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

Sarah Warsi,[1,2] Maria Dahl,[1] Emma M. K. Smith,[1] Anna Rydström,[1] Els Mansell,[1] Valgardur Sigurdsson,[1] Julia Sjöberg,[1] Shamit Soneji,[3,4] Emma Rörby,[1] Kavitha Siva,[1] Tan H. M. Grahn,[1] Yang Liu,[1] Ulrika Blank,[1] Göran Karlsson[3] and Stefan Karlsson[1]

[1]Division of Molecular Medicine and Gene Therapy, Lund Stem Cell Center, Lund University; [2]Skåne University Hospital, Region Skåne; [3]Division of Molecular Hematology, Lund Stem Cell Center, Lund University and [4]Lund University Bioinformatics Core, Lund Stem Cell Center, Lund University, Lund, Sweden

Correspondence: S. Warsi
sarah.warsi@med.lu.se

Received: August 22, 2021.
Accepted: May 18, 2022.
Prepublished: May 26, 2022.

https://doi.org/10.3324/haematol.2021.279799

Abstract

Even though hematopoietic stem cells (HSC) are characterized by their ability to self-renew and differentiate, they primarily reside in quiescence. Despite the immense importance of this quiescent state, its maintenance and regulation is still incompletely understood. Schlafen2 (Slfn2) is a cytoplasmic protein known to be involved in cell proliferation, differentiation, quiescence, interferon response, and regulation of the immune system. Interestingly, Slfn2 is highly expressed in primitive hematopoietic cells. In order to investigate the role of Slfn2 in the regulation of HSC we have studied HSC function in the elektra mouse model, where the elektra allele of the *Slfn2* gene contains a point mutation causing loss of function of the Slfn2 protein. We found that homozygosity for the elektra allele caused a decrease of primitive hematopoietic compartments in murine bone marrow. We further found that transplantation of elektra bone marrow and purified HSC resulted in a significantly reduced regenerative capacity of HSC in competitive transplantation settings. Importantly, we found that a significantly higher fraction of elektra HSC (as compared to wild-type HSC) were actively cycling, suggesting that the mutation in Slfn2 increases HSC proliferation. This additionally caused an increased amount of apoptotic stem and progenitor cells. Taken together, our findings demonstrate that dysregulation of Slfn2 results in a functional deficiency of primitive hematopoietic cells, which is particularly reflected by a drastically impaired ability to reconstitute the hematopoietic system following transplantation and an increase in HSC proliferation. This study thus identifies Slfn2 as a novel and critical regulator of adult HSC and HSC quiescence.

Introduction

Hematopoietic stem cells (HSC) typically reside in a dormant state in the bone marrow (BM). They are kept under tight regulation by both intrinsic factors and extrinsic factors provided by surrounding cells in the BM niche,[1] affecting the various fate options of HSC, such as quiescence, self-renewal, and differentiation. The longevity of adult HSC is in large due to their maintenance in a quiescent (G_0) state.[2] Several factors regulate entry into the proliferating G_1 phase (followed by S/G_2/M phases), including Cyclin-Cdk complexes, the Ink4 proteins (e.g., p15, p16, p18), as well as CIP/KIP family members (p21, p27, p57) and their regulators such as p53.[2] It has been suggested that HSC quiescence correlates with repopulation potential and self-renewal capacity.[3] Considerable work remains in the long-term aim of mapping the HSC regulatory network, in particular regarding the regulation of quiescence.

The Schlafen (Slfn) proteins are a largely uncharacterized protein family with roles in cell proliferation, differentiation, immune system regulation, and interferon (IFN) response.[4-7] Slfn2 belongs to subgroup I, the shortest of the Slfn proteins, and has in cell lines been shown to localize exclusively in the cytoplasm.[8] In previous studies, the function of Slfn2 has primarily been characterized in cell lines and to a lesser extent in immune cells. Recent work identified Slfn2 as a critical regulator of T-cell quiescence and apoptosis. Using a mouse model containing a loss of function point mutation in the *Slfn2* gene (the so called elektra allele, which renders an isoleucine-to-asparagine substitution of residue 135 of the 378 amino acid protein), it was shown that elektra homozygous T cells proliferated excessively and underwent apoptosis upon cytokine-me-

diated activation.[9] Additionally, small interfering RNA (siRNA)-mediated knockdown (KD) of *Slfn2* in Sca1$^+$ mouse BM cells enhances proliferation in colony formation assays.[5] Slfn2 is also known to be expressed in a primitive self-renewing hematopoietic cell line, and its expression is downregulated when these cells are differentiated to various lineages.[10]

Cell proliferation, differentiation, and quiescence are important features for HSC regulation, where quiescence is a fundamental characteristic of HSC. Due to the decrease in expression of Slfn2 with hematopoietic cell differentiation, and the apparent link between Slfn2 and quiescence, we hypothesized that Slfn2 has a role in the regulation of HSC. In order to investigate the role of Slfn2 *in vivo*, we made use of the previously characterized elektra mouse model.[9] Interestingly, we can show that elektra HSC have a severely impaired capacity to regenerate the hematopoietic system of irradiated mice and elektra hematopoietic stem and progenitor cells (HSPC) are in a state of excessive cycling and apoptosis. Our study thus identifies Slfn2 as a critical regulator of adult murine HSC quiescence and function.

Methods

Ethical approval

All animal experimental procedures were approved by the regional Animal Ethical Committee in Lund.

Flow cytometry and fluorescense-activated cell sorting

Flow cytometry analyzes were performed on a Beckton Dickinson CantoII or custom order LSRII at the Lund University FACS core. Sorting was performed on a custom order AriaII or AriaIII. A list of antibodies is provided in the *Online Supplementary Table S1*. HSPC compartments were defined by using markers for lineages (Ter119, B220, CD3, Mac1, Gr1), Sca1, and c-kit, with CD34/Flt3 or CD150/CD48 and CD9.[11] Progenitor analyzis was done as previously described.[12] Apoptotic cells were defined using AnnexinV and cell cycle analysis was done by intracellular staining for Ki67/DAPI (Molecular Probes, Invitrogen). For cell cycle analysis, cells were fixed and permeabilized using 0.4% formaldehyde and 0.2% Triton-X. Flow cytometry data was analyzed in FlowJo (TreeStar software). In this study, analysis of primitive hematopoietic cells was done using LSK (Lineage$^-$Sca1$^+$c-kit$^+$) with either CD34/Flt3 or CD150/CD48. In order to distinguish between these analyzed populations of primitive hematopoietic cells we use the following terminology. LSK CD34$^-$Flt3$^-$ stem cells are called CD34-LTHSC (long-term HSC) or defined by markers if only abbreviated LTHSC. Other populations in this analysis modality are short-term HSC (ST-HSC; LSK CD34$^+$Flt3$^-$) and lymphoid-primed multipotent progenitors (LMPP; LSK CD34$^+$Flt3$^+$). LSK CD150$^+$CD48$^-$ stem cells are called SLAM-LTHSC or defined by markers if only abbreviated LTHSC. Other populations in this modality are MPP (LSK CD150$^-$ CD48$^-$) and lineage restricted progenitor (LRP; LSK CD150$^-$ CD48$^+$). The abbreviation HSC is also used as a broader term for hematopoietic stem cells regardless of cell surface markers.

