Supplementary Data

Supplementary Materials and Methods

Drugs and chemicals

NaCl, Na_3VO_4, phosphate-buffered saline (PBS) phosphate buffer, bicine, benzamidine, β-mercaptoethanol, bromophenol blue, sodium dodecyl sulfate, NaF, ethylenediaminetetraacetic acid, May-Grunwald stain, Giemsa stain, phenylhydrazine hydrochloride, albumin from bovine serum (bovine serum albumin [BSA]), and glycerol were obtained from Sigma/Aldrich; protease inhibitor cocktail tablets were from Roche; dithiotreithol was from Fluka; Triton X-100 was from GE Healthcare Life Sciences; 40% Acrylamide/Bis Solution, 37.5:1 was from Bio-Rad; Dulbecco's Phosphate-Buffered Saline was from Lonza; and Luminata Forte and Luminata Classico Hrp solutions were from Merck Millipore.

Hematological parameters and red cell indices

Blood was collected by retro-orbital venipuncture in anesthetized mice using heparined microcapillary tubes. Hematological parameters were evaluated on a Bayer Technicon Analyzer ADVIA. Hematocrit and hemoglobin were manually determined as previously described (4, 8, 9, 13).

Flow cytometric analysis of mouse bone marrow and spleen precursors and fluorescence-activated cell sorting of murine bone marrow erythroblasts

The flow cytometric analysis of erythroid precursors from the bone marrow and spleen of the mice from the four mouse strains was carried out as previously described (13, 28, 40). CD44-FITC (eBioscience), TER-119 APC (eBioscience), CD45 APC-eFluor 780 (eBioscience), GR1 APC-Cy7 (BD Biosciences), and CD11b APC-Cy7 (BD Biosciences) were used. The 7-aminoactinomycin D Viability staining solution (eBioscience) was used to remove dead cells from the analysis. Flow cytometric analysis was carried out with the FACSCanto^{TM} flow cytometer (Becton Dickinson) that was equipped with the FACSDiva software (Becton Dickinson). Data were analyzed with the FlowJo software (Tree Star) (13, 28, 40).

Population II, III, and IV, corresponding to basophilic, polychromatic, and orthochromatic erythroblasts, were sorted from mouse bone marrows using the FACSAria II cell sorter (Becton Dickinson).

Quantitative real-time–polymerase chain reaction

For the quantitative real-time–polymerase chain reaction (qRT-PCR), mRNA was isolated and reverse transcribed into high-purity cDNA using μMACS One-step cDNA Kit according to the manufacturer's instructions (Miltenyi Biotec). We started from 500,000 sorted polychromatic and orthochromatic erythroblasts from the bone marrow of wild-type, $Prx2^{-/-}$, $Hbb^{3th-/+}$, $Hbb^{3th-/+}$, and $Prx2^{-/-}$ mouse strains. One out of fiftieth of the reactions were added to appropriate wells of the PCR plates. qRT-PCR was performed by SYBR Green PCR Master Mix (Applied Biosystems) by using the Applied Biosystems Model 7900HT Sequence Detection System. Detailed methods and primer sequences are available on request and in Supplementary Table S1. All PCR reactions were performed in triplicate. Relative gene expression was calculated by using the $2^{-\Delta Ct}$ method, in which Ct indicates cycle threshold, the fractional cycle number where the fluorescent signal reaches the detection threshold (13). The ΔCt was computed by calculating the difference of the average Ct between the X-gene and the internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The data are presented as mean $\pm$ the standard deviation.

