# Schlafen2 is a regulator of quiescence in adult murine hematopoietic stem cells

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SUPPLEMENTARY METHODS AND RESULTS

## Supplementary methods

*Mice*

The elektra strain was purchased from the MMRRC Repository (strain ID C57BL/6J-Slfn2$^{m1Btlr}$/Mmucd) and embryo transferred into the Lund University animal facility. Wild type (WT) mice (B6SJL or B6SJLxC57Bl/6) were obtained from in-house breeding or purchased from Taconic. Mice were kept in 12 hour light/dark cycle with food and water *ad libitum*. Ear biopsies from the elektra strain were taken at around 4 weeks of age and genotyped with PCR and sequencing, following protocol from MMRRC. Mice were 8-20 weeks of age at the time of experimentation unless otherwise specified.

*Cells*

Cell preparation from blood and BM was done as previously described[1]. The HT1080 cell line was cultured in DMEM according to standard protocol (ATCC, LGC standards).

*Transfection and immunofluorescence analysis*

For over-expression of Slfn2 and elektra, Myc-tagged proteins were obtained from GenScript and cloned into the pcDNA3.1 vector backbone. HT180 cells were transfected using Lipofectamine (Thermo Fisher Scientific) with pcDNA3.1-Slfn2-Myc or pcDNA3.1-elektra-Myc. Cells were transferred to chamber slides, permeabilised using triton-X-100, and stained with rabbit-anti-Myc and goat-anti-rabbit-AlexaFluor488. After addition of DAPI for localization of nuclei, cells were studied and photographed using an Olympus BX51 microscope and Olympus DP73 camera.

*Colony formation assay*

Whole BM cells were seeded in methylcellulose at a concentration of 30 000 cells/ml. We used MethoCult M3234 (Stem Cell Technologies) supplemented with 10 U/ml penicillin and 5 ug/ml streptomycin (Nordic Biolabs), as well as 50

ng/ml mSCF, 10 ng/ml IL-3 and 10 ng/ml IL-6 (Pepro Tech). Plates were placed in a humidified incubator at 37°C and 5% $CO_2$ and after seven days colonies were counted using bright field microscopy.

*Hematopoietic stress assay with in vivo 5-fluorouracil treatment*

Mice were given an intra-venous bolus injection of 5FU (0.15 mg/g) and kept under supervision the following days for analysis of survival. In a first experiment three mice each (WT and elektra) were given 5FU and analysed for survival. In a second experiment six elektra mice were given 5FU and half of these received a BM transplant three days later before analysis of survival.

*Microarray*

2700–6000 SLAM-LTHSCs or MPPs were sorted as described in methods into 350 μl RLT buffer (Qiagen) and immediately frozen on dry ice. RNA isolation, microarray analysis (Affymetrix Gene Chip; Mouse Gene 2.0 ST array), and calculations of summarized probe set signals in log2 scale using the RMA algorithm[2] was performed by the authorized Affymetrix service provider Kompetenzzentrum Fluoreszente Bioanalytik (KFB) Center of Excellence for Fluorescent Bioanalytics (Regensburg, Germany) as previously described[1]. The gene list generated was additionally analysed by gene set enrichment analysis (GSEA) using an online resource (GSEA 3.0, Broad Institute)[3,4] and the Molecular Signatures Database (MSigDB) hallmark gene sets collection[3,5].

Further processing and analysis of the raw data was performed at the Stem Cell Center Bioinformatics Core Facility (Lund University). The microarray data was normalised using RMA from the R package "affy". Differentially expressed genes were identified with the rank products methods using the RankProd package[6] where a gene was called differential if FPR (false positive rate) was less than 10%. Heatmaps were generated using the pheatmap package. The gene lists generated were analysed using the Database for Annotation, Visualization and Integrated Discovery (DAVID; v6.8)[7,8] to gain insight into gene ontology and clustering.

*qRT-PCR analysis*

1500–5000 cells of various primitive hematopoietic cell populations, as defined by SLAM markers CD48/CD150 with/without CD9 or by markers CD34/Flt3, were sorted into RLT buffer (Qiagen). Cells were also sorted from wild type mice two days after intravenous injection with 5FU (0.15 mg/g). Due to known disruption of c-kit expression following 5FU, primitive hematopoietic cells were sorted as $Lin^-$ $Sca1^+$ $CD150^+$ $CD48^-$. RNA purification was done with Qiagen RNeasy micro kit, according to manufacturer's protocol. cDNA conversion was done using the Superscript III First Strand system (Life Technologies) with in-house modifications. qPCR was performed with Taqman probes (Life Technologies) according to manufacturer's protocol on a 7900 HT Fast Real Time PCR system (Bio-Rad). Data was analysed using the SDS 2.4 software (Applied Biosystems).

