SUPPLEMENTARY APPENDIX

Targeted shRNA screening identified critical roles of pleckstrin-2 in erythropoiesis

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Supplemental Methods

Purification and culture of fetal liver cells for targeted screening

Fetal liver cells were isolated from E13.5 C57BL/6 embryos and mechanically dissociated by pipetting in PBS containing 10% fetal bovine serum (GIBCO). Single-cell suspensions were prepared by passing the dissociated cells through 40 μm cell strainers (BD Biosciences). Total fetal liver cells were labeled with biotin-conjugated anti-TER119 antibody (1:100) (eBioscience), and TER119 negative cells were purified using EasySep column free cell isolation system according to the manufacturer's instructions (Stem Cell Technologies). Purified cells were seeded in round bottom 96 well plates at a cell density of $1x10^3$ cells/200μl media/well. The purified cells were infected by distinct lentiviruses encoding shRNAs against selected genes in each well of the 96-well plate. The infection strategies were schematically illustrated in Figure 1A. For the discovery of genes important for the early stage of terminal erythropoiesis, the cells after 1-hour spin infection (2000 rpm at 37°C) were cultured in Epo free medium containing 50 ng/ml mouse stem cell factor (SCF), 20ng/ml mouse interleukin-6 and 30ng/ml mouse FLT3 ligand (PeproTech). The cells were cultured for 12 hours followed by medium change to Epo (0.5 unit/ml, Amgen) containing medium and puromycin (1 μg/ml) to select the transduced cells. The cells were continued to culture for 48 hours before flow cytometric analysis. For the discovery of genes important for the late stage of terminal erythropoiesis, the cells after 1-hour spin infection were cultured directly in Epo (0.5 unit/ml) medium for 12 hours followed by puromycin treatment. The cells were continued to culture in Epo medium for 48 hours before enucleation flow cytometric analysis.

Retroviral transduction

For infection of the purified TER119-negative fetal liver cells with retroviruses, $2x10^5$ cells were resuspended in 1 ml viral supernatant containing 10 μg/ml polybrene (Sigma) and centrifuged at 800 x g for 1 hour 37°C. The cells were then cultured based on Figure 1A for the analysis of the early and late stages of terminal erythropoiesis.

Mass spectrometry analysis

The samples were loaded directly onto a 10 cm long, 75 μM reversed phase capillary column (ProteoPep(tm) II C18, 300 Å, 5 μm size, New Objective, Woburn MA) and separated with a 70-minute gradient from 5% acetonitrile to 100% acetonitrile on a Proxeon Easy n-LC II (Thermo Scientific, San Jose, CA). The peptides were directly eluted into an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, San Jose, CA) with electrospray ionization at 350 nl/minute flow rate. The mass spectrometer was operated in data dependent mode, and for each MS1 precursor ion scan the ten most intense ions were selected from fragmentation by CID (collision induced dissociation). The other parameters for mass spectrometry analysis were: resolution of MS1 was set at 60,000, normalized collision energy 35%, activation time 10 ms, isolation width 1.5, and the +1 and +4 and higher charge states were rejected. The data were processed using Proteome Discoverer (version 1.3, Thermo Scientific, San Jose, CA) and searched using an in-house MASCOT server. The data were searched against both reviewed and unreviewed sections of Uniprot; Swiss-Prot (version 2011_12) and TrEMBL (version 2011_12). The other parameters were as follows: (i) enzyme specificity: trypsin; (ii) fixed modification: cysteine carbamidomethylation; (iv) variable modification: methionine

oxidation and N-terminal acetylation; (v) precursor mass tolerance was ±10 ppm; and (vi) fragment ion mass tolerance was ±0.8 Da. All the spectra were searched against target/decoy databases and the mascot significance threshold was chosen to achieve a targeted false discovery rate of 1%. The peptide identification was considered valid if its corresponding mascot score was equal to or less then the threshold and any protein identification was deemed valid only if supported by at least one unique peptide.

