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**Review**

Mammalian chromosomes terminate in stretches of repetitive telomeric DNA that act as buffers to avoid loss of essential genetic information during end-replication. A multiprotein complex known as shelterin prevents recognition of telomeric sequences as sites of DNA damage. Telomere erosion contributes to human diseases ranging from BM failure to premature aging syndromes and cancer. The role of shelterin telomere protection is less understood. Mutations in genes encoding the shelterin proteins TRF1-interacting nuclear factor 2 (TIN2) and adrenocortical dysplasia homolog (ACD) were identified in dyskeratosis congenita, a syndrome characterized by somatic stem cell dysfunction in multiple organs leading to BM failure and other pleiotropic manifestations. Here, we introduce the biochemical features and in vivo effects of individual shelterin proteins, discuss shelterin functions in hematopoiesis, and review emerging knowledge implicating the shelterin complex in hematological disorders.

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# The shelterin complex and hematopoiesis

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**Mammalian chromosomes terminate in stretches of repetitive telomeric DNA that act as buffers to avoid loss of essential genetic information during end-replication. A multiprotein complex known as shelterin prevents recognition of telomeric sequences as sites of DNA damage. Telomere erosion contributes to human diseases ranging from BM failure to premature aging syndromes and cancer. The role of shelterin telomere protection is less understood. Mutations in genes encoding the shelterin proteins TRF1-interacting nuclear factor 2 (TIN2) and adrenocortical dysplasia homolog (ACD) were identified in dyskeratosis congenita, a syndrome characterized by somatic stem cell dysfunction in multiple organs leading to BM failure and other pleiotropic manifestations. Here, we introduce the biochemical features and in vivo effects of individual shelterin proteins, discuss shelterin functions in hematopoiesis, and review emerging knowledge implicating the shelterin complex in hematological disorders.**

## Organization of the shelterin complex and telomerase at chromosomal ends

*Telomeres, end-replication, and end-protection.* Eukaryotic cells contain nucleoprotein complexes known as telomeres that organize and regulate the ends of linear chromosomes. Telomeric DNA consists of repetitive sequences with a short single-stranded, G-rich overhang preceded by a much longer double-stranded region. The average length of telomeric DNA varies between species and tends to decrease in aging cells and tissues. Telomeric DNA is associated with the shelterin complex, a group of six proteins that bind specifically to telomeric DNA: telomere repeat factor 1 (TRF1), TRF2, repressor/activator protein 1 (RAP1), TRF1-interacting nuclear factor 2 (TIN2), protection of telomeres 1 (POT1), and adrenocortical dysplasia homolog (ACD, also referred to as TIN2/PTOP/PIP1 [TPP1] based on initial descriptions of this protein) (Figure 1 and ref. 1). Shelterin proteins also recruit the telomerase complex to chromosomal ends through an interaction with the telomerase protein component telomerase reverse transcriptase (TERT) (2–7). Together, the telomerase and shelterin complexes solve unique problems caused by linear chromosomes: the end-replication and end-protection problems.

The end-replication problem arises because the DNA replication machinery requires an RNA primer that provides a 3' hydroxyl group for DNA synthesis at the 5' end of the lagging strand. Upon primer removal, the resulting gap cannot be filled by replicative DNA polymerases, resulting in DNA loss at chromosomal ends after each replication cycle. Telomerase, a specialized ribonucleoprotein complex, counters this DNA loss by adding telomeric repeats to chromosomal ends and provides a molecular buffer to genetic erosion during end-replication. The telomerase complex contains both the enzymatic activity (encoded by its protein sub-

unit TERT) and the RNA template (present in its RNA subunit telomerase RNA component [TERC]) for reverse transcription. In long-lived proliferative cells, telomere length is maintained by telomerase (8–18).

The end-protection problem is the potential recognition of chromosomal ends as double-stranded breaks (DSBs) by the DNA damage response (DDR) machinery, which could result in deleterious chromosomal end-to-end fusions and genomic instability (Figure 1). Shelterin proteins bind to single- and double-stranded components of telomeric DNA and solve the end-protection problem (1). Without shelterin, telomeres evoke a robust DDR. Exposure of single-stranded G-overhangs elicits an ataxia telangiectasia and RAD3-related-mediated (ATR-mediated) DDR, as single-stranded DNA (ssDNA) is bound by replication protein A (RPA) and RPA/ssDNA complexes recruit ATR (19–22). RPA/ssDNA also interacts with the cell cycle checkpoint complex RAD9/RAD1/HUS1 via RAD17 (23). This complex recruits DNA topoisomerase 2-binding protein 1 (TOPBP1), activating ATR through poorly defined mechanisms (24–26). ATR phosphorylates checkpoint kinase 1 (CHK1) and can trigger cell cycle arrest through regulation of cyclin-dependent kinases or p53 activation, allowing cells the opportunity to repair DNA aberrations (27).