Cytospin preparation and immunofluorescence assay for nuclear factor-erythroid 2 and P-Nrf2

Overall, 250,000 sorted basophilic, polychromatic, and orthochromatic erythroblasts from wild-type, $Prx2^{-/-}$, $Hbb^{3th-/+}$, $Prx2^{-/-}$, and $Hbb^{3th-/+}$ were cytospun onto glass slides and either stained with May-Grunwald-Giemsa for morphological analysis or fixed with 4% paraformaldehyde for immunofluorescence assay. Fixed cells were washed thrice with PBS, BSA 1%, permeabilized using Triton X-100 0.3% in PBS, BSA 1%, washed three more times, and incubated in PBS 1% BSA overnight with the anti-nuclear factor-erythroid 2 (Nrf2) andibody (Abcam). Cells were washed with PBS, BSA 1%, Triton 0.1% and stained with Alexa Fluor 594 anti-rabbit IgG antibody (Thermo Fisher Scientific). Nuclei were stained with 4′,6-diamidino-2-phenylindole (Thermo Fisher Scientific). May-Grunwald-Giemsa images were captured using the Nikon EclipseE600 microscope, $\times$100 magnification. Fluorescent samples were imaged using a confocal laser-scanning microscope equipped with a 60$\times$, 1.4 numerical aperture oil immersion lens (Leica Microsystems). Nrf2 quantification was performed using the ImageJ software (http://imagej.nih.gov/ij/) as previously described (13).

Immunoblot analysis of sorted erythroid precursors

Overall, 1.5×10^6 sorted wild-type, $Prx2^{-/-}$, $Hbb^{3th-/+}$, $Prx2^{-/-}$, and $Hbb^{3th-/+}$ were solubilized as previously described (13). Proteins were separated by monodimensional electrophoresis and transferred to a membrane for immunoblot analysis with the specific antibodies anti Nrf2-phospho-S40, anti Nrf2 (Abcam) and anti GAPDH (Sigma/Aldrich), anti beta-Tubulin (clone E7; DSHB), and anti Lamin B (Santa Cruz Biotechnology), which were used as loading controls. Images were acquired using the Image Quant Las Mini 4000 Digital Imaging System (GE Healthcare Life Sciences), and densitometric analysis was performed using the ImageQuant TL software (GE Healthcare Life Sciences).

Supplementary Table S1. Details of Primers for the Specific Quantitative Real-Time–Polymerase Chain Reaction of the Analyzed Genes on Sorted Mouse Erythroblasts

Gene symbol	*Forward primer sequence (5′→3′)*	*Reverse primer sequence (5′→3′)*
Gstm	CAAGCTGGGCCTGGACTTT	CGCAGGATGGCATTGCTC
Hmox1	CAAGCACAGGGTGACAGAAGAG	GTCAGCATCACCTGCAGCTC
Nqo1	TGCTCGTAGCAGGATTTGCC	CCAGTGGTGATAGAAAGCAAGGT
Gapdh	GTAGACAAAATGGTGAAGGTCGG	GCCACTGCAAATGGCAGC
Srxn1	TGCAGAGCCTGGTGGACA	TTTGATCCAGAGGACGTCGAT
Bach1	GGAGCAGGACTGTGAGGTGAAG	GGAAATCATTTCGTGAGAGCG
Txnrd1	CAAGACATGGCCAACAAAATCG	CGATCTGTTCAATTTTCGTTGGG
Sesn2	TTCCGAGATCAAGGGCTACC	CACTGGAATGAAAGCGCTGG
Hb A1	TGCGTGTGGATCCCGTC	TGCTGGAATTTCAGGTGCTG

Supplementary Table S2. Hematological Parameters and Red Cell Indices in Wild-Type and Prx2$^{-/-}$ Mice

	Wild-type mice (n = 15)	*Prx2$^{-/-}$ mice (n = 10)*
Hct (%)	46 ± 1.4	39.8 ± 2.0
Hb (g/dl)	14.9 ± 0.1	12.3 ± 1.5*
MCV (fl)	51.5 ± 0.7	50.4 ± 1.0
MCH (g/dl)	16.4 ± 0.5	15.3 ± 0.2*
CH (pg)	13.4 ± 0.02	13.2 ± 0.02
RDW (%)	12.2 ± 0.2	14,7 ± 0.2*
Retics (%)	2.5 ± 0.8	4.8 ± 0.9*
MCVr (fl)	57.6 ± 1.4	57.7 ± 1.2

*$p < 0.05$ compared with wild-type mice.

Hb, hemoglobin; Hct, hematocrit; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MCVr, mean corpuscular volume reticulocytes; RDW, red cell distribution width; Retics, reticulocytes.