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Wilson Chun Fok
Washington University School of Medicine in St. Louis

Evandro Luis de Oliveira Niero
Washington University School of Medicine in St. Louis

Carissa Dege
Washington University School of Medicine in St. Louis

Kirsten Ann Brenner
Washington University School of Medicine in St. Louis

Christopher Michael Sturgeon
Washington University School of Medicine in St. Louis

See next page for additional authors

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p53 Mediates Failure of Human Definitive Hematopoiesis in Dyskeratosis Congenita

Wilson Chun Fok,[1] Evandro Luis de Oliveira Niero,[1] Carissa Dege,[1] Kirsten Ann Brenner,[1] Christopher Michael Sturgeon,[1,2,3,*] and Luis Francisco Zirnberger Batista[1,2,3,*]
[1]Department of Medicine
[2]Department of Developmental Biology
[3]Center of Regenerative Medicine
Washington University in St. Louis, St. Louis, MO 63110, USA
*Correspondence: csturgeon@wustl.edu (C.M.S.), lbatista@wustl.edu (L.F.Z.B.)
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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 nucleolar 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*$^{-/-}$; Figure S2H). Accordingly, these cells undergo robust apoptosis induction in culture (Figure S2I), similar to *TERT*$^{-/-}$ hESCs (Sexton et al., 2014). Combined, these data suggest that phenotypes caused by disease-associated mutations in telomerase cannot be recapitulated 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-myelo-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^{+} 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^{+} primitive hematopoietic progenitors (Figure 2Bii).

Similarly, KDR^{+}CD235a^{-} mesoderm harboring definitive hematopoietic potential was efficiently obtained on day 3 of differentiation (Figure S2A) following WNT activation,

