SUPPLEMENTARY APPENDIX

A novel role for nuclear factor-erythroid 2 in erythroid maturation by modulation of mitochondrial autophagy

Monika Gothwal,[1] Julius Wehrle,[1] Konrad Aumann,[2] Vanessa Zimmermann,[1] Albert Gründer,[1] and Heike L. Pahl[1]

[1]Clinic for Internal Medicine I, Division of Molecular Hematology; and [2]Department of Pathology, Medical Center, Faculty of Medicine, University of Freiburg, Germany

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Correspondence: pahl@uni-freiburg.de

Supplementary figures

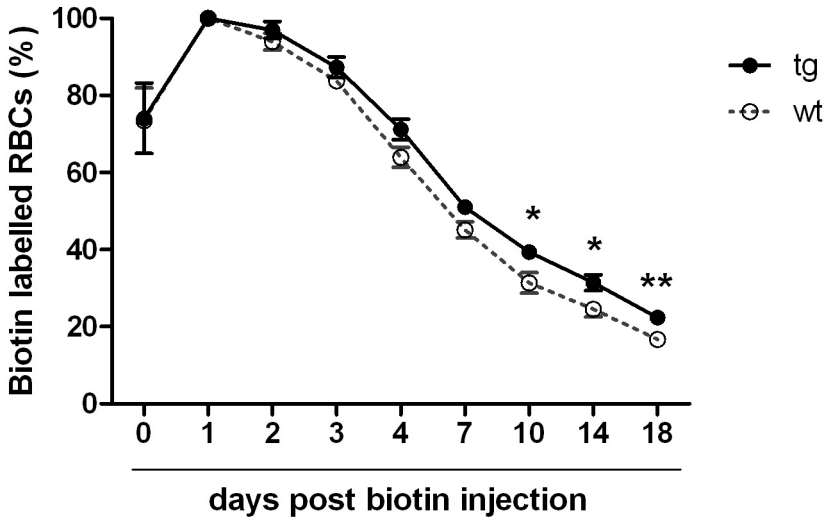

Figure S1: RBC turnover in NF-E2tg and wt mice. Mice were injected intravenously with NHS-Biotin, followed by blood draw on the indicated days and FACS staining with Streptavidin and Ter119 to detect biotin labeled RBCs. Percentage of biotin labeled RBCs are considered 100% on day 1 post biotin injection. Decrease in % labeling is determined relative to day 1. Percent of biotin labeled cells indicates RBCs in circulation. n= 6 wt, n= 6 tg. Each data point represents mean and SEM. (* $p<0.05$, ** $p<0.01$). Statistical significance was calculated by Student's t-tests.

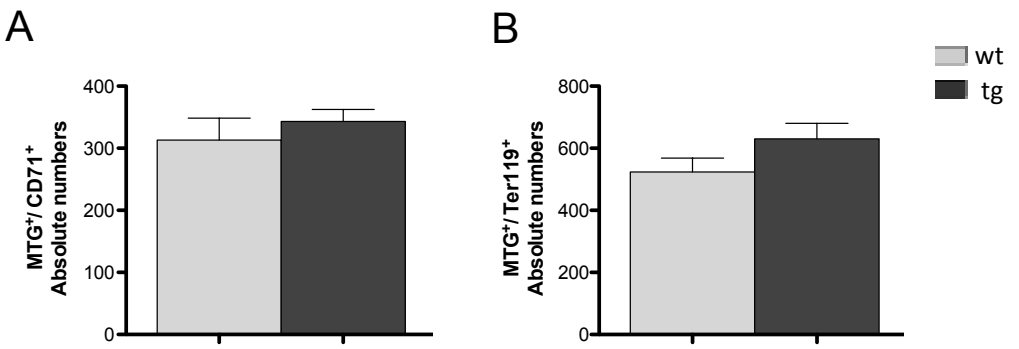

Figure S2: Absolute Numbers of MTG positive erythroid cells in NF-E2tg and wt mice. Whole blood from wt and tg animals, as indicated was stained with CD71 and Ter119 antibodies as well as mitotracker green (MTG) dye. Absolute numbers per 10.000 measured events (per 3 μl whole blood) of **(A)** $MTG^+/CD71^+$ and **(B)** $MTG^+/Ter119^+$ stained cells in NF-E2tg and wt mice. n= 12 wt, n= 21 tg. Histograms show mean and SEM.