*Protein prediction analysis*

Using AlphaFold[9] and PredictProtein[10] we visualized the Slfn2 protein structure and localization of the specific residue substituted by the elektra mutation. With PolyPhen-2[11] the effect of this mutation was predicted.

*Statistical analysis*

Statistical analyses were performed in GraphPad Prism v.6-8 for Mac OS X. Unless otherwise stated we used unpaired, non-parametric tests (Mann-Whitney for comparison of two groups or Kruskal-Wallis with *post hoc* multiple comparisons test for three or more groups). Isolated outliers were detected using Grubbs' test, which identifies one outlier per group and data set (alpha = 0.05). Where indicated (in figure legends), iterative Grubbs' test was used to identify more than one outlier. Statistical analysis of survival was done using the log-rank method. *P*-values of $< 0.05$ were considered statistically significant. All data is presented as mean values ± standard deviation (SD). All experiments in this study were performed two to three times unless otherwise stated in above methods and figure legends.

## Supplementary results

*Normal donor engraftment in the Slfn2 mutant BM niche*

As the elektra mutation can be found in all tissues of this mouse model, we wanted to evaluate the effect of Slfn2 dysregulation on the BM niche, as niche modulations have been reported to affect HSC function[12]. WT cells were transplanted into irradiated elektra mice as well as WT littermates and 16 weeks after reverse transplantation WT cell engraftment was found to be equal in elektra and littermate recipient mice (Supplementary Figure 10A). There was, however, a non-significant increase in the number of engrafted LTHSCs in the BM of elektra recipients (Supplementary Figure 10B). This may be explained by decreased fitness of residual elektra HSCs in the BM following irradiation, giving transplanted WT cells an additional advantage during engraftment and reconstitution. It may also reflect an alteration in the BM niche of elektra mice.

*Homing is not affected by Slfn2 deficiency*

To evaluate the effect of Slfn2 deficiency on HSPC homing ability we analysed recipient BM 24h after BM transplantation. We found no significant differences in frequencies of primitive hematopoietic cells that had homed to recipient BM (Supplementary Figure 10C-E). There is a non-significant trend of increased engraftment of $Lin^-$ $Sca1^+$ $CD150^+$ cells (Supplementary Figure 10E) that may be investigated further in future studies.

**References** (supplementary methods and results)

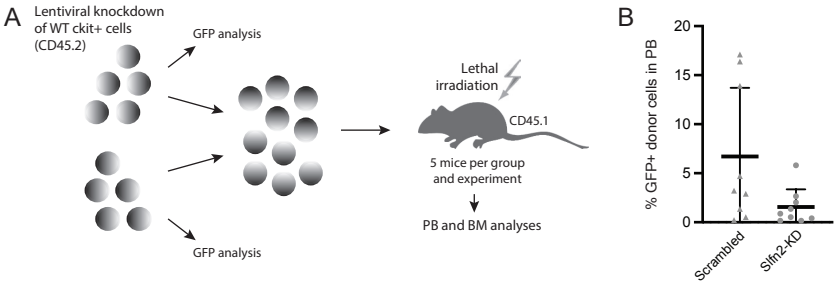

1. Warsi S, Blank U, Dahl M, et al. BMP signaling is required for postnatal murine hematopoietic stem cell self-renewal. Haematologica. 2020.
2. Irizarry RA, Hobbs B, Collin F, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics. 2003;4(2):249-264.
3. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A. 2005;102(43):15545-15550.
4. Mootha VK, Lindgren CM, Eriksson KF, et al. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet. 2003;34(3):267-273.
5. Liberzon A, Birger C, Thorvaldsdottir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular Signatures Database (MSigDB) hallmark gene set collection. Cell Syst. 2015;1(6):417-425.
6. Hong F, Breitling R, McEntee CW, Wittner BS, Nemhauser JL, Chory J. RankProd: a bioconductor package for detecting differentially expressed genes in meta-analysis. Bioinformatics. 2006;22(22):2825-2827.
7. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009;4(1):44-57.
8. Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 2009;37(1):1-13.
9. Jumper J, Evans R, Pritzel A, et al. Highly accurate protein structure prediction with AlphaFold. Nature. 2021;596(7873):583-589.
10. Bernhofer M, Dallago C, Karl T, et al. PredictProtein - Predicting Protein Structure and Function for 29 Years. Nucleic Acids Res. 2021;49(W1):W535-w540.
11. Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. Nat Methods. 2010;7(4):248-249.
12. Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. Nature. 2014;505(7483):327-334.

