SUPPLEMENTARY APPENDIX

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

Laura A. Richards,[1*] Ashu Kumari,[1*] Kathy Knezevic,[2] Julie Al Thoms,[2,3] Georg von Jonquieres,[1] Christine E. Napier,[1] Zara Ali,[4] Rosemary O'Brien,[1] Jonathon Marks-Bluth,[2] Michelle F. Maritz,[1] Hilda A Pickett,[5] Jonathan Morris,[6] John E. Pimanda[2,3] and Karen L. MacKenzie[1,4,7,8]

[1]Children's Cancer Institute Australia, Randwick; [2]Adult Cancer Program, Prince of Wales Clinical School, Lowy Cancer Research Centre, UNSW, Sydney; [3]School of Medical Sciences, UNSW, Sydney; [4]Cancer Research Unit, Children's Medical Research Institute, Westmead; [5]Telomere Length Regulation Unit, Children's Medical Research Institute, Westmead; [6]The University of Sydney School of Medicine, Kolling Institute of Medical Research, St Leonards; [7]School of Women's and Children's Health, UNSW, Sydney and [8]Faculty of Medicine and Health, University of Sydney, Sydney, New South Wales, Australia

**LAR and AK contributed equally as co-first authors*

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Correspondence: *KAREN L MACKENZIE* - kmackenzie@cmri.org.au

SUPPLEMENTARY METHODS

Isolation of CB CD34+ HSPCs and GLYA+ 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 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). The direct CD34+ Progenitor Isolation Kit and CD235a microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) were used to isolate CD34+ and GLYA+ cells respectively from CB or bone marrow mononuclear cells (MNCs) using an autoMACS magnetic separation device (Miltenyi Biotech, Bergisch Gladbach, Germany) as previously described (1).

Cell culture

HEL 92.1.7 erythroleukemia and MRC-5 cell lines were purchased from ATCC (Manassas VA USA). TF-1 cell line identity was verified by STR profiling at CellBank Australia, Westmead Australia. HEL 92.1.7 and TF-1 cell lines were cultured in IMDM with 10% FBS. MRC-5 cells were cultured in α-MEM (Life Technologies) with 10% FBS. Clonogenic progenitors were quantified in triplicate cultures of 1% methylcellulose (Fluka, Seelze, Germany) supplemented with cytokines as described (2).

Quantitative RT-PCR (qRT-PCR)

RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Chadstone Centre, Australia), followed by reverse transcription of 1 µg of RNA using SuperScript® III reverse

transcriptase (Life Technologies) according to the manufacturer's instructions. Quantitative RT-PCR (qRT-PCR) was performed using iQTM SYBR Green (Bio-Rad, Hercules CA) as previously described using primers listed in Supplementary Table S3 (3). Gene expression levels were normalized to ABL mRNA and fold expression calculated using the ΔΔCt method. Relative gene expression was calculated from duplicates and normalized to expression in HeLa tumor cells. Values are expressed as means + standard error of the mean (SEM) derived from at least three experiments.

Western blot analysis

Nuclear lysates were prepared using NE-PER Nuclear and Cytoplasmic extraction kit (Thermo Scientific, North Ryde, Australia) according to the manufacturer's protocol and Western blots carried out as previously described (3). For Western blots of protein from whole cell lysates, cell pellets were resuspended in 1 mL of ice-cold TRAPeze CHAPS lysis Buffer. The cell suspension was incubated on ice for 30 min with occasional vortexing followed by centrifugation at 4300 g at 4^{o}C for 15 minutes. Protein concentration was determined by Bradford protein assay (BioRad, Gladesville, Australia) and Western blot performed as previously described (3). The dyskerin antibody was validated in a previous study (3). The GATA-1 was a gift from Prof Emery Bresnick (University of Wisconsin School of Medicine and Public Health, WI USA) (4). Other antibodies are detailed in Supplementary Table S2. Membranes were stripped for re-hybridization by washing in 25 mM glycine-HCl, pH 2, 1% SDS buffer for 20 min at room temperature.

