SUPPLEMENTARY APPENDIX

Hemoglobin switching in mice carrying the *Klf1*^{Nan} variant

Anne Korporaal,[1] Nynke Gillemans,[1] Steven Heshusius,[1,2] Ileana Cantú,[1] Emile van den Akker,[2] Thamar B. van Dijk,[1] Marieke von Lindern[2] and Sjaak Philipsen[1]

[1]Erasmus MC Department of Cell Biology, Rotterdam and [2]Sanquin Research Department of Hematopoiesis, Amsterdam and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

 doi:10.3324/haematol.2019.239830

Received: October 4, 2019.

Accepted: January 23, 2020.

Pre-published: May 28, 2020.

Correspondence: *SJAAK PHILIPSEN* - j.philipsen@erasmusmc.nl

Hemoglobin switching in mice carrying the *Klf1*^{Nan} variant

Anne Korporaal[1], Nynke Gillemans[1], Steven Heshusius[1,2], Ileana Cantú[1], Emile van den Akker[2], Thamar B. van Dijk[1], Marieke von Lindern[2] and Sjaak Philipsen[1]

[1] Erasmus MC Department of Cell Biology, Rotterdam, The Netherlands

[2] Sanquin Research Department of Hematopoiesis, Amsterdam, The Netherlands, and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

