p53 Mediates Failure of Human Definitive Hematopoiesis in Dyskeratosis Congenita

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SUMMARY

Dyskeratosis congenita (DC) is a bone marrow failure syndrome associated with telomere dysfunction. The progression and molecular determinants of hematopoietic failure in DC remain poorly understood. Here, we use the directed differentiation of human embryonic stem cells harboring clinically relevant mutations in telomerase to understand the consequences of DC-associated mutations on the primitive and definitive hematopoietic programs. Interestingly, telomere shortening does not broadly impair hematopoiesis, as primitive hematopoiesis is not impaired in DC cells. In contrast, while phenotypic definitive hemogenic endothelium is specified, the endothelial-to-hematopoietic transition is impaired in cells with shortened telomeres. This failure is caused by DNA damage accrual and is mediated by p53 stabilization. These observations indicate that detrimental effects of telomere shortening in the hematopoietic system are specific to the definitive hematopoietic lineages. This work illustrates how telomere dysfunction impairs hematopoietic development and creates a robust platform for therapeutic discovery for treatment of DC patients.

INTRODUCTION

Dyskeratosis congenita (DC) is a bone marrow failure (BMF) syndrome whereby patients have telomeres at, or below, the first percentile in length when compared with the rest of the population (Savage and Alter, 2009). Patients with DC usually come to clinical attention during childhood and present with a triad of oral leukoplakia, reticular skin pigmentation, and nail dystrophy. While these patients are at increased risk for developing leukemia and solid tumors, BMF is the major cause of death in DC, commonly in young adulthood (Wilson et al., 2014). While the severity of DC varies across patients, more than 85% of afflicted individuals have cytopenias in one or more lineages in late childhood, and more than 95% will develop pancytopenia by adulthood.

All mutations identified to date in DC occur in genes that regulate different aspects of telomere maintenance (Armanios and Blackburn, 2012). The active telomerase complex, which is responsible for telomere elongation in vertebrates, is composed of the reverse transcriptase TERT, the RNA component of telomerase TERC, the TERC-stabilizing nuclear protein DKC1, and TCAB1, which is responsible for telomerase biogenesis and trafficking (Schmidt and Cech, 2015). Due to the tight regulation of TERT expression, telomerase is only active in stem and progenitor cells (including hematopoietic stem cells [Morrison et al., 1996; Yui et al., 1998]), which suggests that DC is caused by a stem cell failure in highly proliferative tissues, including the bone marrow.

Although there has been extensive biochemical characterization of telomere maintenance mechanisms and their role in maintaining genomic integrity, the connection between telomere dysfunction and the specific clinical phenotypes of BMF in DC patients remains poorly understood. Patient samples are rare and cannot address the effect of telomere deficiency on the genesis of tissue failure that occurs during hematopoietic development. Here, we characterize the primitive and definitive hematopoietic development of isogenic human embryonic stem cells (hESCs) carrying disease-associated mutations in the telomerase components TERT and DKC1, two of the most commonly mutated genes in DC (Armanios and Blackburn, 2012). We show that telomerase expression levels are highly regulated during hESC differentiation toward hematopoietic progenitors, with reduced TERT expression within mesoderm, but restored TERT levels in hematopoietic progenitors. Telomere shortening causes an increase in primitive hematopoietic potential but significantly ablates definitive hematopoietic potential. Furthermore, we demonstrate that p53 mediates the effects of telomere shortening on definitive hematopoiesis, which can be rescued by inhibition of DNA damage signaling or by reactivation of telomerase. Our findings elucidate telomerase regulation patterns during early blood development, indicate a role of DNA damage-induced p53 signaling in hematopoietic specification, and demonstrate the value of in vitro hematopoietic differentiation to study the pathogenesis of BMF in DC patients.
RESULTS

Engineered hESCs Harboring DC-Associated Mutations Have Impaired Telomere Maintenance

To study hematopoietic failure in cells harboring common DC-associated mutations, we generated DKC1_A353V and TERT_P704S mutant hESCs using CRISPR/Cas9-mediated genome editing (Figures 1A–1C). Cells retained expression of pluripotency markers (Figure 1D) and had no chromosomal abnormalities (Figure S1A). We then analyzed the consequences of these mutations on the expression of telomerase components. As expected, TERT expression remained the same in both mutant cell lines while TERC expression was significantly reduced in DKC1_A353V hESCs (Figure 1E), as dyskerin is necessary for TERC stabilization (Mitchell et al., 1999). No difference was observed in the expression of dyskerin and shelterin components in both DKC1_A353V and TERT_P704S hESCs (Figure S1B). Importantly, telomere repeat amplification assays (TRAP; Figure 1F) showed that telomerase activity, in comparison with wild-type control, is reduced by approximately 75% in DKC1_A353V and TERT_P704S hESCs. While DC hESCs did exhibit subtle changes in cell-cycle distribution (Figures S1C and S1D), they do not show apoptosis induction (Figures S1E–S1G), in contrast with hESCs to full deletion of telomerase (Sexton et al., 2014), suggesting that hESCs with residual telomerase activity behave differently from those completely lacking telomerase activity. To confirm this hypothesis, we created hESCs completely lacking TERC (TERC<sup>-/-</sup>; Figure S2H). Accordingly, these cells undergo robust apoptosis induction in culture (Figure S2I), similar to TERT<sup>-/-</sup> hESCs (Sexton et al., 2014). Combined, these data suggest that phenotypes caused by disease-associated mutations in telomerase cannot be recapitated by complete knockout of telomerase components.