Transplantation assays

Recipient mice (C57Bl/6 x B6SJL, CD45.1/45.2) were lethally irradiated (900 cGy or 2x 500 cGy) 16-24 hours (h) prior to transplant. Whole BM competitive transplants contained 200,000 elektra (or wild-type [WT] littermate) BM cells (C57Bl/6, CD45.2) as well as 200,000 WT (B6SJL, CD45.1) competitor BM cells. Transplants of purified HSC contained 20 sorted elektra/littermate LSK CD9^{high}CD48$^-$CD150$^+$ LTHSC and 200,000 competitor cells. Donor cells were identified (in flow cytometry analysis) using the markers CD45.1/45.2. Mice were bled and sacrificed at 16-18 weeks post-transplantation for end point analyses and serial transplantation. Secondary recipients received 2 million cells from primary recipients, and tertiary recipients received 20 million cells. Reverse transplantation assay was performed by transplanting 200,000 whole BM cells (WT B6SJL; CD45.1) into lethally-irradiated elektra mice or littermates (C57Bl/6; CD45.2). Homing analysis was done by transplantation of 20 million whole BM cells with analysis of recipient BM after 24 h. All transplants were done through intravenous bolus injection into the tail vein.

Knockdown of *Slfn2*

For knockdown (KD) of *Slfn2*, lentiviral plasmid pGFP-C-shLenti containing short hairpin RNA (shRNA) targeting *Slfn2* or scrambled (Scr) shRNA (OriGene) was used to produce lentiviral particles at the Stem Cell Center Vector Core Facility (Lund University). C-kit-enriched BM cells from WT mice were placed into virus-loaded plates at an MOI of 50 and incubated overnight (37°C, 5% CO2). Transduced cells were collected and transplanted into lethally-irradiated recipient mice. An aliquot of cells was cultured for flow cytometry analysis of transduction efficiency after 48 h. Experiment schematic is outlined in the *Online Supplementary Figure S1A*. BM of transplanted animals was analyzed at 16-18 weeks after transplantation. Recipient and donor cells were discriminated using the markers CD45.1 *versus* CD45.2.

Additional methods are included in the *Online Supplementary Appendix*.

Results

Slfn2 is highly expressed in long-term hematopoietic stem cells

In order to assess the expression levels of Slfn2 in HSPC

compartments, quantitative polymerase chain reaction (qPCR) analysis was performed on WT murine BM. We found that CD34-LTHSC (LSK CD34$^-$Flt3$^-$) had higher expression of *Slfn2* than short-term HSC (ST-HSC; LSK CD34$^+$Flt3$^-$) and lymphoid-primed multipotent progenitors (LMPP; LSK CD34$^+$Flt3$^+$) (Figure 1A) and hypothesized that Slfn2 has a role in regulation of LTHSC specifically. We also saw a high expression in Lin+ cells (Figure 1A), which can be explained by the role of Slfn2 in lymphocytes and lymphocyte progenitors. Our data is in line with findings presented in the online gene expression tool BloodSpot.[13]

The elektra point mutation does not affect mRNA level or subcellular localization

As previous studies have shown that Slfn2 localizes exclusively in the cytoplasm in cell lines,[8] we investigated whether this was the case also for its mutated form elektra. Myc-tagged *Slfn2* and elektra were overexpressed in transfected HT1080 cells, which were then stained using anti-Myc and DAPI and analyzed for fluorescent signal. In contrast to previous findings, we saw that both Slfn2 and elektra localize to both the cytoplasm and nucleus in the above cell line (*Online Supplementary Figure S2A*).