Retroviral and lentiviral transductions

miR30-styled shRNAs targeting the *DKC1* gene and a non-silencing control oligonucleotide were synthesized (Integrated DNA Technologies, Coralville, IA) and cloned into the LMS retroviral vector backbone (5),(6). Replication-defective retroviral vectors were generated by transfection of Phoenix A packaging cells. *DKC1* was overexpressed from a lentiviral vector pCL10.1-DKC1 generously provided by Prof Inderjeet Dokal (Queen Mary University of London, United Kingdom). The control vector encoded GFP alone downstream from an internal ribosome entry site. Lentiviral vector particles were produced by co-transfection of HEK293T cells with the vector and packaging constructs pKGP, pRT and pVSV-G. All transfections were performed using Lipofectamine 2000 according to the manufacturer's instructions (Life Technologies, California, USA). Supernatant containing viral particles was collected 24 hours after transfection and periodically thereafter for 4 – 6 rounds of infection as previously described (7).

Telomere length measurements

Briefly, 11 μg of genomic DNA was digested with HinfI and RsaI then fractionated through a 0.7% agarose gel for 26 hours at 65 volts, transferred to nylon membranes, hybridized to 1:5,000 dilution of the telomere probe and visualized using CDP-star substrate (Roche) with subsequent exposure of the membrane to x-ray film (Fujifilm, Tokyo, Japan) and imaged on a Gel Doc™ EZ Imager (BioRad). Mean TRF lengths were determined using MacBas software (Fuji) as previously described [(8)].

***DKC1* promoter assay**

HEL 92.1.7 cells were plated at $2.0x10^5$ cells in 500 □L 10% FCS/IMDM in triplicates and co-transfected with 3.0 μg of *DKC1*-pGL2 reporter plasmid and 1.0 μg of pEFBOS-LacZ encoding □-galactosidase (LacZ) using Lipofectamine 2000 according to the manufacturer's instructions. Forty-eight hours after transfection, cell lysates were prepared and assayed for luciferase activity using 25 μL Luciferase Assay Substrate (Promega, Alexandria Australia) and a GloMax luminometer (Promega) as described elsewhere (9). LacZ activity was detected at 405 nm using a 20 μL aliquot of cell extract. Reporter activity was calculated as luciferase activity normalized to LacZ activity and expressed relative to values from control pGL2.

