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DKC1 is a transcriptional target of GATA1 and drives upregulation of telomerase activity in normal human erythroblasts

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ABSTRACT

Telomerase is a ribonucleoprotein complex that maintains the length and integrity of telomeres, and thereby enables cellular proliferation. Understanding the regulation of telomerase in hematopoietic cells is relevant to the pathogenesis of leukemia, in which telomerase is constitutively activated, as well as bone marrow failure syndromes that feature telomerase insufficiency. Past studies showing high levels of telomerase in human erythroblasts and a prevalence of anemia in disorders of telomerase insufficiency provide the rationale for investigating telomerase regulation in erythroid cells. Here it is shown for the first time that the telomerase RNA-binding protein dyskerin (encoded by *DKC1*) is dramatically upregulated as human hematopoietic stem and progenitor cells commit to the erythroid lineage, driving an increase in telomerase activity in the presence of limiting amounts of *TERT* mRNA. It is also shown that upregulation of *DKC1* was necessary for expansion of glycophorin A^+ erythroblasts and sufficient to extend telomeres in erythroleukemia cells. Chromatin immunoprecipitation and reporter assays implicated GATA1-mediated transcriptional regulation of *DKC1* in the modulation of telomerase in erythroid lineage cells. Together these results describe a novel mechanism of telomerase regulation in erythroid cells which contrasts with mechanisms centered on transcriptional regulation of *TERT* that are known to operate in other cell types. This is the first study to reveal a biological context in which telomerase is upregulated by *DKC1* and to implicate *GATA1* in telomerase regulation. The results from this study are relevant to hematopoietic disorders involving *DKC1* mutations, *GATA1* deregulation and/or telomerase insufficiency.

Introduction

Telomerase is a ribonucleoprotein complex that maintains the length and integrity of chromosomal-end structures called telomeres and thereby enables continuous cellular proliferation.[1] The minimum essential components of the human telomerase holoenzyme are a specialized reverse transcriptase (TERT) and a non-coding RNA (TERC) that includes an RNA template domain for priming synthesis of telomeric repeats. Active human telomerase ribonuclear proteins also include the RNA binding and modifying protein, dyskerin. Dyskerin, encoded by *DKC1*, augments telomerase activity by directly binding to TERC to confer the structural rigidity and stability necessary for its accumulation and function.[2,3]

Telomerase enzyme activity underpins the unrestricted proliferation of cancer cells in approximately 80-90% of malignancies, including acute leukemias and lym-

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phomas.[4-7] It also plays an important role in the function of normal hematopoietic stem and progenitor cells (HSPC).[8-11] Telomerase activity is low in quiescent hematopoietic stem cells, and is upregulated by self-renewal cytokines that promote cell cycling.[11,12] It is then downregulated to undetectable levels as HSPC differentiate into granulocytes, monocytes and macrophages.[9,11,12] Telomerase is similarly upregulated in lymphoid cells upon exposure to mitogens but is barely detectable in mature resting lymphocytes and peripheral blood mononuclear cells.[9,13] Studies of telomerase in erythroid lineage cells are limited; however, our previous investigation demonstrated that in contrast to the downregulation of telomerase observed during myelomonocytic differentiation, telomerase was robustly upregulated as umbilical cord blood (CB)-derived HSPC underwent commitment and expansion along the erythroid lineage.[14] Supporting the functional significance of this finding, a strong correlation was demonstrated between the level of telomerase in human HSPC and the proliferative potential of erythroblasts. In contrast, there was no correlation between telomerase in HSPC and expansion along granulocytic or monocytic lineages. A recent study demonstrating that telomerase knockout mice have more pronounced defects in erythroid progenitors than in granulocyte-macrophage progenitors further supports an important role for telomerase in the erythroid lineage.[15] The mechanism responsible for the upregulation of telomerase during erythropoiesis is currently unknown.

Insufficient telomerase due to mutations in telomerase-associated genes is causally involved in inherited bone marrow failure syndromes including dyskeratosis congenita.[16] Dyskeratosis congenita manifests with mucocutaneous symptoms and multiple organ dysfunction; however, anemia is prevalent and bone marrow failure is the most common cause of death among patients with telomerase mutations.[17] Patients with telomerase insufficiency syndromes exhibit pancytopenias and have fewer circulating hematopoietic progenitors than do healthy individuals.[18] Understanding the mechanisms that regulate telomerase activity in human hematopoietic cells is a crucial step toward the development of effective treatment of hematologic conditions associated with insufficient telomerase. Past studies along these lines have attributed the modulation of telomerase activity in hematopoietic cells to transcriptional regulation of TERT, the rate-limiting component of the telomerase holoenzyme.[19-21] These studies focused on telomerase regulation in progenitors, lymphoid and myelomonocytic cells. There is no prior study of telomerase regulation in normal erythroid lineage cells. The prevalence of anemia in telomerase insufficiency syndromes and the need for new treatments for these disorders, provided the impetus for investigation in this area.

Here we show that the increase in telomerase activity that occurs as human HSPC commit to the erythroid lineage is a result of upregulation of the *DKC1* gene in the presence of limiting amounts of TERT mRNA. It is shown for the first time that the *DKC1* gene is a direct transcriptional target of the erythroid-specific transcription factor GATA1 and that high expression of DKC1 is required for efficient production of glycophorin A-positive (GLYA$^+$) erythroblasts. These results provide a novel mechanistic explanation for high levels of telomerase in GLYA$^+$ erythroblasts and the heightened vulnerability of the erythroid compartment to telomerase insufficiency.

Methods

Cord blood cell isolation and culture of CD34$^+$ and glycophorin A$^+$ cells

CB was obtained from the Royal North Shore Hospital and the Australian Cord Blood Bank. Ethical approval for the use of CB was obtained from the Human Research Ethics Committees of the relevant hospitals and the University of New South Wales (approval numbers: HREC 05188, NSCCH 0602-004M, SESIAHS 08/190). Bone marrow mononuclear cells were obtained from Lonza (Mt Waverly Australia). CB processing and isolation of CD34$^+$ HSPC and GLYA$^+$ cells are described in the *Online Supplementary Methods*. CD34$^+$ HSPC were expanded for 1 week in Isocove modified Dulbecco media (Life Technologies, Carlsbad, CA, USA) with 20% fetal bovine serum (Trace Scientific, Melbourne, Australia), 100 ng/mL stem cell factor (SCF, Amgen, Thousand Oaks, CA, USA), 100 ng/mL thrombopoietin (Peprotech, Rocky Hill, NJ, USA), 100 ng/mL Flt-3 ligand (FLT-3L, Amgen) (STF), 50 μg/mL gentamycin and 200 mM glutamine. The cells were then cultured in cytokine combinations that force expansion and differentiation along specific lineages as described in our previous study (*Online Supplementary Table S1*).[14] Differentiation was assessed by fluorescence activated cell sorting (FACS) analysis after staining cells with the conjugated antibodies detailed in *Online Supplementary Table S2*. Green fluorescent protein-positive (GFP$^+$), GLYA$^+$ and CD13$^+$ subpopulations were purified by FACS using a FACS Diva (Becton Dickinson).