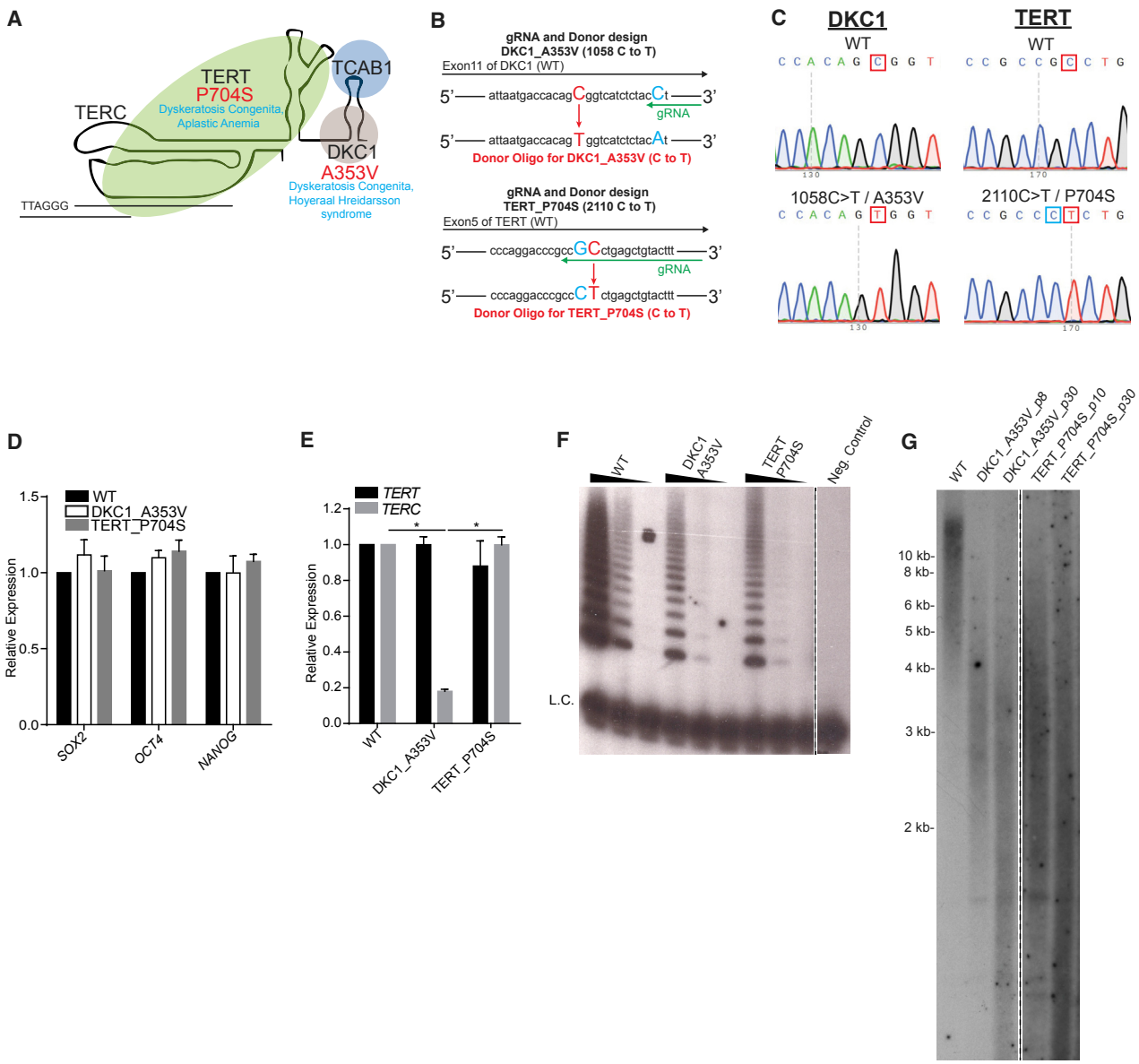

Figure 1. Generation of Isogenic hESCs Harboring DC-Associated Mutations

(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.

