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Review Article

Telomere Dysfunction in Human Diseases: The Long and Short of It!

Kathryn A. Carroll and Hinh Ly

Department of Pathology and Laboratory Medicine, Emory University, Atlanta, GA

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Abstract: It has been over one hundred years since the first reported case of dyskeratosis congenita (DC) and over twenty since the discovery of telomerase, an enzyme that adds telomeric DNA repeats to chromosome ends. Emerging evidence suggests that telomere dysfunction plays an important role in the pathogenesis of DC and other human disorders involving tissues that require rapid repair and renewal capacities. Yet we still do not fully understand how mutations in telomere maintenance genes contribute to disease development in affected individuals. In this review, we provide an up-to-date summary of the topic by discussing the results from genetic screens of patients, *in vitro* mutational analysis of involved molecules, and genetically engineered mouse models. While these data shed important light on the mechanisms underlying disease development, further investigation, particularly in an *in vivo* setting, is needed.
Key Words: Telomere, telomerase, aplastic anemia, dyskeratosis congenita, idiopathic pulmonary fibrosis

Introduction

Human Telomerase

Telomerase is a ribonucleoprotein complex whose main function is to add six nucleotide repeats onto the ends of chromosomes utilizing its reverse transcriptase (hTERT) and its intrinsic RNA template (hTERC), as well as the associated proteins dyskerin, NOP10, NHP2, and GAR1 (**Figure 1**). This DNA elongation is necessary to overcome the "end-replication problem" whereby the conventional DNA polymerases cannot fully replicate linear chromosomes [1, 2]. This phenomenon, coupled with oxidative damage, and other exogenous or endogenous effects, causes our telomeres to be shortened by approximately 50-100bp per cell division. Telomere erosion limits the replicative capacity of the majority of somatic cells which do not express active telomerase [3, 4]. Cells whose telomeres shorten to a "critical length" enter a stage termed replicative senescence whereby cell division is prevented [5, 6]. Stem cells, germ cells, and certain types of somatic cells circumvent this barrier by expressing the telomerase enzyme, allowing them to maintain their telomere length and escape senescence.

The hTERT has been extensively studied and hence several of its functional domains have been mapped [7]. The protein is defined by the catalytic domain, which contains seven reverse transcriptase motifs essential for enzymatic activity. The C terminus is short and highly divergent among different species, and its exact function is not completely clear at this point. However, one region has clearly emerged, termed the C-DAT for the C-terminal region that dissociates the activities of telomerase. Mutations in this domain generate enzymes which are catalytically active *in vitro* but biologically inert. In contrast, the N-terminus contains several evolutionarily conserved regions important for hTERT's cellular localization, RNA interaction, protein-protein multimerization, and enzymatic function. Functionally important regions have also been defined in the hTERC (**Figure 1**) [8]. Most obviously, the template region is absolutely required for the hTERT protein to reverse transcribe it into telomeric DNA repeats. In addition, the pseudoknot domain is required for telomerase activity, hTERT binding, and hTERC RNA dimerization, while the Box

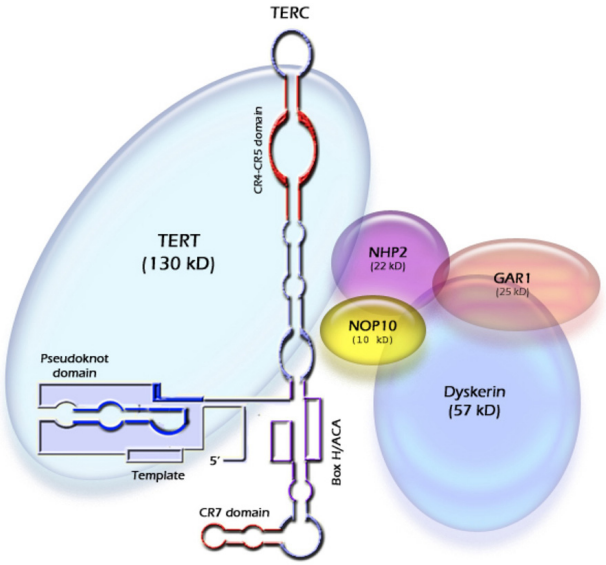

Figure 1 Telomerase holoenzyme. Simplified illustration of the telomerase holoenzyme showing its main components: hTERT, hTERC, Dyskerin, NOP10, NHP2, and GAR1. Functional regions of the hTERC RNA (template, pseudoknot, CR4-CR5, Box H/ACA, and CR7) are indicated.