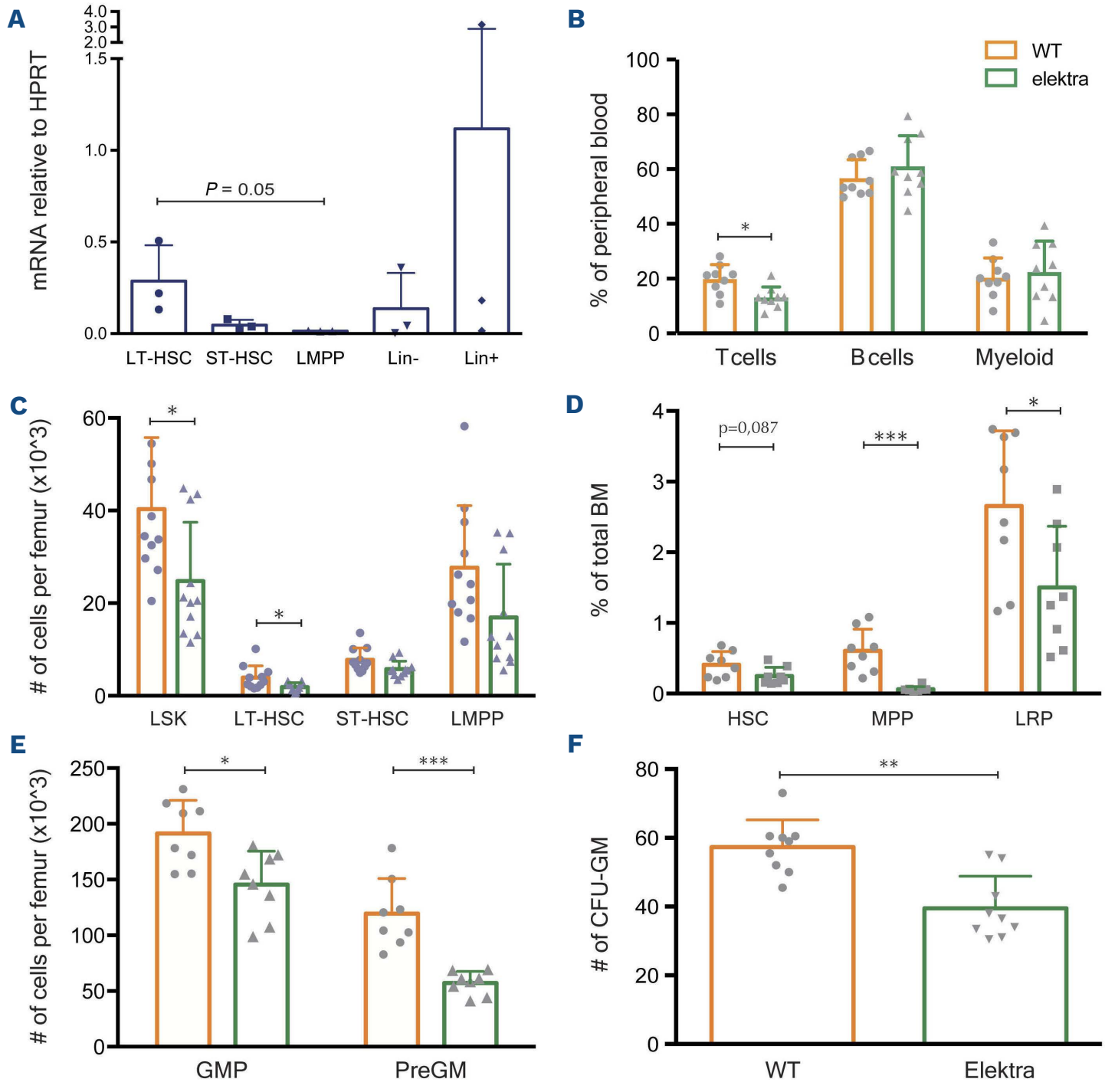


Figure 1. A mutation in *Slfn2* perturbs steady state hematopoietic parameters. (A) Quantitative polymersase chain reaction (qPCR) data showing that *Slfn2* is expressed at a higher level in long-term hematopoietic stem cells (LTHSC) (LSK CD34$^-$Flt3$^-$) than in short-term hematopoietic stem cells (ST-HSC) (LSK CD34$^+$Flt3$^-$) or lymphoid-primed multipotent progenitors (LMPP) (LSK CD34$^+$Flt3$^+$) (n=3 per group). (B) Mice homozygous for the elektra allele have decreased T cells and otherwise normal steady state hematopoiesis, as measured by flow cytometry analysis of lineage markers CD3, B220, and Mac1/Gr1 (n=6-7). (C and D) Elektra bone marrow (BM) shows reduced immunophenotypic hematopoietic stem and progenitor cells (HSPC) fractions (n=7-11), analyzed by both LSK CD34/Flt3 (C) and CD48/CD150 (D) expression. (E) There is a reduced number of granulocyte-macrophage progenitors (GMP) (L$^-$S$^-$K$^+$ CD41$^-$FcR$^+$CD150$^-$) and PreGM (L$^-$S$^-$K$^+$ CD41$^-$FcR$^-$CD150$^-$CD105$^-$) cells in elektra mice (n=7-8). (F) Elektra BM cells show reduced colony formation capacity in CFU-GM assays (n=6-9). Steady state analyses were also done in mice heterozygous for the elektra allele; these data are not shown, as they did not differ significantly compared to wild-type (WT) mice.

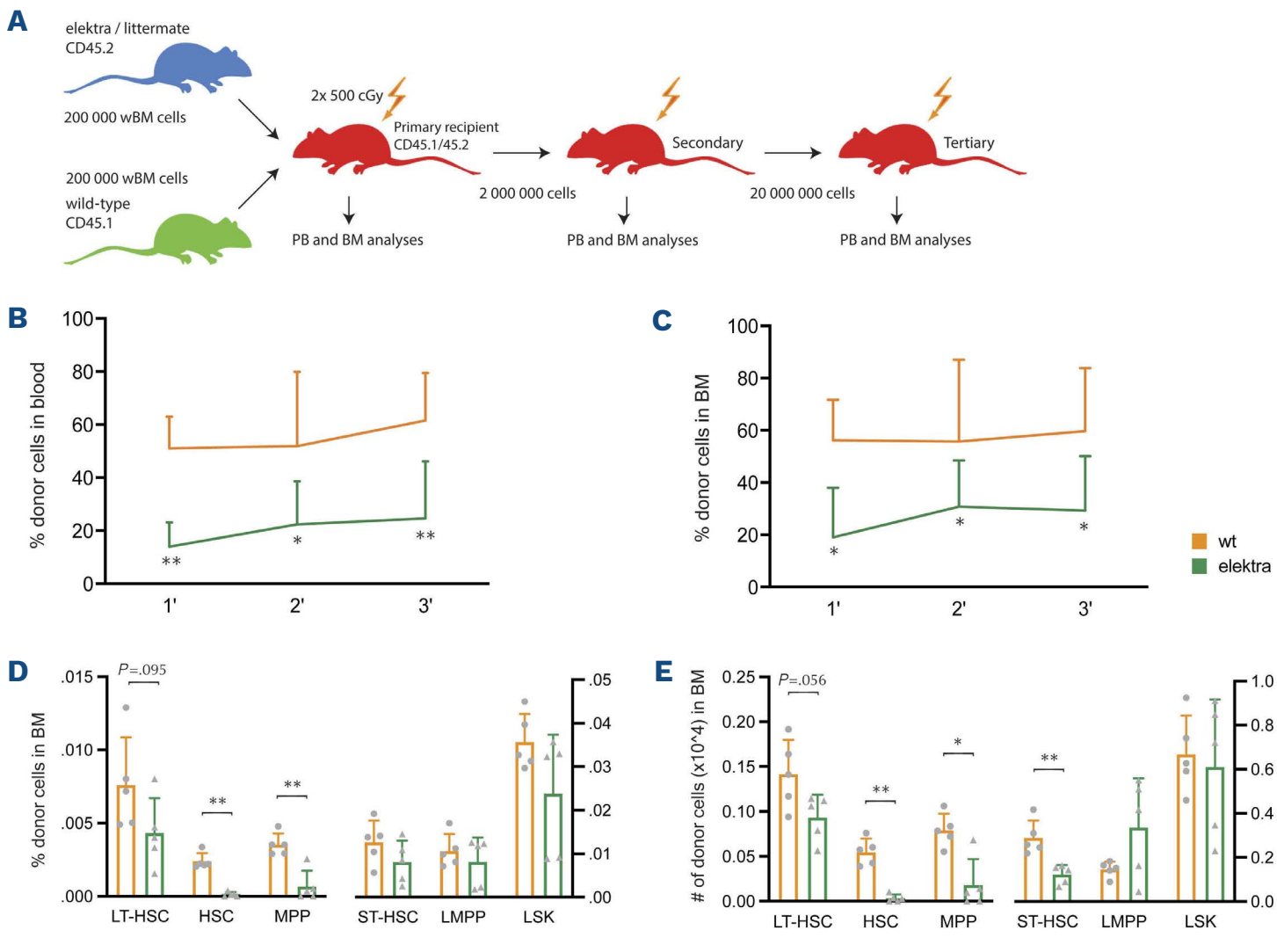
Previous research also suggests a possible, though debated, connection between the Schlafen proteins and CyclinD1.[14,15] Quantitative PCR analysis of mRNA in *Slfn2* WT or deficient (elektra) murine SLAM-LTHSC (LSK CD48$^-$ CD150$^+$) showed no difference in levels of *CyclinD1* mRNA. We further confirmed that the *Slfn2* mRNA levels are not changed, i.e., the elektra mutation does not cause degradation or dysregulation at the mRNA level (*Online Supplementary Figure S2B*).

