the SNM1 nuclease domain of MRT-1 in telomere maintenance is presently unclear, these results highlight the crucial role for the POT1-derived OB-fold domain of MRT-1 in promoting telomerase activity. Consistently, AtPot1a promotes telomerase RNP activity in Arabidopsis and POT1/TPP1 may promote telomerase activity in a context-dependent manner in human cells.[47,50]

Together, studies of POT1 homologs in diverse organisms suggest that reduced or altered hPOT1 function in certain human tissues might cause progressive telomere shortening, via effects on either telomere capping or telomerase function, which could precipitate lethal failure of the hematopoietic or pulmonary systems.

SNM1B/Apollo is Involved in the Pathology of Bone Marrow Failure Syndrome Hoyeraal-Hreidarsson

Genes responsible for almost half of DC cases remain molecularly undefined, as is the case for even greater proportions of idiopathic pulmonary fibrosis and aplastic anemia patients, where no causal polymorphisms were found in described telomere maintenance genes (*hTR*, *hTERT*, *NOP10*, *NHP2*, *DKC1* and *TINF2*). Recently, one of the SNM1 family genes in humans, *SNM1B/Apollo*, which possesses the ancestral SNM1 function of interstrand cross-link (ICL) repair and interacts with the shelterin component TRF2, has been implicated as a causal factor in the symptoms of telomere dysfunction in a patient diagnosed with Hoyeraal-Hreidarsson (HH) syndrome.[54-57] HH syndrome is characterized by more severe and earlier-onset DC-associated abnormalities as well as additional phenotypes including cerebellar hypoplasia, microcephaly, immunodeficiency, AA and growth retardation.[58] While HH can be caused by homozygous mutations in *hTERT* and hemizygous mutations in *DKC1*, this patient harbors a dominant-negative splice form of Snm1B/Apollo, "Apollo-Δ", and fibroblasts from this patient exhibited similar telomere lengths to that of normal age-matched controls.[59] Thus, critically shortened telomeres may not be the direct cause of deterioration of affected tissues in this patient, consistent with reports for other HH patients where telomere shortening was not reliably observed.[60] However, additional forms of telomere dysfunction, including shortened 3' overhangs, Telomere Dysfunction-Induced Foci (TIFs), end-to-end chromosome fusions and unusual telomere doublet structures were apparent in patient fibroblasts, suggesting that (one of) these phenotypes, rather than telomere shortening, may be directly relevant to disease.[56,60] Specifically, the higher frequency of telomere doublets, as compared to telomere chromatid fusions, in both fibroblasts transformed with Apollo-Δ as well as in cultured fibroblasts from this HH patient, implicates these doublets as a causal factor in the observed dysfunctional phenotypes.[56] Moreover, an independent study has indicated that cells transformed with nuclease-dead versions of SNM1B/Apollo also exhibit significant increases in telomere doublets, confirming a role for SNM1B/Apollo in preventing the formation of these structures, whose nature remains uncertain.[61]

Recently, a role has been proposed for SNM1B/Apollo in telomere protection through generation of 3' single-stranded overhangs via 5' C-strand resection at newly replicated blunt leading-strand telomeres, which are subjected to non-homologous end joining (NHEJ)-mediated fusion in the absence of SNM1B/Apollo.[62,63] *SNM1B/Apollo*$^{-/-}$ MEFs exhibit leading-strand chromatid-type fusions and reduced overall 3' overhang intensity, phenotypes that are suppressed when cells are reconstituted with wild-type SNM1B/Apollo, but not with SNM1B/Apollo lacking its TRF2-interacting domain or nuclease activity. Thus, the 5' to 3' nuclease activity of SNM1B/Apollo, as well as its physical presence at telomeres, are crucial elements of telomere protection and overhang maintenance.[62,63] The lack of increased sensitivity of cells transformed with Apollo-Δ to IR or ICL-inducing agents indicates that these cells likely retain proper Apollo nuclease activity, which is required for its DNA repair functions, but that this activity is unable to function properly at the telomere, probably due to the inability of Apollo-Δ to bind TRF2, which not only tethers SNM1B/Apollo to the telomere but also stimulates its nuclease activity.[61] Overexpression of nuclease-inactive SNM1B/Apollo in wild-type human cells resulted in an increase in TIFs and accelerated onset of senescence, similar to what is seen in cells transformed with Apollo-Δ.[61]

It is likely that the dysfunction caused by the splice variant Apollo-Δ manifests in the HH patient as a defect in telomere capping, or in the process that prevents telomere doublets, rather than a defect in telomerase itself. The maladies of this Hoyeraal-Hreidarsson patient present as more severe in penetrance and age-of-onset than those exhibited by DC patients, similar to the severity of disease that patients with mutations in *TINF2* experience and allude to the importance of telomere processing and capping in early onset forms of telomere dysfunction.

Analysis of the other two SNM1 family genes has revealed that *SNM1A* functions in ICL repair and has been implicated as a tumor suppressor, but has no known role at telomeres.[53] *SNM1C/Artemis* has been extensively studied

due to its role in V(D)J recombination, which is dependent on its collaboration with DNA-PKcs.[53] *Artemis* deficiency in humans leads to human severe combined immunodeficiency (SCID) syndrome, which is mimicked in mice that exhibit SCID-like symptoms when *Artemis* is deficient.[53] *Artemis* has been implicated in telomere maintenance through several observations where *Artemis*-null mouse ES lines exhibited telomeric fusions, general genomic instability and telomere shortening.[64,65] However, this is a mild telomere fusion phenotype in comparison to that seen for lethal SNM1B/Apollo mutations. Although a single SNM1 homolog exists in *C. elegans*, the MRT-1 POT1 OB-fold/SNM1 fusion protein, null alleles of the *mrt-1* gene yield a progressive telomere erosion phenotype that is indistinguishable from that of telomerase mutants. Significant levels of end-to-end fusions are not apparent in early generation *mrt-1* strains with long telomeres (Ahmed S, unpublished), suggesting that a role in telomere capping or leading-strand resection is derived for or otherwise specific to SNM1B in vertebrates. Nevertheless, a role in telomere biology is suggested for the SNM1 domain of MRT-1 given its fusion to the telomerase-promoting POT1 OB-fold.

Conclusion

The discovery of causal mutations in proteins involved in telomerase-mediated telomere maintenance, *hTR*, *hTERT*, *DKC1*, *NOP10*, *NHP2*, as well as in proteins that promote telomere capping, *TINF2* and *Apollo/SNM1B*, in patients with debilitating bone marrow failure syndromes contributes to our understanding of how telomere biology confers disease pathology in humans. Unusual telomere capping abnormalities that do not perturb telomere length but affect replication and DDR signaling at telomeres can result in the culling of stem or progenitor cell populations, particularly in hematopoietic and pulmonary systems, with lethal consequences. Mutations in telomere binding proteins or proteins that transiently interact with telomeres to promote telomere capping, can lead to rapid and severe symptoms in affected individuals.[66-68] Likewise, mutations in the many genes that function in telomerase complex stability, biogenesis or recruitment to telomeres can yield either a subset or the full spectrum of DC-like symptoms that are typically milder than those seen in HH patients and take multiple generations to cause strong effects (**Fig. 1**).[69-72] Elucidation of the respective disease contributions of telomeric doublets, which commonly occur in cells that harbor Apollo-Δ, in comparison to minor but possibly significant levels of chromosome fusion, represent important questions in the field. These questions may be solved by continued analysis of cells from a spectrum of human patients with dysfunctional telomeres in conjunction with basic research in telomere biology.

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