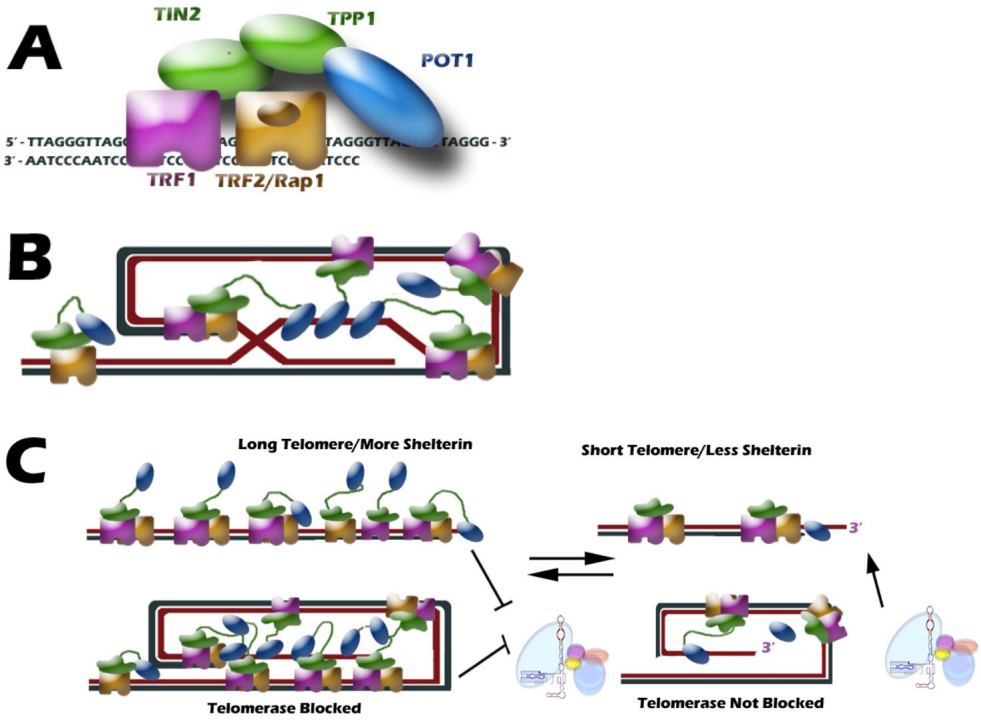

Figure 2 Shelterin Complex. **A.** Six components of the telomere-binding complex, shelterin, and their DNA and protein binding abilities. **B.** A schematic of the shelterin complex bound to the telomere t-loop structure. **C.** A proposed model for how shelterin can function to control telomere length in *cis*. Long telomeres allow for more shelterin binding, which can block access of telomerase to the telomere end. In contrast, a short telomere with less shelterin bound is preferentially elongated by telomerase.

H/ACA domain is important for hTERC RNA processing and stability. Despite extensive work to map the aforementioned motifs, it is still relatively unclear which particular residues are absolutely required for the activity of either of the telomerase core components.

Telomere Structure and the Shelterin Complex

The tips of chromosome ends consist of a long strand overhang composed of the G-rich strand (TTAGGG; as opposed to the C-rich strand CCCTAA). In order to avoid being recognized as a double-strand break and corrected by DNA repair machineries, a fate quite detrimental to the cell, the single-stranded region folds back upon itself and tucks into the adjacent double-stranded telomeric region, forming a telomeric loop (t-loop) (**Figure 2B**; [9]). This structure is formed and protected by a collection of six proteins, termed the shelterin complex, which with the telomeric DNA repeats compose the entire nucleoprotein structure commonly referred to as telomeres. The shelterin complex is formed by the double-stranded DNA binding proteins, TRF1 and TRF2; a binding partner of TRF2, RAP1; a single-stranded DNA binding protein POT1; and the two bridging proteins, TIN2 and TPP1 (**Figure 2A**; [9]). Not only do these proteins function in protecting the chromosome end, they also function in telomere length regulation. Telomere length is maintained within a strict range throughout cell division, suggesting a negative feedback loop involving the shelterin complex. Due to the exquisite specificity of these DNA binding proteins, the amount of shelterin protein bound to telomeres is roughly proportional to their length (**Figure 2C**; [9]). Thus, a long telomere would have a greater ability to inhibit telomerase activity, while a short telomere, with less bound protein, would be more accessible to telomerase elongation.

Telomerase/Telomere and Human Diseases

Bone marrow failure syndromes (BMFS) represent a diverse group of diseases with similar presentations, including dyskeratosis congenita, aplastic anemia, myelodysplatic syndromes (MDS), and others [10]. Overlapping symptoms and lack of concrete disease characterization make early diagnosis extremely difficult, especially when the few definitive phenotypes do not usually manifest until later in the disease progression. The prognosis for affected individuals can be bleak as the most prominent treatment is bone marrow transplant and frequently matching bone marrow donors are difficult to find. The fact that patients with BMFS have shortened telomeres led us and other researchers to screen these patients for mutations in telomerase and some protein components of the shelterin complex. These efforts yielded mutations in hTERT (**Figure 3**; [11-24]), hTERC (**Figure 4**; [20, 22, 25-43]), the telomerase-associated proteins dyskerin [44-57], NOP10 [58], and NHP2 [59], and the shelterin components TRF1 [60], TRF2 [60], and TIN2 (**Figure 5**; [61-64]). These findings support the hypothesis that dysfunctional telomeres due to mutations in telomere maintenance genes lead to exhaustion of the stem cell compartment and hence to various defects in cell types with a high turnover rate such as the hematopoietic system. In addition to hematopoietic malignancies, mutations in these components can also contribute to idiopathic pulmonary, liver, and heart fibroses [12-14, 19, 41]. Why mutations in the same proteins can be found in diseases with similar yet different phenotypes is unclear. It is likely that other factors (exogenous and/or endogenous) might be involved in the pathogenesis of these diseases, but the influence of telomere length regulation on cell proliferation cannot be discounted. Thus, a more thorough study of these molecules, their functions, and their regulation is necessary in order to fully understand them and to possibly allow more targeted therapies for these ailments.