## Supplementary Table 1

Antibodies used for immunofluorescence microscopy, flow cytometry, and cell sorting.

| *Antigen* | *Fluorochrome* | *Clone* | *Company* |
|---|---|---|---|
| anti-Myc | - | rabbit polyclonal | Abcam |
| anti-rabbit | AlexaFluor-488 | goat polyclonal | Abcam |
| B220 | PE/Cy5 | RA3-6B2 | BioLegend |
| B220 | PE | RA3-6B2 | BioLegend |
| c-kit | APC-eFl780 | 2B8 | AH Diagnostics |
| CD3 | PE/Cy5 | 145-2C11 | BioLegend |
| CD9 | PE | 145-2C11 | BioLegend |
| CD16/32 | APC | 93 | BioLegend |
| CD34 | FITC | RAM34 | Beckton Dickinson |
| CD41 | FITC | MWReg30 | BioLegend |
| CD45.1 | PE/Cy7 | A20 | BioLegend |
| CD45.1 | Biotin | A20 | Beckton Dickinson |
| CD45.1 | PE | A20 | BioLegend |
| CD45.1 | PE/Cy5 | A20 | eBioscience |
| CD45.2 | APC | 104 | BioLegend |
| CD45.2 | BV510 | 104 | BioLegend |
| CD48 | FITC | HM48-1 | BioLegend |
| CD105 | Biotin | MJ7/18 | BioLegend |
| CD150 | PE/Cy7 | TC15-12F12.2 | BioLegend |
| Flt3 | PE | A2F10.1 | Beckton Dickinson |
| Gr1 | PE | RB6-8C5 | BioLegend |
| Gr1 | PE/Cy5 | RB6-8C5 | BioLegend |
| Ki67 | PE | B56 | Beckton Dickinson |
| Mac1 | PE | M1/70 | BioLegend |
| Mac1 | PE/Cy5 | M1/70 | BioLegend |
| Sca1 | BV421 | D7 | BioLegend |
| Streptavidin | Qdot605 | - | Life Technologies |
| Ter119 | PE/Cy5 | TER-119 | BioLegend |

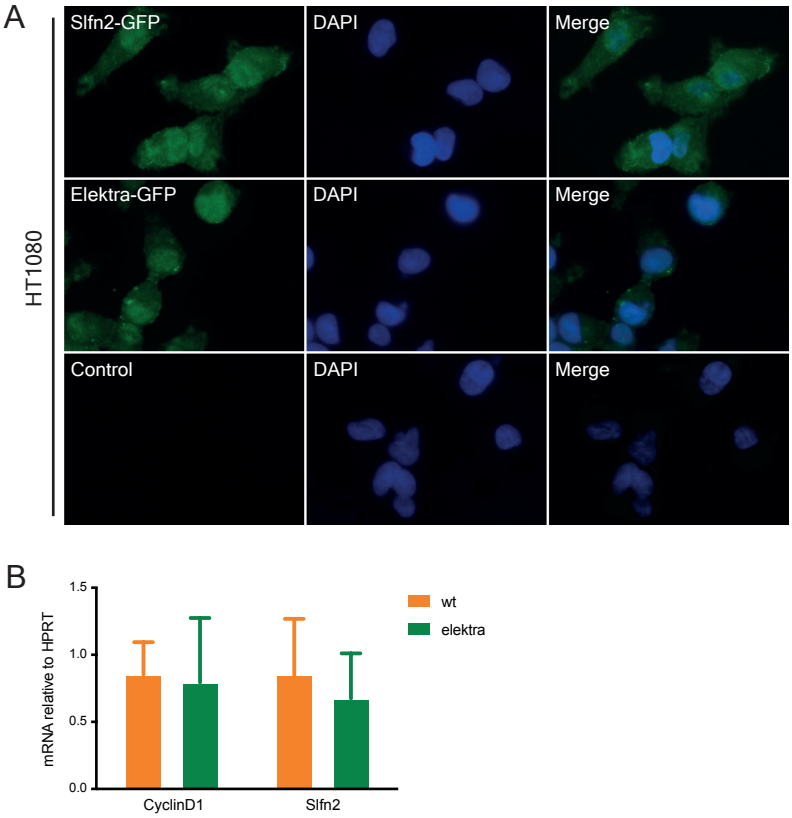
# Supplementary figure 1




A. Schematic of Slfn2 knock-down transplantation experiments. For optimal transduction efficiency in cell culture two wells of cells were transduced. Transduction efficiency (GFP) was analyzed by flow cytometry. Pooled cells were transplanted into lethally irradiated mice and analyzed by flow cytometry after 16-18 weeks.