Importantly, both DKC1_A353V and TERT_P704S hESCs have significantly impaired telomere maintenance (Figure 1G), the major characteristic of telomere syndromes. Telomere length in DKC1_A353V and TERT_P704S hESCs averaged <4 kb at passage 30 (compared with >10 kb in the isogenic, parental wild-type; Figure 1G). Collectively, these results indicate that the DKC1_A353V and TERT_P704S point mutations interfere directly with the catalytic activity of telomerase, similar to what is observed in patients. Thus, hESCs harboring patient-specific mutations allow us to directly interrogate the consequences of telomere dysfunction during human tissue development in vitro.

Telomerase Expression Is Stringently Regulated during Human Mesoderm and Hematopoietic Development

While we and others have made great strides in demonstrating that induced pluripotent stem cells (iPSCs) can serve as a valuable resource to study the biochemical regulation of mutant telomerase, intrinsic clonal variability (Gu et al., 2015; Winkler et al., 2013) and conflicting results arising from different reprogramming methodologies (Agarwal et al., 2010; Batista et al., 2011) have precluded the use of these cells to understand hematopoietic disease development. Vertebrate hematopoietic development comprises at least two distinctive hematopoietic programs: a transient, erythro-myeloid restricted primitive hematopoietic program, and the definitive hematopoietic program, which gives rise to all blood cell lineages found in the adult (Medvinsky et al., 2011). Clinical symptoms of DC are usually not present at birth, with patients not having indications of in utero hematopoietic deficiency, suggesting that aplastic anemia in DC clinically manifests as defective postnatal definitive hematopoiesis. However, most hESC/iPSC differentiation strategies to date have yielded hematopoietic progenitors that are heterogeneous for both programs (reviewed in Ditadi et al., 2017), confounding their use in studying the consequences of telomere dysfunction during human definitive hematopoietic development.

We have recently demonstrated that stage-specific manipulation of canonical WNT-β-catenin signaling allows for the generation of exclusively primitive or exclusively definitive hematopoietic progenitors from hESCs (Figure 2A; Sturgeon et al., 2014). This methodology gives rise to multipotent NOTCH-dependent clonal definitive erythro-myeloid-lymphoid progenitors (Ditadi et al., 2015), a hallmark of definitive hematopoiesis (Clements and Traver, 2013). With this approach, we asked whether we could dissect the consequences of dysfunctional telomere maintenance in human definitive hematopoietic progenitors, independently from primitive hematopoietic development.

We first asked whether we could detect telomerase expression during hESC hematopoietic differentiation. We analyzed the expression of the telomerase core components TERT and TERC during early primitive and definitive hematopoietic specification. Mesoderm harboring primitive hematopoietic potential, identified by KDR and CD235a expression, was efficiently obtained on day 3 of differentiation (Figure S2A) following WNT inhibition. By day 8 of differentiation, CD43<sup>+</sup> primitive hematopoietic progenitors were identified by flow cytometry (Figure S2A). We isolated these different populations by fluorescence-activated cell sorting (FACS) and found that TERC expression was sustained in all populations (Figure 2Bi). Intriguingly, TERT was transiently repressed in primitive hematopoietic mesoderm, but then its expression was restored in CD43<sup>+</sup> primitive hematopoietic progenitors (Figure 2Bii). Similarly, KDR<sup>−</sup>CD235a<sup>−</sup> mesoderm harboring definitive hematopoietic potential was efficiently obtained on day 3 of differentiation (Figure S2A) following WNT activation,
Figure 1. Generation of Isogenic hESCs Harboring DC-Associated Mutations

(A) Model depicting the telomerase complex with specific amino acid modifications in red: DKC1_A353V and TERT_P704S. Diseases associated with these mutations are described in blue.

(B) Strategy for introduction of disease-specific mutations in DKC1 and TERT. Guide RNAs (gRNAs) targeting exon 11 (DKC1) and exon 5 (TERT) were used in combination with specific single-strand DNA donor oligo templates for introduction of DKC1 (A353V; C → T) and TERT (P704S; C → T). In blue are silent mutations introduced to facilitate CRISPR/Cas9-mediated genome modification.

(C) Sequencing traces confirming genome modification: red boxes indicate nucleotide modifications that lead to the desired amino acid change. Blue box in TERT indicates a silent mutation introduced for increased genome-editing efficiency.

(D) Quantification of SOX2, OCT4, and NANOG levels by qRT-PCR analysis.

(E) Quantification of TERC and TERT expression by qRT-PCR in wild-type and telomerase-mutant hESCs. Expression is shown relative to parental, wild-type cells. Data are plotted as mean ± SEM, n = 3 independent experiments. *p ≤ 0.05, Student’s t test.

(F) Telomerase activity by TRAP in wild-type, DKC1_A353V, and TERT_P704S mutants. Range of concentrations represents 4-fold serial dilutions. L.C., loading control; negative control: NP40 buffer.

(G) Telomere length analysis by telomere restriction fragment (TRF) of wild-type, DKC1_A353V, and TERT_P704S hESCs at different cell passages, demonstrating progressive telomere shortening in mutant cells. Molecular weight is shown on the left.

WT, wild-type. See also Figure S1.
Figure 2. Progressive Telomere Shortening Differentially Regulates Primitive and Definitive Hematopoietic Specification

(A) Schematic of in vitro definitive and primitive hematopoietic differentiation. WNT modulation at day 2 of differentiation determines primitive or definitive hematopoietic specification. WNT activation is achieved by treatment with the WNT agonist CHIR99021, and WNT activation leads to the expression of TERT and TERC, and the differentiation of CD43+ CD184- CD45+ cells into Myeloid and BFU-E colonies. (legend continued on next page)
which then gave rise to a CD34+CD43-CD73-CD184+ hemogenic endothelial population (Figure S2A). When isolated and further cultured under hemato-endothelial conditions, this definitive population gives rise to CD45+ definitive hematopoietic progenitors (Choi et al., 2012; Ditadi et al., 2015). After these populations were isolated by FACS, we observed that TERC expression was retained in all populations (Figure 2Biii). Similar to what was observed in primitive mesoderm, TERT levels were strongly reduced in mesoderm harboring definitive hematopoietic potential (Figure 2Biv), indicating that canonical WNT signaling had no effect on TERT expression. However, following this transient repression, TERT expression within hemogenic endothelium, and its resultant CD45+ definitive hematopoietic progenitors, was significantly upregulated (Figure 2Biv). When taken together, these observations indicate that TERT expression is highly regulated during mesoderm/blood development, with robust telomerase expression being a conserved characteristic of both primitive and definitive hematopoietic progenitors, highlighting the importance of telomere maintenance during hematopoiesis.