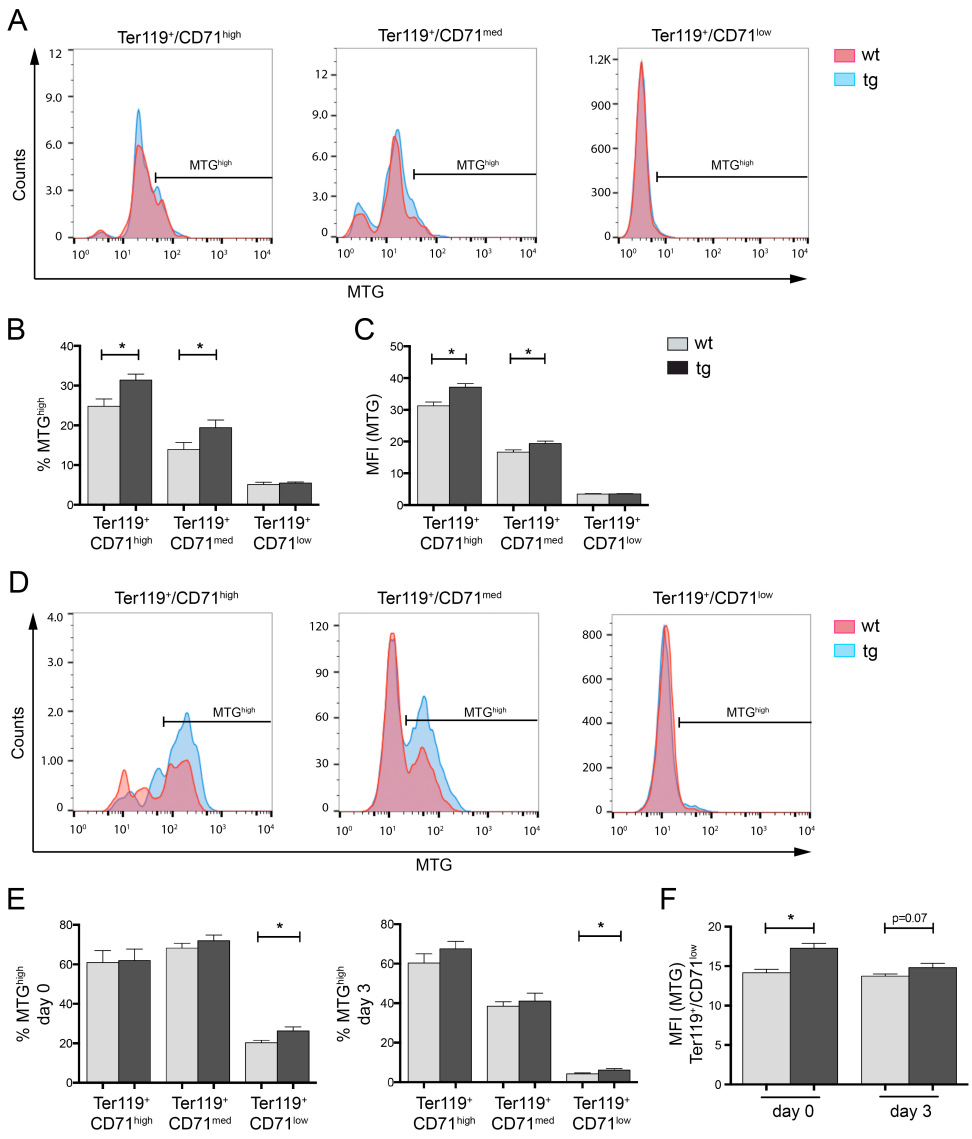

Figure S3: Analysis of MTG staining on Ter119 and CD71 subpopulations. Whole blood was collected and analysed either in the native state or after PHZ treatment. **(A-C)** Analysis in native state. **(A)** Representative overlay histograms of MTG staining in the Ter119/CD71 subpopulations as depicted in Fig. 1A. n= 4 wt, n= 6 tg (6 strain "9"). **(B, C)** Quantification of MTG staining in the defined erythroid populations: % MTG^{high} (B) and mean fluorescence intensity (MFI) (C). **(D-F)** Analysis after PHZ treatment. **(D)** Representative overlay histograms of MTG staining on day 0 in the Ter119/CD71 subpopulations as depicted in Fig. 2B. n= 4 wt, n= 4 tg (4 strain "9"). **(E)** Quantification of MTG staining in the defined erythroid populations depicted as % MTG^{high}. **(F)** Determination of MFI for MTG in the $MTG^{+}/CD71^{low}$ populations on days 0 and 3. Histograms show mean and SEM. *p<0.05. Statistical significance was calculated by Student's t-tests.