Exposure of the double-stranded portion of telomeres results in aberrant recognition of the telomere as a DSB. DSBs are sensed by the MRE11/RAD50/NBS1 (MRN) complex (28). MRN recruitment triggers ataxia telangiectasia mutated (ATM) kinase binding and enhances ATM interaction with CHK2 and p53 (28–30). Activated CHK2 enforces cell cycle arrest directly and through p53 activation (27, 31, 32). ATM may also activate ATR (33). DDR activation at telomeres primarily activates nonhomologous end joining (NHEJ) and homologous recombination (HR), although additional pathways may be activated (22). Exposure of double-stranded telomeric DNA activates NHEJ, while exposure of ssDNA activates HR (19, 20, 34). NHEJ-mediated repair at telomeres leads to fusion of different chromosomes or sister

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chromatids, while HR-mediated repair triggers recombination between homologous sister chromatid templates followed by exchange of material between sister chromatids (telomere sister chromatid exchange).

*Individual functions of shelterin components as revealed in vitro and in mouse models.* The shelterin proteins TRF1 and TRF2 bind double-stranded telomeric DNA; POT1 binds to the single-stranded G-overhang; TIN2 and TPP1 do not bind DNA, but interact with TRF1/2 and POT1, respectively; and RAP1 binds to TRF2 (Figure 1 and ref. 1).

Shelterin components play multiple roles in suppressing DDR activation and regulating telomere length. Initial biochemical studies largely used transformed cells and mouse embryonic fibroblasts (MEFs) to elucidate the specific functions of shelterin components. However, while cell culture-based studies provided valuable insights, until recently, the *in vivo* significance of shelterin dysfunction remained uncharacterized. We now know that individual shelterin proteins play critical roles *in vivo* in both mice and humans. The unique roles of shelterin proteins in mammalian development and tissue homeostasis are more complex than predictions based on previously reported biochemical functions alone. This profound *in vivo* significance is most prevalent in tissues that require active turnover and robust stem cell compartments, such as hematopoietic tissues. In addition to developmental defects, several mouse models of shelterin dysfunction revealed features reminiscent of the human syndrome dyskeratosis congenita (35–45).

## TRF1/TRF2

TRF1 and TRF2 play unique roles in telomere protection and length regulation (46–49). TRF1 overexpression results in telomere shortening, while dominant-negative TRF1 leads to telomere lengthening (50). Though TRF1 and TRF2 are not known to directly interact with telomerase, they facilitate formation of higher-order telomeric structures (51). TRF1 isoforms have also been linked to cell cycle control (52). In MEFs, *Trf1* inactivation results in widespread stalling of the DNA replication machinery at telomeres and the formation of gaps, known as fragile telomeres (53, 54). *Trf1* inactivation and subsequent replicative dysfunction induces robust DDR activation involving ATM, ATR, p53, and retinoblastoma (RB), leading to cellular senescence (53, 54). *Trf1* is essential for embryonic development, as germline *Trf1* inactivation results in lethality before E6.5 (55). While p53 inactivation rescued MEFs from the effects of *Trf1* deletion, a similar rescue was not observed in embryos, suggesting that differences exist between the effects of shelterin deficiencies in cell culture as compared with the entire organism.

*Trf1* inactivation in the BM results in hematopoietic failure within weeks (45). *Trf1*<sup>−/−</sup> hematopoietic stem cells (HSCs) had impaired repopulation potential in transplantation assays that was linked to p53 activation in the absence of a significant apoptotic response. Instead, p21-mediated senescence with associated telomere shortening was documented. In addition, *Trf1* inactivation in the skin resulted in severe epidermal defects and neonatal mortality due to loss of skin barrier function (54). Interestingly, p53 inactivation in the skin was sufficient to prevent lethality. However, this rescue strategy resulted in squamous cell carcinomas, demon-

strating that genomic instability due to *Trf1* loss is tumorigenic. The ability of p53 inactivation to rescue developmental abnormalities in the skin, but not in the entire embryo, suggests that defined cell types and tissues may differentially activate DDR signaling in response to shelterin defects.

TRF2 is structurally related to TRF1 and specifically prevents ATM activation at telomeric ends (19, 20, 56, 57). *Trf2* inactivation results in NHEJ-driven telomeric fusion events independent of replication fork stalling (34, 57–59). TRF2 helps form higher-order telomeric structures known as t-loops, which have been proposed to be instrumental for TRF2's functions (51, 60, 61). TRF2 overexpression in telomerase-positive human cells leads to telomere shortening, while telomere elongation is observed when TRF2 levels decrease (51). As with TRF1, this finding is not related to regulation of telomerase expression or activity (51).