Immunofluorescence stain

The harvested cells were washed with ice-cold serum free media (IMDM) and plated over a poly-L-lysine coated cover-slips (BD Biosciences) ($1x10^5$ cells/80 µl medium/slide). The cells were then incubated at 37°C in a humidified incubator for 5 minutes. Cells attached to the coverslips were washed by ice cold PBS (pH 7.2), fixed by 4% paraformaldehyde for 15 minutes at room temperature and permeabilized by 0.1% Triton-X 100 in PBS for 10 minutes at room temperature. After three times wash in PBS, the cells were blocked by 3% bovine serum albumin in PBS with 0.05% Triton-X 100 for an hour at room temperature. The cells were then stained with respective primary and secondary antibodies for an hour, each followed by three times wash cycles (PBS for 5 minutes). After final wash, the cells were treated with 1 µg/ml DAPI to stain the nucleus followed by PBS wash. The cells were then mounted over a glass slide in Slowfade Antifade reagent (Invitrogen). For the staining of mitochondria, the cells were co-stained with 50 nM MitoTracker Deep Red (Invitrogen) for 15 minutes at room temperature. For the microscopic analysis, Nikon A1R laser scanning confocal microscope was used to acquire confocal images of the stained erythroblasts.

(Objective lens used: Plan Apo TIRF 100X). Nikon N-SIM super-resolution structural illumination microscope was used to acquire super-resolution images of erythroblasts through 100X Apo objective with NA 1.49. The captured images were analyzed using NIS Elements software.

Hemoglobin quantification

Hemoglobin was quantified by lysing $1x10^6$ cells in 200 ml Drabkin's reagent according to the manufacture's instruction (Sigma Aldrich). Spectrophotometric reading was then performed at 540 nm to quantify the hemoglobin concentration.

Protein immunoprecipitation

Endogenous plek2 was immunoprecipitated from $1x10^7$ mouse E13.5 TER119 positive fetal liver cell lysate (~1mg/500 μl) (Lysis buffer: 250 mM NaCl, 50mM HEPES pH 7.0, 5 mM EDTA, 1% NP-40 and protease inhibitors). The purified proteins were separated by SDS-PAGE denaturing gel electrophoresis and silver stained according to the manufacture's instruction (Pierce). The protein bands of interest were excised from silver stained gels and destained using 30mM potassium ferricyanide and 100 mM sodium thisosulfate. After destaining, gel bands were washed followed by in gel digestion with sequencing grade trypsin (Promega Inc., Madison WI).

Supplemental Tables

Table S1. Selected genes for the targeted shRNA screening.

Query Refseq	Gene symbol	NCBI geneId
NM_025912	*2010011I20Rik*	67017
NM_028320	*Adipor1*	72674
NM_010347	*Aes*	14797
NM_177275	*Amigo3*	320844
NM_027144	*Arhgef12*	69632
NM_024184	*Asf1b*	66929
NM_001003917	*Atg9a*	245860
NM_009733	*Axin1*	12005
NM_080708	*Bmp2k*	140780
NM_009761	*Bnip3l**	12177
NM_007570	*Btg2*	12227
NM_138678	*Butr1*	192194
NM_026192	*Calcoco1*	67488
NM_007652	*Cd59a*	12509
NM_007656	*Cd82*	12521
NM_023117	*Cdc25b*	12531
NM_009895	*Cish*	12700
NM_007713	*Clk3*	102414
NM_012000	*Cln8*	26889
NM_026252	*Cpeb4*	67579
NM_009975	*Csnk2b*	13001

NM_207677	*Dedd2*	67379
NM_019670	*Diap3**	56419
NM_021422	*Dnaja4*	58233
NM_053246	*Dok4*	114255
NM_199322	*Dot1l*	208266
NM_008748	*Dusp8*	18218
NM_145508	*Dyrk3*	226419
NM_138953	*Ell2*	192657
NM_010149	*EpoR**	13857
NM_007971	*Ezh2*	14056
NM_013890	*Fbxw2*	30050
NM_001033301	*Fhdc1*	229474
NM_019740	*Foxo3*	56484
NM_172475	*Frmd4a*	209630
NM_022721	*fzd5*	14367
NM_026693	*Gabarapl2*	93739
NM_007836	*Gadd45a*	13197
NM_008089	*Gata1**	14460
NM_010277	*Gfap*	14580
NM_030258	*Gpr146*	80290
NM_023168	*Grina*	66168
NM_008207	*H2-T24*	100045444
NM_144919	*Hdac11*	232232
NM_008229	*Hdac2**	15182
NM_138753	*Hexim1*	192231
NM_133662	*Ier3*	15937