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SUPPLEMENTARY TABLES

Table S1 Cytokines for lineage-specific expansion

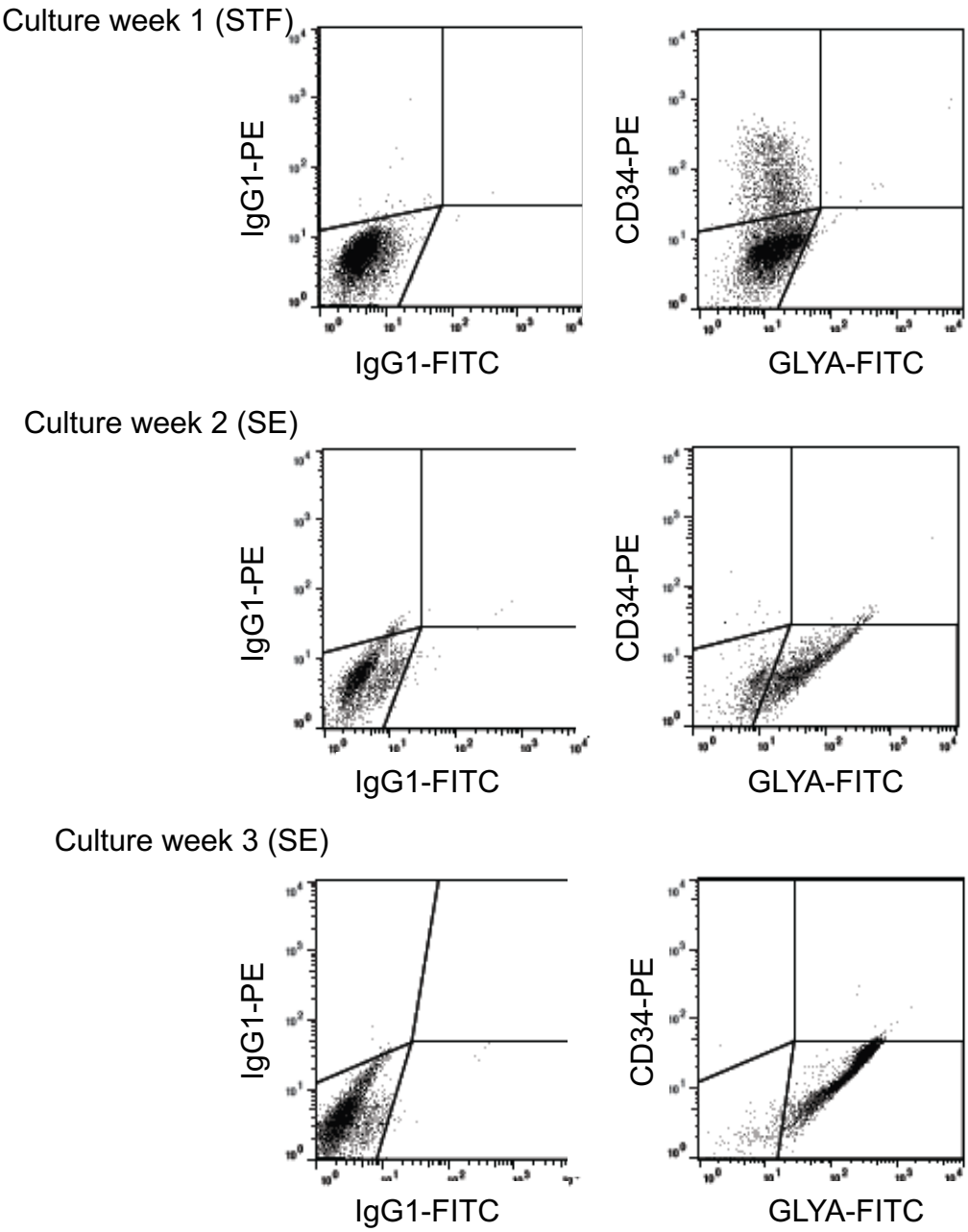
Cell type	Cytokines[1]
Erythroid	SE; 20ng/mL SCF, 6U/mL EPO
Monocytic	SFM; 20ng/mL SCF, 50ng/mL FLT-3, 50ng/mL M-CSF
Granulocytic	SFG; 20ng/mL SCF, 50ng/mL FLT-3, 100ng/mL G-CSF
Megakaryocytic	ST6; 20ng/mL SCF, 50ng/mL TPO, 50ng/mL IL-6

Table S2 Commercial antibodies

Antibody	Manufacturer/Source	Catalogue #
Western blot and ChIP		
AcH3	Millipore	06-599
Actin	Sigma	A2066
H3K27me3	Millipore	07-449
H3K4me3	Millipore	04-745
Lamin B1	Abcam	ab16048
MYC	Santa Cruz	sc764
Normal Rabbit IgG	Sigma	12370
TAL-1	Santa Cruz	sc12984
Flow cytometry		
CD114-PE	Becton Dickinson	554538
CD14-PE	Becton Dickinson	347497
CD235a-PE	Becton Dickinson	555570
CD34-PE	Becton Dickinson	348057
CD61-PE	Becton Dickinson	555754
GlyA-FITC	Dako Cytomation	N/A