**Telomere Attrition Specifically Impairs Definitive Hematopoiesis in DC**

With this in vitro model to study the role of telomerase during hematopoietic development, we differentiated our DC hESCs toward primitive and definitive hematopoietic progenitors (Figure 2A). Telomere shortening did not affect the specification of mesoderm, as late-passage DKC1_A353V and TERT_P704S hESCs gave rise to KDR+CD235a+ (primitive) and KDR+CD235a- (definitive) mesodermal populations with efficiency similar to that of wild-type hESCs (Figures S2B and S2C). However, we observed striking differences at later times in hematopoietic development. In comparison with wild-type cells and early-passage (longer telomeres) DC hESCs, late passage (shorter telomeres) had significantly enhanced CD43+ primitive hematopoietic progenitor expansion (Figures S2D and S2E) and primitive erythroid colony-forming cell potential (EryP-CFC; Figures 2Ci and 2Di). These observations indicate that telomere shortening does not broadly abrogate hematopoietic differentiation potential, as DC hESCs with short telomeres are still able to efficiently generate primitive hematopoietic progenitors.

Similarly, early stages of definitive hematopoietic specification were not affected by telomere shortening, as early- and late-passage DKC1_A353V and TERT_P704S hESCs generated a CD34+CD43+ population on day 8 of differentiation with efficiency similar to that of control hESCs (Figure S3A). From these, we isolated the definitive CD34+CD43+ population and cultured them under hemato-endothelial conditions, to assess their ability to undergo the endothelial-to-hematopoietic transition and yield definitive erythro-myoeloid progenitors (Ditadi et al., 2015). Strikingly, late-passage DKC1_A353V and TERT_P704S hESCs exhibited a severe defect in definitive hematopoietic potential, giving rise to approximately 5-fold less definitive erythroid burst-forming units (BFU-E), and 3-fold less myeloid colony-forming units (CFU-M) when compared with wild-type or DC hESCs in early passage (Figures 2Cii and 2Dii). Furthermore, while these cells still retained the ability to give rise to CD4+CD8+ T cell progenitors (Figure 2E), late-passage DC hESCs exhibited a severe reduction in cellularity, suggesting that definitive hematopoiesis is impaired with progressive telomere shortening. The definitive hematopoietic profile of our differentiations was further confirmed by their hemoglobin expression profile (Figure S3B). Importantly, the difference observed is not caused by differential regulation of WNT canonical activity between wild-type and DC mutant cells, as treatment with CHIR and IWP2 led to identical levels of activation repression of the WNT target gene AXIN2 at day 3 of differentiation, between the wild-type and different isogenic lines.
(Figure S3C). Combined, these observations suggest that progressive telomere shortening in telomerase-mutant hESCs causes a specific impairment of definitive hematopoietic potential, which may share similar mechanism(s) with the severe pancytopenia observed in DC patients.

**p53 Stabilization Mediates Hematopoietic Failure in DC Cells**

Next, we interrogated the mechanism behind the differential regulation of hematopoiesis in hESCs with dysfunctional telomeres. Telomere uncapping due to progressive telomere shortening is a potent inducer of DNA damage. Accordingly, we detected phosphorylated H2AX (γH2AX) accumulation in DKC1_A353V and TERT_P704S hESCs in late passage (Figure 3A). Additionally we observed a cumulative increase in p53 levels in DC-mutant hESCs with progressively shorter telomeres (Figures 3B and S4A), a hallmark of cells harboring dysfunctional telomeres (Palm and de Lange, 2008). As p53-dependent DNA damage response causes BMF in murine models of Fanconi anemia (Ceccaldi et al., 2012), we hypothesized that telomere dysfunction-mediated p53 stabilization could also regulate the specification of hematopoiesis in hESCs. To test this hypothesis, we ablated p53 in our DKC1_A353V hESCs (DKC1_A353V_p53−/−; Figures S4B–S4D). Similar to those with functional p53, these hESCs continue to exhibit progressive telomere shortening with extended passage, as telomerase activity remains impaired (Figure 3C).

We observed that primitive hematopoietic potential was restored to normal levels following p53 deletion, as late-passage DKC1_A353V_p53−/− hESCs exhibited significantly less CD43+ primitive hematopoietic progenitors (Figures 3D and 3E) and concomitant EryP-CFC potential (Figure 3F). Conversely, when we specified these hESCs toward definitive hematopoiesis, we observed that definitive hematopoietic potential was restored in late-passage DKC1_A353V_p53−/− hESCs, as they gave rise to definitive erythro-myeloid and lymphoid progenitors (Figures 3G and S4E) with efficiency similar to that of controls. Furthermore, this functional increase in definitive hematopoiesis was not due to an increase in phenotypic hemogenic endothelium specification, as there was no increase in the observed CD34+CD43+CD73+CD184+ population in DKC1_A353V_p53−/− cells compared with DKC1_A353V cells (Figures 3H and 3I). Taken together, these data establish p53 as a regulator of human hematopoietic potential, eliciting positive and negative effects on primitive and definitive hematopoiesis, respectively.