DKC1 gene suppression and overexpression

The viral vectors and methods used for suppression and overexpression of DKC1 are described in the *Online Supplementary Methods*.

Telomerase enzyme assays and telomere length measurements

Telomerase enzyme activity was quantified using the real-time polymerase chain reaction (PCR)-based telomeric amplification protocol (qTRAP) as described elsewhere.[22] Mean telomeric restriction fragment length was measured using the TeloTAGGG Telomere Length Assay kit (Roche, Mannheim, Germany) as previously described and detailed in the *Online Supplementary Methods*.[23]

Gene and protein expression analyses

Quantitative real-time PCR (qRT-PCR) and western blot analysis were performed according to standard protocols described in the *Online Supplementary Methods*.

Chromatin immunoprecipitation and reporter assays

Chromatin immunoprecipitation (ChIP) assays were performed as previously described.[24] Briefly, 2 x 10^7 cells were treated with 1% formaldehyde, then cross-linked chromatin was sonicated to obtain 300-500 bp fragments. Chromatin was immunoprecipitated with antibodies detailed in *Online Supplementary Table S2* and subjected to qRT-PCR using Express SYBR Green (Life Technologies) and the primers described in *Online Supplementary Table S3*. Values were normalized to products from immunoprecipitation with control IgG antibody.

A *DKC1* promoter reporter construct (pGL2-DKC1L) was made by cloning a *DKC1* sequence spanning +211 to -1113 bp from the *DKC1* transcription start site into Xho1 and HindIII sites of the pGL2 vector encoding luciferase. Two proximal GATA sites were mutated by site-directed mutagenesis using the QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with the primers listed in *Online Supplementary Table S3*. The mutated

nucleotides were verified by Sanger sequencing. Luciferase assays were performed using HEL 92.1.7 cells as described in the *Online Supplementary Methods*.

Statistics

All statistics were performed using GraphPad Prism 6.0b (La Jolla, CA, USA). Results were considered statistically significant when $P<0.05$.

Results

DKC1 is upregulated with erythroid lineage commitment

It was previously shown that telomerase activity is upregulated when CB-derived HSPC were switched to conditions promoting erythroid differentiation.[14] To verify this finding in a pure population of erythroid cells, GLYA$^+$ cells were sorted by FACS from cultures generated by *ex vivo* expansion of HSPC. CD34$^+$ cells were first expanded in medium supplemented with STF for 1 week, then switched to medium containing SCF and erythropoietin (SE) for a further 2 weeks. FACS analysis using antibodies for GLYA and CD34 confirmed differentiation of HSPC and enrichment for GLYA$^+$/CD34$^-$ erythroid cells (>80% of the viable population) at week 2 and week 3 (Figure 1A and *Online Supplementary Figure S1*). Erythroid cell populations were further purified from week 2 cultures by FACS sorting cells based on either low or high expression of GLYA and lack of expression of the myeloid cell marker CD13. CD13$^+$/GLYA$^-$ myeloid cells were also purified from the week 2 cultures for comparison with the erythroid cells (Figure 1B). Telomerase activity was quantified in the FACS-sorted populations of GLYA^{+high}, GLYA^{+low} erythroid cells and CD13$^+$ myeloid cells, as well as CD34$^+$ cells and an unpurified population of cells cultured in SE. The results demonstrated that telomerase activity was upregulated in GLYA^{+high} erythroblasts relative to both uncultured CD34$^+$ HSPC and unsorted SE-cultured cells (Figure 1C). In contrast, no significant telomerase activity was detected in CD13$^+$ myeloid cells. These data confirm that telomerase activity was confined to the GLYA$^+$ erythroid subset of cells in the SE culture, and was downregulated in differentiated myeloid cells.

To investigate the regulation of telomerase during erythroid commitment, telomerase enzyme activity and expression of *TERT*, *TERC* and *DKC1* was assessed at weekly time points over the 3-week culture period. As previously reported, telomerase was modestly upregulated upon initial cytokine stimulation with STF,[11,12] then further increased during the second week of culture after switching to SE ($P<0.01$) (Figure 2A).[14] In parallel with the initial induction of telomerase activity, there was a measurable increase in *TERT* expression during the first week