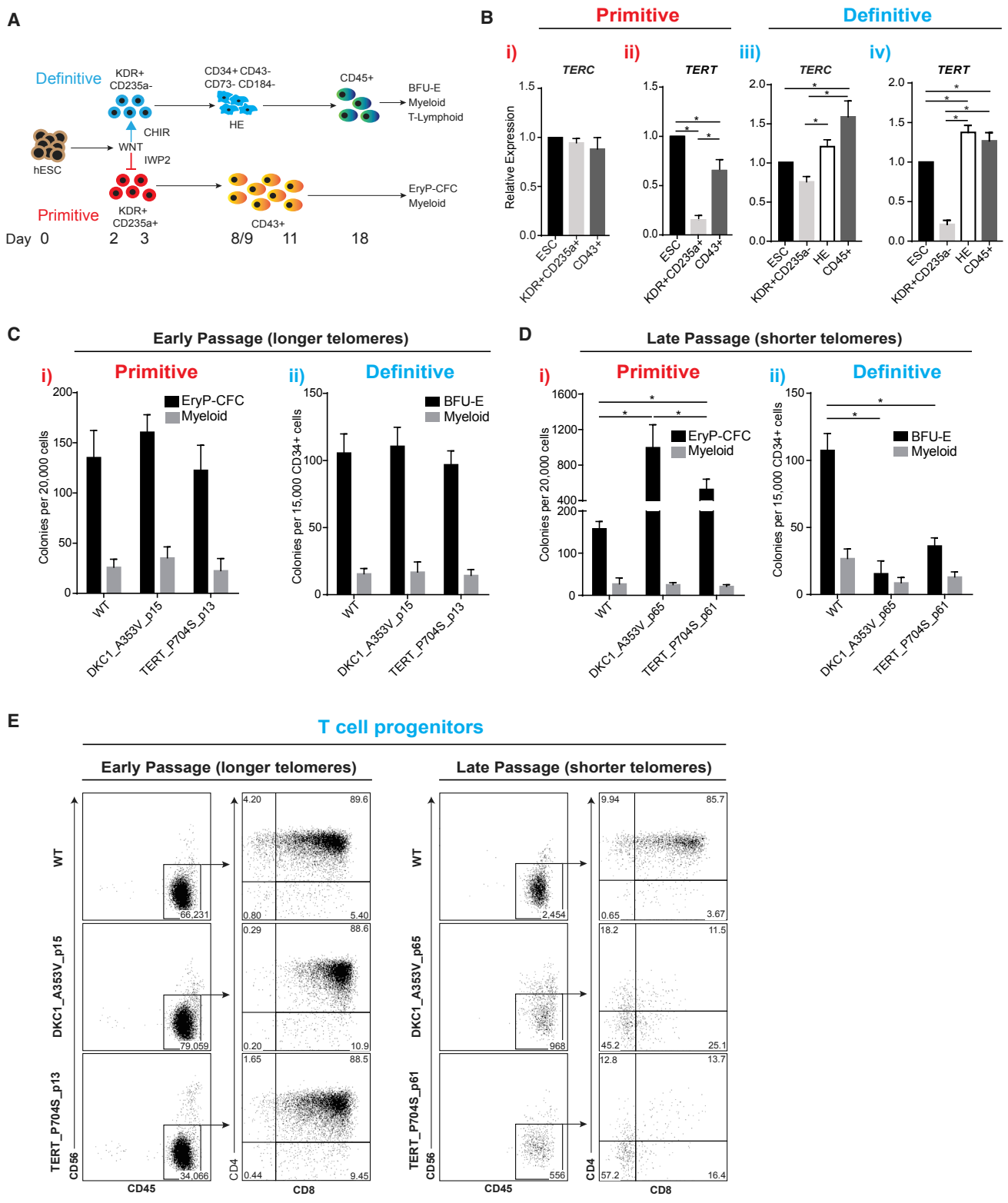

Figure 2. Progressive Telomere Shortening Differentially Regulates Primitive and Definitive Hematopoietic Specification

(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

(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-myeloid 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

repression is achieved by treatment with the WNT antagonist IWP2. Mesoderm specification is assessed at day 3 by flow-cytometric analysis of KDR and CD235a expression. During definitive hematopoietic specification, hemogenic endothelium (H&E) is identified at day 8 of differentiation as a $CD34^+CD43^-CD184^-CD73^-$ population. $CD45^+$ cells are identified after isolated day-8 H&E cells undergo an endothelial-to-hematopoietic transition. For primitive hematopoietic specification, $CD43^+$ cells are observed beginning at day 8.

(B) *TERC* and *TERT* expression during primitive (i and ii) and definitive (iii and iv) hematopoietic specification, as measured by qRT-PCR from isolated populations, as in (A). n = 3 independent experiments; mean ± SEM. *$p \leq 0.05$, Student's t test.

(C) Primitive EryP-CFC (i) and definitive colony-forming cell (ii) potential of DKC1_A353V and TERT_P704S hESCs in early passages (indicated in the figure).

(D) Primitive Ery-P-CFC (i) and definitive colony-forming cell (ii) potential of DKC1_A353V and TERT_P704S hESCs in late passages (indicated in the figure). n = 3 independent experiments; mean ± SEM. *$p \leq 0.05$, Student's t test.

(E) T cell potential of $CD34^+CD43^-$ populations derived from wild-type, DKC1_A353V, and TERT_P704S hESC lines at early or late passages (indicated in the figure), obtained following CHIR99021 treatment. T cell potential is assessed by the development of $CD4^+CD8^+$ cells within a $CD45^+CD56^-$ gate following culture on OP9-DL4 stromal cells for 28 days. CD56 and CD45 are compared on the left with number of events shown for $CD45^+CD56^-$. On the right is the subset of cells that were $CD45^+CD56^-$, and the percentage of CD4 versus CD8 cells is shown.

WT, wild-type. See also Figures S2 and S3.

(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 homeostasis 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