Supplementary Table 1

Supplementary Figures 1 - 3

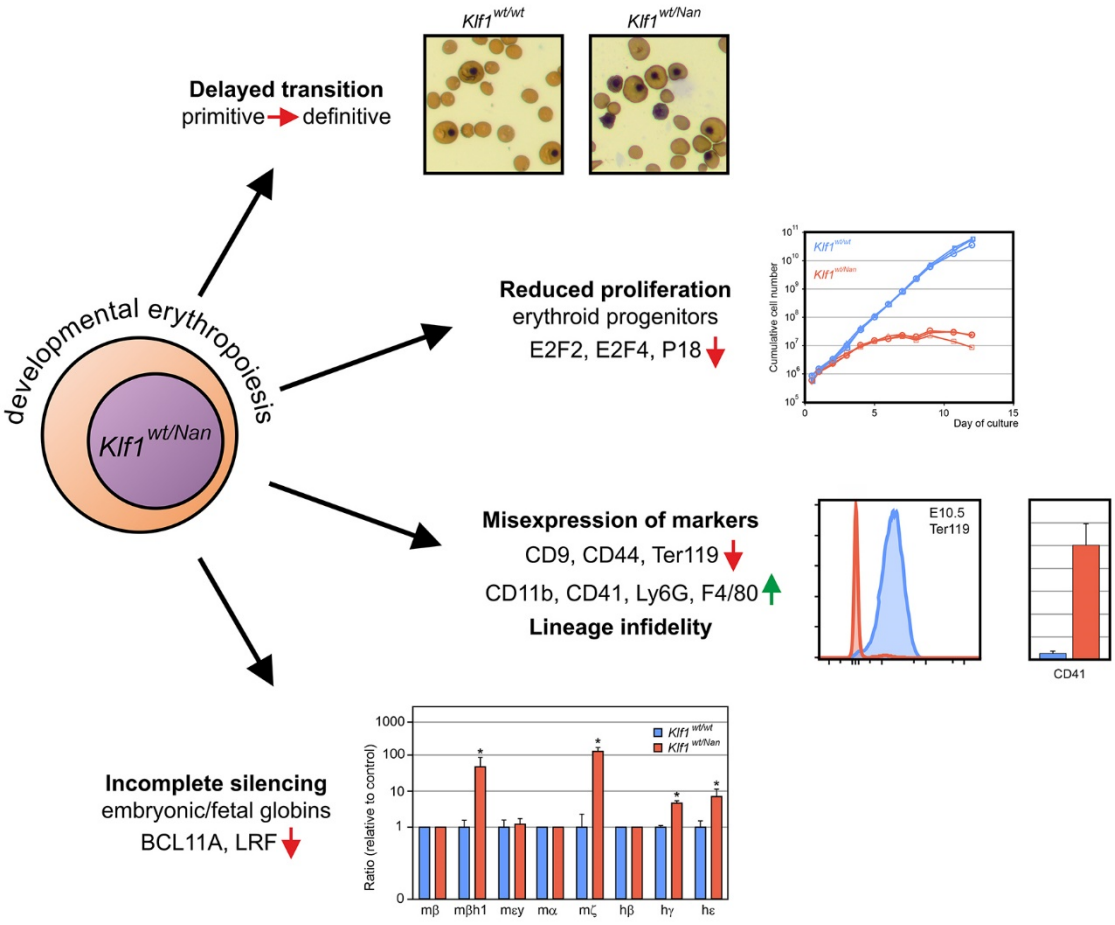
Supplementary Methods

Supplementary Table 1

	Klf1 wt/wt (n=4)	stdev	*Klf1* wt/Nan (n=4)	stdev	fold-change
mεy	0.0001	0.00003	0.0032	0.00232	54.06
mβh1	0.0000	0.00002	0.0006	0.00048	13.39
mβ	0.9999	0.00002	0.9961	0.00278	1.00
mζ	0.0000	0.00000	0.0010	0.00207	307.84
mα	1.0000	0.00000	0.9990	0.00207	1.00
hε	0.0000	0.00000	0.0007	0.00068	165.86
hγ	0.0049	0.00239	0.0494	0.01064	10.05
hβ	0.9951	0.00239	0.9498	0.01122	0.95

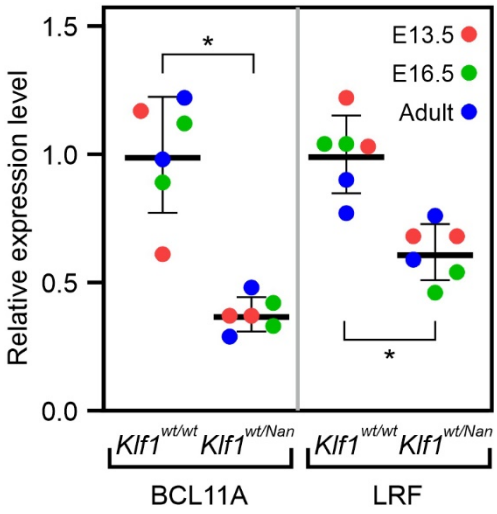
Globin expression in cultured E12.5 fetal liver cells (Day 6 of culture) was assessed by RT-qPCR. Globin expression was calculated as fraction of total mβ-like, mα-like or hβ-like globin. Fold-change is calculated as expression in *Klf1*$^{wt/Nan}$ cells divided by expression in *Klf1*$^{wt/wt}$ cells.

Supplementary Figure 1



Supplementary Figure 1. Visual abstract summarizing the main observations reported in this paper