**Telomerase Reactivation Rescues Definitive Hematopoietic Specification in DC Mutants**

Currently, DC has no cure. However, murine models have shown that reactivation of telomerase is able to rescue hematopoiesis across different tissues in telomerase-mutant mice (Bar et al., 2016; Jaskelioff et al., 2011). We therefore asked whether telomerase reactivation via genetic correction could restore human definitive hematopoietic potential in DC hESCs. Moreover, by specifically restoring TERC levels in hESCs that retain mutant dyskerin, defects caused specifically by telomere dysfunction can be isolated from defects potentially caused by dyskerin’s role in ribosomal biology (Meier, 2005). We therefore inserted a U3-driven TERC within the AAVS1 “safe-harbor” locus (Hockemeyer et al., 2009; Sim et al., 2015), creating DKC1_A353V + TERC hESCs (Figure 4A). Both TERC expression levels (Figure 4B) and TRAP analysis (Figure 4C) indicate restored telomerase activity, which is corroborated by a significant increase of telomere length in DKC1_A353V + TERC hESCs (Figure 4D). We next differentiated these hESCs toward primitive and definitive hematopoiesis. As observed with p53 deletion, telomerase reactivation decreased primitive hematopoietic potential (Figures 4E–4G) and successfully restored definitive erythro-myeloid potential in late-passage DKC1_A353V hESCs (Figure 4H). Thus, reactivation of telomerase can restore normal primitive and definitive hematopoietic output in DC hESCs.

**DISCUSSION**

The results presented here demonstrate the essential role of telomerase during human hematopoietic development. Our stage-specific directed differentiation approach demonstrated that telomere shortening elicits a differential effect on primitive and definitive hematopoiesis, mediated by p53.

While a positive role of p53 in erythropoietic differentiation has been observed during malignant hematopoietic differentiation (Molchadsky et al., 2010), our results indicate that p53 can also regulate non-malignant hematopoietic development. Our observed near-complete failure of definitive hematopoietic output from phenotypic hemogenic endothelium, which is restored by p53 deletion, suggests that p53 directly regulates definitive hematopoietic potential in DC cells. Hemogenic endothelium is the direct precursor to the hematopoietic stem cell (Julien et al., 2016), raising the possibility that our observations and clinical BMF may both be due to conserved mechanism(s) governed by p53. As our understanding of human definitive hemogenic endothelium improves, we will be able to better define the role of p53 in this complex developmental process.

Importantly, while telomerase-deficient mice have abnormal blood phenotypes, overt cytopenias due to telomere dysfunction have never been fully recapitulated in murine models (Calado and Young, 2008), which suggests
Figure 3. DNA Damage Accrual and p53 Stabilization Impair Definitive Hematopoietic Development in DC Cells

(A) Representative immunoblot analysis of γH2AX in wild-type and mutant hESCs at different cell passages.
(B) Representative immunoblot analysis of p53 levels in DKC1_A353V (i) and TERT_P704S (ii) hESCs with progressive cell passage number (P.N.). GAPDH is shown as loading control.
(C) Telomere length analysis by TRF of wild-type, DKC1_A353V, and DKC1_A353V_p53−/− hESCs at different passages.
(D) Representative flow-cytometric analysis of CD34 and CD43 expression in day-11 differentiation cultures treated with IWP2, as in Figure 2A. In red, population of interest.
(E) Primitive erythroid colony-forming cell (EryP-CFC) potential in day-11 differentiation cultures treated with IWP2, as in (D).
(F) Colony-forming cell potential of definitive hematopoietic progenitors, generated as in Figure 2A.
(G) Representative flow-cytometric analysis of CD73 and CD184 expression within CD34+CD43− cells. In red, population of interest.
(H) Quantification of CD34+CD43+CD73−CD184− population, as in (H).

In all panels, wild-type (WT) is compared with DKC1_A353V (p66) and DKC1_A353V_p53−/− (p70) hESCs. n = 3 independent experiments, mean ± SEM; *p ≤ 0.05; Student’s t test. NS, not significant. See also Figure S4.
Figure 4. Telomerase Reactivation Restores Hematopoietic Potential in DKC1_A353V hESCs

(A) Model of AAVS1 targeting in DKC1_A353V hESCs.

(B) Quantification of TERC levels by qRT-PCR in wild-type, DKC1_A353V, and DKC1_A353V + TERC hESCs.

(C) TRAP analysis measuring telomerase activity in wild-type, DKC1_A353V, and DKC1_A353V + TERC hESCs. Range of concentrations represents 4-fold serial dilutions. L.C., loading control; negative control: NP40 buffer.

(D) Telomere length analysis by TRF of wild-type, DKC1_A353V, and DKC1_A353V + TERC hESCs.

(E) Representative flow-cytometric analysis of CD34 and CD43 expression in day-11 differentiation cultures treated with IWP2, as in Figure 2A. In red, population of interest.

(F) Quantification of CD43^+ population obtained from day-11 differentiation cultures treated with IWP2, as in (E).

(legend continued on next page)
that our observed pan-hematopoietic defect specifically in definitive derived lineages more broadly recapitulates those that found in patients. While the exact mechanism(s) exerted by p53 in the regulation of definitive hematopoiesis remains elusive, this in vitro platform of hematopoietic differentiation can be used to address this question. Importantly, however, DC patients are clinically diverse in terms of genetic mutations, inherited telomere length, age of onset, and disease severity. Therefore, it remains to be demonstrated that p53 activation is a universal marker for hematopoietic failure in this disease.

The ability to restore hematopoietic output by reactivation of telomerase lends further support for recent clinical trials demonstrating that increased telomerase function has a positive effect on hematopoiesis in adult aplastic anemia patients (Townesley et al., 2016). Our observations suggest that similar approaches may be used in DC children suffering from hypoplastic marrow. Moreover, the restoration of hematopoietic potential in DKC1 mutant hESCs by rescuing TERC levels, while not interfering with dyskerin’s role in ribosomal biology (Meier, 2005), conclusively establishes X-linked DC as a telomere-dysfunction disease.