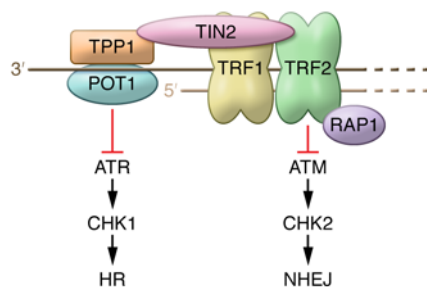
In mice, constitutive *Trf2* deficiency led to embryonic lethality that was not rescued by p53 deficiency (56). *Trf2* overexpression in the skin resulted in hyperpigmentation and predisposed mice to squamous cell carcinomas (41). Epithelial cells from mice with *Trf2* overexpression had prominent telomere shortening that was not related to telomerase dysfunction. The combination of telomerase deficiency and *Trf2* overexpression led to accelerated tumorigenesis as the result of enhanced telomere dysfunction and fusion events (40).

## RAP1

RAP1 (encoded by the gene *TERF2IP*) is localized at telomeres via its interaction with TRF2 (62, 63). *Terf2ip* is the only shelterin gene whose inactivation does not cause embryonic lethality in mice, suggesting no essential function in end-protection or replication (37). Initial studies indicated that RAP1 was involved in preventing NHEJ at telomeres, while later work suggested a role in preventing HR (37, 62, 64–67). Recently, RAP1 was also found to perform important extratelomeric functions that provide protection against obesity in mice (68, 69). In addition, *Terf2ip* gene inactivation results in hyperpigmentation, telomere shortening, and increased DDR activation in adult mice (37). Together, these findings suggest that RAP1 plays an important role in telomere homeostasis. Studies of *Terf2ip* illustrate the concept that *in vivo* observations are required to advance our understanding of shelterin protein functions.

## POT1

POT1 binds the single-stranded G-overhang of telomeric DNA, which requires stable heterodimer formation with TPP1 *in vivo* (19, 70–76). POT1 is required for suppression of ATR-mediated DDR and for regulation of 3' G-strand overhang length (77). In mice, this function is achieved through complex regulation of 5' C-strand resection and subsequent fill-in (78). The mouse *Pot1* gene underwent duplication, creating *Pot1a* and *Pot1b* (79). *Pot1a* is critical for ATR inhibition, while *Pot1b* is involved in telomere length regulation and prevents excessive 5' resection (42, 43, 79, 80). *Pot1a* loss results in p53-dependent senescence induction (80). In the presence of telomerase, only combined *Pot1a/b* loss leads to increased telomeric instability (77, 79). Furthermore, *Pot1a/b* loss causes endoreduplication, resulting in MEFs with >4N DNA (79). However, combined *Pot1b* deficiency and telomerase



**Figure 1. Organization and molecular functions of the shelterin complex at telomeric ends.** Telomeric DNA contains repetitive sequences forming a long double-stranded region (right) followed by a shorter G-rich, single-stranded overhang (left). The shelterin complex consists of six proteins (TRF1, TRF2, RAP1, TIN2, POT1, and TPP1) that bind the double-stranded and single-stranded regions of telomeric DNA. TPP1 is encoded by the gene *ACD*. Shelterin proteins play important, nonredundant roles in preventing activation of DDR pathways at chromosome ends, including ATR/CHK1 activation by exposed ssDNA and ATM/CHK2 activation by double-stranded DNA. Shelterin proteins prevent attempted repair by HR and NHEJ mechanisms. Additional pathways and crosstalk between pathways exist but are not represented here for the sake of simplicity.

haploinsufficiency result in telomere shortening and increased chromosomal fusions (42, 43). By virtue of binding tightly to the 3' overhang at chromosome ends, POT1 serves as a natural inhibitor of telomerase. Indeed, disruption of POT1's DNA binding domain results in excessive telomere elongation, suggesting unregulated access of telomerase to chromosome ends (81).

*Pot1a* deletion causes early embryonic lethality, while *Pot1b*-deficient mice are born in normal Mendelian ratios (43, 79, 80). This suggests a critical role for *Pot1a* but not *Pot1b* during embryonic development. Despite the normal development of *Pot1b*-deficient mice, males eventually became infertile with reduced sperm production. Subsequent genetic models in which *Pot1b* deficiency was combined with *Terc* haploinsufficiency resulted in the first in vivo models with a phenotype reminiscent of dyskeratosis congenita as a result of shelterin dysfunction (42, 43). In contrast, complete loss of telomerase activity in the setting of *Pot1b* deficiency resulted in embryonic lethality, perhaps due to excessive chromosomal shortening during early development. Mice deficient for *Pot1b* with combined *Terc* haploinsufficiency demonstrated a spectrum of phenotypes reminiscent of dyskeratosis congenita patients, including skin hyperpigmentation, testicular atrophy, increased apoptosis in intestinal crypts, and reduced lifespan in the context of telomere shortening. Reduced life expectancy was attributed to the development of hematopoietic failure. Mechanistically, *Pot1b*<sup>-/-</sup>*Terc*<sup>+/-</sup> HSCs were reduced in number and failed to compete with WT HSCs in transplantation assays (44). Hematopoietic progenitor cells had increased p53 activation with a bias toward apoptosis rather than cell cycle arrest. p53 loss markedly rescued HSC function, demonstrating that p53 plays a critical role in *Pot1b*<sup>-/-</sup>*Terc*<sup>+/-</sup> HSC dysfunction.