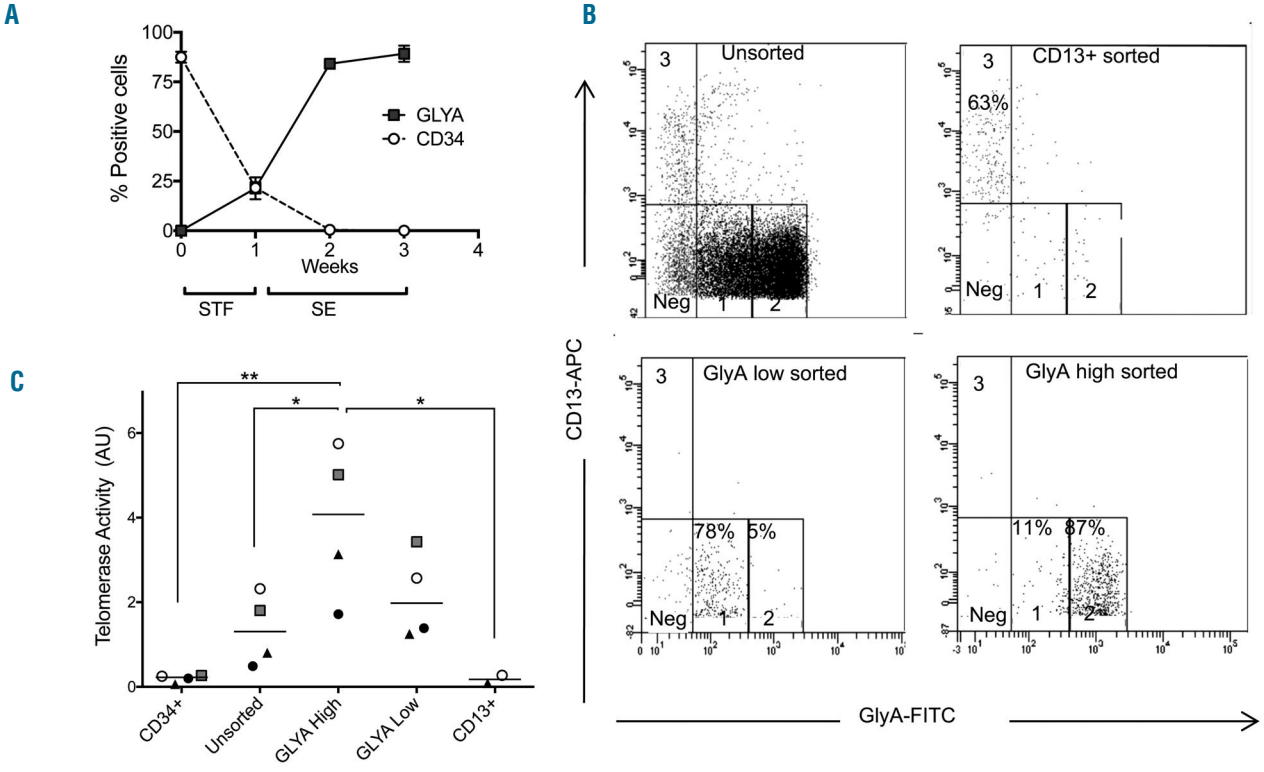

Figure 1. Telomerase enzyme activity is upregulated in glycophorin A$^+$ erythroblasts. Cord blood (CB)-derived CD34$^+$ hematopoietic stem and progenitor cells (HSPC) were expanded for 7 days in medium supplemented with stem cell factor, thrombopoietin and Flt-3 ligand (STF), then cultured for an additional 2 weeks in medium with stem cell factor plus erythropoietin (SE). (A) Graphical representation of the proportions of CD34$^+$ and glycophorin-positive (GLYA$^+$) cells in the total viable cell fraction, shown as the mean ± standard error of the mean (SEM) calculated from nine independent CB cultures. (B) Cell populations enriched for erythroblasts with high or low GLYA expression, or CD13$^+$ myeloid cells were isolated from SE cultures by fluorescence activated cell sorting (FACS). Panels show gates used for sorting cell subsets expressing low (1) or high (2) levels of GLYA and CD13 before and after FACS. (C) qTRAP measurement of telomerase enzyme activity in uncultured HSPC cells (CD34$^+$), unfractionated cells from SE cultures (Unsorted) and FACS-sorted cell populations. Each symbol represents the mean from three measures of telomerase activity in independent assays of cells from four CB cultures. *$P<0.05$, **$P<0.01$, Dunnett multiple comparisons test.

of culture in STF. *TERT* expression then returned to the low basal levels detected in unstimulated HSPC when the culture was switched to SE (weeks 2-3). In contrast to *TERT*, *DKC1* expression was induced after cultures were switched to SE. The kinetics of *DKC1* upregulation varied among the CB cultures established from different individuals, but invariably increased after the switch to erythroid conditions at week 1 and peaked at either week 2 or 3 of culture (*Online Supplementary Figure S2*). The *DKC1* expression pattern closely paralleled erythroid commitment and expansion, as indicated by expression of GLYA$^+$ (Figure 1A). Expression of *TERC* did not alter dramatically over the time course, apart from a modest increase from week 1 to 2 of culture.

To determine whether the upregulation of *DKC1* expression was specific for the erythroid lineage, *DKC1* expression was analyzed in cell populations enriched for monocytic, granulocytic and megakaryocytic cells. Cultures enriched for these lineages were produced by switching STF cultures at week 1 from STF to SCF, FLT-3L