B. Engraftment of GFP+ donor cells in peripheral blood (PB) 16-18 weeks after transplantation of transduced cells (n=9). The difference seen is near-significant at p=0.059.

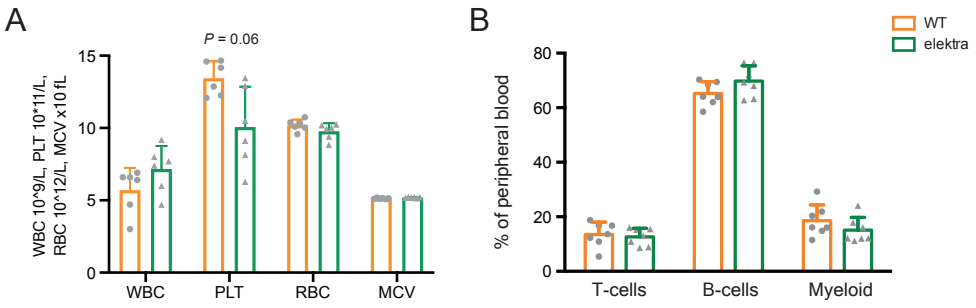
## Supplementary Figure 2




A. Immunofluorescence signal in HT1080 cells following transfection with plasmids over-expressing GFP-conjugated Slfn2 or Elektra. DAPI staining used to localize nuclei.

B. mRNA levels of *CyclinD1* and *Slfn2*, assessed by qPCR on LT-HSCs (LSK CD48- CD150+ CD9-high) purified by FACS (n=3).

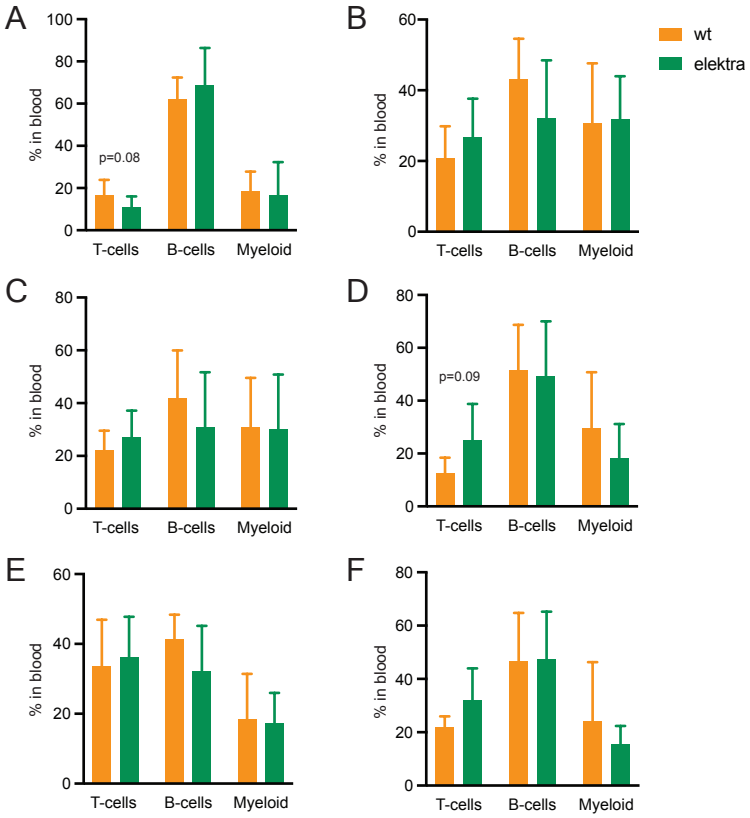
# Supplementary Figure 3




A. Sysmex analysis of peripheral blood from steady state mice. Graph depicts levels of white blood cell (WBC), platelets (PLT), red blood cells (RBC), and mean cell volume (MCV) (n=6). Platelet analysis may be underpowered, i.e. future studies should add more mice to this analysis.