NM_010587	*Itsn1*	16443
NM_153128	*Klhl12*	240756
NM_010697	*Ldb1**	16825
NM_201519	*Map4k5*	399510
NM_010773	*Mbd2*	17191
NM_020007	*mbnl1*	56758
NM_134163	*mbnl3*	171170
NM_172424	*Med13l*	76199
NM_025569	*Mgst3*	66447
NM_010751	*Mxd1*	17119
NM_016662	*Mxd3*	17121
NM_175441	*Mylk3*	633594
NM_001033988	*Ncoa4*	27057
NM_172495	*Ncoa7*	211329
NM_008692	*Nfyc*	18046
NM_008774	*Pabpc1*	18458
NM_026018	*Pdzk1ip1*	67182
NM_008842	*Pim1*	18712
NM_008846	*Pip5k1b*	18719
NM_139269	*Pla2g16*	225845
NM_011116	*Pld3*	18807
NM_013738	*Plek2*	27260
NM_022318	*Popdc2*	64082
NM_008911	*Ppox*	19044
NM_008975	*Ptp4a3*	19245
NM_133717	*Rab43*	69834

NM_023130	*Raly*	19383
NM_019547	*Rbm38*	56190
NM_009069	*Rit1*	19769
NM_011882	*Rnasel*	24014
NM_009822	*Runx1t1*	12395
NM_021372	*Sertad2*	58172
NM_133210	*Sertad3*	170742
NM_030241	*Setd8*	67956
NM_022432	*Sirt2*	64383
NM_153056	*Sirt7*	209011
NM_009193	*Slbp*	20492
NM_026542	*Slc25a39*	68066
NM_008135	*Slc6a9*	14664
NM_026912	*Snx15*	69024
NM_178362	*Sorbs1*	20411
NM_178362	*Sorbs1*	20411
NM_172656	*Stradb*	227154
NM_199196	*Suz12*	52615
NM_181546	*Syt14*	329324
NM_173038	*Tbcel*	272589
NM_178874	*Tmcc2*	68875
NM_023440	*Tmem86b*	68255
NM_021883	*Tmod1*	21916
NM_178931	*Tnfrsf14*	230979
NP_081568.1	*Tpso2*	70026
NM_172406	*Trak2*	70827

XM_908452	*Trim33*	2137357
NM_146010	*Tspan8*	216350
NM_177545	*Vangl1*	229658
NM_175231	*Whsc1*	107823
NM_023045	*Xpo7*	65246
NM_026417	*Yipf4*	67864

* Positive control genes.

Table S2. List of hit genes that affect enucleation when down regulated in the late stage of terminal erythropoiesis.

Enucleation index (EI) indicates the percentage of enucleated cells (TER119 high, Hoechst low) analyzed by flow cytometry. The shRNA sequences were listed.