Telomerase Mutations in Human Blood Disorders

Dyskeratosis Congenita

Dyskeratosis congenital (DC) is a rare inherited disorder characterized by a triad of clinical symptoms: mucosal leukoplakia, nail dystrophy, and abnormal skin pigmentation [65]. The majority of the cases (>80%) occur in children, who experience BMFS and the aforementioned physical anomalies generally by the age of 10. Other symptoms indicative of premature aging, including pulmonary diseases, dental abnormalities, esophagostenosis, and alopecia, are often associated with the disease in 15-25% of the cases. Solid tumors of the gastrointestinal tract, nasopharynx and skin, and

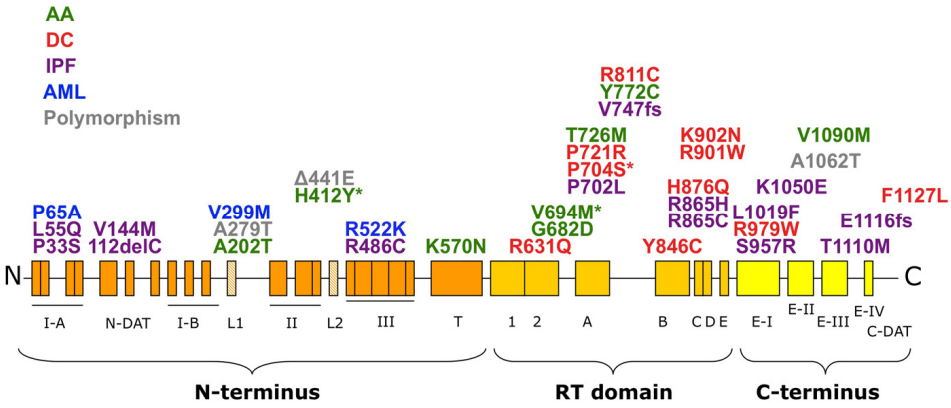

Figure 3 Natural hTERT mutations. All exonic sequence changes identified in patients and/or controls are shown. Mutations are color-coded according to the disease in which they were first identified. Those denoted with an asterisk (*) have been found in multiple telomere dysfunction disorders (H412Y: AA, AML; V694M: AA, IPF; P704S: DC, IPF). AA: aplastic anemia; DC: dyskeratosis congenita; IPF: idiopathic pulmonary fibrosis; AML: acute myeloid leukemia.

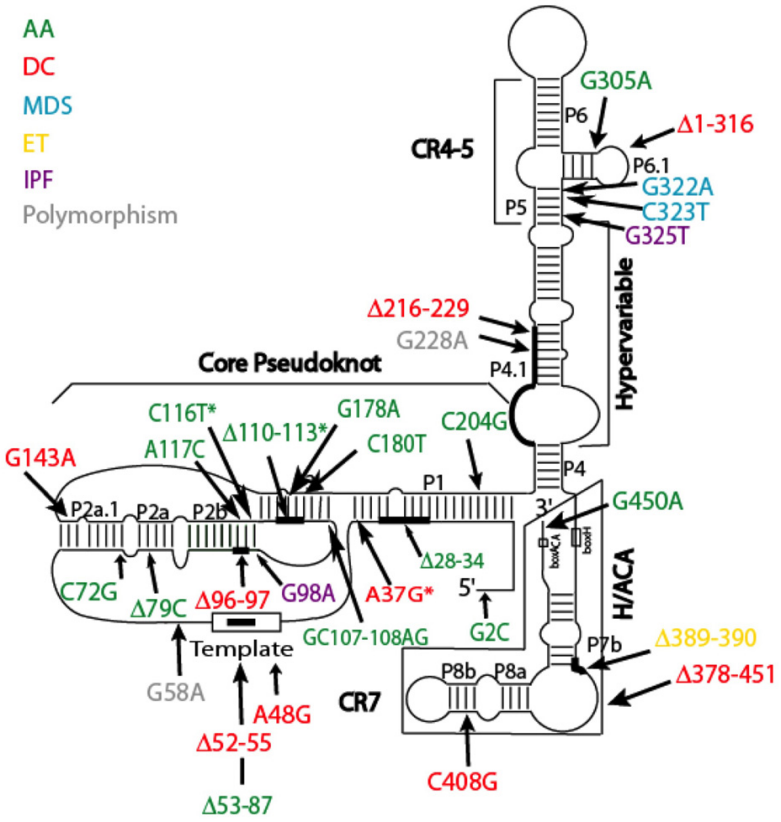

Figure 4 Natural hTERC mutations. All naturally-occurring sequence changes in the hTERC coding region are shown. Mutations are color-coded according to the disease in which they were first identified. Those denoted with an asterisk (*) have been found in multiple telomere dysfunction disorders (A37G: DC, IPF; Δ110-113: AA, MDS; C116T: AA, MDS). AA: aplastic anemia; DC: dyskeratosis congenita; MDS: myelodysplastic syndromes; ET: essential thrombocythemia; IPF: idiopathic pulmonary fibrosis.