B. Flow cytometry analysis of peripheral blood from steady state mice at 12 weeks of age (n=6).

# Supplementary Figure 4

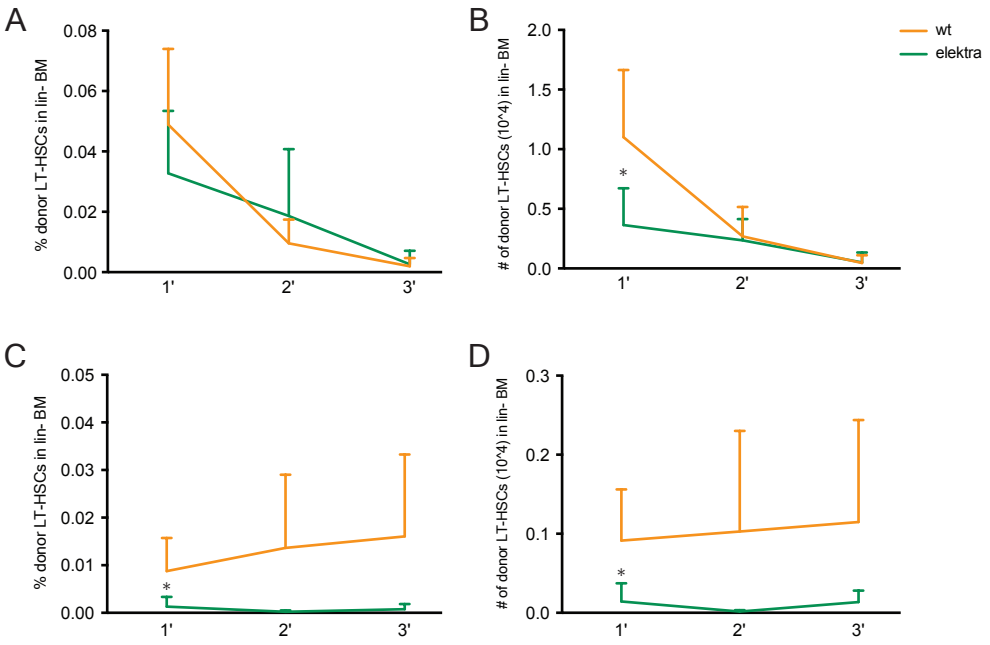



Lineage repopulation in blood of transplanted mice.

A-C. Primary (A), secondary (B), and tertiary (C) recipient mice transplanted with whole bone marrow.

D-F. Primary (D), secondary (E), and tertiary (F) recipient mice transplanted with sorted long term HCSs.