Slfn2 deficiency causes perturbations in steady state hematopoietic parameters

In order to investigate the effect of Slfn2 on steady state hematopoietic parameters we performed flow cytometry analyses on blood and BM of elektra mice. Mice homozygous for the elektra allele had unaltered steady state blood parameters (Figure 1B; *Online Supplementary Figure S3A*), except for a significant reduction in T cells (Figure 1B) at 8 weeks of age. This was, however, not observed in blood of 12-week-old mice (*Online Supplementary Figure S3B*) or in BM (data not shown). We found reduced HSPC compartments in BM, with a significant reduction in the LSK and LMPP fraction (Figure 1C) and when staining for SLAM markers we similarly saw a reduction in the multipotent progenitor (MPP) fraction (Figure 1D). We also found that elektra mice had a significantly lower number of granulocyte-macrophage progenitors (GMP) and PreGM cells (Figure 1E). Accordingly, when assessing progenitor colony formation in methylcellulose (CFU-GM) assays there was a reduced colony-forming activity in elektra BM cells (Figure 1F). Heterozygous mice did not show any significant differences in steady state hematopoietic parameters (data not shown). Together, our data show that dysregulation of Slfn2 causes perturbed steady state hematopoietic parameters in BM, such as reduced HSPC compartments and colony-forming activity.

Reduced regenerative capacity of hematopoietic stem cells following loss of Slfn2 function

In order to assess the role of Slfn2 in long-term repopulation potential of HSC we performed serial transplantations of whole BM as well as of purified SLAM-LTHSC (LSK CD9^{high}CD150$^+$CD48$^-$) (schematic in Figures 2A and 3A). Following competitive whole BM transplantation into irradiated recipient mice, we found that elektra homozygosity causes a significant and robust reduction in reconstitution of blood and BM compared to WT littermates 16 weeks after transplantation (Figure 2B and C). There were no significant differences in lineage repopulation of blood (*Online Supplementary Figure S4A* to *C*). When analyzing reconstitution of HSPC compartments in primary recipients we observed a significant decrease in frequency of immunophenotypic LTHSC and MPP as defined by LSK expressing CD150 and CD105 (endoglin), but not based on



Continued on following page.

Figure 2. Elektra hematopoietic stem cells have reduced reconstitution following whole bone marrow transplantation. (A) Schematic overview of serial transplantation assays using whole bone marrow. (B and C) Whole bone marrow (BM) competitive transplantation assays with elektra BM cells showing reduced reconstitution capacity (blood and BM at 16 weeks, n=7-8). Primary, secondary, and tertiary transplantations are indicated by 1', 2', and 3' respectively. (D and E) Flow cytometry data showing frequency (D) and absolute numbers (per one femur) (E) of engrafted donor LSK cells, LSK CD34/Flt3 hematopoietic stem and progenitor cells (HSPC) (LTHSC, ST-HSC, LMPP), and LSK CD150/CD105 HSPC (HSC, MPP) in BM of primary recipients 16 weeks after whole BM competitive transplants (n=7-8).

expression of CD34 and Flt3 (Figure 2D). When comparing absolute numbers of engrafted HSPC we saw similar results, but with a near-significant decrease in CD34-LTHSC as well as a significant decrease in ST-HSC (Figure 2E). When following CD34-LTHSC engraftment through primary, secondary, and tertiary transplantations, we continued to find no significant differences in either frequency or number of engrafted WT *versus* elektra LTHSC (*Online Supplementary Figure S5A* and *B*).

The FACS gating strategy used for purifying HSC for transplantation is shown in Figure 3B. The above-described reduced regenerative capacity was recapitulated and exacerbated following transplantation of purified SLAM-LTHSC (Figure 3C and D), indicating that Slfn2 is essential for LTHSC specifically and that reconstitution levels following whole BM transplant is partially rescued by downstream progenitor cells. There were again no significant differences in lineage repopulation of blood (*Online Supplementary Figure S4D* and *F*). All primitive HSPC populations as defined by LSK with SLAM markers CD150 and CD48 were significantly reduced in both frequency and number in the BM of primary recipients (Figure 3E and F). When analyzing primitive cells by the LSK CD34 Flt3 immunophenotype we found a significant decrease in LSK cells (frequency and absolute numbers engrafted) as well as frequency of ST-HSC and engrafted number of LMPP (Figure 3G and H). Though LSK $CD48^{-}CD150^{+}$ LTHSC continued to show a clear decrease in secondary and tertiary recipients, the differences no longer reached statistical significance (*Online Supplementary Figure S5C* and *D*).

These data establish an essential role for Slfn2 in HSC transplantation, where loss of Slfn2 function leads to a dramatic decrease in engrafting LTHSC.

Gene expression analysis reveals elektra-induced changes in molecular programs important for hematopoietic stem cell function

In order to map the mechanism through which Slfn2 exerts its effect on HSC, we purified SLAM-LTHSC (LSK $CD9^{high}CD48^{-}CD150^{+}$) and performed microarray analysis. Differentially-expressed genes in elektra SLAM-LTHSC are depicted in the heat map in Figure 4A. Interestingly, both gene ontology analysis (DAVID) and gene set enrichment analysis (GSEA) rendered several clusters of enriched genes in elektra LTHSC, where many of these were associated with HSC fate decision-related processes like cell cycling, differentiation, and apoptosis (Table 1; *Online Supplementary Figure S6*).

As the MPP population was also decreased in frequency in elektra homozygous mice (Figure 1D), we similarly purified LSK $CD150^{-}CD48^{-}$ cells for microarray analysis. The number of differentially-expressed genes caused by the elektra mutation in MPP (visualized in the heat map in Figure 4B) was substantially lower compared to that in HSC and only very few genes were involved in functions related to primitive hematopoietic cells or self-renewal (*Online Supplementary Figure S7*). Together, this data supports a relevant function for Slfn2 in the most primitive HSC and less of a role in the downstream progenitor cells. However, note that these results may be affected by a high fraction of apoptotic MPP in elektra mice (see below) and future studies may need to investigate differentially-expressed genes specifically in the subpopulation consisting of apoptotic MPP.