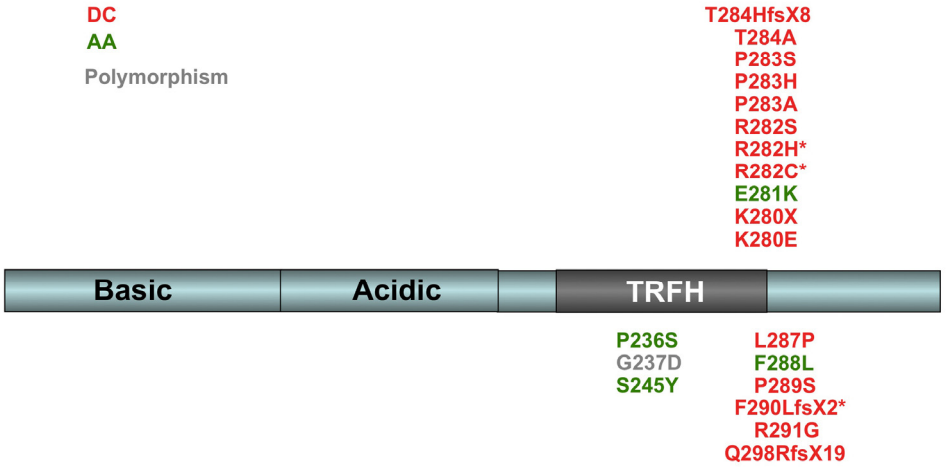

Figure 5 Natural TIN2 mutations. All known sequence changes in the TIN2 coding region are shown. Acidic and basic domains are denoted based on the amino acid content in these regions. The TRFH domain is the general region that has been shown to interact with TRF1. Mutations are color-coded according to the disease in which they were first identified. Those denoted with an asterisk (*) have been found in multiple telomere dysfunction disorders (R282C: DC, AA; R282H: DC, AA; F290LfsX2: DC, AA). DC: dyskeratosis congenita; AA: aplastic anemia.

hematopoietic malignancies (e.g., MDS, Hodgkin lymphoma and acute myelogenous leukemias) have also been observed in some DC patients [66]. Since this disease affects rapidly renewing tissues, it has been speculated that DC is a telomerase disease in all three different patterns of inheritance: X-linked recessive, autosomal dominant, and autosomal recessive. In support of this theory, most DC patients have short telomeres [67, 68] and carry mutations in the three main components of the telomerase holoenzyme complex: dyskerin, hTERT (protein), and hTERC (RNA) [69].

The X-linked form of DC is the most severe and is caused by mutations in the *DKC1* gene on chromosome Xq28. *DKC1* encodes dyskerin, a 514 amino acid, nucleolar protein in the H/ACA family, which is highly conserved throughout evolution. As is the case with other H/ACA proteins, dyskerin is predicted to function in ribosomal RNA processing, in addition to its predicted role in the biogenesis of the telomerase holoenzyme. Most *DKC1* mutations are missense mutations and include a 3' deletion, suggesting that both frameshift and null mutations are incompatible with life [44-47, 49-53, 55, 57]. Indeed, a *DKC1*-null mouse model is embryonically lethal [70]. In humans, one mutation (A353V) is seen quite frequently in both X-linked DC and a more severe form of the disease, the Hoyeraal-Hreidarsson (HH) syndrome (see below), and accounts for approximately 30% of all X-linked DC cases. A number of intronic mutations have also been found, in addition to a promoter mutation (-141C→G), which destroys an Sp1 binding site. Female carriers of *DKC1* mutations show skewed X inactivation patterns due to the fact that cells expressing the normal allele have an inherent growth advantage. Yet, it remains to be determined the extent to which each of dyskerin's functions (rRNA processing or telomere maintenance) contributes to the X-linked DC phenotype.

Now considered a more severe allelic form of X-linked DC, the Hoyeraal-Hreidarsson (HH) syndrome is a multisystemic disorder characterized by mental retardation, microcephaly, intrauterine growth retardation, and aplastic anemia [71]. More recently, progressive combined immune deficiency has come to be regarded as another common symptom in this disease [72]. Missense mutations in dyskerin found in HH families segregate with the disease [48, 54, 56]. However, as of yet, there is no explanation for why different mutations in the same protein can cause such diverse phenotypes. The situation is further complicated by the identification of a female HH patient from a

consanguineous marriage of asymptomatic parents who carries a homozygous mutation in hTERT [17]. However, it is quite possible that is in fact a very severe case of autosomal dominant DC due to the inheritance of moderately shortened telomeres from both parents.