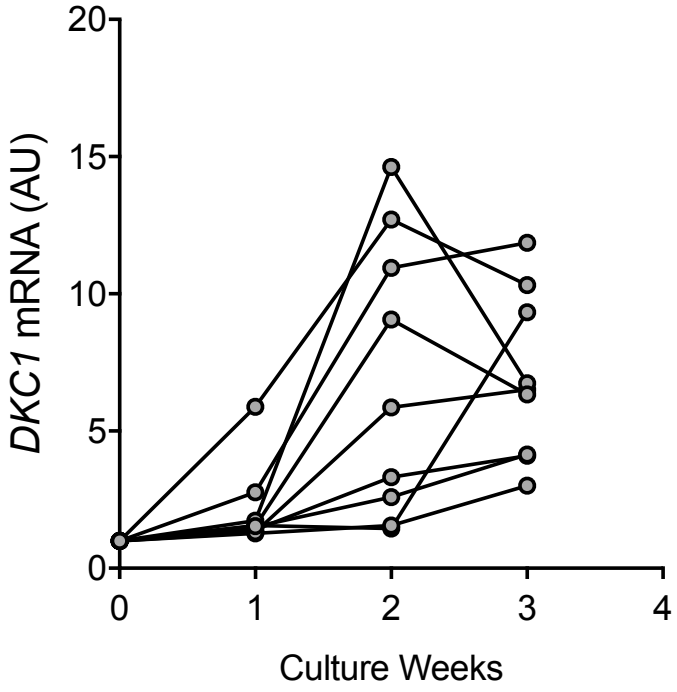
Table S3 Primer sequences

Primers	Sequence 5'-3'
qRT-PCR	
DKC1 forward	CATGGCGGATGCGGAAGTAAT
DKC1 reverse	GTCAAGATTAATGAAACCTG
TERC forward	CGCTGTTTTTCTCGCTGACTT
TERC reverse	TGCTCTAGAATGAACGGTGGAA
TERT forward	TGACACCTCACCTCACCCAC
TERT reverse	CACTGTCTTCCGCAAGTTCAC
ABL forward	TGGAGTAACACTCTAAGCATAACTAAAGGT
ABL reverse	CCATTTTTGGTTTGGGCTTCACACCATT
qTRAP	
ACX primer	GCGCGG[CTTACC]3CTAACC
TS primer	AATCCGTCGAGCAGAGTT
ChIP	
GATA forward	ATCCCTGGGGAGAAATCAGT
GATA reverse	CAAACAGCAGAGACAAAGCACTG
JP151 (-ve control region) forward	GAAATAAATATCTCCACTGTCCTG
JP151 (-ve control region) reverse	CTATCTGCCTATCTCTCATCTATC
Site-directed mutagenesis	
JP543 forward (site 440-450)	CAGCCCTGCTGCCCCCAGGCTCATCC
JP543 reverse (site 440-450)	GGATGAGCCTGGGGGCAGCAGGGCTG
JP544 forward (site 650-660)	TCATACTGTTGTGTTTTGCCTATATGTGGAAATC GCTGGGTTAAAAAAAT
JP544 reverse (site 650-660)	ATTTTTTTAACCCAGCGATTTCCACATATAGGCA AAACACAACAGTATGA



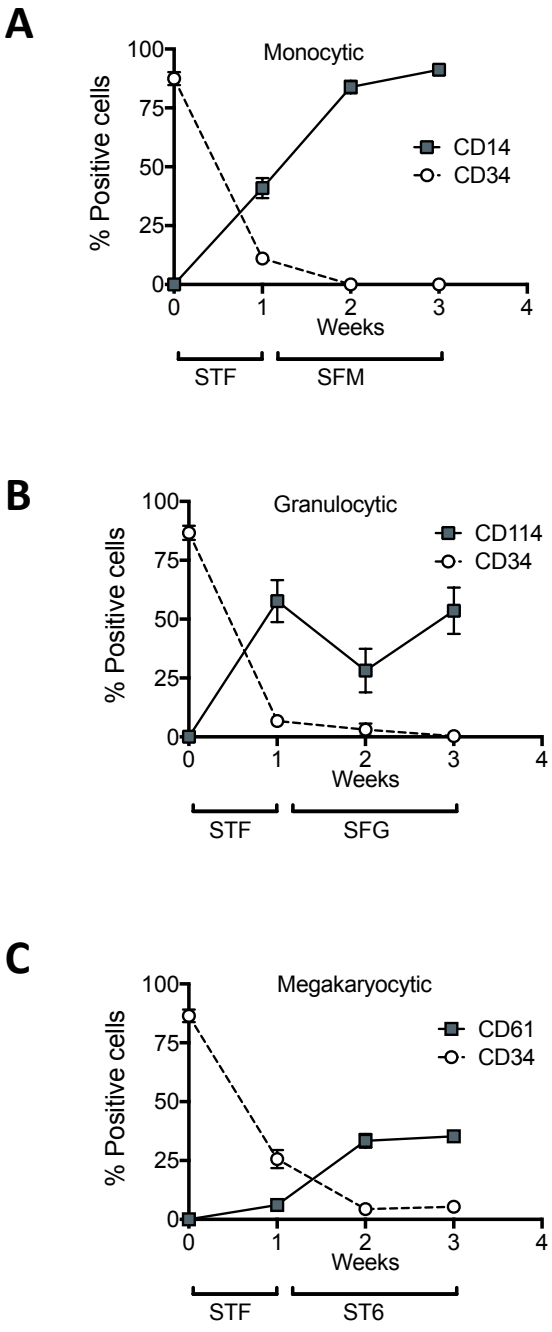
Supplementary Figure S1: Generation of GLYA+ erythroblasts by ex vivo expansion of cord-blood derived hematopoietic progenitor cells (HPCs)