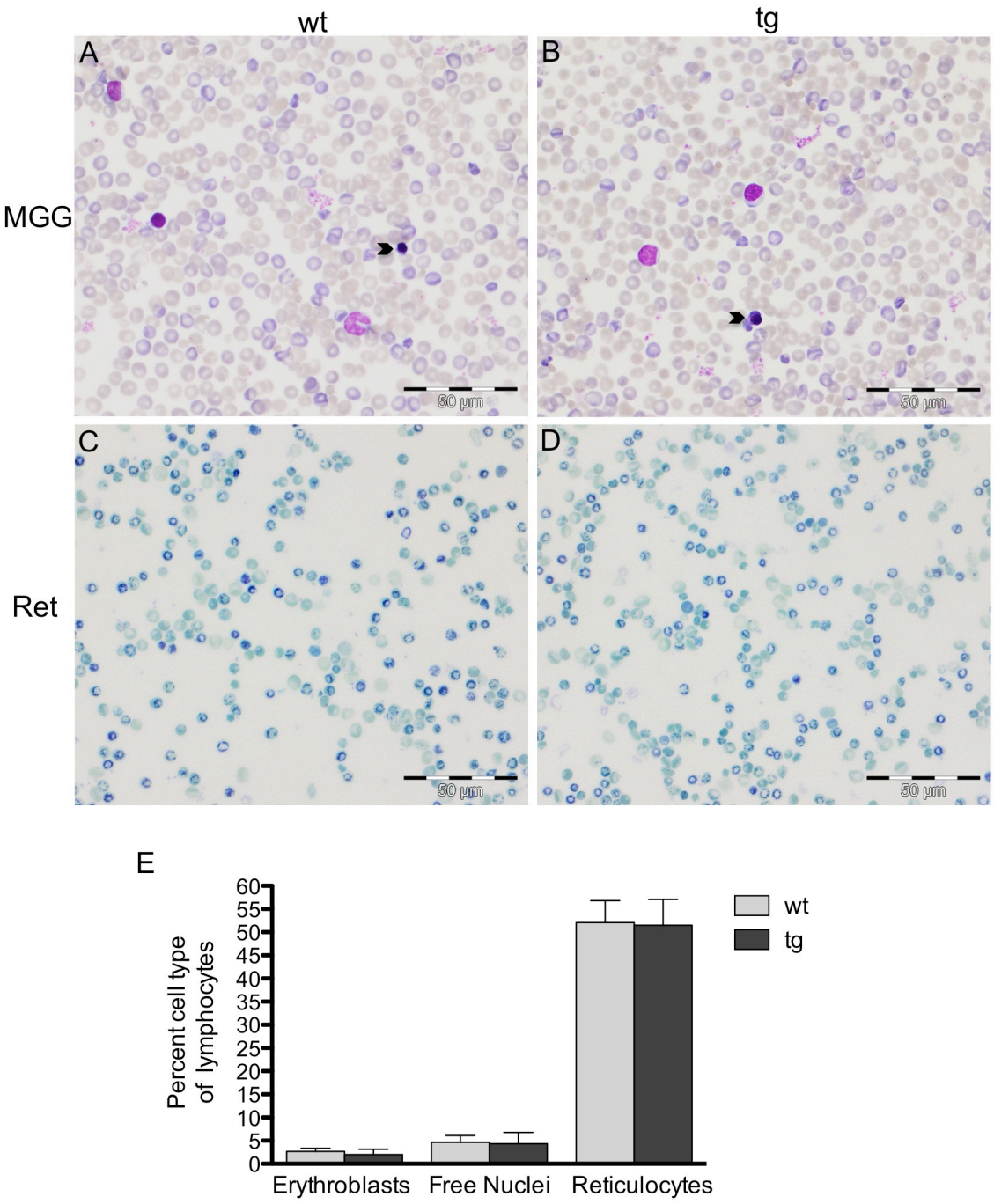

Figure S4: Peripheral blood cytology in NF-E2tg and wt mice following PHZ treatment. Mice were injected with PHZ to induce reticulocytosis, as depicted in Fig. 2A, and peripheral blood smears were stained with either **(A, B)** May-Grünwald–Giemsa (MGG) or **(C, D)** reticulocyte (Ret) stain and the cell populations counted per 100 lymphocytes. n= 6 wt, n= 6 tg. **(E)** Histograms