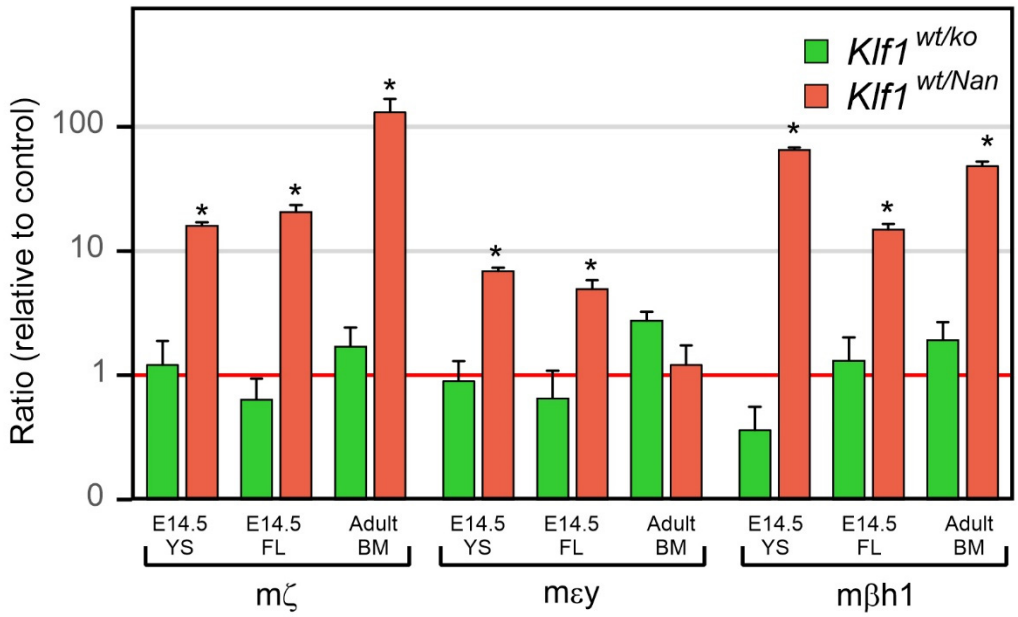
Supplementary Figure 2



Supplementary Figure 2. Expression of BCL11A and LRF in erythroid tissues of control and *Klf1*$^{wt/Nan}$ embryos

Expression of BCL11A and LRF in fetal livers (E13.5 and E16.5) and adult bone marrow was determined by RT-qPCR. Expression of P112 was used as a reference to normalize the data; the average expression level in the control samples was set to 1. Asterisks indicate p-values<0.05; error bars indicate standard deviations.

Supplementary Figure 3



Supplementary Figure 3. Expression of mouse embryonic globins in erythroid tissues of *Klf1*$^{wt/ko}$ and *Klf1*$^{wt/Nan}$ embryos

Expression of mouse embryonic globins in E14.5 yolk sac and fetal liver and adult bone marrow was determined by RT-qPCR. The average expression level in samples from control littermates (indicated by the red line) was set to 1. Asterisks indicate p-values<0.05 between *Klf1*$^{wt/ko}$ and *Klf1*$^{wt/Nan}$ samples; error bars indicate standard deviations. Note logarithmic scale. Data for *Klf1*$^{wt/ko}$ samples were obtained from [1].

SUPPLEMENTARY METHODS

Animals

Mice were maintained by breeding *Klf1*$^{wt/Nan::HBB}$ males with C57BL/6 females. Genotyping was performed by PCR using DNA isolated from toe biopsies. For *Klf1*^{Nan} genotyping, the PCR product was digested with DpnII [2].

Primers used for genotyping

All primer sequences are given in 5' to 3' direction.

Klf1^{Nan}

Fw: CTGCAGGATTGCAGCTGTAGATAC

Rv: AGTCCTTGTGCAGGATCACTCAGA

Approximately 340 bp PCR product for the wildtype *Klf1* allele, and 240 + 100 bp for the *Klf1*^{Nan} allele after DpnII digestion [2].

PAC8.1 human HBB locus single copy transgene [3]

Fw: GCTGCTGTTATGACCACTAGAGGG

Rv: AGACAGGGAAGGAGGTGTGG

PCR product 500 bp.

PCR conditions: 3 min 94°C, 28 cycles [30 sec 94°C, 30 sec 56°C, 30 sec 72°C], 2 min 72°C.

For timed pregnancies, *Klf1*$^{wt/Nan::HBB}$ males were mated with C57BL/6 and C57BL/6^{HBB} females. The day of vaginal plug discovery was considered E0.5. Embryos were collected between E11.5 and E16.5; head DNA was used for genotyping. Adult mice were analyzed at >18 weeks of age.

RT-qPCR

To synthesize cDNA, 2 µg of total RNA was used together with oligo dT, RNase OUT, and SuperScript reverse transcriptase II (all Thermo Fischer Scientific) in a total volume of 20 µl for 1 hour at 42°C. 0.2 µl of cDNA was used for amplification by RT-qPCR.

Primers were designed using Primer-BLAST, available through the NCBI web site at https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome.

PCR product size was set to 'Maximum 150 bp'. To avoid inter-globin and inter-species cross-specificity, the primer pair specificity checking parameters were set to 'Enable search for primer pairs specific to the intended PCR template' and 'Database-Genomes for selected organisms (primary reference assembly only)-Homo sapiens, Mus musculus'. Exon/intron selection was set to 'Primer pair must be separated by at least one intron on the corresponding genomic DNA' and Intron length range 'Minimum 100 bp'. All other settings were left at default values.