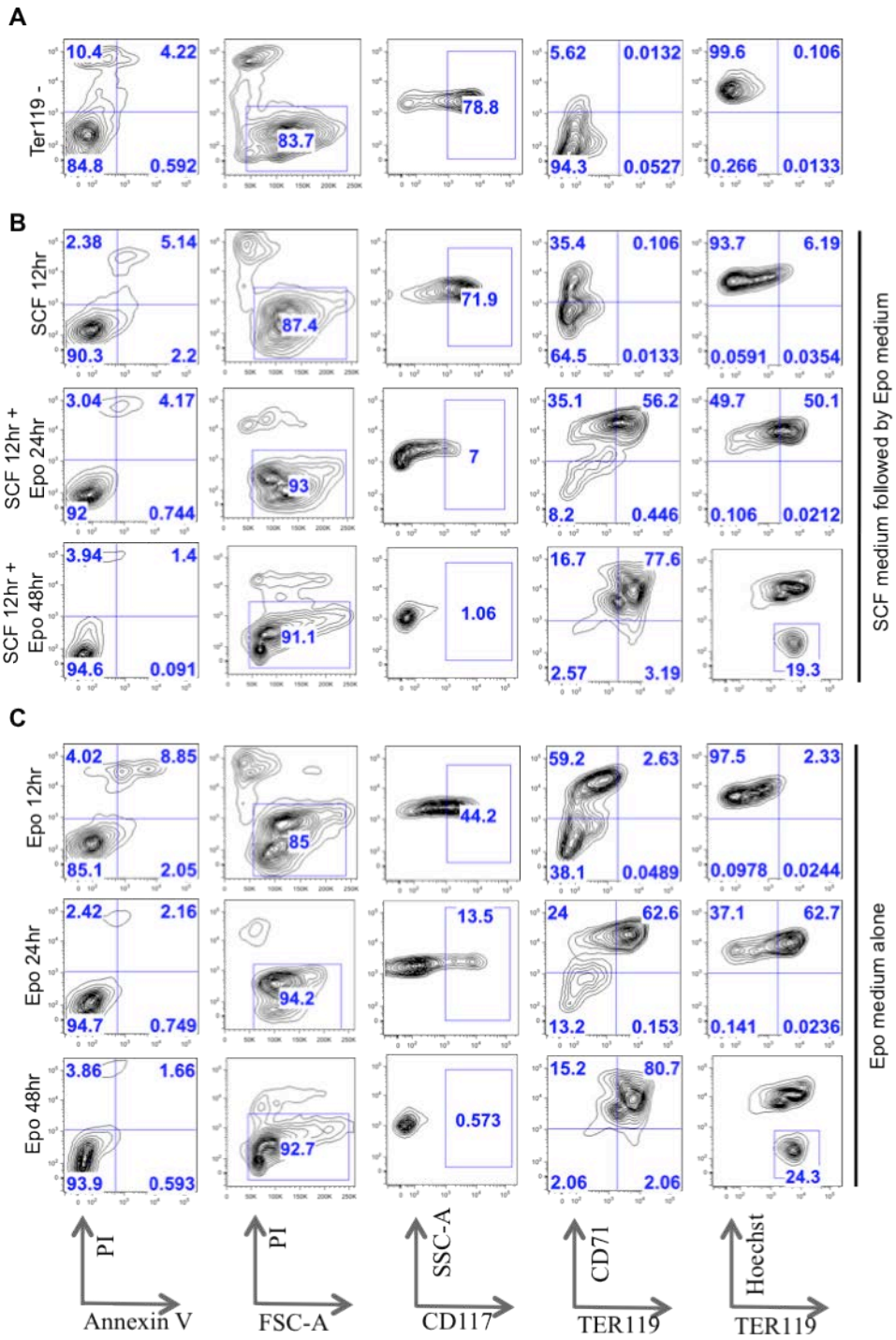
Gene Symbol	ID	Target Sequence	EI (%)	Description
Ezh2	14056	ACTTGCCCACCTCGGAAATTT	7.1	Enhancer of zeste homolog 2
		AGTCGCCTCGGTGCCTATAAT	5.6	
		TTGAGTACTGTGGGCAATTTA	11.9	
		CGGCTCCTCTAACCATGTTTA	9.2	
HDAC11	232232	TGGTCCGAGCCCATGATATAC	13.4	Histone deacetylase 11
		TTGGCTTACTTCCTCACTTTA	10.3	
		GCCACCATCATTGATCTCGAT	18.1	
		CATGGGTGACAAGCGAGTATA	14.9	
		AGAGTCGTTTGCTGTTCATAT	1.6	
Dyrk3	226419	GCGGGTAGTTAACCCTACAAA	8.1	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 3
		CAAGCGCTGAAGCAGTATAAA	7.8	
		AGACCTGTATGAGCTTATTAA	8.9	
PLEK2	27260	AGGATGACACACACTATTATA	8.2	Pleckstrin 2
		CCAGCTTTCCTGCACTACTAT	14.8	
		GCATCGAATTGTGGACAAGAT	18.4	
SERTAD2	58172	TCCCTTATGAAACTGTATAAC	17.8	SERTA domain-containing protein 2
		TTGCAGAAGACTGTCTTAATT	18.8	
		ACCTTACAGCGCCAGACTATC	9.1	
Pdzk1ip1	67182	TCTACAGGAATCTACTGAAAC	16.4	PDZK1 interacting protein 1
		GATGGCAGATACTCCTCAATG	12.1	
		GGAGCACAGTGATGATCATTG	10.1	