show mean and SEM. Arrowheads represent erythroblasts.

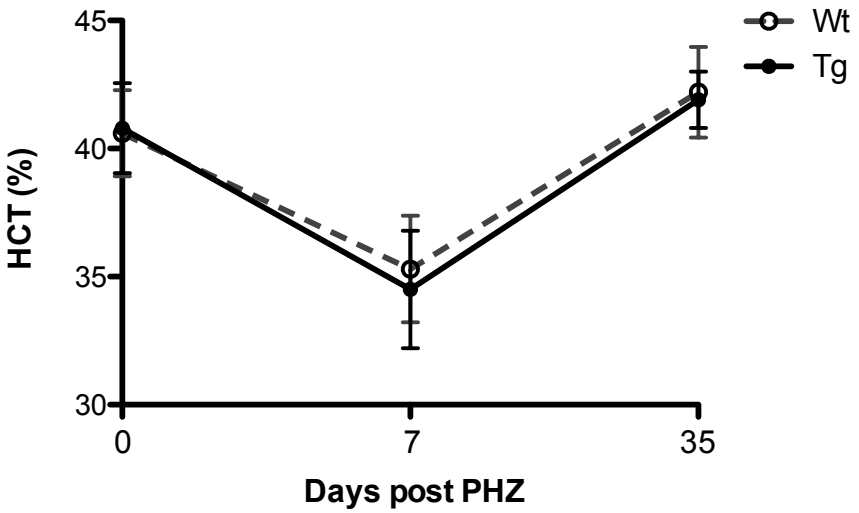

Figure S5: Hematocrit recovery in NF-E2tg and wt mice. Mice were injected intra-peritoneously with PHZ on d0, 1 and 3. Whole blood was analyzed on an Advia 120 hematology analyzer before PHZ injection (d0) and 7 and 35 days after the first injection. n= 4 wt, n= 4 tg. Each data point represents mean and SEM.