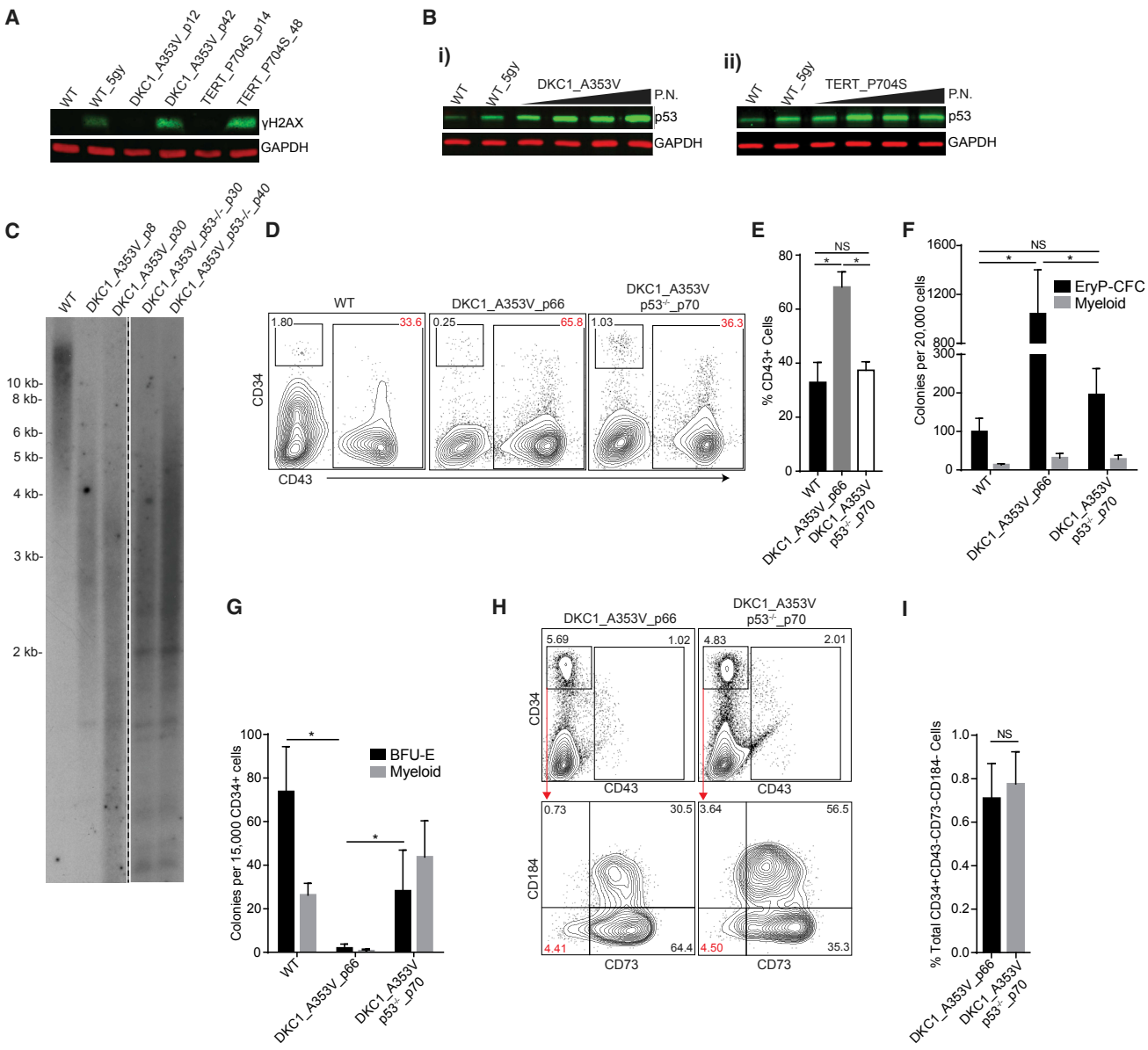

Figure 3. DNA Damage Accrual and p53 Stabilization Impair Definitive Hematopoietic Development in DC Cells

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

(F) Primitive erythroid colony-forming cell (EryP-CFC) potential in day-11 differentiation cultures, treated with IWP2 as in (D).

(G) Colony-forming cell potential of definitive hematopoietic progenitors, generated as in Figure 2A.

(H) Representative flow-cytometric analysis of CD73 and CD184 expression within CD34^{+}CD43^{-} cells. In red, population of interest.

(I) 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.