## ACD/TPP1

In vivo, POT1 binding requires TPP1, the protein product of the *ACD* gene (19, 36, 73–76, 82). Distinct functions of TPP1 can be traced to biochemical functions of TPP1's protein domains, which

form binding interfaces with specific partners: POT1, TIN2, and TERT. With these characteristics, TPP1 functions as a molecular hub coordinating end-protection and end-replication functions of the shelterin/telomerase complex.

Initial studies in *Acd*<sup>-/-</sup> MEFs revealed that many consequences of *Acd* deficiency phenocopied *Pot1a/b* inactivation. These studies indicated that the suppression of ATR signaling and HR through POT1 is the central function of TPP1 (74). Additional studies implicated TPP1 in preventing ATM activation and suppressing NHEJ, suggesting a more complicated role in end-protection (19, 83, 84). MEF-based studies showed that most effects of *Acd* inactivation in cultured cells are driven by p53-mediated growth arrest (19, 84). However, subsequent work suggested that p53-independent effects of *Acd* loss exist, as MEFs immortalized with SV40 large T antigen (which inactivates p53 and RB) still demonstrate growth arrest upon *Acd* loss (74).

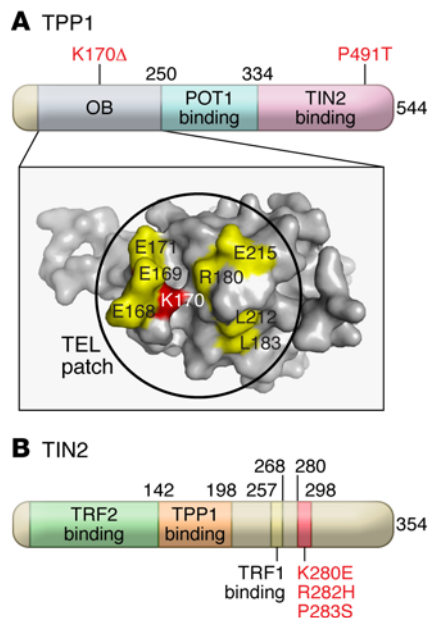
Apart from its end-protection function, TPP1 also plays complex roles in regulating telomerase activity. Work in human cell lines demonstrated that TPP1 is required for recruitment of both telomerase and POT1 (2, 3, 73). While POT1 recruitment is expected to negatively regulate telomere lengthening, recruitment of telomerase by TPP1 would result in telomere lengthening. Indeed, the telomere length phenotype of TPP1 knockdown is variable and context dependent. While TPP1 knockdown in cultured cancer cell lines resulted in telomere lengthening, telomere shortening was observed in skin keratinocytes from newborn mice with keratinocyte-specific deletion of *Acd* (*K5-Cre*<sup>+</sup>*Acd*<sup>fl/fl</sup>) (76, 84). How TPP1 coordinates its contrasting telomerase-regulating activities remains to be determined.

The telomerase recruitment activity of TPP1 was attributed to the oligonucleotide/oligosaccharide binding (OB) fold domain of TPP1. Recent studies identified a small region in the TPP1 OB fold (the TEL patch) that is both necessary and sufficient for telomerase recruitment (Figure 2 and refs. 4–6). Thus, TPP1 is not required solely for DDR suppression but also for telomere elongation, suggesting the need to study TPP1 function in telomerase-expressing cells, including stem cells and cancer cells. In addition to telomerase recruitment, TPP1 promotes telomerase processivity in the presence of POT1 (85). How POT1, a negative regulator of telomerase, teams up with TPP1 to stimulate telomerase processivity is not fully understood, although models based on telomerase enzyme kinetics have been proposed (86).

A recent study showed that TPP1 interacts with another ssDNA-binding complex known as the CST complex (CTC1, STN1, and TEN1) to limit excessive telomere elongation (87). At early steps of telomere replication, TPP1 recruits telomerase and promotes telomere extension. As replication progresses, CST binds the elongating G-strand, preventing further association with telomerase. Additionally, the presence of CST inhibits TPP1's ability to enhance telomerase processivity.