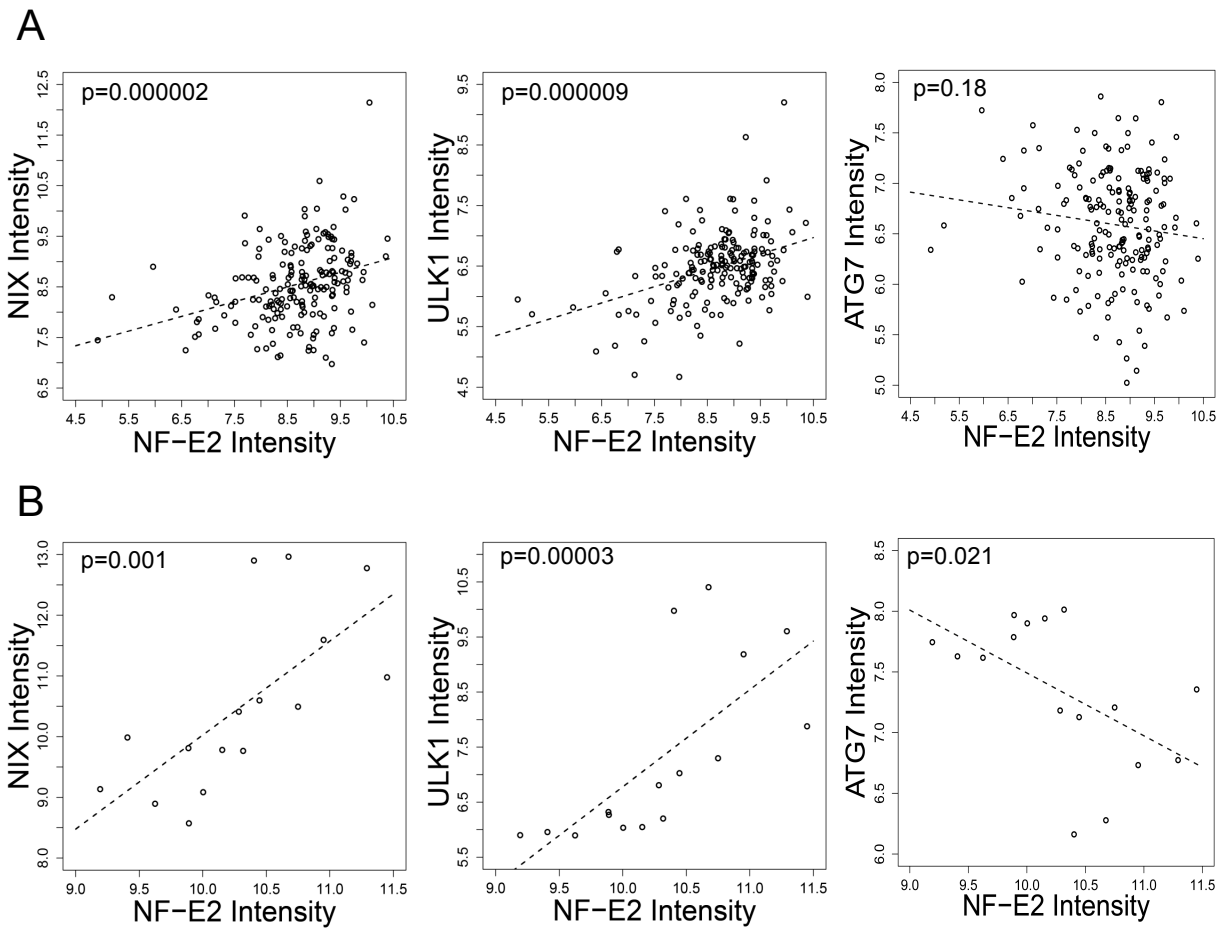

Figure S6: Correlation analysis of *NF-E2* mRNA expression levels with either *NIX*, *ULK1* or *ATG7* expression levels. **(A)** Expression data from 200 AML patients was used (TCGA AML dataset) **(B)** Correlations in human erythroid precursors: expression data are pooled from cells of four distinct stages in human erythropoiesis ranging from colony-forming units (CFU-Es) to late erythroblasts (7). Dashed lines represent the fitted linear regression. Statistical significance was determined using Spearman's correlation coefficient.

Supplementary methods

Mice

Establishment of the VAV-HA-hNF-E2 transgenic animals has been described elsewhere (1). All animal experiments were performed in compliance with the German animal protection law (TierSchG). The mice were housed and handled in accordance with good animal practice as defined by FELASA (www.felasa.eu/guidelines.php) and the national animal welfare body GV-SOLAS (www.gv-solas.de/index.html). The animal welfare committees of the University of Freiburg as well as the local authorities (Regierungspraesidium Freiburg) approved all animal experiments.