Together, these results provide evidence that human BMF syndromes can be modeled in vitro, by directed differentiation strategies of human PSCs that recapitulate in vivo development. This opens the exciting possibility of identifying novel therapeutics against BMF in a high-throughput, controlled manner.

**EXPERIMENTAL PROCEDURES**

**Human Embryonic Stem Cells**

H1 (WA01) hESCs were acquired from the WiCell Research Institute (Madison, WI), following all institutional guidelines determined by the Embryonic Stem Cell Research Oversight Committee (ESROC) at Washington University. hESCs were routinely cultured with mouse embryonic fibroblasts (MEFs) that had been previously γ-irradiated (30 Gy). The cellular passages described as “early” represent the shortest telomeres that still allow efficient hematopoietic differentiation.

**Generation of Isogenic Telomerase-Mutant hESCs**

DKC1_A353V (X-linked), TERT_P704S (homozygous mutation), DKC1_A353V_p53−/−, and TERC−/− were generated using CRISPR/Cas9 genome-editing technology. CRISPR guides were designed using the Massachusetts Institute of Technology (MIT) CRISPR Design tool (http://crispr.mit.edu/) and generated as described previously (Ran et al., 2013).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.06.015.

**AUTHOR CONTRIBUTIONS**

W.C.F., E.L.d.O.N., C.D., C.M.S., and L.E.Z.B. designed the experiments and analyzed the data; W.C.F., E.L.N., C.D., and K.A.B. performed the experiments; W.C.F., C.M.S., and L.E.Z.B. wrote the manuscript.

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Supplemental Information

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Figure S1. Extended characterization of DKC1_A353V and TERT_P704S and TERC-/- hESCs. Related to Figure 1. (A) G-band analysis in wild-type, DKC1_A353V, and TERT_P704S hESCs. No chromosomal abnormalities were detected. (B) Representative immunoblot analysis of dyskerin, TRF1, TRF2, and RAP1 in wild-type and mutant cells. ACTB is shown as a loading control. (C) Representative histograms of cell cycle distribution in wild-type, DKC1_A353V, and TERT_P704S hESCs at different passages. (D) Quantification of cells in G1 phase in wild-type, DKC1_A353V, and TERT_P704S hESCs at different passages. (E) Apoptosis levels in wild-type and telomerase mutant cells, analyzed by sub-G1 population scoring. (F) Quantification of caspase 3, 8, and 9 activation in early passage (EP: <p15) and late passage (LP: >p50) hESCs. (G) BCL2 and BCL-XL expression in wild-type, DKC1_A353V, and TERT_P704S at different passages, as indicated. (H) Schematic of TERC deletion in hESCs. Two different guide RNAs were utilized, flanking the entire TERC region. Clones were selected through targeted sequencing (not shown). Accordingly, selected TERC-/- hESCs clones have no detectable TERC levels or telomerase activity (data not shown). (I) Cell death levels in wild-type and TERC-/- hESCs, analyzed by propidium iodide staining. For (E), (F), (G) and (I): Positive control represents wild-type hESCs irradiated with 50J/m2 of ultraviolet-light C (UV-C). n=3, mean ± SEM. Statistical analysis were performed using Student’s t-test.
Figure S2. Mesoderm and early primitive hematopoietic progenitors in telomerase mutant hESCs. Related to Figure 2. (A) Representative flow cytometric analysis of primitive and definitive hematopoietic differentiation of wild-type hESCs. Primitive hematopoietic differentiation at Day 3 (i), demonstrating the presence of a KDR+CD235a+ population, and Day 8 (ii), demonstrating the presence of a CD43+ population. Definitive hematopoietic differentiation at Day 3 (iii), demonstrating the presence of a KDR+CD235a- population, and lack of a CD235a+ population, and Day 8 (iv), demonstrating the presence of a hemogenic endothelium (CD34+CD43-CD73-CD184-) population. (B) Representative flow cytometric analysis of Day 3 KDR and CD235a expression following IWP2 (primitive mesoderm) or CHIR99021 (definitive mesoderm) treatment, as in Figure 2A. (C) Quantification of KDR+CD235a+ (primitive mesoderm) and KDR+CD235a- (definitive mesoderm) populations on Day 3 of differentiation, as in (B). (D) Representative flow cytometric analysis on Day 11 of differentiation showing CD34 and CD43 expression following IWP2 treatment in early and late passages DKC1_A353V and TERT_P704S hESCs (indicated in the figure). (E) Quantification of CD43+ population obtained from Day 11 differentiation cultures treated with IWP2 (primitive progenitors). n=3, mean ± SEM, Statistical analysis was performed using Student’s t-test.
Figure S3. Definitive hematopoietic differentiations in telomerase mutant hESCs. Related to Figure 2. (A) Representative flow cytometric analysis of Day 8 CD34 and CD43 expression following CHIR99021 treatment in early and late passages DKC1_A353V and TERT_P704S hESCs (indicated in the figure). (B) Ratio of gamma globin (HBG) and epsilon globin (HBE) in various erythroid colonies, as measured by qRT-PCR. (C) qRT-PCR analysis of AXIN2 expression at Day 3 of differentiation following CHIR and IWP2 treatments. n=3, mean ± SEM, *p≤0.05. Statistical analysis were performed using Student’s t-test.
Figure S4: p53 stabilization and ablation in hESCs harboring DC-associated. Related to Figure 3.
(A) Immunoblot analysis of p53 levels quantified in relation to GAPDH levels in DKC1_A353V and TERT_P704S at the indicated passages using the Li-COR Image Studio software. Protein extracts from wild-type hESCs were run and quantified in every independent gel analyzed. (B) Schematic of p53 ablation using CRISPR/Cas9 genome editing. Model shows approximate gRNA binding site on Exon 2 of p53. Targeted cells had a premature stop codon on Exon 2. (C) Representative immunoblot of p53 and γH2AX expression in hESCs 4 hours following 5 Gy gamma irradiation. (D) qRT-PCR analysis of p21 expression in DKC1_A353V and DKC1_A353V p53-/- hESCs 4 hours following 5 Gy of gamma irradiation. n=3, mean ± SEM, *p≤0.05. Statistical analysis was performed using Student’s t-test. (E) T-cell potential from CD34+CD43- populations derived from wild-type and DKC1_A353V_p53-/- at passage 70 hESCs, obtained following CHIR99021 treatment. T-cell potential is measured by the development of CD4+CD8+ cells within a CD45+CD56- gate following culture on OP9-DL4 stromal cells for 28 days. CD56 vs. CD45 is compared on the left with number of events shown for CD45+CD56-. On the right are the subset of cells that were CD45+CD56- and the percentage of CD4 vs. CD8 cells are shown.
Table S1. CRISPR guide RNA and donor oligo sequences used to generate DC point mutants and p53 ablated cells. In red, bases that were modified for insertion of desired point-mutation. In blue, bases that were modified for introduction of silent CRISPR blocking mutations or to facilitate efficient screening.