In mice, complete *Acd* inactivation leads to embryonic lethality (74). However, a spontaneously occurring splice variant resulting in a hypomorphic allele (*acd*) revealed essential developmental functions for *Acd* (36). Depending on the genetic background, mice homozygous for hypomorphic *acd* displayed adrenocortical dysplasia (giving the gene its original name), caudal truncation, genitourinary abnormalities, infertility, skin





**Figure 2. Structure of TPP1 and TIN2 shelterin proteins, highlighting key mutations identified in patients with dyskeratosis congenita. (A)** TPP1 (encoded by *ACD*) contains an OB fold domain (OB), which recruits telomerase through an exposed TEL patch, as well as a POT1-binding domain and a TIN2-binding domain. Human variants identified in dyskeratosis congenita families are shown in red (K170Δ, P491T). Essential surface-exposed amino acids in the TEL patch are shown in yellow, while the mutation identified in dyskeratosis congenita is shown in red. Numbers indicate amino acid positions. **(B)** TIN2 (encoded by *TINF2*) contains an N-terminal TRF2-binding region, followed by TPP1-binding and TRF1-binding domains. Selected recurrent mutations identified in dyskeratosis congenita patients are indicated in red (K280E, R282H, P283S). Multiple other mutations affecting the 280–300 amino acid cluster were reported but are not depicted. Note that the hotspot for dyskeratosis congenita mutations lies outside of all known protein-binding regions of TIN2. Numbers indicate amino acid positions.

hyperpigmentation, and strain-dependent prenatal or perinatal lethality. A subsequent study showed that *Acd* inactivation in the skin caused hyperpigmentation, epidermal stem cell defects, and neonatal mortality due to loss of barrier function (84). With both skin-specific *Acd* deletion and *acd*-homozygous mice, most phenotypic abnormalities could be rescued by *p53* inactivation (35, 84, 88). Interestingly, *p53* inactivation failed to rescue strain-specific embryonic lethality in *acd* homozygotes (35). These data reveal critical tissue-specific and developmental functions for *Acd* and suggest that *p53*-dependent and *p53*-independent mechanisms underlie these effects.

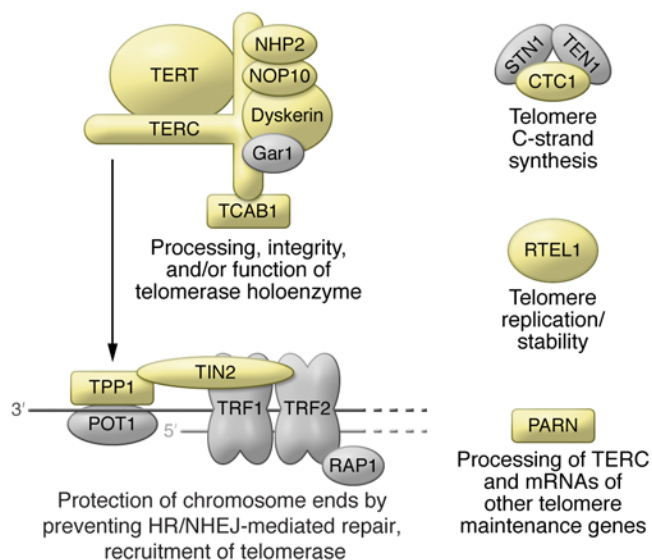
We recently demonstrated that *Acd* critically regulates HSC homeostasis (39). In mice with hypomorphic *acd* alleles, we observed G2/M arrest in fetal hematopoietic progenitors. These cells could not provide hematopoietic reconstitution in irradiated recipients, demonstrating marked HSC dysfunction. When *Acd* was inactivated using a conditional allele, HSCs were rapidly depleted within less than one week in adult mice, and these cells were unable to provide stable engraftment after competitive or noncompetitive transplantation. In addition to G2/M arrest, hematopoietic progenitors demonstrated chromosomal instabil-

ity, as indicated by fusion events and robust caspase activation within 48 hours of *Acd* deletion. Interestingly, *p53* deletion did not rescue *Acd*-deficient HSCs, even transiently. These findings contrast with the rescue of *Acd*-deficient epidermal stem cells by *p53* inactivation, suggesting that distinct stem cell populations respond differently to *Acd* loss. Moreover, the functional rescue of *Pot1b*-deficient HSCs by *p53* inactivation suggests that *Acd*'s effects in HSCs are not solely due to POT1B destabilization (44, 84). Thus, the relationship between shelterin deficiency and *p53* activation is more complicated than previously anticipated.