The autosomal dominant form of DC (AD-DC) is much less severe and less common than the X-linked form. Like aplastic anemia (see below), mutations in hTERT and hTERC, as well as the telomere binding protein TIN2 have been associated with AD-DC (**Figures 3**, **4**, and **5**). As the vast majority of these mutations are heterozygous, it is possible that they could function as either haploinsufficient, with a single copy of the normal telomerase component being insufficient to maintain telomere length, or dominant negative, with the mutant telomerase component negatively affecting the wild-type copy. To determine which of these is the case, researchers transfect cells with both a wild-type and a mutant copy of the given telomerase component and perform the telomere-repeats amplification assay (or TRAP) in order to detect for the effect of the mutated copy on normal telomerase enzymatic function. If the mechanism is haploinsufficiency, the TRAP activity of the doubly-transfected cells will either only be slightly reduced or be the same as the activity of cells transfected with a single wild-type copy. In contrast, cells transfected with a wild-type copy and a dominant negative mutant would exhibit a complete abolishment of TRAP activity. While most of the mutations have been found to exert their effect by a haploinsufficiency mechanism, 2 mutations in the RNA component located in the template region (Δ52-55 and A48G) seem to act as dominant-negatives [22]. It remains to be seen, however, whether this is truly the case *in vivo*. In fact, in a different system recently developed by Errington *et al*, these mutations do not show the same dominant negative effect [73]. Interestingly, disease anticipation has been observed in families with AD-DC [38], a phenomenon whose mechanism has thus far always involved a genetic change, such as the expansion of triplet repeats in severe neurological disorders [74]. In AD-DC families, the genetic lesion does not change, yet the onset of disease features occurs, on average, 20 years earlier in the children than in their parents. Telomere length appears to play a role in this accelerated presentation as telomeres were significantly shorter in the second generation of affected families as compared to normal families. This trend is echoed in TERC knockout mice, where clinical features of telomere shortening and DC do not develop until the fourth generation, with sixth generation mice becoming infertile [75-78].

The causal gene for the autosomal recessive form of DC remains elusive. A recent study by Walne *et al* aimed at uncovering the genetic basis for the disease concluded that there is no single locus responsible [58]. Nevertheless, a homozygous mutation in the NOP10 protein was found in all 3 affected members of a single family and is predicted to alter protein structure and may affect endogenous hTERC RNA levels as NOP10 is a telomerase-associated protein that is predicted to aid in hTERC processing and assembly. This mutation (R34W) in NOP10 appears to segregate with the disease as unaffected family members are heterozygous and both patients and unaffected carriers do in fact have significantly shorter telomeres than controls. However, this mutation was not identified in any of the other 15 families screened, suggesting that it may be a very rare genetic risk factor of this form of the disease.

Aplastic Anemia

Aplastic anemia (AA) is a rare but serious bone marrow disorder, characterized by hypocellular bone marrow and low blood cell counts [79]. As patient leukocytes have significantly shorter telomeres than age-matched controls, we and other researchers have screened AA patients for mutations in telomerase components. Heterozygous mutations have been found in both the protein and RNA components of telomerase (**Figures 3** and **4**) as well as the telomere-binding proteins TRF1, TRF2 [60], and TIN2 (**Figure 5**; [62, 63]). It appears that the AA-associated RNA mutations tend to cluster in the conserved pseudoknot region, which is required for telomerase enzymatic activity and hTERT binding. All hTERC mutations identified in AA patients that have been examined thus far function as haploinsufficient, as opposed to dominant negative, at least *in vitro*. However, as patients with telomerase mutations present with highly varying symptoms, it remains to be seen if mutations at specific residues can explain the differing degrees of severity or if there are some other genetic or environmental factors at

play. It has also been suggested that some cases of AA may be classified as cryptic and atypical form of DC as they develop slowly over time and do not show the characteristic triads of physical anomalies as frequently observed in X-linked cases. Recently, Calado *et al* identified a mutation in the *SBDS* gene, the causative gene for another bone marrow failure syndrome, Shwachman-Diamond Syndrome, in some AA patients [80]. The significance of this mutation in AA has yet to be determined.

Myelodysplastic Syndromes

Myelodysplatic syndromes (MDS) encompass a group of diseases caused by abnormal blood-forming cells, such that the bone marrow cannot effectively produce blood cells, resulting in low blood cell counts. MDS is a clonal disease, meaning that the abnormal cell population arises from a single, abnormal cell. As such, some consider MDS a form of cancer, and, in fact, about 30% of MDS cases progress into acute myeloid leukemia (AML). Despite the bone marrow defects, mutations in telomerase components are extremely rare, with no mutations in hTERT or dyskerin having been identified to date. Only 4 isolated hTERC mutations have been reported (**Figure 4**; [29, 31, 33, 43]), in addition to two promoter region mutations, one of which is located in a putative Sp1 binding site [33, 42]. The significance of these mutations in the disease pathogenesis is unclear, however.

Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a heterogeneous disorder of hematopoietic progenitor cells, causing abnormal proliferation and differentiation, and can evolve from AA and MDS [81, 82]. In addition, a predisposition to developing cancer, including AML, is a characteristic of DC patients [82]. As genomic instability has been shown to be important for the development of the disease, Calado *et al* examined three cohorts of AML patients who show no physical signs of DC for sequence variation in the hTERT and hTERC genes [24, 81]. They identified three novel missense mutations in hTERT (**Figure 3**), and, while the V299M sequence change did not seem to affect telomerase enzymatic activity when tested by the TRAP assay, both the P65A and R522K mutations conferred dramatic defects. Surprisingly, they also identified three AML patients who are homozygous for sequence changes previously identified in a heterozygous state in AA patients and controls (A1062T and del441E) [23]. Thus, it appears that hTERT gene variants have low penetrance and are carried in patients with a wide variety of disorders. This phenomenon can be explained if short telomeres, as opposed to mutation status of telomerase, mediate disease pathogenesis, a hypothesis consistent with the fact that the median age at presentation for AML is 70 [81]. In corroboration with this, abnormal karyotypes were present in 18 of the 21 patients who were carriers of hTERT mutations, suggesting that these patients' excessively short telomeres have contributed to genomic instability and their development of AML. However, this correlation still needs to be validated *in vivo*.

Paroxysmal Nocturnal Haemoglobinuria

Paroxysmal nocturnal haemoglobinuria (PNH) is a clonal blood disorder arising from a defective blood cell lacking glycosylphosphatidylinositol (GPI)-anchored proteins due to a mutation in the *PIGA* gene [83, 84]. This disorder is commonly associated with aplastic anemia and as such, patients have been screened for mutations in telomerase components. While no mutations in dyskerin, hTERT, or hTERC have been found, a single mutation (-99C→G) within the Sp1 binding site in the promoter region of hTERC was isolated in a PNH patient [85]. Interestingly, this mutation was also found in patients with MDS [33]. While the effect of disrupting this site has not yet been determined *in vivo*, its activity *in vitro* seems to vary depending on the exact promoter context used for the luciferase reporter assay. In the minimal promoter context (nucleotides -107 to +10), the -99C/G mutation results in an increase in luciferase activity, suggesting a repressive role for the Sp1 binding site. However, a similar experiment performed by the same group, using a longer hTERC promoter sequence (-107 to +69) and a double substitution in the same site (C-101A/C-100A), identified this site as a positive regulator of hTERC transcription. Furthermore, preliminary data from our lab suggests that the -99C/G sequence change may not confer a dramatic defect when considered in the context of a much larger promoter construct of

1457bp (unpublished data). As both of these mutations have been shown to disrupt Sp1/Sp3 binding, these results suggest that this site may act as both positive and negative regulatory element to control hTERC gene expression.

Essential Thrombocythemia

Essential thrombocythemia (ET) is a rare chronic myeloproliferative neoplasm (CMPN), usually characterized by the overproduction of platelets by megakaryocytes in the bone marrow which generally affects middle-aged to elderly individuals [86]. An ET patient was recently identified who carries an hTERC allele with a two-nucleotide deletion [29]. This mutation (Δ389-390) failed to reconstitute telomerase activity *in vitro*, suggesting that telomerase may play a role in the disease pathogenesis of some patients. Since this disease tends to have a later age of onset, progressive telomere shortening and resulting genomic instability could possibly contribute to the ET phenotype. We have undertaken an effort to screen 90 patients who have been clinically diagnosed with CMPN, including 43 patients with ET, and found no mutations in the hTERC gene [26]. It is possible that other genetic factors may play a role in this group of diseases with highly diverse features. Indeed, recent studies have shown that a single acquired (somatic) mutation (V617F) in the tyrosine kinase *JAK2* gene seems to be strongly associated with CMPNs, found in more than half of patients with either ET or chronic idiopathic myelofibrosis and in almost all patients with polycythemia vera [87-89]. The mutation leads to constitutive activation of JAK2, which promotes cytokine hypersensitivity [90]. It may cause constitutive activation of an erythropoietin receptor (EpoR) even in the absence of stimulation by its natural ligand erythropoietin, which has been shown to induce erythocytosis in a mouse model.