## Supplementary Figure 5



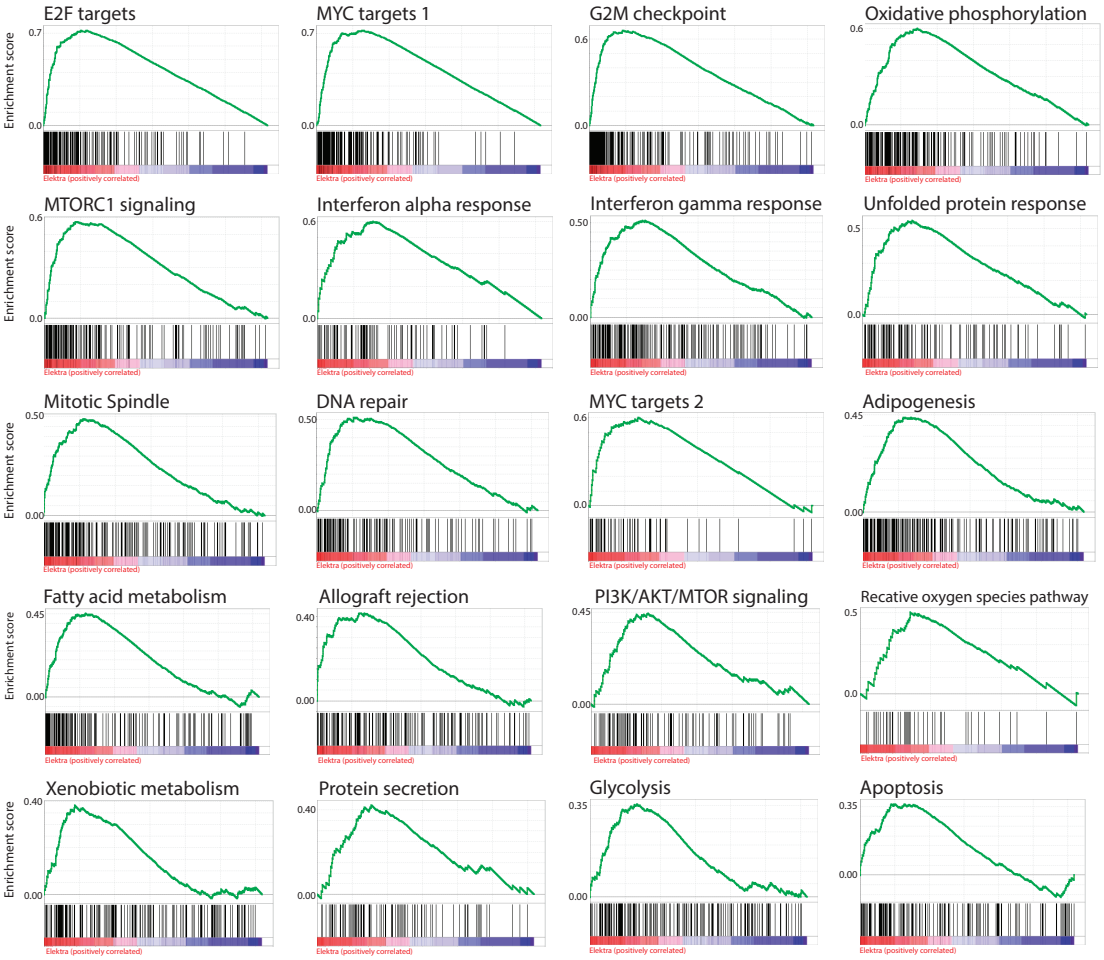

A-B. Flow cytometry analysis of engrafted LT-HSCs (LSK CD34- Flt3-) shown as frequency of lineage negative BM cells (A) or absolute numbers of LT-HSCs in lineage negative BM (one femur) (B). Analysis 16-18 weeks post-transplantation in BM of mice transplanted with whole BM (n=5-8). Iterative Grubb's test used for outlier identification and removal in dataset #LTHSC 1' (B); two outliers were identified and removed among technical replicates.

C-D. Flow cytometry analysis of engrafted LT-HSCs (LSK CD48- CD150+) shown as frequency of lineage negative BM cells (A) or absolute numbers of LT-HSCs in lineage negative BM (one femur) (B). Analysed 16-18 weeks post-transplantation in BM of mice transplanted with sorted HSCs (n=4-5).

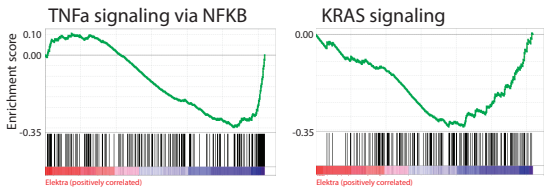
Primary, secondary, and tertiary recipients indicated with 1', 2', and 3' respectively. * $p < 0.05$

# Supplementary Figure 6

Results of the gene set enrichment analysis of the LT-HSC microarray data.



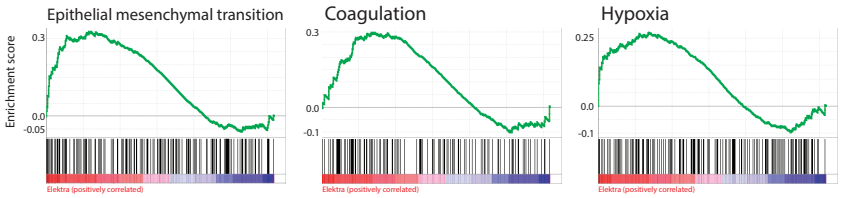

Plots are showing the 20 most significant gene sets upregulated in elektra LT-HSCs (LSK CD9hi CD48- CD150+). In total 28 gene sets were significantly enriched ($p<0.05$) in elektra LT-HSCs; aside from the above also P53 pathway, peroxisome, bile acid metabolism, IL2/STAT5 signaling, spermatogenesis, inflammatory response, heme metabolism, and complement.



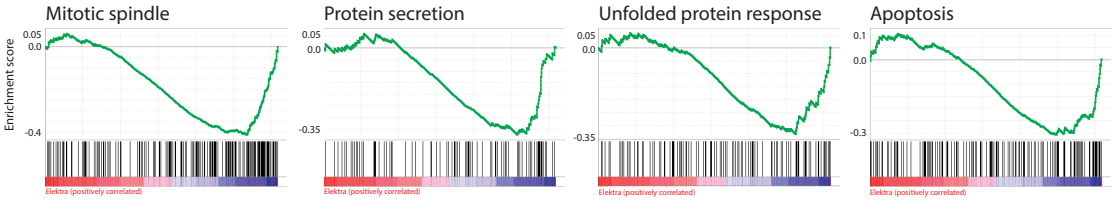

Plots are showing the two significantly downregulated gene sets in elektra LT-HSCs (LSK CD9hi CD48- CD150+).