Analysis of RBC turnover

NHS-Biotin (20217, Thermo Scientific) was injected intravenously at a concentration of 0.15mg/g body weight. Heparinized whole blood was collected from the sub-mandibular vein and stained with Streptavidin-PE (405203, BioLegend) and Ter119-PerCP (116226, BioLegend) for 30 minutes in dark at room temperature. Cells were resuspended in PBS for flow cytometry on a FACS Calibur.

Induction of reticulocytosis in mice

Reticulocytosis was induced by intra-peritoneal injections of phenylhydrazine hydrochloride (PHZ-HCl; P6926, Sigma) (2). PHZ was injected at a concentration of 40µg/g body weight on days 0, 1 and 3. On day 7 post PHZ injection, blood was drawn retro-bulbar and percentage of reticulocytes was assessed on an Advia 120 hematology analyzer (Siemens).

Ex vivo maturation of reticulocytes

Cultures were set up according to Zhang *et al*. (3). Briefly, heparinized whole blood from PHZ treated mice was diluted 1:500 in 1.5ml maturation medium consisting of 60% IMDM (21980, Gibco) [containing 2mM L-glutamine (25030, Gibco) and 100U penicillin-streptomycin (DE17-602E, Lonza)], 30% FCS (10270, Gibco), 1% BSA (A1595, Sigma), 0.001% monothioglycerol (M6145, Sigma) and 1x Mycozap (VZA-2022, Lonza) and cultured in a 24-well tissue culture plate in a cell culture incubator with 5% CO_2, at 37 ^{o}C until staining.

For the culture of reticulocytes in the presence of depolarizing agents, carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (FCCP) (C2920, Sigma) and ABT-737 (S1002, Selleckchem) were added to the culture medium just before seeding. FCCP and ABT-737 were used at a final concentration of 10μM and 1μM, respectively.

Staining

Whole blood was cultured in the maturation medium (native blood for 2 hours and after PHZ for 0 to 3 days). The cells in culture were stained with 100nM mitotracker green dye (MTG) (M7514, Invitrogen) for 30 minutes in a cell culture incubator with 5% CO_2, at 37^{o}C. Subsequently the cells were collected, washed in PBS, resuspended in 100μl PBS and incubated with Ter119-PE (22155234, ImmunoTools) and CD71-APC (C355, Leinco Technologies) antibodies for 30 minutes in dark at room temperature. For thiazole orange (TO) (390062, Sigma) staining, cells were collected and resuspended in 1ml of TO in PBS (2μg/ml) followed by washing and staining with Ter119 and CD71 antibodies. JC1 (65-0851-38, eBioscience) staining was done according to Cossarizza *et al.* (4). JC1 working dilution (10μg/ml) was prepared immediately before use in IMDM media containing 30% FCS while continuously vortexing. The cultured reticulocytes were collected at different time points (0h, 4h, 12h, 48h), washed and incubated with 1ml JC1 solution for 20 minutes at 37^{o}C in dark, followed by washing, resuspension in PBS and FACS analysis. FACS analysis was done on a FACS Calibur (BD Biosciences).

Quantitative RT-PCR

qRT-PCR was performed using Assays on Demand (Applied Biosystems) for gene expression analysis. The assay IDs used are: *Nix*: Mm01625871, *Ulk1*: Mm00437245, *Atg7*: Mm00512209. Bone marrow RNA (400ng) or RNA isolated from Ter119^{+}/CD71^{+} sorted peripheral blood cells (100ng) was reverse transcribed using the TaqMan Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed on a PRISM 7000 Cycler (Applied Biosystems). Relative expression levels were calculated with respect to murine β-2-microglobulin (*B2m*) using the ΔΔCt method. *B2m* primer and probe sequences have been described in Jutzi *et al.*, 2013 (5).