Telomerase Mutations in Non-Hematological Disorders

Idiopathic Pulmonary Fibrosis

Idiopathic pulmonary fibrosis (IPF) is a progressive disorder with an autosomal dominant pattern of inheritance and variable degrees of penetrance and accounts for greater than 70% of all cases of idiopathic interstitial pneumonias (IIPs). It is characterized by symptoms of chronic cough and shortness of breath, as well as diffuse interstitial fibrosis [91]. Approximately 20% of DC patients also have some form of pulmonary disease, which can sometimes lead to permanent scarring of the lungs. It has been hypothesized that since there is an inverse relationship between caveolin-1 and TGF-β1 expression and TGF-β1 negatively regulates telomerase activity, there may be a link between a genetic predisposition and the actual molecular signaling. Wang *et al* has shown that patients with IPF have reduced expression of caveolin-1, providing a possible mechanism by which a change in gene expression may lead to telomere shortening in certain lung tissue progenitor cells [92]. In addition, different groups have independently isolated heterozygous mutations in both hTERT and hTERC in patients with IPF, which appear to function via haploinsufficiency [12, 14, 19, 41]. Both patients and carriers have shorter telomeres than age-matched controls. In fact, a recent paper by Alder *et al* shows that telomere shortening, in the presence or absence of mutations in telomerase components, may contribute to disease risk in IIP patients who have no family history [41]. Although the mutations appear to impair telomerase activity to different extents in *in vitro* TRAP assays, they confer a dramatic increase in susceptibility to this adult-onset and fatal disease. The significance of telomerase mutations in the development of IPF still needs to be demonstrated *in vivo*.

Cri du Chat Syndrome

Cri du chat syndrome (CdCS) is a disease in infants, which is characterized by a distinct cat-like cry, in addition to other physical anomalies, including microcephaly, widely spaced eyes, low set ears, a low broad nasal bridge, and palmar creases [93]. It results from loss of the distal portion of chromosome 5p, a region that encompasses the hTERT and several other genes. Indeed, fluorescence in situ hybridization (FISH) analysis on patient lymphocytes and fibroblasts showed only a single copy of hTERT, indicating that cells may be haploinsufficient for telomere maintenance [94]. The accelerated telomere shortening predicted by this hypothesis was confirmed in patient lymphocytes by a reduction in Q-FISH signal and shorter telomere restriction fragments (TRFs) as compared to those of age-

matched controls. While it has been shown that patient lymphocytes and fibroblasts have only one copy of hTERT and that dermal fibroblasts have an impaired replicative capacity, it is unclear how loss of the catalytic component of telomerase could cause all the symptoms associated with CdCS. It has been proposed that accelerated telomere shortening and subsequent progenitor cell death could adversely affect normal fetal development. However, since other genes are also deleted from chromosome 5p in this disease, it remains to be seen whether hTERT is truly the causal gene or just one of many genetic factors leading to CdCS.

Mouse Models of DC

X-Linked DC

Two approaches have been taken in order to generate a mouse model of X-linked DC: (1) targeted C-terminal deletion of the *Dkc1* gene utilizing the *Cre-Lox* system and (2) hypomorphic allele in which the wild-type *Dkc1* gene is expressed at reduced levels. While null dyskerin mutations are embryonically lethal, Gu *et al* have constructed a mouse which carries a dyskerin mutation designed to mimic a mutation found in a family with X-linked DC [55, 70, 95]. From studies on these mice and embryonic stem (ES) cells, they have shown that cells expressing wild-type dyskerin have a growth advantage over those expressing a truncated version, a phenomenon that is telomerase-, but not telomere length, dependent. In addition, mutant ES cells exhibit an enhanced DNA damage response via the classical p53/ATM pathway and the damage foci colocalize with telomeres [95]. Interestingly, this model does not show any alterations in ribosome biogenesis nor any characteristic phenotypes of DC. On the contrary, mice expressing a hypomorphic allele of Dkc1 exhibit several phenotypes observed in DC patients, including bone marrow failure, dyskeratosis of the skin, lung abnormalities, and an increased susceptibility to cancer development [96]. Moreover, these pathological features were observed in first- and second-generation mice, suggesting that they arose independent of telomere length. Telomere shortening was not observed in these mice until generation 4 (G4), accompanied by a decrease in telomerase activity caused by decreased mouse telomerase RNA (mTERC) stability. The X-linked DC phenotypes in these mice seem to be initiated by decreased ribosomal RNA (rRNA) processing and an impairment in internal ribosomal entry site (IRES)-mediated translation [97]. While each of these models sheds important light on dyskerin's various functions in the cell, it seems most likely that a combination of the observed defects contributes to X-linked DC pathogenesis in humans. In fact, mice carrying two different mutations identified in patients exhibit varying defects in mTERC and small nucleolar RNA (snoRNA) accumulation, telomerase activity, telomere length, and rRNA processing [98]. More careful mapping of specific domains and residues necessary for each of dyskerin's cellular activities should help to shed some light on a possible mechanism underlying this disease.