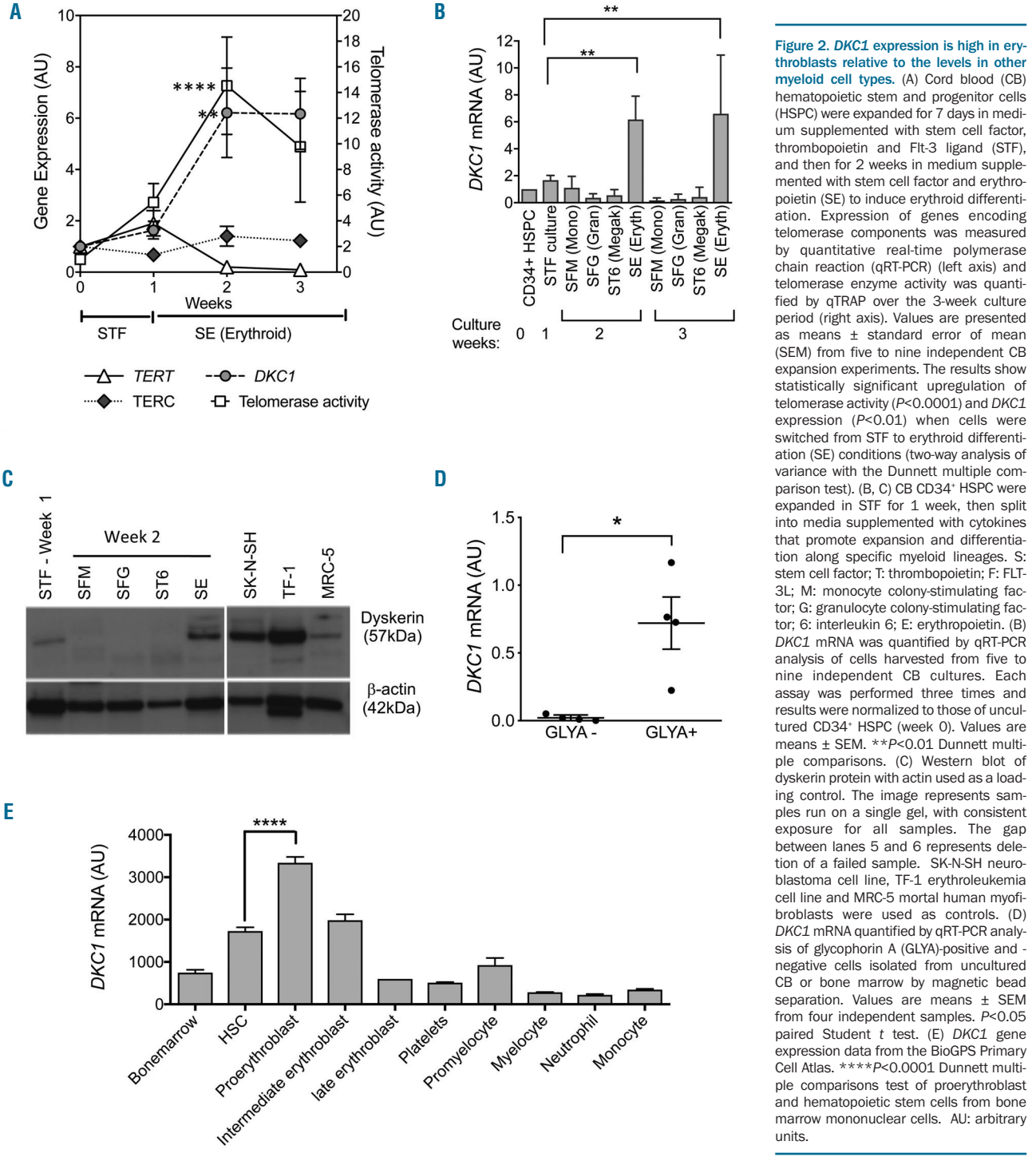

Figure 2. *DKC1* expression is high in erythroblasts relative to the levels in other myeloid cell types. (A) Cord blood (CB) hematopoietic stem and progenitor cells (HSPC) were expanded for 7 days in medium supplemented with stem cell factor, thrombopoietin and Flt-3 ligand (STF), and then for 2 weeks in medium supplemented with stem cell factor and erythropoietin (SE) to induce erythroid differentiation. Expression of genes encoding telomerase components was measured by quantitative real-time polymerase chain reaction (qRT-PCR) (left axis) and telomerase enzyme activity was quantified by qTRAP over the 3-week culture period (right axis). Values are presented as means ± standard error of mean (SEM) from five to nine independent CB expansion experiments. The results show statistically significant upregulation of telomerase activity (P<0.0001) and *DKC1* expression (P<0.01) when cells were switched from STF to erythroid differentiation (SE) conditions (two-way analysis of variance with the Dunnett multiple comparison test). (B, C) CB CD34$^+$ HSPC were expanded in STF for 1 week, then split into media supplemented with cytokines that promote expansion and differentiation along specific myeloid lineages. S: stem cell factor; T: thrombopoietin; F: FLT-3L; M: monocyte colony-stimulating factor; G: granulocyte colony-stimulating factor; 6: interleukin 6; E: erythropoietin. (B) *DKC1* mRNA was quantified by qRT-PCR analysis of cells harvested from five to nine independent CB cultures. Each assay was performed three times and results were normalized to those of uncultured CD34$^+$ HSPC (week 0). Values are means ± SEM. **P<0.01 Dunnett multiple comparisons. (C) Western blot of dyskerin protein with actin used as a loading control. The image represents samples run on a single gel, with consistent exposure for all samples. The gap between lanes 5 and 6 represents deletion of a failed sample. SK-N-SH neuroblastoma cell line, TF-1 erythroleukemia cell line and MRC-5 mortal human myofibroblasts were used as controls. (D) *DKC1* mRNA quantified by qRT-PCR analysis of glycophorin A (GLYA)-positive and -negative cells isolated from uncultured CB or bone marrow by magnetic bead separation. Values are means ± SEM from four independent samples. P<0.05 paired Student t test. (E) *DKC1* gene expression data from the BioGPS Primary Cell Atlas. ****P<0.0001 Dunnett multiple comparisons test of proerythroblast and hematopoietic stem cells from bone marrow mononuclear cells. AU: arbitrary units.

and monocyte colony-stimulating factor (SFM) for monocytic differentiation, to SCF, FLT-3L and granulocyte colony-stimulating factor (SFG) for granulocytic expansion and to SCF, thrombopoietin and interleukin-6 (ST6) to promote megakaryocyte differentiation (*Online Supplementary Figure S2*). qRT-PCR analysis showed that in contrast to the upregulation of *DKC1* observed in cells cultured with SE ($P<0.01$), there was no significant induction of *DKC1* expression in cells grown under conditions favoring monocytic, granulocytic or megakaryocytic differentiation (Figure 2B). Western blot analysis further demonstrated high expression of dyskerin protein only in cell populations produced under erythroid conditions (Figure 2C).

Upregulation of *DKC1* bolsters telomerase activity and promotes telomere lengthening

To confirm that *DKC1* expression was upregulated in erythroblasts produced *in vivo* as well as in *ex vivo*-generated erythroid cultures, *DKC1* expression was assessed in GLYA$^+$ cells isolated from uncultured CB and bone marrow mononuclear cells. Results from qRT-PCR analysis consistently showed higher *DKC1* mRNA in the GLYA$^+$ fraction than in the GLYA$^-$ fraction of cells isolated from four samples (Figure 2D). Consistent with these results, gene expression data, collected through meta-analysis of multiple independent studies (available through the BioGPS Primary Cell Atlas dataset), showed *DKC1* expression to be upregulated in proerythroblasts relative to hematopoietic stem cells and myeloid lineage cells (Figure 2E).[25] The upregulation of *DKC1* mRNA in bone marrow proerythroblasts appeared to be transient, as *DKC1* levels in intermediate erythroblasts were similar to those in hematopoietic stem cells. Collectively these data provide strong evidence of *DKC1* upregulation during erythroid commitment of human HSPC.

To test whether induction of *DKC1* was sufficient for the upregulation of telomerase activity observed in erythroblasts, HSPC were transduced with lentiviral vectors encoding *DKC1* cDNA plus GFP (MSCV-DKC1) or GFP alone (MSCV-GFP). Robust upregulation of *DKC1* mRNA in MSCV-DKC1-transduced cells was demonstrated by qRT-PCR analysis of GFP$^+$ cells isolated by FACS ($P<0.01$, Student *t* test) (Figure 3A). Telomerase enzyme activity was also substantially increased in MSCV-DKC1 cells relative to control vector-transduced cells ($P<0.01$, Student *t* test) (Figure 3B). There were no significant differences in the expression of *TERT* and *TERC* between MSCV-DKC1 and control vector-transduced cells (Figure 3A), although *TERC* expression tended to be higher in the MSCV-DKC1 cells.

To enable analysis of the effect of *DKC1* upregulation over a time course sufficient for assaying telomere length changes, we also overexpressed *DKC1* in the immortal erythroleukemia cell line, HEL 92.1.7 (Figure 3C). Consistent with the CB experiments, overexpression of *DKC1* in HEL 92.1.7 cells caused a substantial elevation of telomerase activity without an apparent change in *TERT* mRNA expression (Figure 3C, D). Southern blot-based analysis of telomere length showed that ectopic expression of *DKC1* resulted in telomere lengthening at a rate of approximately 500 bp over a 2-month period, and 900 bp over 7 months (Figure 3E). To verify that *DKC1* upregulation is sufficient for telomere extension, we also overexpressed *DKC1* in HL-60 cells. Consistent results were obtained, showing that *DKC1* overexpression resulted in robust upregulation of telomerase and elongated telomeres (*Online Supplementary Figure S4*). These data show that upregulation of *DKC1* results in an accumulation of functional telomerase complexes capable of telomere elongation.

High expression of *DKC1* is necessary for erythroblast proliferation

We next tested whether elevated expression of *DKC1* was necessary for erythroid cell proliferation and differentiation. For these investigations CB-derived HSPC were transduced with retroviral vectors encoding one of two different shRNA targeting *DKC1* mRNA (D2 and D3) or a non-silencing shRNA (NS) plus GFP. HSPC were pre-stimulated and transduced in medium supplemented with STF and then FACS-sorted for GFP$^+$ cells, which were then cultured in SE. qRT-PCR analysis confirmed effective suppression of *DKC1* and corresponding downregulation of telomerase activity in erythroid cells transduced with D2 and D3 vectors relative to control vector-transduced cells (Figure 4A, B). The suppression of telomerase activity was not attributable to reduced expression of *TERT*, which was expressed at equivalent levels in D2-, D3- and NS-transduced cells (Figure 4C). TERC levels varied among the independent experiments, although they tended to be lower in D2- and D3-transduced cells relative to control cells (Figure 4D), consistent with the known role of dyskerin as a stabilizing scaffold for TERC.