Cord blood-derived CD34+ hematopoietic progenitor cells (HPCs) were expanded for seven days in media supplemented with stem cell factor, thrombopoietin and Flt-3 ligand (STF), then cultured for an additional two weeks in media with stem cell factor plus erythropoietin (SE). FACS analysis was performed at weekly intervals to monitor CD34+ progenitor cells and erythroid differentiation defined by expression of Glycophorin A (GLYA). FACS plots are shown from a representative experiment. Quantitative FACS data from expansion of seven cord blood samples are summarised in Figure 1.



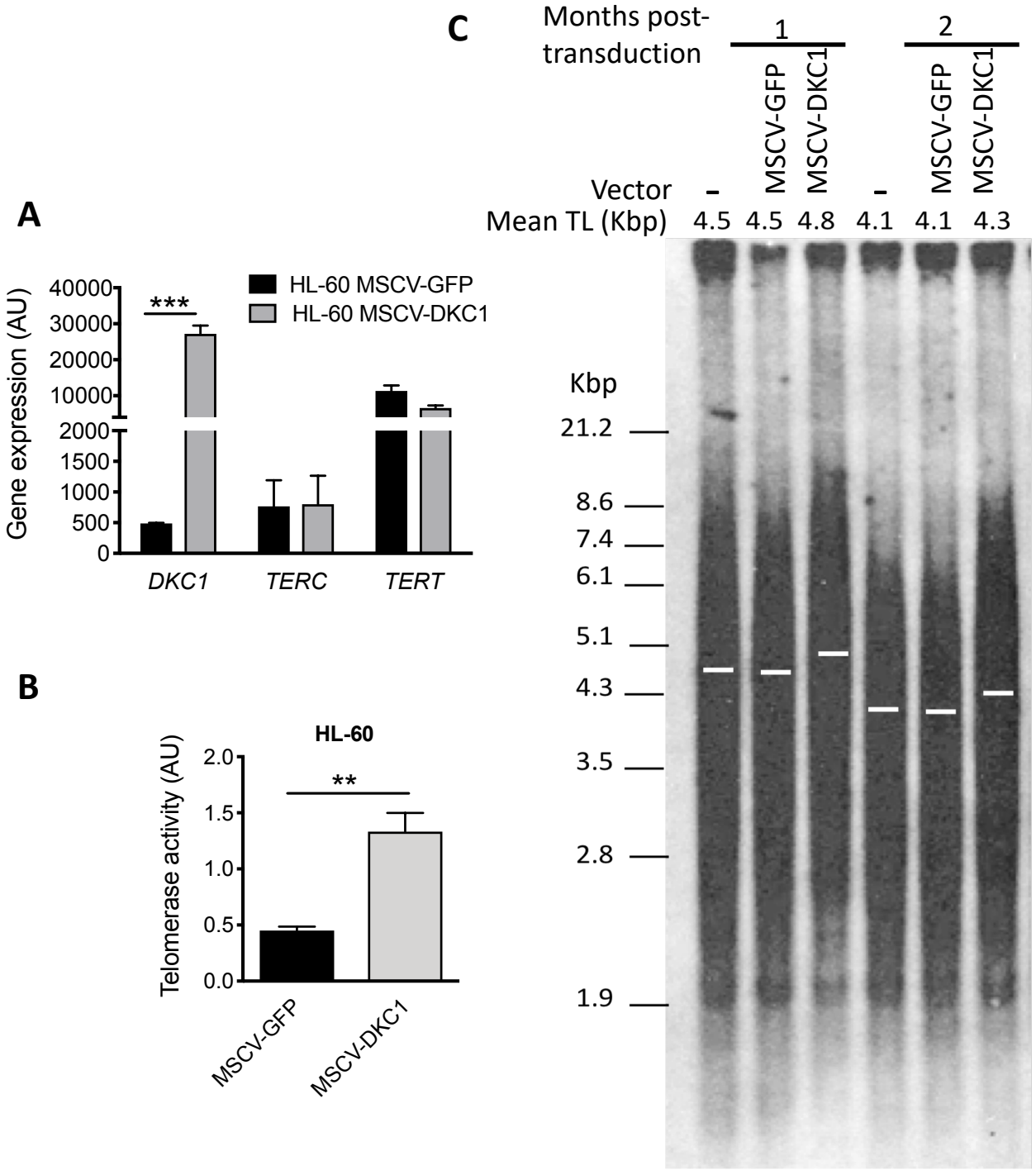
Supplementary Figure S2: Induction of *DKC1* expression in independent cord blood cultures