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<tr>
<th>Mutant</th>
<th>Sequence (5’-&gt;3’)</th>
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<tr>
<td>DKC1_A353V gRNA</td>
<td>GGCTACTATACCATGGTGCNGG</td>
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<tr>
<td>TERT_P704S gRNA</td>
<td>TTGACAAAGTACAGCTCAGGNGG</td>
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<tr>
<td>p53^-/- gRNA</td>
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<td>TERC^-/- gRNA1</td>
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Table S2: Primer sequences for quantitative real-time PCR analysis.

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<tr>
<td>TERC_Rev</td>
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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell Culture. Briefly, hESCs were kept in a humidified incubator at 37°C in 5% CO2 and 5% O2 levels and maintained in DMEM/F12/glutamax media (Gibco, Waltham, MA) supplemented with 20% knockout serum replacement (Invitrogen, Waltham, MA), 1% non-essential amino acids, 1% pen strep (Gibco), 1x 2-Mercaptoethanol (EMD Millpore, Billerica, MA), and 10ng/ml bFGF (R&D, Minneapolis, MN). Wild-type and DC mutant hESCs were maintained and passaged onto new 6-well plates every 5 days at a split ratio of 1:12. The passage number of cells used in different experiments is indicated in each figure. Experiments involving genome engineering, cell cycle analysis, immunoblots, and real-time PCR experiments were performed on matrigel-coated plates (Corning, Tewksbury, MA) with mTESR1 media (Stem Cell Technologies, Vancouver, Canada) supplemented with 1% pen strep (Gibco).

Gene Editing. CRISPR gRNAs were inserted into the MLM3636 plasmid (Addgene 43860) and co-transfected with a plasmid carrying Cas9 (Addgene 43945) using the 4D-Nucleofector with the P4 Primary Cell 4D-Nucleofector kit (Lonza, Allendale, NJ). For generation of point mutants, single-stranded DNA donor oligos were co-transfected with plasmids carrying specific gRNAs and Cas9 (Table S1). For DKC1_A353V_p53-/-, one gRNA sequence was co-transfected with Cas9 to induce non-homologous end-joining (NHEJ) resulting in a frame shift and early termination, which was verified by targeted sequencing and protein expression analysis. For TERC+ hESCs, 2 gRNA sequences were co-transfected with Cas9 to induce NHEJ, resulting in the total removal of the TERC gene, which was verified by targeted sequencing. Nucleofected cells were seeded on matrigel at low density and manually picked when colonies reached an appropriate size. Clones were then screened and sequenced. DKC1_A353V+TERC cells were generated by zinc finger nuclease (ZFN) targeted integration at the AAVS1 (Adeno-Associated Virus Integration Site 1) locus. AAVS1 ZFNs were generated as previously described (Hockemeyer et al., 2009) and U3-TERC was cloned into CompoZR AAVS1 donor plasmid (Sigma-Aldrich, St. Louis, MO). Transfection targeting the AAVS1 locus was performed with X-TremeGene 9 following the manufacturer’s instructions (Roche, Indianapolis, IN).

Definitive Hematopoietic Differentiation. Hematopoietic differentiation was performed as previously described (Ditadi and Sturgeon, 2016). Briefly, confluent hESCs were dissociated with 0.25% trypsin (Gibco) for 1 minute and then placed onto matrigel coated plates for 1 day to remove MEFs. On Day 0 of hematopoietic differentiation, cells were dissociated with 0.05% trypsin (Gibco) for 1 minute and cultured in serum-free media (SFD: IMDM supplemented with Hams F12 (25%), BSA (0.05%), B27 supplement (1x, Gibco), N2 supplement (0.5x, Gibco), L-glutamine (2 mM, Gibco), ascorbic acid (50 µg/mL, Gibco), monothioglycerol (400 µM, Sigma), transferrin (150 µg/mL)) supplemented with BMP-4 (10 ng/mL), on coated 6 well plates coated using a 5% poly(2-hydroxyethyl methacrylate) solution. On Day 1, one additional volume of SFD was added, containing BMP4 (10 ng/mL) and bFGF (adjusted to a final concentration of 5 ng/mL). On Day 2, media was changed to fresh SFD, supplemented with BMP4 (10mg/mL), Activin A (1 ng/mL), bFGF (5 ng/mL), and CHIR99021 (3 µM). On Day 3, media was changed to StemPro-34 (LifeTechnologies) media (SP-34: supplemented with L-glutamine (2 mM, Gibco), ascorbic acid (1 mM, Gibco), monothioglycerol (400 µM, Sigma), transferrin (150 µg/mL)), supplemented with VEGF (15 ng/mL) and bFGF (5 ng/mL). On Day 6, one addition volume of SP-34 was added, supplemented with VEGF (15 ng/mL), bFGF (5 ng/mL), IL-6 (20 ng/mL), IGF-1 (50 ng/mL), IL-11 (10 ng/mL), SCF (200 ng/mL), and EPO (4 IU). All cultures were maintained in a 5% CO2, 5% O2 incubator. All cytokines were purchased from R&D BioSystems (Minneapolis, MN) with the exception of EPO (Peprotech, Rocky Hill, NJ).