Weekly counts of transduced cells revealed that shRNA-mediated suppression of *DKC1* expression inhibited proliferation in SE-driven cultures (Figure 4E). Since there was no apparent difference in the proportion of GLYA$^+$ cells in D2 and D3 cultures compared to NS cultures, the reduction in GLYA$^+$ cell number did not appear to be due to impaired erythroid differentiation (Figure 4F). When plated in methylcellulose, D2- and D3-transduced cells formed erythroid colonies with normal burst-forming unit-erythroid (BFU-E) size and morphology; however, significantly fewer colonies were generated by D2 and D3 cultures than by NS (Figure 4G). In contrast to the effect of *DKC1* knockdown on BFU-E colony numbers, there was no discernible effect on colony-forming units of granulocyte-monocyte or granulocyte-erythrocyte-macrophage-megakaryocyte. Together, the results demonstrate a critical role for *DKC1* expression in erythroblast proliferation that is independent of differentiation.

GATA1 contributes to transcriptional regulation of *DKC1* in erythroblasts

The *DKC1* promoter was previously shown to be a target of *MYC* family oncoproteins in *MYC*-driven cancers.[26,27] Since *MYC* is also expressed in erythroid progenitors,[28] we next investigated whether MYC or other erythroid transcription factors, namely GATA1 and TAL1, play a role in the regulation of *DKC1*. Western blot analysis of uncultured CB CD34$^+$ HSPC and *ex vivo*-expanded CB cells revealed MYC expression in unstimulated CB HSPC and in cells harvested from STF, STM and SE cultures (Figure 5A). In contrast, expression of GATA1 and TAL1 was confined to cells cultured in SE. Consistent with the qRT-PCR results (Figure 2B), dyskerin protein was detectable in undifferentiated cells cultured in STF and erythroid cells at weeks 2 and 3, but was not detected in cells from monocyte-enriched cultures (Figure 5A).

Canonical E-boxes have previously been identified in

the *DKC1* promoter.[26,27] ChIP sequencing data from peripheral blood erythroblasts made available through ENCODE on the UCSC browser also provide evidence of GATA 1 binding at the *DKC1* promoter, in the vicinity of -1097 to -493 relative to the transcription start site (chrX: 153,991,030, hg19) (Figure 5B). Guided by these data, we identified putative GATA sites at positions: -679 to -668 and -453 to -468 and designed PCR primers to interrogate the transcription factor binding by ChIP. First, using antibodies to trimethylated H3K4 (H3K4me3), which occupies transcriptionally active chromatin, and H3K27me3, which identifies repressed sites, we verified that chromatin at the *DKC1* promoter was in an open configuration conducive to transcriptional activation in both STF-stimulated cells and erythroblastic cells (Figure 5C).[29] ChIP analysis also confirmed MYC binding to the *DKC1* promoter, although this appeared to diminish progressively as undifferentiated CB cells underwent erythroid differentiation in SE culture. Conversely, GATA1 binding at the *DKC1* promoter appeared most robust at week 3, corresponding with the accumulation of erythroblasts expressing high levels of GLYA (*Online Supplementary Figure S5*) and peak expression of *DKC1* in six out of nine CB cultures (*Online Supplementary Figure S2*). Consistent with GATA1 binding in GLYA^{high} CB-derived erythroblasts (Figure 5C), ChIP sequencing results from three independent investigations, accessed using CistromeDB, showed GATA1 binding at the DKC1 promoter of erythroblasts derived from bone marrow and peripheral blood (*Online Supplementary Figure S6*).[30-32] Although TAL1 can bind DNA via E-boxes, no TAL1 binding at the *DKC1* promoter was detected in CB cells at any stage of culture. Collectively, these data suggest a model whereby MYC binds the *DKC1* promoter in undifferentiated cells and is replaced by GATA1 during erythroid differentiation.

Since GATA1 regulation of *DKC1* has not previously been described, a luciferase reporter assay was conducted to confirm that GATA sites contribute to *DKC1* transcription. Mutations were induced in two potential GATA1 binding sites of the *DKC1* promoter construct by site-directed mutagenesis and luciferase activity was measured