Increased cycling and apoptosis of Slfn2-deficient hematopoietic stem cells

The previously established role of Slfn2 in cell proliferation together with our microarray findings indicate that dysregulation of Slfn2 would have an effect on apoptosis and cell cycle status of HSC. Indeed, flow cytometry analysis of Ki67 expression and DAPI staining in LSK SLAM compartments (Figure 5A) showed that elektra HSPC had an increased fraction of cycling cells. We found a significantly lower fraction of cells in G_0 in LSK, MPP, and LRP (LSK $CD150^{-}CD48^{+}$) compartments, as well as a higher fraction of G_1 cells in the SLAM-LTHSC and MPP compartments (Figure 5B to E). In order to determine the apoptotic status of elektra HSPC we similarly analyzed Annexin V expression in LSK SLAM populations (Figure 5A), and found a significantly higher fraction of apoptotic HSC and MPP (Figure 5F). In order to further assess the effect of Slfn2 on HSPC cycling, we investigated recovery after treatment with the myelotoxic agent 5FU. In accordance with the already increased fraction of cycling elektra SLAM-LTHSC, 10-11 days after a 0.15 mg/g dose of 5FU, two of three elektra mice died while all WT mice survived (Figure 5G). We also found that elektra mice treated with 5FU could be rescued by a subsequent transplantation of fresh WT BM cells (Figure 5G). Finally we analyzed *Slfn2* gene expression 2 days after 5FU injection, where *Slfn2* is found to be upregulated (Figure 5H). This establishes a role for Slfn2 in HSC stress response.

Taken together, our data shows excessive cycling and apoptosis of cells with dysregulated Slfn2. We also find

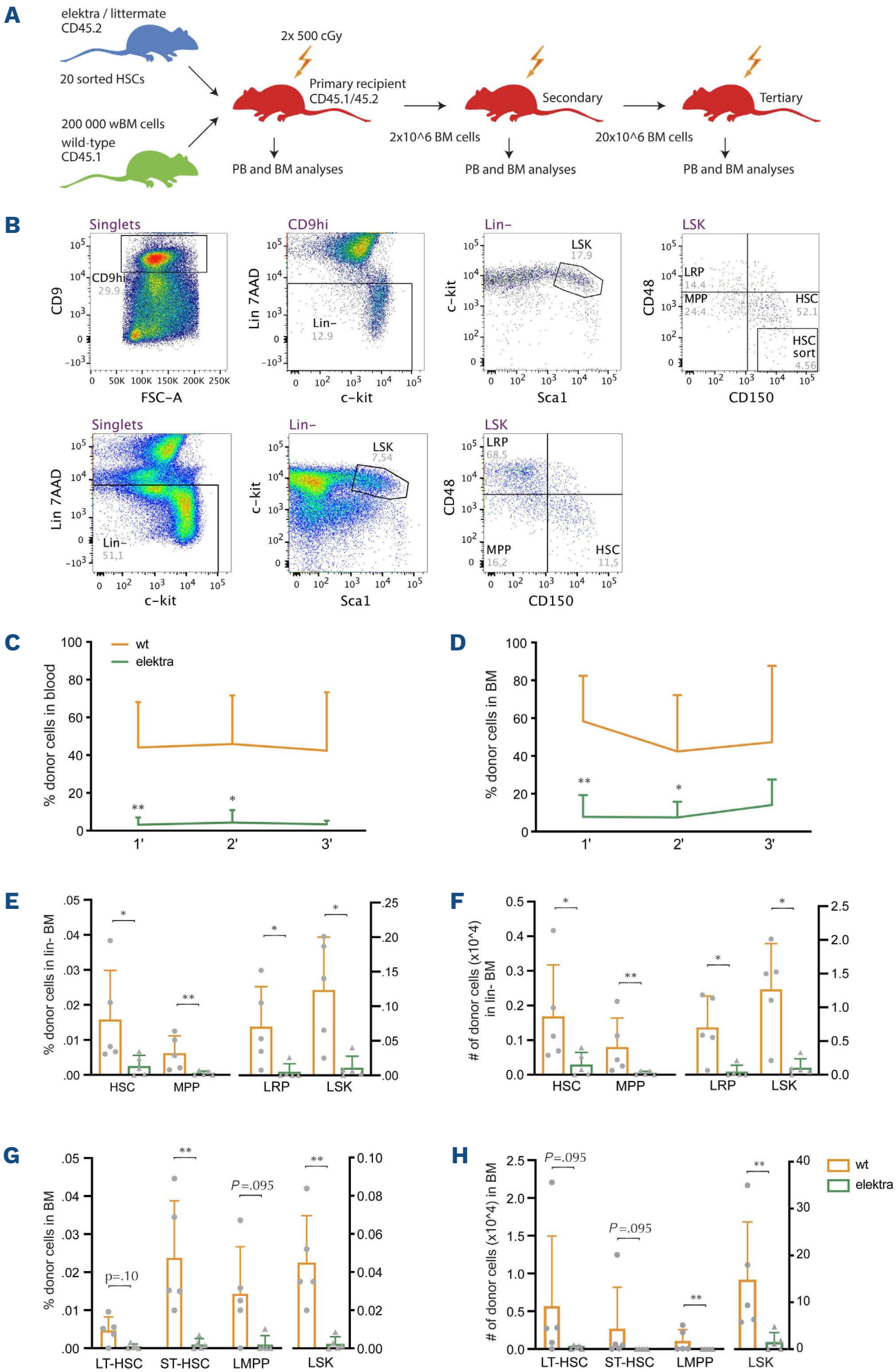


Figure 3. Elektra hematopoietic stem cells have reduced reconstitution following purified SLAM-long-term hematopoietic stem cell transplantation. (A) Schematic overview of serial transplantation assays where primary recipient mice were transplanted with sorted hematopoietic stem cells (HSC). (B) Schematic overview of FACS gating strategy. Upper panel shows gating when sorting long-term hematopoietic stem cells (LTHSC) (LSK CD48^{-}CD150^{+}CD9^{high}). Wider gates are based on FMO (for comparison), but to obtain a pure sorted population a much stricter gate is set. Lower panel shows, for comparison, the same specimen in conventional analysis gating (when CD9^{high} gating is not used). (C and D) Reduced reconstitution following transplantations of sorted elektra LTHSC (blood and bone marrow [BM] at 16 weeks, n=4-5). Primary, secondary, and tertiary transplantations are indicated by 1', 2', and 3' respectively. (E and F) Frequency (E) and absolute numbers (per one femur) (F) of engrafted donor LSK CD48/CD150 hematopoietic stem and progenitor cells (HSPC) in BM of primary recipients 16 weeks after sorted HSC competitive transplants (n=4-5). (G and H) Frequency (G) and absolute numbers (per one femur) (H) of engrafted donor LSK CD34/Flt3 HSPC in BM of primary recipients 16 weeks after sorted HSC competitive transplants (n=4-5).