## TIN2

TIN2 is an adaptor protein that plays critical roles in stabilizing the shelterin complex. TIN2 binds TRF1 and TRF2, stabilizing TRF1 and TRF2 at telomeres (89). TIN2 also binds TPP1 and is required for TPP1/POT1's recruitment to the shelterin complex (90). TIN2 loss results in ATR activation and excessive 3' overhang generation (from TPP1/POT1 loss) and ATM activation (from TRF2 destabilization). These data highlight the critical function of TIN2 in organizing the shelterin complex (38). TIN2 knockdown also results in decreased telomerase recruitment to telomeres, most likely because TIN2 is required for telomeric recruitment of TPP1 (3). TIN2 may have additional roles outside of telomere homeostasis. TIN2 was detected in the mitochondria in a human cancer cell line, and TIN2 knockdown led to abnormal mitochondrial morphology (91). TIN2 loss promoted increased oxidative metabolism and reduced glycolysis in human cancer cells. These data indicate that TIN2 may have telomeric and extratelomeric functions, and they suggest the possibility that other shelterin components may also function away from telomeres.

*Tin2* loss causes embryonic lethality in mice (92). Conditional *Tin2* inactivation has not been characterized in vivo. However, *TINF2* mutations were identified in human dyskeratosis congenita patients, providing an impetus for in vivo modeling of TIN2 function (93–97). To this end, a knock-in approach in which a *Tin2* allele with lysine 267 replaced with glutamate (K267E, synonymous with the dyskeratosis congenita-causing K280E mutation in humans) was utilized (Figure 2 and ref. 38). Homozygous mice were not viable, but heterozygous mice demonstrated reductions in neutrophil, reticulocyte, and platelet counts that worsened with increasing generations. This type of disease anticipation is observed in mice or patients with telomerase defects, as they inherit shortened telomeres through the germline. In addition to the hematopoietic phenotype, heterozygous mice were smaller than WT littermates and born in reduced numbers, with worsening of the phenotype in later generations. Male mice harboring a single mutant allele had reduced fertility. This model also revealed apparent telomerase-independent telomere shortening. Mice harboring the *Tin2*-K267E allele had shorter telomeres than WT littermates. When this allele was crossed onto a *Terc*-deficient background, telomeres shortened to a greater extent than in mice null for *Terc* alone. Given that the hotspot for dyskeratosis congenita mutations in *TINF2* encodes a region of the protein that is dispensable for TRF1, TRF2, and TPP1 binding, it is unclear how these mutations result in telomere shortening. More work is needed to understand the molecular and cellular consequences of *TINF2* mutations.



**Figure 3. Schematic representation of human proteins affected by germline mutations in dyskeratosis congenita and related disorders.** Mutations affecting 11 different proteins have been reported to date in dyskeratosis congenita and related disorders, as indicated by colored structures. TERT, TERC, dyskerin, NHP2, NOP10, and TCAB1 are important for the processing, integrity, and/or function of the telomerase holoenzyme, a ribonucleoprotein complex containing TERC RNA and the catalytic TERT protein with reverse transcriptase activity. TIN2 and TPP1 (encoded by *ACD*) are members of the shelterin complex (see Figure 1). CTC1 belongs to a complex that regulates telomere C-strand synthesis and telomere replication. RTBL1 is important for telomere replication and stability. PARN was recently described to control processing of TERC RNA as well as mRNAs of other telomere maintenance genes.

## Shelterin and human BM failure syndromes

Recent studies in dyskeratosis congenita highlight the importance of the shelterin complex in hematopoiesis. Dyskeratosis congenita is a human telomere biology disorder with pleiotropic manifestations that frequently causes BM failure (98, 99). Hematopoietic stem and progenitor cells from patients with dyskeratosis congenita have defective self-renewal, suggesting that these hematopoietic cell-intrinsic defects are the primary cause of hematopoietic failure in these patients. In addition, HSC niche defects could also contribute to the phenotype (100).

Dyskeratosis congenita was first linked to mutations in *DKC1* — which encodes dyskerin, an accessory protein required for telomerase stability and function — and then in genes encoding components of telomerase itself: *TERC*, encoding the telomerase RNA subunit, and *TERT*, encoding the telomerase enzymatic protein subunit (Figure 3 and refs. 101–104). Other mutations were described in *NOP10* (encoding a small nucleolar ribonucleoprotein), *NHP2* (encoding a small nucleolar ribonucleoprotein subunit), and *WRAP53* (encoding TCAB1), which affect telomerase trafficking, assembly, or stability (105–107); in *RTBL1* (encoding an RNA helicase that regulates telomere unwinding and replication) (108–112); in *CTC1* (encoding a component of the CST complex) (113–115); and in *PARN* (encoding a poly[A]-specific ribonuclease) (116, 117). In addition, mutations in two genes encoding elements of the shelterin complex (*TINF2* and *ACD*) were identified in families with dyskeratosis congenita and dyskeratosis congenita-related

disorders, as discussed in more detail below (93, 94, 118, 119). Altogether, mutations in a total of 11 genes have been reported to date, including X-linked, autosomal dominant, and autosomal recessive inheritance patterns.