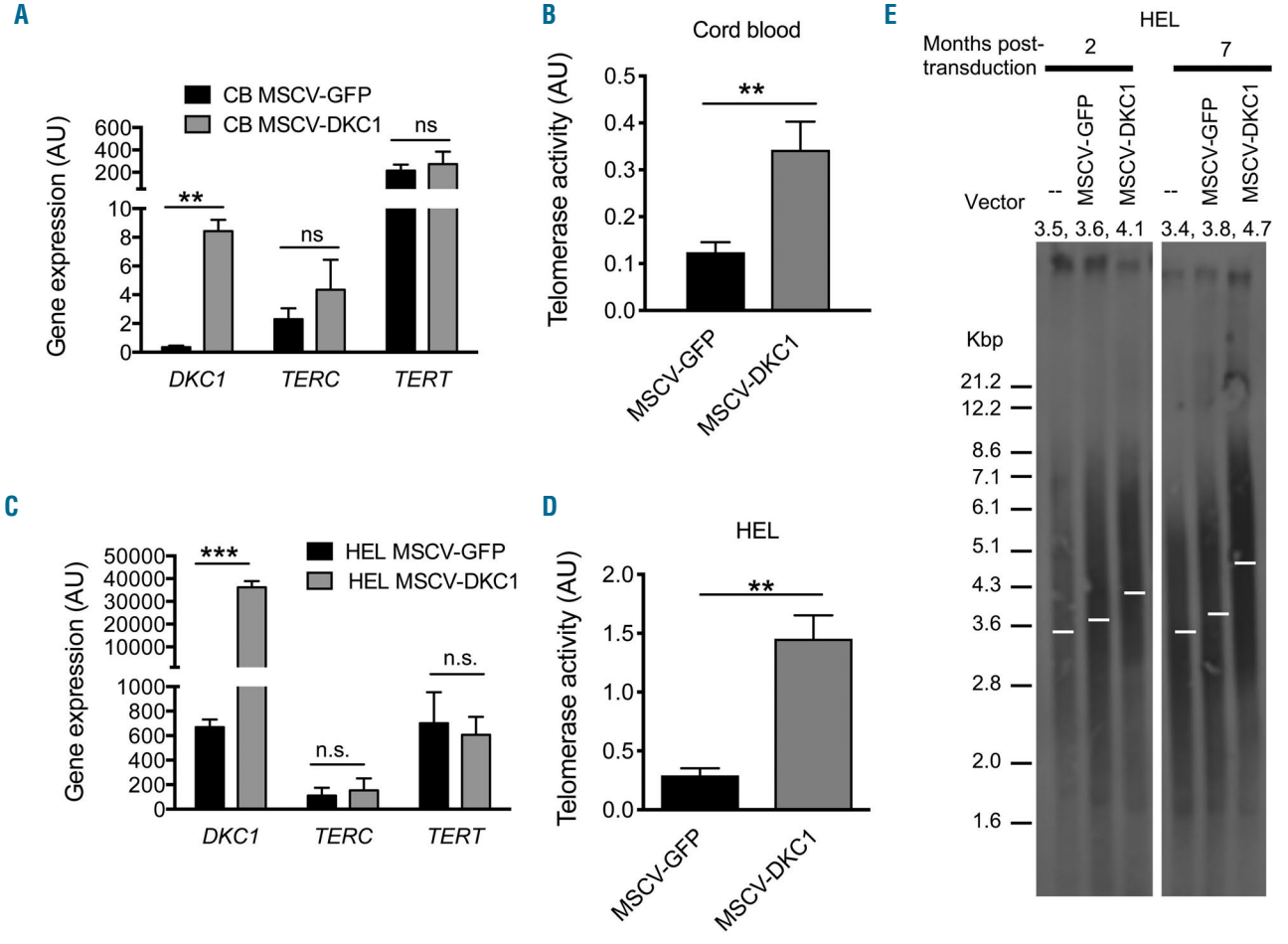

Figure 3. Upregulation of *DKC1* is sufficient for induction of telomerase activity in normal cord blood progenitors and erythroleukemia cells. (A-D) Cord blood (CB) CD34$^+$ hematopoietic stem and progenitor cells (HSPC) (A, B) and HEL 92.1.7 leukemia cells (HEL) (C, D) were transduced with a lentiviral vector encoding *DKC1* and green fluorescence protein (GFP) (MSCV-DKC1) or a control vector expressing GFP alone (MSCV-GFP). Transduced cells were enriched by fluorescence activated cell sorting (FACS) for GFP expression 72 h after the last round of lentiviral infection. (A) Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of *DKC1*, *TERC* and *TERT* expression in transduced CB cells collected immediately after FACS. (B) Telomerase activity in transduced CB cells measured using the qTRAP assay. (C) qRT-PCR analysis of *DKC1* mRNA, TERC and *TERT* mRNA in HEL 92.1.7 cells. (D) Telomerase activity in transduced HEL 92.1.7 cells measured in cells harvested 7 days after FACS using the qTRAP assay. Results for (A-D) are mean values ± standard error of mean calculated from three assays from each of two to four independent lentiviral transduction experiments. $^{**}P<0.01$, $^{***}P<0.001$ from a two-tailed paired Student *t* test. (E) Telomeric restriction fragment Southern blot showing telomere lengths in transduced HEL 92.1.7 cells at 2 months and 7 months after transduction.

in transfected HEL 92.1.7 erythroleukemia cells. The results demonstrated that both GATA sites contributed to promoter activity, and that concurrent ablation of the two GATA sites substantially diminished promoter activity (Figure 5D). Collectively the results provide strong evidence that the erythroid-restricted transcription factor GATA1 contributes to the regulation of *DKC1*.

Discussion

This report is the first to implicate GATA1 in the regulation of telomerase and to describe a biological context in which telomerase activity is upregulated by induction of *DKC1*. Here it is shown that GATA1 binds the *DKC1* promotor and contributes to the upregulation of *DKC1*,