Slfn2 upregulated following 5FU treatment *in vivo*, and decreased survival of 5FU-treated elektra homozygous mice.

Slfn2 knockdown is similar to *Slfn2* elektra, a loss of function mutation

The elektra mutation is considered a Slfn2 loss-of-function.[9] However, since both Slfn2 elektra mRNA and protein levels are readily detected in elektra mice, we further investigated the effect of loss of *Slfn2* using shRNA KD transplantation experiments. Here, ckit-enriched WT BM cells underwent viral transduction with either a *Slfn2*-KD or a Scr shRNA vector prior to transplantation. The transduction efficiency at transplantation was significantly higher for the *Slfn2*-KD vector (Figure 6A). Despite this, 16 weeks post-transplantation we found a trend towards decreased engraftment of *Slfn2*-KD cells in most analyzed

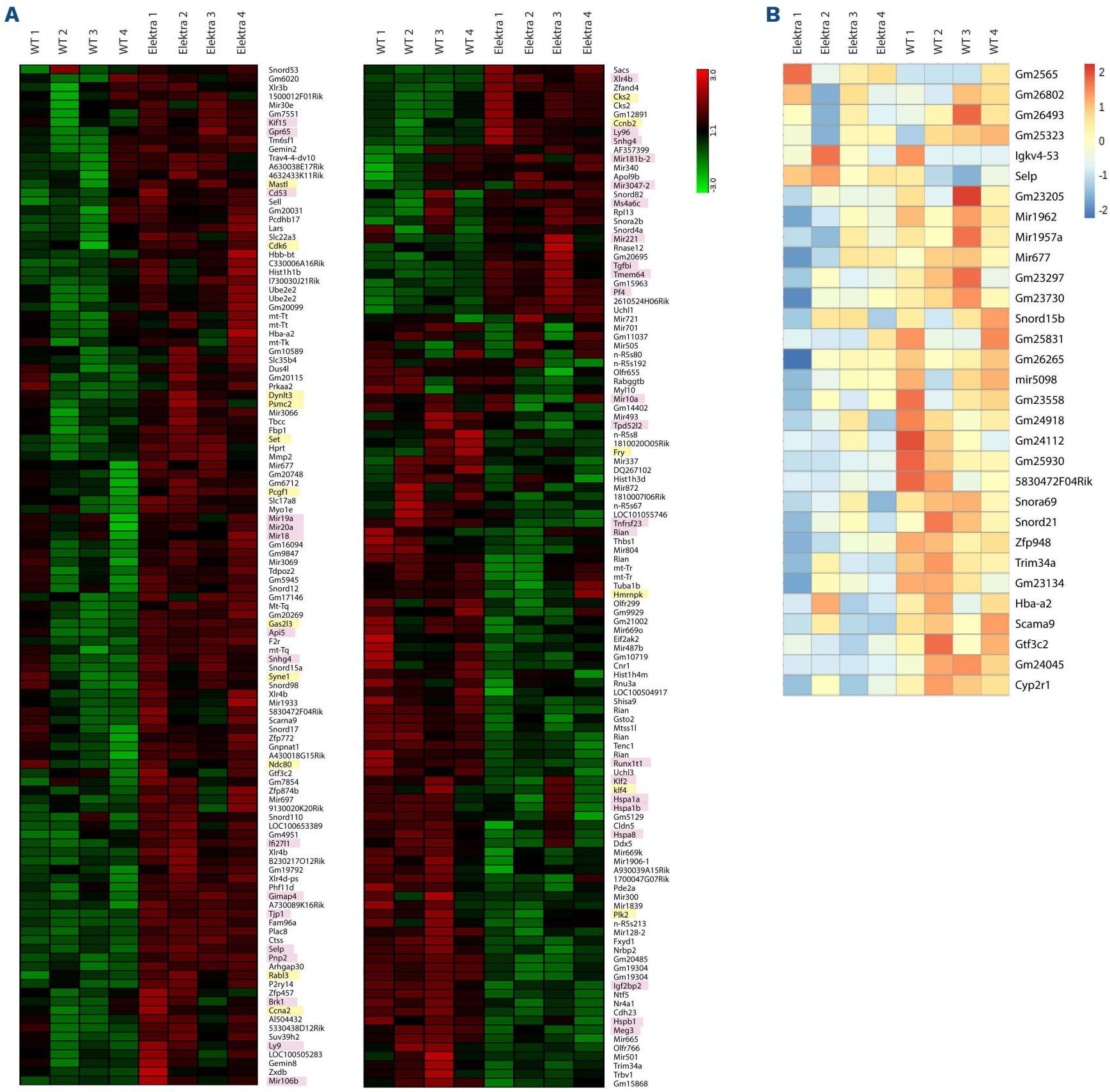


Figure 4. Microarray of elektra hematopoietic stem cells shows several differentially expressed genes. (A) Heat map showing differentially expressed genes in elektra long-term hematopoietic stem cells (LTHSC) (LSK CD9^{hi}CD48^{-}CD150^{+}) in comparison to wild-type (WT) LTHSC. A select number of genes of interest are indicated: yellow highlights genes associated with cell proliferation, cell division, and/or stem cell functions. Pink highlights genes associated with hematopoiesis, stem cell functions, stress response, inflammatory response and/or leukemia. (B) Heat map of differentially expressed genes in elektra multipotent progenitors (MPP) (LSK CD48^{-}CD150^{-}) in comparison to WT MPP, resulting in a much shorter list.

Table 1. Gene ontology analysis of the microarray data shows several cell cycle-associated clusters upregulated in elektra long-term hematopoietic stem cells.