Dyskeratosis congenita patients are classically diagnosed by the “mucocutaneous triad” of oral leukoplakia, abnormal skin pigmentation, and nail dystrophy. However, the clinical manifestations are pleiotropic, and penetrance is variable; thus, the classic triad is not present in all patients. Up to 85% of dyskeratosis congenita patients eventually develop hematopoietic failure, establishing dyskeratosis congenita as an inherited BM failure syndrome (99, 102, 120). Dyskeratosis congenita patients are also at risk of liver disease and pulmonary fibrosis, suggesting that somatic stem/progenitor cell populations in these organs are susceptible to telomere dysregulation (121). Additionally, dyskeratosis congenita is a cancer predisposition syndrome, as approximately 40% of patients with dyskeratosis congenita develop cancer by the age of 50, with myelodysplastic syndromes, acute myeloid leukemia, and squamous cell carcinoma being most common (102). Up to 70% of dyskeratosis congenita cases have an identifiable mutation in one of the above genes, while the remaining 30% remain of unknown genetic origin (102). Thus, our knowledge of the genetic causes of dyskeratosis congenita remains incomplete, and additional contributing genes must be considered.

## TINF2

The first shelterin gene implicated in dyskeratosis congenita was *TINF2* (93, 94). Patients with *TINF2* mutations usually have shorter telomeres with symptom onset at an earlier age than patients with *TERC* or *TERT* mutations (93, 96, 122, 123). *TINF2* mutations are associated with Hoyeraal-Hreidarsson syndrome (a dyskeratosis congenita variant with cerebellar hypoplasia and immunodeficiency) and Revesz syndrome (with bilateral exudative retinopathy) (93, 94, 124, 125). To date, approximately 20 mutations have been identified in *TINF2* in patients, affecting a recurrent hotspot in the gene (Figure 2 and ref. 125). *TINF2* mutations act in an autosomal dominant fashion but often appeared to arise de novo in dyskeratosis congenita patients (93). At least two alternative mechanisms of telomere dysfunction have been proposed for *TINF2* mutations. Biochemical analysis of a subset of disease-causing *TINF2* mutations showed reduced telomerase recruitment to telomeres in a TPP1-dependent fashion (126). Another study using a knock-in approach reported decreased telomerase recruitment without changes in TPP1 localization to telomeres (127). These data suggest a mechanism for progressive telomere shortening due to defective telomerase recruitment, although they fail to explain the early age of dyskeratosis congenita onset, increased clinical complications, or exceedingly short telomeres in the setting of *TINF2* mutations as compared with other dyskeratosis congenita etiologies. Another study showed that dyskeratosis congenita-associated mutations in TIN2 interfere with binding of TIN2 to heterochromatin protein 1γ (HP1γ), which results in defective sister telomere cohesion in cultured human cells (128). Recent work focusing on the K280E mutation, one of the initially characterized disease-causing *TINF2* mutations, suggests that at least part of the disease phenotype can be mediated by telomerase-independent telomere shortening and DDR activation (38, 94).

**Table 1. Germline and somatic shelterin gene mutations reported in human cancer**

Gene	Mutation	Type of cancer	Nature	References
<i>TERF2IP</i>	M5I	Familial Melanoma	Germline	135
	D10H			
	Q191R			
	R364X			
<i>ACD</i>	A200T	Familial Melanoma	Germline	135
	N249S			
	V272M			
	Q320X			
	I322F			
<i>POT1</i>	G223V	Acute Lymphoblastic Leukemia	Somatic	138
	M1L	CLL	Somatic	132, 133
	Y36N			
	Y66X	Familial melanoma	Germline	136
	K90Q			
	Q94R			
	Y223C			
	S250X			
	H266L			
	G272V			
	C591W			
	Y89C	Cutaneous Malignant Melanoma	Germline	137
	Q94E			
	R273L			
	R137H			
	D224N			
	S270N	Familial Glioma	Germline	139
	A532P			
	Q623H			
	G95C			
	E450X	Cardiac Angiosarcoma	Germline	140
	D617E			
	R117C			