Chromatin Immunoprecipitation (ChIP)

Human Erythroleukemia (HEL) cells were harvested and cross-linked in 1% formaldehyde for 10 min at room temperature followed by quenching the reaction with 0.125M glycine. Cells were lysed in a lysis buffer containing 50mM Tris pH 8.0, 1% SDS and 10mM EDTA and chromatin sheared by sonication in a Bioruptor Plus (Diagenode). The chromatin solution was diluted five times in a buffer containing 20mM Tris pH 8.0, 0.01% SDS, 2mM EDTA, 150mM NaCl and 1% Triton X-100. Further, the samples were precleared with Protein A agarose (Millipore) and incubated overnight with the primary antibody against NF-E2 or an IgG control. The immunocomplexes were further bound to Protein A agarose, washed, eluted and decross-linked. DNA was purified by phenol/chloroform extraction and subjected to PCR analysis. Primers were designed based on UCSC Genome Browser (genome.ucsc.edu/) NF-E2 binding site prediction on the human *NIX* and *ULK1* genes. Primers used are:

NIX gene

Binding site	FP	RP	Amplicon (bp)
NF-E2	5'-CATCACTCATCCCATCCGGTC-3'	5'-GACCGTCTCCGGCACTCAG-3'	255
Random	5'-CAGGTAGCGATGGAGTTTGGAG-3'	5'-GACAGTATGCTAAACATGGGC-3'	219

ULK1 gene

Binding site	FP	RP	Amplicon (bp)
NF-E2	5'-CTGGCTTTCGTGCTGCCTC-3'	5'-CCAAGGCCACAGCAAAGCTC-3'	245
Random	5'-CTTTCCAGCCCAGATCCTCA-3'	5'-CCTGAGATGATCCCGGTTGG-3'	198

Western Blotting

Whole cell extracts from human granulocytes were prepared using the Trizol reagent according to the manufacturer's instructions. Isolated proteins were resuspended in 1% SDS supplemented with 1x Complete (Roche). Samples were prepared by protein reduction and alkylation, as described elsewhere (6), and subjected to SDS-PAGE and western blotting. Lysates (60µg) were resolved on a 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Blocking was performed with 5% skimmed milk dissolved in PBS + 0.1%Tween-20. The antibodies used are: NIX (ab109414, Abcam) and ULK1 (ab128859, Abcam). NF-E2 antibody was generated in association with Eurogentec. GAPDH (G8795, Sigma) was used as a

loading control. Secondary antibodies were: anti-rabbit-IgG HRP (#7074) and anti-mouse-IgG HRP (#7076) from Cell Signaling. The immunocomplexes were detected with enhanced chemiluminescence western blotting reagents from Perkin Elmer. Densitometric analyses were performed using ImageJ software (NIH).

Cytology

Blood smears were performed with whole blood from PHZ treated animals. Reticulocytes were stained by incubating an equal volume of whole blood and the reticulocyte stain (R4132, Sigma) for 30 minutes at room temperature followed by preparation of smears. May-Grünwald-Giemsa staining was performed on native blood smears after overnight drying. Smears were stained with May-Grünwald solution (101424, Merck), washed in buffered water, pH 6.8 and counterstained with 1:20 Giemsa solution in buffered water (109204, Merck).

Bioinformatic data analysis

Microrarray data were obtained from NCBI Gene Expression Omnibus under accession number GSE22552 for the dataset of human primary erythropoiesis (7) and from the TCGA data platform (http://cancergenome.nih.gov/) for the TCGA AML dataset. Data preprocessing and statistical analysis was performed using the Bioconductor packages in R (R version 3.2.0 / www.bioconductor.org). Data were loaded and normalized by the Robust Multiarray Average (RMA) method of Irizarry *et al* (8) using the "oligo" package. 2-log expression value for *NF-E2*, *NIX*, *ULK1* and *ATG7* were extracted and used for correlation analysis using the Spearman correlation method reporting the respective p-value.

Lentiviral constructs and transduction

Lentiviral constructs were used as described in Roelz *et al*. (9). UKE-1, SET-2 and HEL cell lines were maintained and transduced with the lentiviral constructs as described in Wehrle *et al.* (10).

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