Mouse globins

Gene	globin encoded	Primer pair	PCR product (bp)
Hba-x	mζ	CCGGTCAACTTCAAGCTCCT	103
		TGAACTTGTCCCAGGCTTCG	
Hba-a1	mα1	GCTGAAGCCCTGGAAAGGAT	82
		CAGAGCCGTGGCTTACATCA	
Hba-a2	mα2	Identical to *Hba-a1*	
Hbb-y	mεy	TTGGCTAGTCACTTCGGCAAT	145
		GCATAGCGGACACACAGGAT	
Hbb-bh1	mβh1	TGGGCTTGGGGGTTAAGAAC	118
		AACATGTTGCCCAGGAGCTT	
Hbb-b1	mβmajor	GCTGCATGTGGATCCTGAGA	112
		CTTCTGGAAGGCAGCCTGTG	
Hbb-b2	mβminor	Identical to *Hbb-b1*	

Human globins

Gene	globin encoded	Primer pair	PCR product (bp)
HBE	hε	CCCTGGCCCATAAGTACCAC	105
		TTTCTCTCAAGGCCAAGCCC	
HBG1	hAγ	GGTGACCGTTTTGGCAATCC	106
		GTATCTGGAGGACAGGGCAC	
HBG2	hGγ	Identical to *HBG1*	
HBB	hβ	GCCCTGGCCCACAAGTATC	109
		GCCCTTCATAATATCCCCCAGTT	

Mouse transcription factors

Gene	factor encoded	Primer pair	PCR product (bp)
Bcl11a	BCL11A	CGTGTGCAGACCGAGGAGAGG	129
		GCATCCAGGTCACGCCAGAGG	
Zbtb7a	LRF	AGAAGGTGATTCAGGGTGCC	112
		AGCTTGTCCTGTCTGGTGAAT	

Mouse cell cycle regulators

Gene	factor encoded	Primer pair	PCR product (bp)
E2f2	E2F2	CTGAATTCCGGACCCCCAAG	101
		CGACGTGTCATAGCGTGTCT	
E2f4	E2F4	GCTTGGCCTACGTGACTCAT	100
		ATGGGCACCTCTAGACTGGT	
Cdkn1a	P21	GAATTGGAGTCAGGCGCAGA	89
		GAACAGGTCGGACATCACCA	
Cdkn2c	P18	AATGGATTTGGGAGAACTGCG	70
		GGAGAAGCCTCCTGGCAATC	

Mouse reference gene

Gene	protein encoded	Primer pair	PCR product (bp)
Psmd1	P112	AATGTTCCAGCGATGTCTCG	150
		GACATGCAGAGTTTGAGGCTG	

Quantitative PCR

PCR amplification was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using Platinum Taq DNA polymerase (Invitrogen). PCR conditions were 3 min 95°C, 40 cycles [30 sec 95°C, 25 sec 60°C, 15 sec 72°C], 5 sec 60°C, 5 min 95°C. Specific PCR product accumulation was monitored by SYBR Green dye fluorescence (Sigma-Aldrich, Saint Louis, MO). Using dilution series of yolk sac and fetal liver cDNAs, we found that the linear dynamic range of all globin primer pairs exceeded 256-fold dilution of the initial cDNA concentration. Dissociation curves were used to assess the homogeneity of PCR products. In addition, PCR product sizes were checked by agarose gel electrophoresis. To obtain expression values, Ct values were transformed by computing 2^{-Ct}. Total mα-like (mζ+mα), mβ-like (mεy+mβh1+mβ) and hβ-like globins (hε+hγ+hβ) expression was used to calculate the contribution of individual globins to the total for each globin in each sample, e.g. mζ/(mζ+mα) and mα/(mζ+mα) for mα-like globins. These fractional values were used to calculate the average and standard deviation for all samples belonging to the same group (e.g. *Klf1*$^{wt/Nan}$ E12.5 fetal liver). Analysis of RNA-seq data revealed that, compared to commonly used reference genes such as *GAPDH* and *ACTB*, expression of *PSMD1* (encoding P112, a proteasome 26S subunit) was relatively stable in primary human erythroblasts cultured under self-renewal or differentiation conditions (Heshusius *et al*, manuscript in preparation). We therefore used *Psmd1* as a reference gene to normalize expression levels of the *Bcl11a* and *Zbtb7a* genes.