Mxd1	17119	CTGTGCCTAGAGAAGCTAAAG	5.5	MAX dimerization protein
		AGTAGCAGATCAACTCACAAT	7.5	1
		GCACCAGTAGCAGATCAACTC	13.3	
Whsc1	107823	AGAGCTGACTTTCAACTATAA	7.8	Wolf-Hirschhorn
		CATAGACAAGGACCGCATAAT	15.8	syndrome candidate 1
		CTGTGAGAGAAGAGGATATTC	12.7	
		CTGATGGTGCAGGCTTCTATT	15.2	
Rbm38	56190	GCCATGTCCTGCTGGGAATTT	13.7	RNA binding motif
		TGCCAAGCCTAGGAGCTTACA	13.7	protein 38
		GCTGTTGGTGTGCAGCAACTA	8.9	
		CCTCGCCCTACCTTGAGTATA	15.8	
Itsn1	16443	TAGACTACTTGGCCTATAATA	9.6	Intersectin 1 (SH3 domain
		GATACTCAGTGACCAGTTAAA	6.3	protein)
		TGAAGCCGATAGCGGGATTTA	10.9	
CD59a	12509	CGGAATGCAAGTGTATCAAAG	13.8	CD59a antigen
		CGGTGGTTTCTTCATGCAATA	6.2	
		GGTGGTTTCTTCATGCAATAT	5.1	
*Setd8**	67956	AGGCATGAAGATTGATCTAAT	15.4	SET domain containing
		AGGAACACCGGGAACGTTATA	13.8	(lysine methyltransferase)
		TGGCTGCTACATGTACTATTT	8.2	8

* Setd8 functions solely in late erythropoiesis/enucleation

Hit genes that lead to increased enucleation index after knockdown

Fhdc1	229474	CCGGCAATGCCGTAGGATTTA	54.8	FH2 domain containing
		AGTATGAACATTGGCATATTT	53.1	protein 1
EpoR	13857	GCATCTCAACAGATTACAGTT	56.5	Erythropoietin receptor
		CGCATCATCCATATCAATGAA	47.2	


A
Ter119 -
10.4
4.22
84.8
0.592
83.7
78.8
5.62
0.0132
94.3
0.0527
99.6
0.106
0.266
0.0133
B
SCF 12hr
2.38
5.14
90.3
2.2
87.4
71.9
35.4
0.106
64.5
0.0133
93.7
6.19
0.0591
0.0354
SCF 12hr + Epo 24hr
3.04
4.17
92
0.744
93
7
35.1
56.2
8.2
0.446
49.7
50.1
0.106
0.0212
SCF 12hr + Epo 48hr
3.94
1.4
94.6
0.091
91.1
1.06
16.7
77.6
2.57
3.19
19.3
SCF medium followed by Epo medium
C
Epo 12hr
4.02
8.85
85.1
2.05
85
44.2
59.2
2.63
38.1
0.0489
97.5
2.33
0.0978
0.0244
Epo 24hr
2.42
2.16
94.7
0.749
94.2
13.5
24
62.6
13.2
0.153
37.1
62.7
0.141
0.0236
Epo 48hr
3.86
1.66
93.9
0.593
92.7
0.573
15.2
80.7
2.06
2.06
24.3
Epo medium alone
PI
Annexin V
PI
FSC-A
SSC-A
CD117
CD71
TER119
Hoechst
TER119

Supplemental Figures

Figure S1. Characterization of the culture system that dissects the early and late stages of terminal erythropoiesis.

(A) TER119 negative mouse fetal liver erythroblasts were purified from E13.5 fetus. Flow cytometric analysis was performed on these cells for apoptosis using Annexin V and propidium iodine (PI), progenitor status using CD117, differentiation using TER119 and CD71, and enucleation using Hoechst 33342 and TER119. (B) Cells from A were cultured in SCF medium for 12 hours followed by Epo medium culture for additional 48 hours. Flow cytometric analysis was performed as in A. For the purpose of demonstrating the culture system, puromycin was not added at 12 hour in culture in B and C. (C) Cells from A were cultured directly in Epo medium for 60 hours. Flow cytometric analysis was performed as in A.