Primitive hematopoietic differentiation. The primitive hematopoietic differentiation protocol is as described in (Ditadi and Sturgeon 2016). Briefly, confluent hESCs were dissociated with 0.25% trypsin (Gibco) for 1 minute and then placed onto matrigel coated plates for 1 day to remove MEFs. On Day 0 of hematopoietic differentiation, cells were dissociated with 0.05% trypsin (Gibco) for 1 minute and cultured in serum-free media (SFD: IMDM supplemented with Hams F12 (25%), BSA (0.05%), B27 supplement (1x, Gibco), N2 supplement (0.5x, Gibco), L-glutamine (2 mM, Gibco), ascorbic acid (50µg/mL, Gibco), monothioglycerol (400 µM, Sigma), and transferrin (150 µg/mL)), supplemented with BMP-4 (10 ng/mL), on 6 well plates coated with a 5% poly(2-hydroxyethyl methacrylate) solution. On Day 1, one additional volume
of SFD media was added, containing BMP4 (10 ng/mL) and bFGF (adjusted to a final concentration of 5 ng/mL). On Day 2, media was changed with fresh SFD containing BMP4 (10 ng/mL), Activin A (1 ng/mL), bFGF (5 ng/mL), and IWP2 (3 μM). On Day 3, cells were changed to StemPro-34 (LifeTechnologies) media (SP-34: supplemented with L-glutamine (2 mM, Gibco), ascorbic acid (1 mM, Gibco), monothioglycerol (400 μM, Sigma), and transferrin (150 μg/mL), supplemented with VEGF (15 ng/mL) and bFGF (5 ng/mL). On Day 6, one addition volume of SP-34 supplemented with VEGF (15 ng/mL), bFGF (5 ng/mL), IL-6 (20 ng/mL), IGF-1 (50 ng/mL), IL-11 (10 ng/mL), SCF (200 ng/mL), and EPO (4 IU). On Day 8, the media was replaced with SP-34 supplemented with IL-6 (10 ng/mL), IGF-1 (25 ng/mL), IL-11 (5 ng/mL), SCF (100 ng/mL), EPO (2 IU), TPO (30 ng/mL), IL-3 (30 ng/mL) and Flt-3L (10 ng/mL). All cultures were maintained in a 5% CO₂, 5% O₂ incubator for the first 8 days then transferred to a 5% CO₂, normoxic incubator after media change on Day 8.

Hematopoietic Colony Assay. Primitive and definitive colony assays were performed as previously described (Ditadi et al., 2015). For analysis of primitive-derived colonies, 20,000 cells from day 11 IWP2-derived cells were plated in 1 mL of MethoCult H4034 Optimum (Stem Cell Technologies) and quantified after 10 days. For definitive-derived colonies, 10,000 sorted CD34+CD43- Day 8 CHIR-derived cells were aggregated overnight at a density of 2 × 10⁵ cells/ml, in a 50 μl volume, in SP-34 containing TPO (30 ng/mL), IL-3 (30 ng/mL), SCF (100 ng/mL), IL-6 (10 ng/mL), IL-11 (5 ng/mL), IGF-1 (25 ng/mL), EPO (2 IU), VEGF (5 ng/mL), bFGF (5 ng/mL), BMP4 (10 ng/mL), Flt-3L (10 ng/mL), and SHH (20 ng/mL). Aggregates were then transferred onto individual wells of a 24-well matrigel coated plate, and 1 ml of the same SP-34 supplemented media was added 6 hours post-transfer, following attachment of the cells to the plate. Eleven days after the isolation of CD34+CD43- cells, 2 wells were harvested and placed in 1.3 mL of MethoCult H4034 Optimum (Stem Cell Technologies) of which 1 mL (15,000 Day 8 CD34+ cells) was plated and quantified after 10 days. All cytokines were purchased from R&D Biosystems with the exception of EPO (Peprotech).

T-Cell Assay. T-Cell assays were performed as previously described (Kennedy et al., 2012; La Motte-Mohs et al., 2005). Briefly, 20,000 sorted CD34+CD43- isolated from CHIR-treated differentiation cultures were co-cultured with OP9-DL4 stroma cells for 28 days, with cells passaged by trituration onto new OP9-DL4 stroma every 5 days. Cells were cultured in α-MEM (Gibco) supplemented with 20% HyClone FBS (GE, Logan, UT), 1% Pen Strep (Gibco), SCF (30 ng/mL; first 6 days only), IL-7 (5 ng/mL) and FLT-3L (5 ng/mL). All cytokines were purchased from R&D Biosystems.