Category	Term	Enrichment score	*P*-value
Upregulated			
Annotation Cluster 1		3.00	
GOTERM_BP_DIRECT	GO:0071241~cellular response to inorganic substance		<0.001
GOTERM_CC_DIRECT	GO:0035068~micro-ribonucleoprotein complex		0.009
Annotation Cluster 2		2.63	
GOTERM_BP_DIRECT	GO:0001701~in utero embryonic development		<0.001
KEGG_PATHWAY	mmu05206:MicroRNA in cancer		<0.001
GOTERM_BP_DIRECT	GO:0002329~pre-B-cell differentiation		0.001
GOTERM_BP_DIRECT	GO:0001783~B-cell apoptotic process		0.001
GOTERM_BP_DIRECT	GO:0021522~spinal cord motor neuron differentiation		0.006
GOTERM_BP_DIRECT	GO:0060412~ventricular septum morphogenesis		0.011
Annotation Cluster 3		1.77	
GOTERM_BP_DIRECT	GO:0051301~cell division		0.004
GOTERM_BP_DIRECT	GO:0007049~cell cycle		0.013
GOTERM_BP_DIRECT	GO:0007067~mitotic nuclear division		0.027
Annotation Cluster 4		1.30	
GOTERM_BP_DIRECT	GO:0007049~cell cycle		0.013
Downregulated			
Annotation Cluster 1		2.26	
GOTERM_CC_DIRECT	GO:0005737~cytoplasm		0.043
Annotation Cluster 2		1.84	
KEGG_PATHWAY	mmu03040:Spliceosome		0.001
GOTERM_MF_DIRECT	GO:0031072~heat shock protein binding		0.006
GOTERM_MF_DIRECT	GO:0098641~cadherin binding involved in cell-cell adhesion		0.023
GOTERM_CC_DIRECT	GO:0005913~cell-cell adherens junction		0.030
GOTERM_CC_DIRECT	GO:0005925~focal adhesion		0.051
Annotation Cluster 3		1.80	
KEGG_PATHWAY	mmu03040:Spliceosome		0.001
KEGG_PATHWAY	mmu04010:MAPK signaling pathway		0.001
KEGG_PATHWAY	mmu05162:Measles		0.010
KEGG_PATHWAY	mmu05134:Legionellosis		0.016
KEGG_PATHWAY	mmu04141:Protein processing in endoplasmic reticulum		0.018
KEGG_PATHWAY	mmu05164:Influenza A		0.019
KEGG_PATHWAY	mmu04612:Antigen processing and presentation		0.031
KEGG_PATHWAY	mmu04915:Estrogen signaling pathway		0.043
KEGG_PATHWAY	mmu05145:Toxoplasmosis		0.049
Annotation Cluster 4		1.29	
GOTERM_CC_DIRECT	GO:0031012~extracellular matrix		<0.001
KEGG_PATHWAY	mmu03040:Spliceosome		0.001
GOTERM_MF_DIRECT	GO:0005515~protein binding		0.008
GOTERM_MF_DIRECT	GO:0044822~poly(A) RNA binding		0.009
GOTERM_BP_DIRECT	GO:0045444~fat cell differentiation		0.013
GOTERM_CC_DIRECT	GO:0005681~spliceosomal complex		0.034
GOTERM_BP_DIRECT	GO:0045893~positive regulation of transcription, DNA-templated	0.038	
GOTERM_MF_DIRECT	GO:0008134~transcription factor binding		0.038
GOTERM_CC_DIRECT	GO:0070062~extracellular exosome		0.051

List of gene clusters (gene ontology terms or KEGG pathway terms) upregulated or downregulated in elektra long-term hematopoietic stem cells (LTHSC, LSK $CD9^{hi}CD48^{-}CD150^{+}$) in comparison to wild-type (WT) LTHSC. This list was created from online analysis (using the tool DAVID) of our in-house generated list of genes up- and downregulated in the microarray of elektra LTHSC.

BM populations (Figure 6B-I) with a near significant decrease in engrafted GFP+ donor LTHSC (LSK CD34$^-$Flt3$^-$) (Figure 6I; *P*=0.0501). We also found a near significant decrease of engraftment of *Slfn2*-KD cells in blood (*Online Supplementary Figure S1B*). In conclusion, *Slfn2*-KD cells behave, in a transplantation setting, similarly to Slfn2 elektra cells, further supporting the hypothesis that Slfn2 is critical for HSC function.

The elektra isoleucine-to-asparagine substitution is predicted to be damaging

The elektra mutation is a so-called missense mutation where mutation of a single nucleotide leads to a single amino acid substitution at residue 135 of the 378 amino acid protein (*Online Supplementary Figure S8A* and *B*). Analysis of protein secondary structure using AlphaFold[16] shows that residue 135 is localized in a β sheet structure and is buried, not exposed (*Online Supplementary Figure*