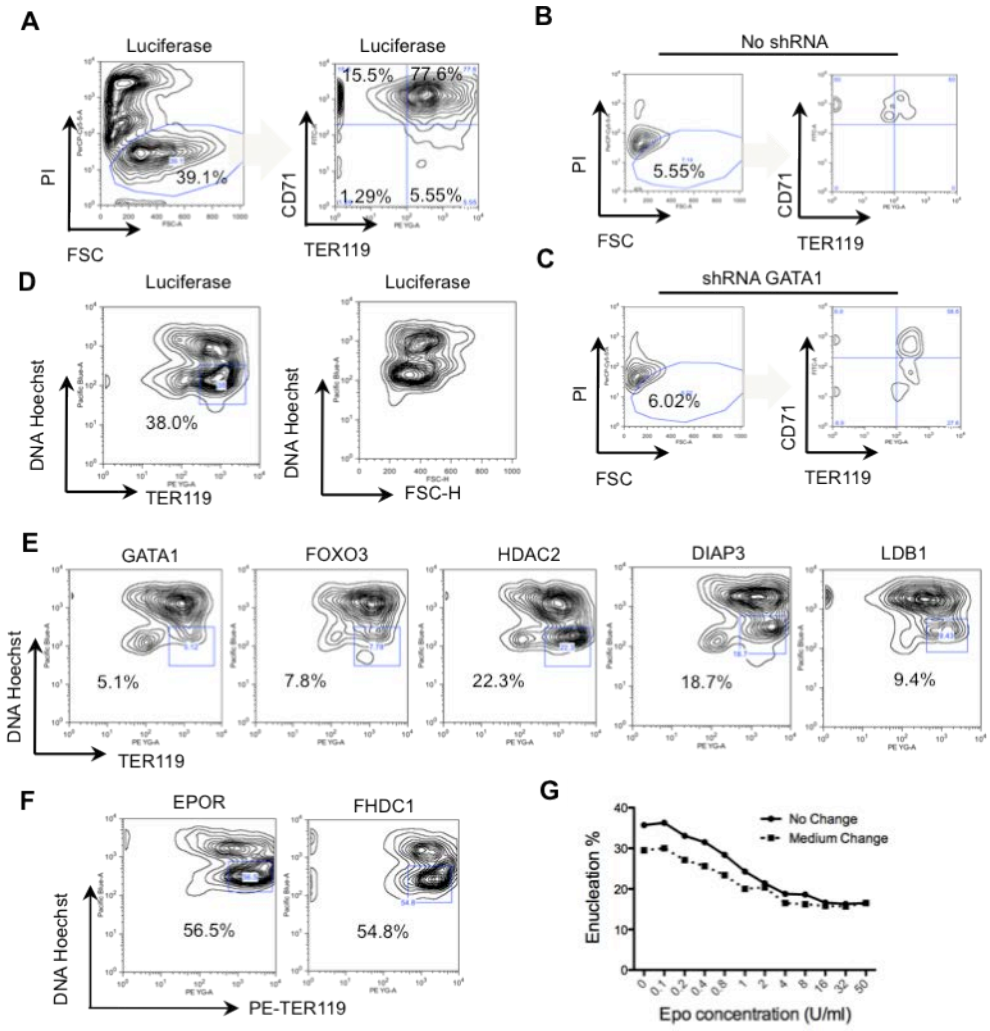


Figure S2. Validation of the targeted shRNA screening system.

(A) The TER119 negative mouse fetal liver erythroblasts were purified. $1x10^3$ cells in each well of a 96-well plate were infected with 10 μl lentiviruses encoding shRNA against luciferase. The cells were cultured in SCF medium for 12 hours followed by puromycin treatment to select the transduced cells. The cells were continued to culture for 48 hours in

Epo medium followed by flow cytometric analysis of apoptosis and differentiation using PI and forward scatter (FSC), and CD71 and TER119, respectively. (B-C) Same as A except a mock shRNA infection in B, and a shRNA against GATA1 in C, were used. (D) The TER119 negative mouse fetal liver erythroblasts were purified and infected with lentiviral shRNA against luciferase. The cells were directly cultured in Epo medium for 12 hours followed by puromycin selection. The cells were then continued to culture for 48 hours in Epo medium followed by flow cytometric analysis of enucleation using Hoechst and TER119 (left panel). Forward scatter (FSC) was used to compare the size of the enucleated reticulocytes with immature erythroblasts (right panel). (E) Same as D except shRNAs targeting genes that play well-known functions in terminal erythropoiesis were used. The percentages of enucleation are presented. (F) Flow cytometric analysis of hit genes that promoted enucleation when knocked down by shRNA. (G) The percentages of enucleation in different concentration of Epo. TER119 negative erythroblasts were purified from E13.5 mouse fetal liver. The cells were cultured in different concentration of Epo as indicated for two days. Enucleation rate was determined by flow cytometry using Hoechst and TER119. The dashed and solid lines indicate culture with or without medium change on day one of culture, respectively.