Immunoblots. Protein extraction was performed using NP-40 buffer (25 mM HEPES-KOH, 150 mM KCl, 1.5 mM MgCl₂, 10% glycerol, 0.5% NP40, and 5 mM 2ME [pH 7.5]) supplemented with protease and phosphatase inhibitors (Roche, Indianapolis, IN) for 20 minutes on ice. Quantification of proteins was performed by Bradford assay (Bradford, 1976). Proteins were resolved in 10% polyacrylamide gels in 1X Tris/glycine/SDS buffer and transferred onto nitrocellulose membrane at 400 milliamps for 1:45 hours in 1X Tris/glycine buffer with 20% methanol. Membranes were blocked in either 5% BSA or 5% milk in TBS buffer. Primary antibody incubation was performed overnight at 4°C in 5% BSA in TBS- buffer supplemented with 1% Tween-20 (TBS-T). Membranes were washed (3X 10 minutes) with TBS-T buffer and incubated in 1% milk in TBS-T with secondary antibodies (Li-COR, Lincoln, NE) for 1 hour. Membranes were then washed with TBS-T and scanned using the odyssey IR scanner (Li-COR). Image capture and signal analysis was done using the Image Studio software (Li-COR). Primary antibodies used in this study were: DKC1 (1:200), BCL-2 (1:200), BCL-XL (1:200), p53 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), Actin (1:2000, Sigma), RAP1 (1:1000), TRF1 (1:1000), TRF2 (1:200), γH2AX (1:1000, Abcam, Cambridge MA), and GAPDH (1:2000, Cell Signaling, Danvers, MA).

Detection of Telomerase Activity. Telomerase activity was measured by Telomere Repeat Amplification Protocol (TRAP) and performed as previously described (Batista et al., 2011). Briefly, cells were lysed in NP-40 buffer for 20 min on ice and extracts clarified by centrifugation at 16,000g for 10 min. Protein quantification was performed by Bradford assay. Telomere extension reactions were performed using 2.0 μg, 0.5 μg and 0.125 μg of protein and resulting products were amplified by PCR, following a modified 2-step TRAP protocol from the manufacturer (TRAPeze, EMD Millipore).
Telomere length analysis. Telomere length was quantified by Telomere Repeat Fragment Analysis (TRF), as previously described (Middleman et al., 2006). Isopropanol-extracted DNA was digested (10µg) overnight with RSA and HINF1 restriction enzymes (New England Biolabs, Ipswich, MA) and resolved (2.5µg for each analysis) on a 0.8% agarose gel for 16 hours at 85 volts in TBE (Tris/Borate/EDTA) buffer. The gel was then soaked in denaturing buffer (1.5M NaCl and 0.5M NaOH) for 45 minutes followed by neutralizing buffer (1.5M NaCl, 1M Tris-HCl at pH 7.4) for 1 hour. DNA was transferred to a nitrocellulose membrane by capillarity for at least 16 hs in 20x saline-sodium citrate (3M NaCl, 0.3M sodium citrate dehydrate at pH 7.0). After cross-linking, the membrane was hybridized with a 32P-labelled probe (TTAAGGG)4 and exposed overnight to Kodak BioMax MR film (Sigma).

Flow Cytometry and Cell sorting. Flow cytometry analysis was done on BD LSR II and BD LSR Fortessa, and sorts were done on BD FACSArria-II at the Department of Pathology & Immunology Flow Cytometry Core (Washington University in St. Louis). Antibodies used were the following: KDR (clone 89106), CD235a-APC (clone HIR2), CD34-APC (clone 8G12), CD43-PE or FITC (clone 1G10), CD184-BV421 (clone 12G5), CD73-PE (clone AD2), CD4-PerCP-Cy5.5 (clone RPA-T4), CD8-PE (clone RPA-T8), CD56-APC (clone B159), and CD45-APC-Cy7 (clone 2D1). All antibodies were purchased from BD biosciences (San Diego, CA) except for KDR (R&D, Minneapolis, MN).

RNA extraction, cDNA synthesis, and quantitative real time PCR analysis. RNA extraction was performed using Trizol (Invitrogen) following manufacturer’s instructions. cDNA synthesis was made using Superscript III First Strand synthesis kit (Invitrogen) following manufacturer’s instructions. Quantitative real-time PCR was performed using a StepOne Plus (Applied Biosystems, Waltham, MA) instrument. For transcriptional analysis of all coding genes, Evagreen master mix (Lambda Biotech, St Louis, MO) was used and reactions were performed in duplicates with 100ng of cDNA per reaction. For TERC qRT-PCR analysis, Brilliant II 1-step qPCR master mix (Agilent, Santa Clara, CA) was used following manufacturer’s instructions. Reactions were performed in duplicates with 100ng of RNA per reaction. Sample size for all experiments was at least $n=3$. Expression levels were calculated by $\Delta\Delta CT$ (Livak and Schmittgen, 2001). β-actin was used as the reference loading gene. All primer sequences can be found in Table S2.

Caspase Activity Measurement. Caspase activity was quantified using dedicated Caspase 3, 8, and 9 colorimetric detection kits following manufacturer's instructions (Abcam).

Cell Cycle Analysis. Cells for cell cycle analysis were collected and fixed in cold 70% ethanol at a density of 1 x 10⁶ cells/mL, overnight at 20°C. One million cells were then washed with PBS buffer and stained with 50µg/mL of propidium iodide (Molecular Probes, Waltham, MA) and 100µg/mL RNase A (Invitrogen), in PBS for three hours at 4°C. Cells were then analyzed using a BD LSR Fortessa cell analyzer at the Washington University Flow Cytometry Core.

Apoptosis analysis by sub-G1-flow cytometry. Cells for sub-G1 analysis were analyzed using a protocol from Riccardi and Nicoletti (2006). Briefly, cells were collected and fixed in cold 70% ethanol at a density of 1 x 10⁶ cells/mL, overnight at 20°C. One million cells were then washed with PBS buffer and resuspended in 500µl of PBS and 500µl of DNA extraction buffer (0.2M Na2HPO4 and 0.004% Triton X-100, Sigma) for 5 minutes. Cells were then stained with 20µg/mL of propidium iodide (Molecular Probes) and 200µg/mL RNase A (Invitrogen) in PBS for 30 minutes at room temperature. Analysis of cells was performed using a BD LSR Fortessa cell analyzer at the Washington University Flow Cytometry Core and 20,000 cells was analyzed per sample.
SUPPLEMENTAL REFERENCES