Culture of mouse erythroid progenitors

E12.5 fetal livers were disrupted and single cell suspensions were seeded into StemPro medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 1 U/ml human recombinant erythropoietin (EPO; Cilag, Schaffhausen, CH), 10 ng/ml murine recombinant stem cell factor (SCF; Supernatant of cells harbouring an expression construct) and 10^{-6} M dexamethasone (Dex; Sigma-Aldrich, Saint Louis, MO) [4]. The cultures of erythroid progenitors were subjected to daily partial medium changes and addition of fresh factors. Cell numbers and size distributions were determined daily, using an electronic cell counter (CASY; Roche, Basel, CH). Cell density was kept between 0.5-2.0x10^6 cells/ml. To differentiate erythroid progenitors, cells were washed twice in phosphate-buffered saline (PBS) and seeded at 1.5x10^6 cells/ml in differentiation medium: StemPro supplemented with 10 U/ml EPO and 1 mg/ml iron-saturated human transferrin (Sigma-Aldrich). Cells were maintained at densities of 2-4x10^6 cells/ml.

Flow cytometry analysis

Single-cell suspensions of peripheral blood samples were washed twice with PBS and resuspended in FACS buffer (PBS containing 1% (w/v) bovine serum albumin, 2 mM EDTA). Approximately 10^6 cells were incubated for 30 minutes at room temperature with CD71-BV421 (562716, dilution 1:400; BD Biosciences, San Jose, CA), CD9-PE (12-0019-81, dilution 1:300; Thermo Fischer Scientific) and Ter119-APC (17-5921-82, dilution 1:200; Thermo Fischer Scientific) antibodies in a final volume of 100 µl. Antibodies were diluted in FACS buffer. The cells were washed, and living cells were distinguished negatively by live/dead Aqua staining (L34965; dilution 1:400; Thermo Fischer Scientific). Cultured cells were washed with PBS and incubated in for 30 minutes at 4 ºC in FACS-buffer with CD71-APC (130-091-727, Miltenyi Bergisch Gladbach DE, dilution: 1:200), Ter119-BV450 (48-5921-82 Thermo Fischer Scientific, dilution 1:200), Ly6G-PE (RB6-8C5, Thermo Fischer Scientific, dilution 1:100), F4/80-FITC (11-4801-85, BD Biosciences, dilution 1:200), CD11b-AF488 (101217, Biolegend San Diego CA, dilution 1:100), CD41PE (12-0411-82, Thermo Fischer Scientific, dilution: 1:100).Data were acquired on a Fortessa instrument (BD Biosciences), and analyzed with FlowJo software v10 (Tree Star, Ashland, OR).

References

1. Esteghamat F, Gillemans N, Bilic I, et al. Erythropoiesis and globin switching in compound Klf1::Bcl11a mutant mice. Blood. 2013;121(13):2553-2562.
2. Siatecka M, Sahr KE, Andersen SG, Mezei M, Bieker JJ, Peters LL. Severe anemia in the Nan mutant mouse caused by sequence-selective disruption of erythroid Kruppel-like factor. Proc Natl Acad Sci U S A. 2010;107(34):15151-15156.
3. de Krom M, van de Corput M, von Lindern M, Grosveld F, Strouboulis J. Stochastic patterns in globin gene expression are established prior to transcriptional activation and are clonally inherited. Mol Cell. 2002;9(6):1319-1326.
4. Dolznig H, Kolbus A, Leberbauer C, et al. Expansion and differentiation of immature mouse and human hematopoietic progenitors. Methods Mol Med. 2005;105:323-344.