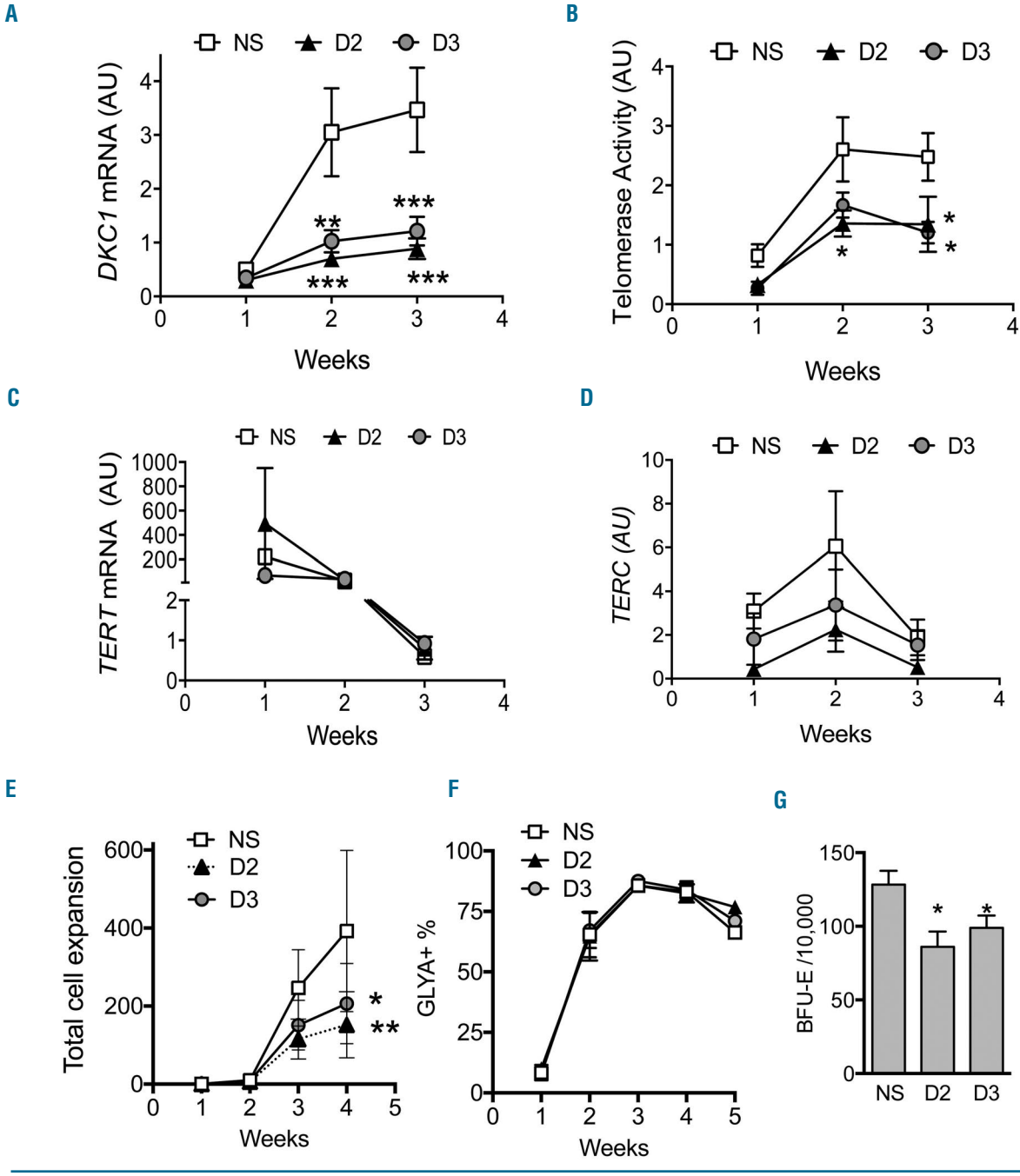

Figure 4. Downregulation of *DKC1* expression suppresses telomerase activity and inhibits erythroid cell proliferation but not differentiation. Cord blood-derived CD34⁺ hematopoietic stem and progenitor cells were transduced with retroviral vectors encoding *DKC1*-targeted shRNA (D2 and D3) or non-silencing RNA (NS) and green fluorescent protein (GFP). GFP⁺ cells were sprted by fluorescent activated cell sorting (FACS) (week 1), then cultured with stem cell factor plus erythropoietin (SE) for a further 2 weeks. Expression of telomerase genes and telomerase enzyme activity were measured by quantitative real-time polymerase chain reaction and the qTRAP assay, respectively. (A) *DKC1* mRNA, (B) telomerase enzyme activity, (C) *TERT* mRNA and (D) *TERC* abundance. (E) Expansion of FACS-sorted GFP⁺ cells determined from cell counts using typan blue exclusion of dead cells. (F) Percentage of glycophorin A-positive cells in cultures determined by weekly FACS analysis. (G) Erythroid progenitors in the GFP⁺ sorted fraction were quantified as burst-forming units-erythroid in methylcellulose assays. Values are means ± standard error of mean from six independent experiments. **P*<0.05, ***P*<0.01, *****P*<0.0001 from the Dunnett multiple comparison of D2 and D3 to NS. AU: arbitrary units; BFU-E: burst-forming unit-erythroid; GLYA⁺: glycophorin A-positive cells.

which drives telomerase activity levels in erythroid cells. These results are notable in relation to past studies that established the paradigm of TERT transcriptional regulation as the primary determinant of telomerase regulation in hematopoietic and cancer cells.[19,20,33-35]

Past investigations of telomerase regulation in hematopoietic cells focused on lymphoid cells, myeloid progenitors and myeloid leukemia cell lines.[11-13,36] These studies revealed that mitogen-induced upregulation of telomerase was followed by telomerase downregulation during differentiation. Studies of lymphoid and myeloid cells also established a direct role for MYC in the transcriptional regulation of *TERT* and telomerase re-activation in hematopoietic cells.[19,37] Consistent with this paradigm, the present study found parallel upregulation of *TERT* and telomerase activity in the presence of MYC protein in CB progenitor cells stimulated with STF. However, there was a clear dissociation of this pathway when cultures were switched to erythroid conditions. Upon switching cultures from STF to SE, the abundance of MYC protein was sustained, but *TERT* expression declined to an apparently rate-limiting level while telomerase enzyme activity escalated. Rather than correlating with *TERT* expression, the increase in telomerase activity detected in erythroblastic cells correlated with upregulation of endogenous *DKC1* mRNA. Modulation of *DKC1* expression by overexpression or targeting with shRNA confirmed that *DKC1* regulated telomerase activity in erythroid cells without altering *TERT* gene expression. The functional significance of these observations was further supported by evidence of telomere lengthening following upregulation of *DKC1* in the HEL 92.1.7 erythroid cell line. *DKC1* mRNA was shown to be abundant in CB-derived CD34^{-}/GLYA^{+} erythroid cells irrespective of