# Supplementary Figure 7

Results of the gene set enrichment analysis of the MPP microarray data.




Plots are showing the three significantly (p<0.05) upregulated gene sets in elektra MPPs (LSK CD9hi CD48- CD150-).




Plots are showing the four significantly downregulated gene sets in elektra MPPs (LSK CD9hi CD48- CD150-).

# Supplementary figure 8

A

CCTGGACTTCACGGAGAGCGAAGGCTACATTTATA**T**CTACGTGAAATCGT

↓

**A** Slfn2 elektra allele

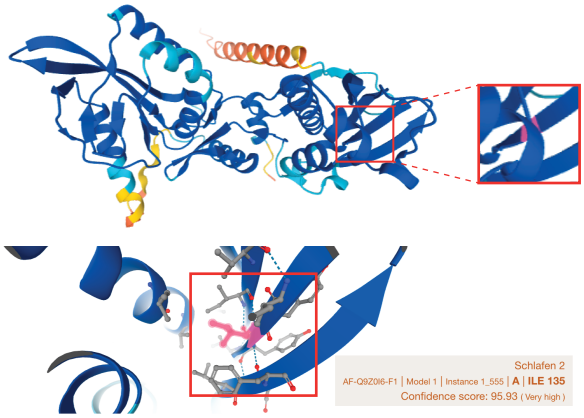
B

FTESEGYIY**I**YVKSWSQEIF

↓

**N** Slfn2 elektra protein

C

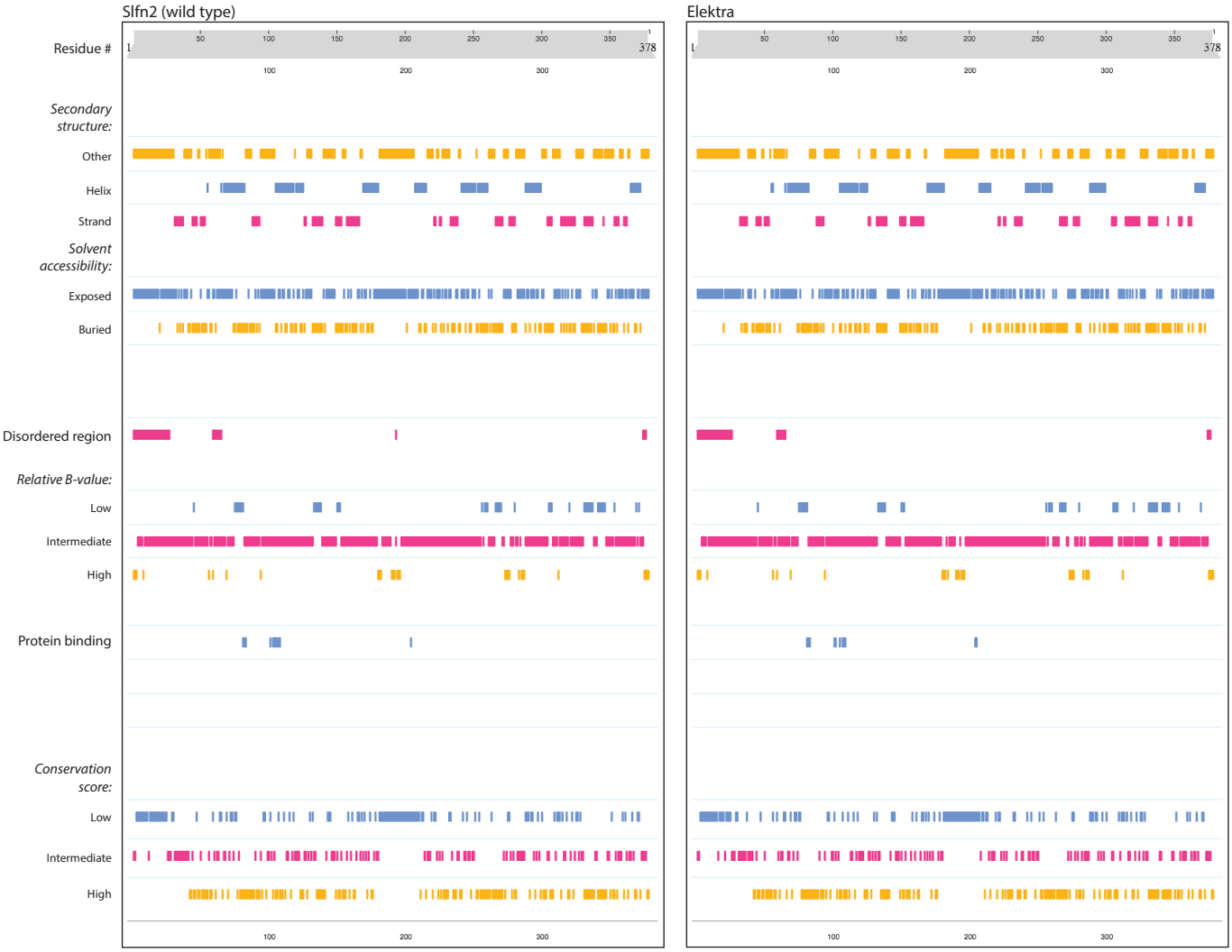



A. Nucleotides 551-600 in the Slfn2 gene and schematic of the single point mutation of the elektra allele.

B. Amino acid residues 125-145 of the Slfn2 protein and the isoleucine-to-asparagine substitution at residue 135 that is caused by the elektra mutation. The protein contains a total of 278 amino acids.

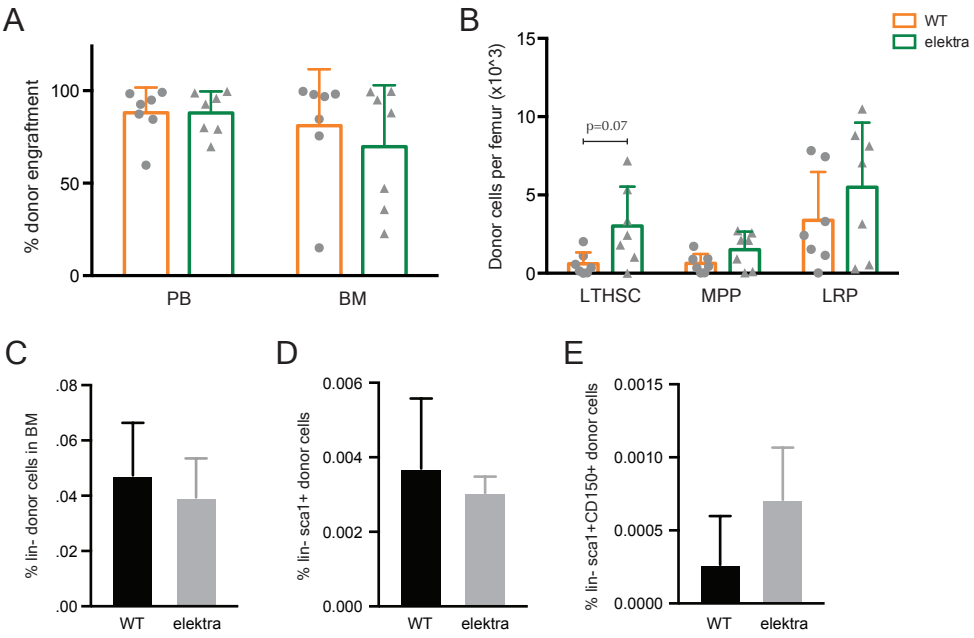
C. Secondary structure of wild type Slfn2 protein. Colour indicates model confidence. Dark blue sections have a very high model confidence with a per residue confidence score of 90-100. Light blue sections are confident (score of 70-90), yellow sections low (score 50-70) and orange very low (score <50). Marked pop-out box indicates location of residue 135. This model was made using the AlphaFold tool.

# Supplementary figure 9




Protein structure and function prediction calculated using the PredictProtein tool. Secondary structure is changed around residues (approximately) 30-60, 170-190, and 350-360. Solvent accessibility is altered around residues (approximately) 30-60, 90-110, and 130-150. A disordered region around residues 190-195 in the wild type protein is no longer viewed in the elektra prediction. Protein binding is altered around residues (approximately) 100-110 and 200-210. Potential alterations in conservation score and relative B-value have not been studied in detail but are depicted above.

# Supplementary Figure 10




Reverse transplantations:

A. Immunophenotypic analysis of CD45.1/CD45.2 in peripheral blood (PB) and bone marrow (BM) showing equal WT cell engraftment in elektra and WT littermate recipients (16 weeks after reverse transplant) (n=7).

B. Flow cytometry data showing engrafted numbers of LSK CD150/CD48 HSPCs in reverse transplanted mice (n=7). LTHSC = LSK CD150+CD48-; MPP = LSK CD150-CD48-; LRP = LSK CD150-CD48+ cells.

Analysis of homing:

C-E. Flow cytometry data showing frequency of donor cells in BM 24h after transplantation. Data is from one experiment, n=3.