CB HPSCs were expanded in STF for 1 week then transferred to KE to promote erythroid differentiation and expansion. *DKC1* expression was analyzed at weekly time points by qRT-PCR. The results show values calculated from triplicate assays for each of nine independent cord blood cultures.

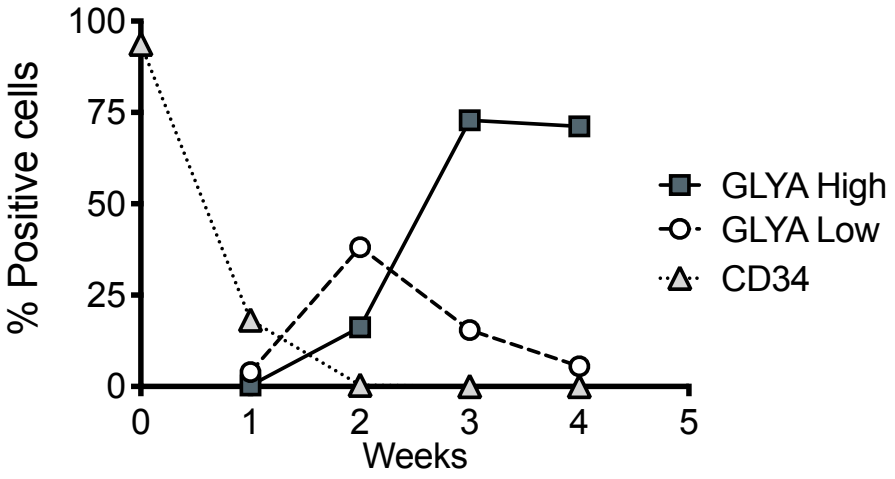


Supplementary Figure S3: Differentiation of cord-blood derived HPCs along myeloid lineages

Cord blood-derived CD34+ HPCs were expanded for one week in STF, then switched to SCF, Flt3L and M-CSF (SFM) (for monocytic differentiation) **(A)** SCF, Flt3L and G-CSF (SFG) for granulocytic differentiation **(B)** and SCF, TPO and IL-6 (ST6) to promote megakaryocyte differentiation **(C)**. FACS analysis was performed using fluorescence labelled antibodies to CD34 and lineage markers at weekly intervals to quantify differentiating cells. Values are means +/- SEM from 5-9 independent experiments