ACD

Two recent reports identified germline *ACD* mutations in families with inherited BM failure and dyskeratosis congenita spectrum disorders (Figure 2 and refs. 118, 119). One proband presented with features of dyskeratosis congenita by the age of 10 months and blood cytopenias by 20 months, requiring matched unrelated allogeneic hematopoietic cell transplantation by 3 years of age (118). This patient was ultimately diagnosed with Hoyeraal-Hreidarsson syndrome due to concomitant cerebellar hypoplasia and dyskeratosis congenita features. In this study, an *ACD* mutation causing in-frame loss of lysine 170 in TPP1 was present in the proband and other family members with very short telomeres. This mutation resulted in a structural change in the TEL patch that abrogated TPP1’s ability to bind telomerase, with functional consequences similar to those of previously reported experimental mutations affecting the TEL patch (4–6). Interestingly, the proband in this study also carried a mutation substituting threonine for proline

at position 491 in the TIN2-interacting domain of TPP1. While this mutation did not affect telomerase recruitment, it resulted in modest disruption of the TPP1/TIN2 interaction, the significance of which remains unknown. Another study characterized a family in which several generations were affected by aplastic anemia (119). In this family, the proband presented at age 8 with worsening pancytopenia. Despite being unrelated to the family in the other study, affected individuals had the same deletion of lysine 170 in TPP1. These independent observations highlight the critical role of TPP1’s TEL patch region in telomere homeostasis. The variability in disease presentation in these two studies was consistent with dyskeratosis congenita caused by mutations in other genes, including *TERT* and *RTEL1*, where autosomal dominant and recessive inheritance patterns have been reported.

In individuals with TEL patch mutations, defective recruitment of telomerase to telomeric ends is predicted to result in telomere shortening, and indeed very short telomeres were observed in affected individuals (118, 119). These conclusions were supported by the study of engineered mutations in embryonic stem cells (129). However, TEL patch mutations have not been modeled in vivo, and the suggested pathogenic mechanisms remain to be fully investigated. In particular, we do not know if human shelterin gene mutations exert all their effects via their impact on telomere length or if other mechanisms such as end-deprotection are involved. Development of mouse models to characterize how focal shelterin defects affect telomere homeostasis could provide fundamental insights into dyskeratosis congenita pathogenesis and the role of shelterin proteins in tissue aging.

Shelterin and hematological malignancies

Poncet and colleagues reported alterations in the expression pattern of several shelterin genes in chronic lymphocytic leukemia (CLL) (130). Expression of *TRF1*, *RAP1*, and *POT1* was reduced, while *ACD* expression was higher than in normal lymphocytes. Subsequent work sought to determine if shelterin dysfunction could play a pathogenic role in CLL. Patients with early-stage CLL had an increased frequency of dysfunctional telomeres, as indicated by the localization of DDR-associated proteins at telomeric ends (telomere dysfunction-induced foci, TIFs) (131). TIFs were identifiable before telomere shortening could be observed. After surveying shelterin genes and genes associated with telomeric maintenance, it was found that expression of *TINF2* and *ACD* was reduced. Thus, telomere deprotection may take place in CLL in addition to subsequent telomere erosion, although the significance of these findings remains to be fully explored.

Further supporting the idea that telomere deprotection may play a role in CLL, *POT1* point mutations were reported in 5% of CLL cases (132, 133). Most of these mutations were in regions encoding the two OB fold-containing domain of POT1, which may hinder POT1’s ability to bind telomeric DNA. Patients harboring these mutations had increased frequencies of sister chromatid fusions, chromosome fusions, and multitelomeric signals or fragile telomeres (a feature associated with replication fork stalling at telomeric ends). It is interesting to speculate whether CLL has unique characteristics that make it particularly sensitive to shelterin dysfunction as compared with other cancers. Although telomere erosion and genomic instability have been documented in CLL (134), similar investi-

gations have not yet been performed in detail in other hematological malignancies. Of note, mutations in the shelterin genes *POT1*, *ACD*, and *TERF2IP* have been described in familial melanoma and other cancers, indicating a broad potential relevance in oncogenesis (135–137). Table 1 summarizes all the germline or somatic mutations in shelterin genes described to date in human cancer, although their mechanisms of action and significance remain to be fully explored.

## Conclusions and perspectives

With the identification of *ACD* and *TINF2* mutations in dyskeratosis congenita patients, two out of six shelterin genes have now been associated with human hematopoietic failure syndromes (Figure 3). At least 30% of patients presenting with dyskeratosis congenita or dyskeratosis congenita-like syndromes still have no known genetic defects; thus, it is possible that additional shelterin gene mutations will be discovered in these disorders. In addition, shelterin dysfunction or mutations may underlie other human diseases, including developmental defects and cancer. Much progress has been made in understanding the complex biology of telomere homeostasis, particularly in terms of how end-elongation and end-protection are coordinately regulated. In the future, targeted genetic interventions could be particularly informative in studying the function of individual shelterin proteins, as global loss or downregulated expression of shelterin components can trigger widespread defects that mask

their precise functions. For example, engineering human mutations and polymorphisms — as well as point mutations targeting specific protein-protein interfaces — could reveal important, clinically relevant phenotypes. In vivo studies will be necessary to fully understand the molecular functions of shelterin proteins in individual tissues, including in the regulation of hematopoietic homeostasis.

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