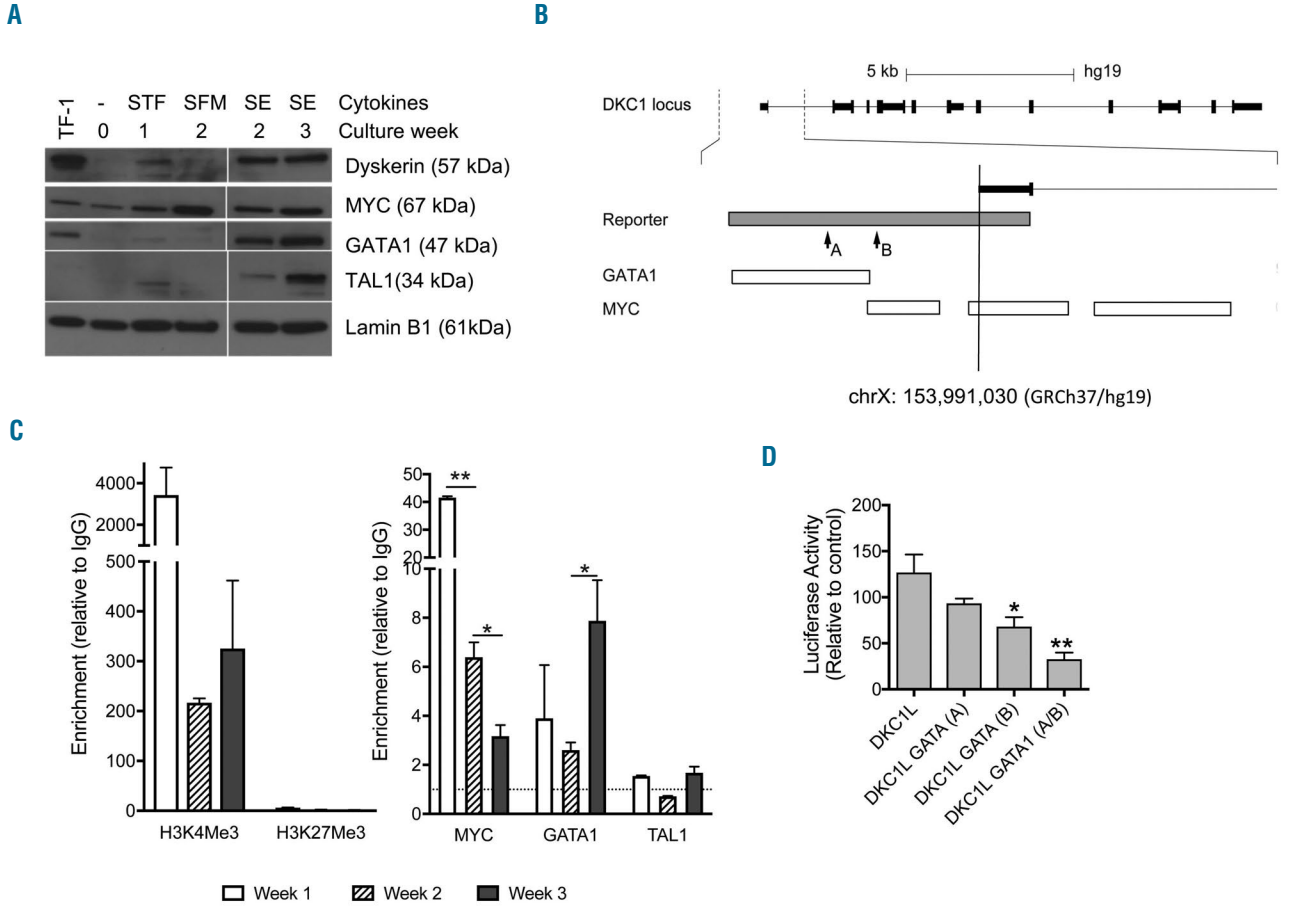

Figure 5. GATA1 interaction and regulation of the *DKC1* promoter in erythroid cells. (A) Immunoblot of nuclear extracts showing expression of dyskerin and the transcription factors MYC, GATA1 and TAL1 in cord blood (CB) cells cultured for 1 week in medium supplemented with stem cell factor (SCF), thrombopoietin and Flt-3 ligand, followed by culture with SCF plus erythropoietin or SCF, Flt-3 ligand and monocyte colony-stimulating factor. The erythroleukemia cell line TF-1 was used as a control for dyskerin expression. The immunoblot was hybridized to Lamin B1 antibody as a loading control. The image shows samples run on a single gel, with consistent exposure for all samples. The gap between lanes 4 and 5 represents deletion of a failed sample. (B) Schematic figure of the *DKC1* promoter showing the region included in the pGL2-*DKC1L* reporter construct, as well as canonical GATA motifs (indicated by arrows labeled A and B), and regions of GATA1 and MYC binding in peripheral blood erythroblasts and other cell types as reported in ENCODE chromatin immunoprecipitation sequencing traces (UCSC browser GRCh37/hg19). (C) Chromatin immunoprecipitation was performed on *ex vivo*-expanded CB cells using antibodies to MYC, GATA1, TAL1 and trimethylated histones or a control IgG antibody. Transcription factor binding was quantified as fold-enrichment of quantitative real-time polymerase chain reaction (qRT-PCR) products amplified from the region of interest relative to control region in an unrelated gene. Data were normalized to results from the IgG control antibody. **P*<0.05, ***P*<0.01, analysis of variance followed by the Bonferroni multiple comparisons test. (D) Site-directed mutagenesis was performed to introduce point mutations at canonical GATA sites within a pGL2-*DKC1L* luciferase reporter construct. HEL 92.1.7 cells were co-transfected with the reporter constructs and control vector pEFBOS-LacZ for normalization. Promoter activity was detected as luciferase activity and measured 48 h after transfection. Values are means ± standard error of mean from three independent experiments. **P*<0.05, ***P*<0.01, ns; not significant, from the Dunnett multiple comparisons test. STF: stem cell factor, thrombopoietin and Flt-3 ligand; SE: stem cell factor plus erythropoietin; SFM: stem cell factor, Flt-3 ligand and monocyte colony-stimulating factor.

whether they were generated *ex vivo* or *in vivo*. Consistent with these findings, publicly available microarray data from fractionated bone marrow showed high levels of *DKC1* expression in proerythroblasts.

In addition to regulating *TERT*, MYC has been shown to bind and activate the *DKC1* promoter in tumor cell lines.[26,27] The present study adds to this knowledge by demonstrating that MYC binds the *DKC1* promoter in primary human hematopoietic cells. Notably however, MYC binding at the *DKC1* promoter appeared to decline, while *DKC1* was upregulated during erythroid commitment and expansion. These results suggest that *MYC* plays a less prominent role in driving *DKC1* expression in erythroblasts compared with undifferentiated HSPC. Instead, our study highlights a potential role for GATA1 in the regulation of *DKC1* in the erythroid lineage. This was evidenced by enrichment of GATA1 at the *DKC1* promoter in GLYA$^+$ erythroblasts, and an apparent transcriptional requirement for GATA binding sites in the proximal region of the *DKC1* promoter.

Consistent with the known role of dyskerin in stabilizing TERC,[38] we consistently observed an increase in TERC in parallel with upregulation of endogenous *DKC1*. The magnitude of this effect was moderate, yet consistent with results from ectopic overexpression of *DKC1*, and converse to the observed effect of shRNA-mediated downregulation of *DKC1*. Overexpression of *TERC* was previously shown to be sufficient to elevate telomerase and hyper-extend telomeres.[39-41] Nevertheless it seems unlikely that the modest elevation in endogenous TERC observed in primary erythroblasts was the singular cause of the dramatic increase in telomerase activity observed in the erythroblasts. In addition to stabilizing TERC, dyskerin may hyperactivate telomerase through its intrinsic pseudouridine synthase activity. This could involve targeting and enzymatically modifying TERC moieties with structural or functional roles, or indirectly through functional modification of rRNA or splicesomal RNA.[42-46] There is also scope for dyskerin to modulate telomerase activity through direct interactions with H/ACA box RNA that have been ascribed roles in post-transcriptional regulation of gene expression.[47-53] Clarification of the functional significance of pseudouridylation of TERC and other non-coding RNA that interact with dyskerin will be valuable in understanding the full extent of dyskerin's influence on telomerase activity.

Gene suppression experiments reported here demonstrate that high *DKC1* expression is required for proliferation of erythroblasts but is dispensable for erythroid differentiation. The apparent requirement for high expression of *DKC1* to sustain erythroblast proliferation may reflect dependence on telomerase as well as the telomerase-independent function of dyskerin in ribosome biogenesis.[2] During erythropoiesis, erythroblasts undergo a period of intense ribosome biogenesis that is necessary for synthesis of large quantities of hemoglobin.[54] An abundance of dyskerin may be necessary to support this process. Consistent with this possibility, zebra fish and murine models have shown that hypomorphic *DKC1* mutations impaired rRNA processing.[50-53] Ribosomal stress has also been demonstrated in *MYC*-transformed cancer cells subjected to shRNA-mediated suppression of dyskerin.[27] The implications of dyskerin's function in ribosome biogenesis are yet to be fully elucidated in relation to the pathogenesis of dyskeratosis congenita when *DKC1* is mutated. However, it is worth noting that the hematologic deficiencies observed in dyskeratosis congenita are also primary characteristics of the bone marrow failure disorders referred to as ribosomopathies, which feature impaired ribosome biogenesis as an underlying cause.[55] Ribosome dysfunction in these disorders is usually attributed to mutations in genes with known roles in ribosome biogenesis. However, the discovery of *GATA1* mutations in the ribosomopathy Diamond Blackfan anemia raises the possibility that dyskerin insufficiency may contribute to the pathogenesis of this genetic subtype.[56]

Collectively the results from these investigations reveal a novel mechanism of telomerase regulation in primary human erythroblasts which contrasts with the established paradigm centered on MYC-mediated regulation of *TERT* expression in HSPC, lymphocytes and myelomonocytic cells. Notwithstanding the requirement for a rate-limiting amount of TERT for telomerase activity,[57] this study shows that *DKC1* expression levels are a critical determinant of telomerase enzyme levels in proliferating erythroblasts. Evidence provided herein that GATA1 contributes to the regulation of *DKC1* has implications in hematopoietic disorders that feature *DKC1* mutations, *GATA1* deregulation and/or telomerase insufficiency.

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