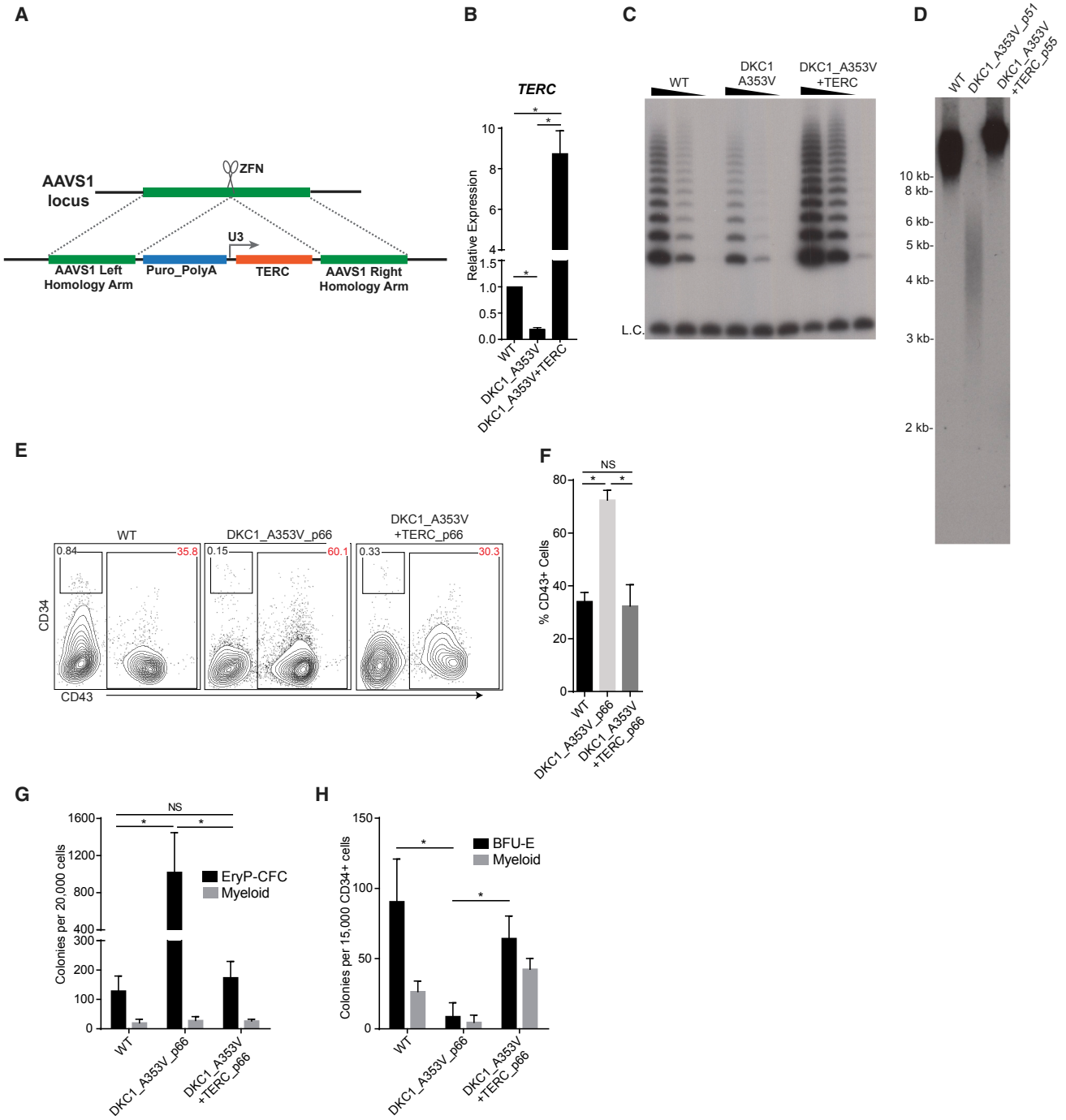

Figure 4. Telomerase Reactivation Restores Hematopoietic Potential in DKC1_A353V hESCs

(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).

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that our observed pan-hematopoietic defect specifically in definitive derived lineages more broadly recapitulates 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 (Townsley 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 (ESCRO) 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.F.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.F.Z.B. wrote the manuscript.

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(G) EryP-CFC potential in day-11 differentiation cultures, as in (E).
(H) Colony-forming cell potential of definitive hematopoietic progenitors, generated as in Figure 2A.
In all panels, wild-type (WT) is compared with DKC1_A353V (p66) and DKC1_A353V + TERC (p66) hESCs. n = 3 independent experiments, mean ± SEM; *p ≤ 0.05; Student's t test. NS, not significant.

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