Autosomal Dominant DC

Knock-outs of *mTERC* and *mTERT* exhibit very similar phenotypes. Neither component, although absolutely essential for telomerase enzymatic activity, is essential for embryonic development, and disease states do not manifest until later generations when telomeres have significantly shortened [75-78, 99-102]. In confirmation of this finding, Hao *et al* have shown that, even in the presence of telomerase activity, short telomeres can limit tissue renewal in the bone marrow, intestines, and testes [103]. In order to generate mice with sufficiently short telomeres, they backcrossed an $mTERC^{+/-}$ C57BL/6 mouse with a CAST/EiJ mouse, which is known to have very short telomeres, for five generations in order to generate a heterozygous generation 1 (HG1) mouse. These heterozygotes were intercrossed to obtain successive generations of $mTERC^{+/+}$ (wt*), $mTERC^{+/-}$ (HG), and $mTERC^{-/-}$ (KO) animals. Several tissue renewal defects were observed in the mTERC null mice, including small intestine atrophy, hematopoietic defects, and impaired wound healing, and were found to follow the disease anticipation phenomenon observed in humans, whereby disease phenotypes appear at an earlier age in later generations due to the inheritance of shortened telomeres. Interestingly, despite the presence of telomerase activity, late generation HG and wt* mice also exhibited telomere shortening and signs of occult genetic disease. Despite these exciting findings, none of these mice exhibited the characteristic triad of features

associated with DC [76, 78].

A couple other intriguing results have been obtained through studies on *mTERT* knock-out mice. First, Rajaraman *et al* conducted a study on telomere dysfunction-induced apoptosis in the intestinal crypts of late generation mTERT$^{-/-}$ mice [104]. In doing so, they found that gastrointestinal (GI) progenitor cells undergo apoptosis due to shortened telomeres shortly after S-phase, but before mitosis, suggesting that telomere uncapping in these cells occurs in late S-phase or in G_2. This timing is consistent with the timing of telomere replication and supports a mechanism whereby disruption of telomere end structure induces apoptosis directly without the need for a fusion-bridge breaking cycle. Secondly, in addition to its roles in telomere maintenance, TERT has been proposed to perform other "extracurricular activities" in the cell (summarized in [105, 106]). Consistent with this idea, conditional induction of mTERT in mouse skin epithelium causes rapid proliferation of hair follicle stem cells independent of hTERC, suggesting that TERT may directly support the processes of differentiation and proliferation [107].

In contrast with the *mTERC* and *mTERT* knock-outs, inactivation of the telomere binding protein TIN2 results in early embryonic lethality which is not rescued by telomerase deficiency [108]. Embryos die before day 7.5 of their embryonic development, suggesting that TIN2 serves telomerase-independent roles in the cell which are absolutely required for life. Unfortunately, the exact cause of death could not be analyzed due to the rapid death of TIN2$^{-/-}$ ES cells in culture. The embryonic lethality of this mouse model mirrors that of TRF1- and TRF2-deficient mice [109, 110]. Further analysis of the *in vivo* functions of these proteins will require conditional or tissue-specific knock-outs.

By far the most successful genetically engineered model of DC is a telomere degradation mouse generated by Hockemeyer *et al* and Wu *et al* [111-114]. Interestingly, while human telomeres are protected by one single-stranded DNA binding protein POT1, mouse telomeres contain two POT1 paralogs, POT1a and POT1b [112, 114]. Lack of POT1a results in embryonic lethality, activation of the DNA damage response, and aberrant homologous recombination. In contrast, POT1b knock-out mice are viable and fertile, but exhibit an increase in C-strand degradation. Despite the independent functions of POT1a and POT1b in repressing a DNA damage signal and in regulating the structure of the telomere end, respectively, full protection of telomeres requires both factors. The most exciting finding is that POT1b-deficient mice display several distinctive features of DC patients: abnormal skin pigmentation, nail dystrophy, and bone marrow failure [111, 113]. Furthermore, these phenotypes are exacerbated by haploinsufficiency for mTERC and double knock-outs for POT1b and mTERC are embryonic lethal. These symptoms arise in the background of normal telomerase activity, strengthening the argument that DC is due to dysfunctional telomeres. It is interesting to note that whereas mutations in the shelterin components TRF1, TRF2, and TIN2 have been identified in patients with bone marrow failure syndromes, mutations in POT1 have not yet been reported.

Conclusions

Although it has been over a century since dyskeratosis congenita was first described [115] and over two decades since the discovery of telomerase [116], this disease and its specific etiology as it relates to telomere dysfunction retain their mystery. *In vitro* studies have been helpful in dissecting the potential roles of telomere maintenance genes in disease pathogenesis, but much of this data still needs to be validated *in vivo*. Unfortunately, while it would be ideal to study patient tissues, this is difficult due to the limited availability of samples and the nature of the desired cells. It is potentially problematic to obtain sufficient bone marrow or blood cells from an already hematologically compromised patient. Thus, several mouse models have been developed, both genetically and chemically (reviewed in [117]), to study the physiological effects of deficiencies in the telomere maintenance pathway. Despite the large telomere reserve of mice and the inherent differences in shelterin complex composition between mice and humans, mouse studies have not only strengthened the *in vitro* findings, but also shed light on some interesting new phenomena. Whether these conclusions will extend themselves to humans remains to be seen.

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Please address all correspondences to Dr. Hinh Ly, PhD, Emory University Pathology Department, 105L Whitehead Bldg., 615 Michael St., Atlanta, GA 30322. Tel: 404-712-2841; Fax: 404-727-8538. Email: hly@emory.edu

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