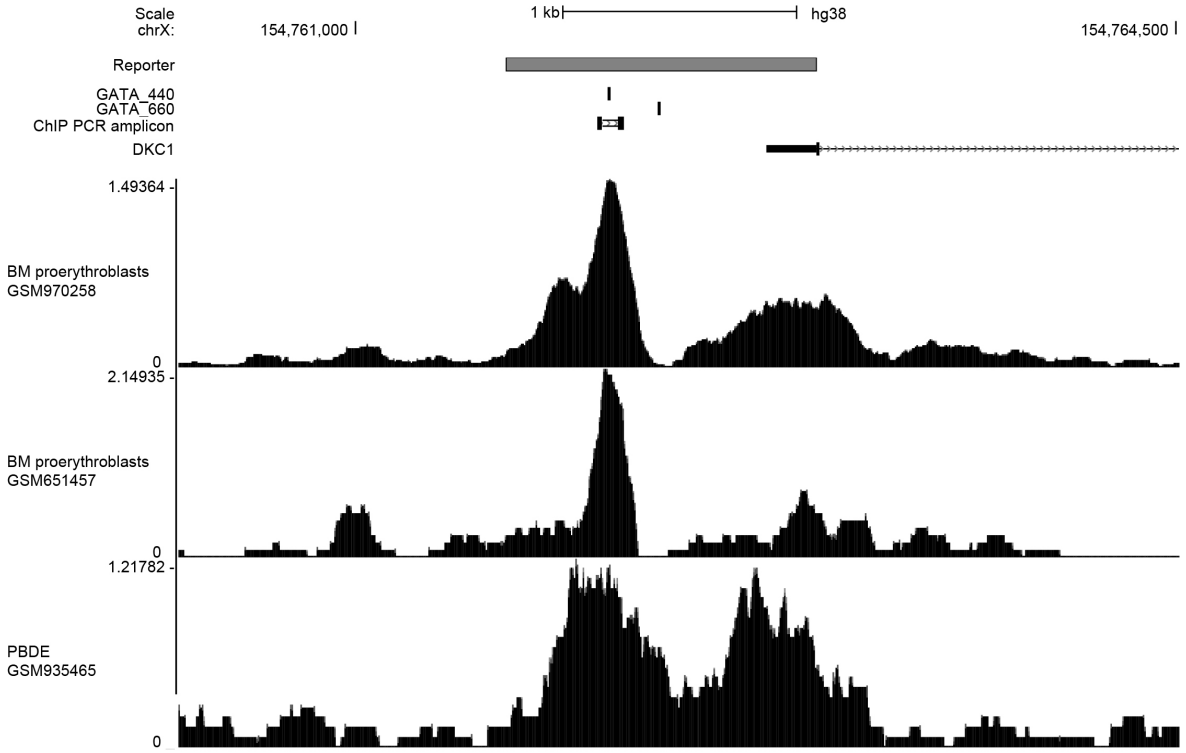


Supplementary Figure S4: HL-60 cells were transduced with a lentiviral vector expressing the *DKC1* gene (MSCV-DKC1) or a control vector (MSCV-GFP). Transduced cells were assayed for expression of telomerase genes by qRT-PCR **(A)** and telomerase enzyme activity by qTRAP **(B)**. Values are means +/- SEM from three assays. ** $P < 0.01$, *** $P < 0.001$. **(C)** Telomere length was measured in transduced HL-60 cells by Southern blot analysis of telomeric restriction fragments. Mean telomere length (TL) is indicated by the white bars and at the top of each lane.



Supplementary Figure S5: Accumulation of cells expressing high levels of Glycophorin A (GLYA)

CB HPSCs were expanded in STF for 1 week then transferred to KE for a further 3 weeks for erythroid cell differentiation and expansion. The cultures were sampled at weekly time points for FACS analysis of Glycophorin A (GLYA) and CD34 expression.



Supplementary Figure S6: GATA1 ChIPseq at the DKC1 locus

Upper: UCSC browser schematic of the *DKC1* promoter (chrX:154,760,244-154,764,516 [hg38]) showing the reporter region and GATA motifs mutated in Figure 5D (GATA-440 and GATA 660) along with the region tested by ChIP PCR in Figure 5C. *Lower*: GATA1 ChIPseq data from three previously published datasets showing GATA1 enrichment at the DKC1 promoter during erythrocyte development. Data from bone marrow (BM) proerythroblasts (GSM970258, GSM651457) and peripheral blood-derived erythroblasts (PBDE; GSM935465) was visualised using CistromeDB.