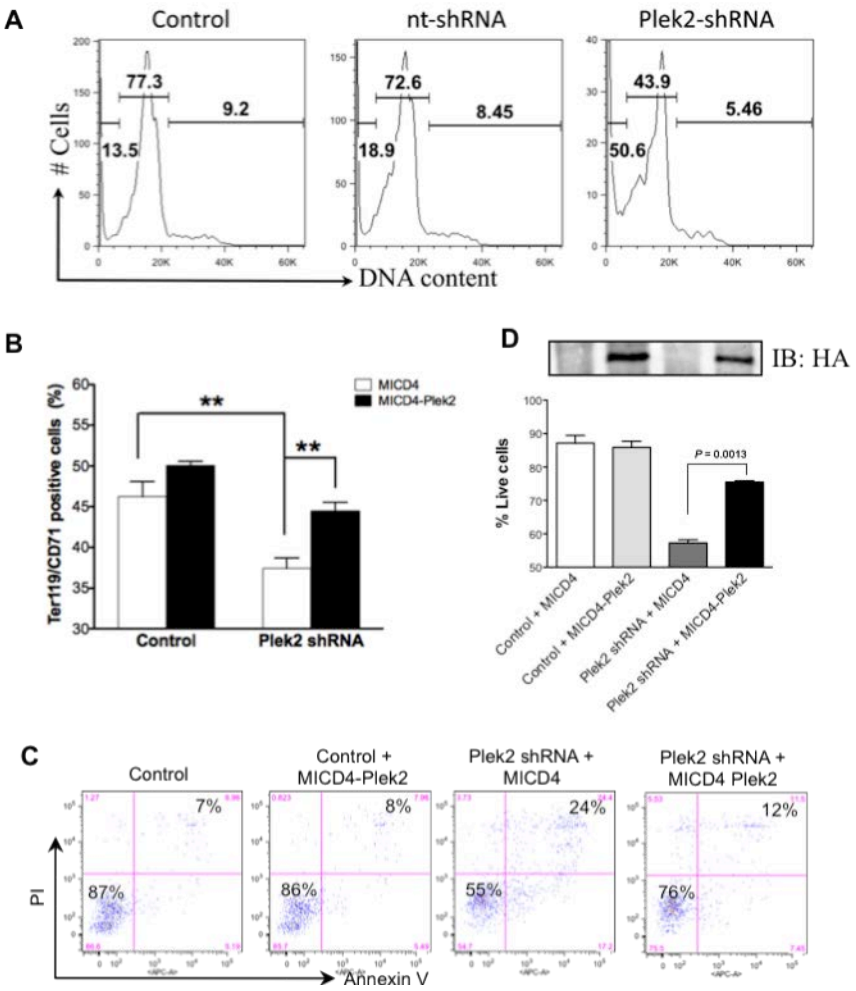


Figure S3. Knockdown of plek2 in the early stage of terminal erythropoiesis blocks cell differentiation and induces cell death.

(A) The cells were the same as Figure 2C. The cells were harvested and cell cycle was analyzed using flow cytometry of cells stained with propidium iodide. (B-D) Over-expression of wild type plek2 rescued early stage erythroblasts differentiation inhibition and apoptosis induced by plek2 knockdown. The TER119 negative mouse fetal liver erythroblasts were purified and infected with indicated retroviruses. MICD4 and MICD4 Plek2 indicate MSCV-

IRES-CD4 vector and MSCV-IRES-HA-Plek2, respectively. For the rescue experiments, the cells were infected simultaneously with two retroviruses as indicated. The cells were cultured for 12 hours in SCF medium followed by 24-hour culture in Epo medium. The cells were then harvested followed by flow cytometric analysis of apoptosis (C-D) and differentiation (B) using PI and Annexin V, and CD71 and TER119, respectively. ** $P < 0.01$.