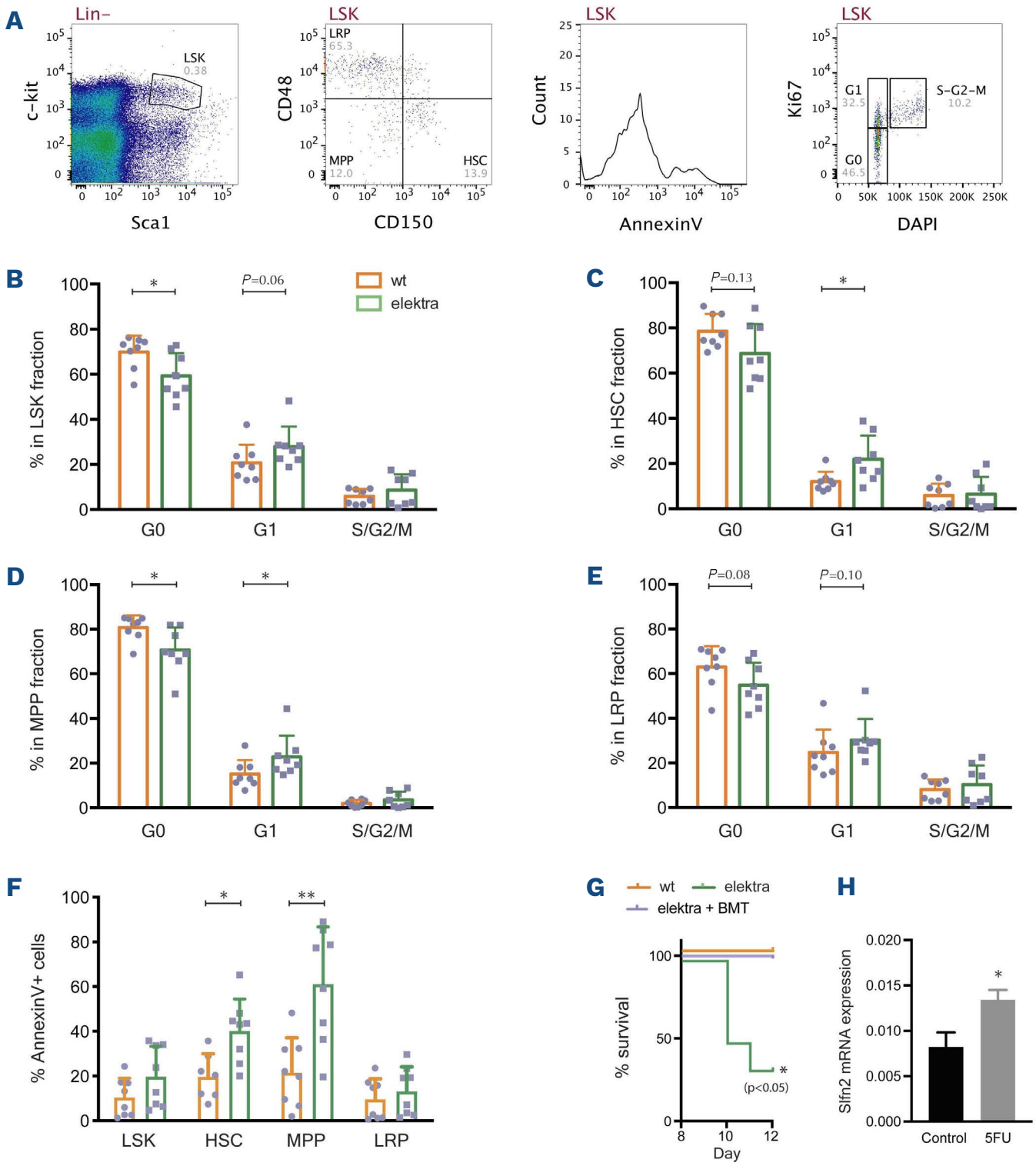


Figure 5. Elektra hematopoietic stem and progenitor cells have increased cycling and apoptosis. (A) Schematic overview of LSK CD48/CD150 HSPC compartments with Annexin V staining for apoptotic cells and cell cycle status assessed using Ki67 and DAPI. (B to E) Elektra hematopoietic stem and progenitor cells (HSPC) show increased proliferation, with a generally lower fraction of cells in G_0 and an increased G_1 fraction (n=8). (F) Elektra HSPC are apoptotic to a higher extent than cells of littermate mice (n=7-8). (G) Kaplan-Meier plot showing the effect of full dose (0.15 mg/g) 5FU-treatment, with a significantly lower survival of elektra mice. A full dose of 5FU causes death of 2/3 elektra mice before day 12 after treatment, while 5FU-treated elektra mice that were subsequently given a bone marrow transplant (BMT) did not die (n=3-6). (H) Slfn2 mRNA expression, relative to B2M expression, analyzed in sorted hematopoietic stem cells (HSC) 2 days after full dose 5FU treatment of wild-type (WT) mice. Control cells sorted as LSK CD150$^+$CD48$^-$. Cells from 5FU-treated mice sorted as LS CD150$^+$CD48$^-$. Slfn2 expression is upregulated following 5FU.

S8C). The substitution is predicted to affect both secondary structure and protein binding as analyzed by PredictProtein[17] (*Online Supplementary Figure S9*). It is also predicted to have a damaging effect in PolyPhen-2[18] analysis with a high likelihood score of 0.998. This is in line with our data and previous studies.
Additional results in are included in the *Online Supplementary Appendix*.

Discussion

Knowledge of the role of Slfn2 in hematopoiesis is limited to studies on immune cells and osteoclastogenesis. RANKL-mediated osteoclast differentiation has been shown to induce Slfn2 expression in BM-derived monocytes/macrophages and in an osteoclast precursor cell line.[6] Slfn2 is also a critical regulator of T-cell quiescence, as it has been shown that the dysregulation of Slfn2 causes increased proliferation and apoptosis in T cells.[9] Thus, there is a clear need for further investigations of the role of Slfn2 in hematopoiesis and stem cell biology. Slfn2 is known as a regulator of quiescence, proliferation, and differentiation – properties that are all fundamental for HSC function. The novel data presented here implicates Slfn2 as a critical regulator of adult murine HSC, particularly regarding cell cycle status, apoptosis, and hematopoietic regeneration. We initially found that Slfn2 is expressed in LTHSC at a higher level than in ST-HSC and LMPP, supporting our hypothesis that this protein has a role in LTHSC. We then investigated the function of Slfn2 by using the loss-of- function elektra mouse model, where the so-called elektra allele contains a point mutation in the *Slfn2* gene.[9]
Here, we report that elektra homozygosity leads to reduced fractions of HSPC *in vivo*, including significantly reduced LSK CD34$^-$Flt3$^-$ LTHSC. We also found decreased amounts of GMP and PreGM cells and a corresponding decrease in colony formation capacity following loss of Slfn2 function. These data indicate a role for Slfn2 in the regulation of early hematopoietic cells. In order to assess the role of Slfn2 in HSC function, elektra BM cells and purified SLAM-LTHSC (LSK CD9^{high}CD48$^-$CD150$^+$) were serially transplanted. We found a drastic reduction in reconstitution in comparison to WT littermate mice, in particular following transplantations of sorted LTHSC, suggesting a role for Slfn2 specifically in LTHSC. The comparatively higher engraftment following whole BM transplantation, in comparison to transplantation of purified LTHSC, could be explained by residual activity of intermediate term HSC at 16 weeks post-transplantation;[19] cells which may not have a similar dependence on Slfn2 as LTHSC.
In order to assess the effect of Slfn2 dysregulation on the BM niche we performed reverse transplantations, which showed no differences in overall engraftment in elektra or WT littermate recipients. However, we found a trend towards increased engraftment of LTHSC in the BM of elektra recipients. This can also be due to decreased fitness of elektra HSC, with fewer competing residual HSC in the elektra BM after irradiation.
The mechanism through which Slfn2 acts is still largely unexplored. Previous studies have suggested (and debated) a connection between Slfn proteins and CyclinD1,[14,15] which would fit with our observations. However, we found that CyclinD1 is not differentially expressed in elektra LTHSC. Instead we found, in a microarray analysis of elektra LTHSC, dysregulation of several other cell cycle-associated genes. Gene ontology analysis and GSEA of the microarray hits show upregulation of clusters of cell cycle and cell division genes in elektra HSC. In order to exemplify, in our list of genes upregulated in elektra HSC we found *Cdk6* (involved in G_1/S transition and regulated by CyclinD proteins), *Ccna2* (involved in S/G_2/M progression), *Cks2* (involved in CDK regulation and cell cycle progression), and *Ccnb2* (suggested involvement in TGFβ-mediated cell cycle control). Interestingly, *Meg3* and *Rian*, known to be expressed in quiescent long-term HSC,[20] are downregulated in elektra HSC. We also found down-regulation of *Igf2bp2*, which has recently been shown to be important for the function of young HSC in mice.[21] Additionally *Ccnb2* along with the also upregulated gene *Ifi27l1* have previously been found upregulated in HSC of IFN-treated mice.[22] We also found upregulation of *Cd53*, which is involved in IL2 signaling and thus also connected to inflammation and IFN. The putative connection between Slfn2 and IFN is discussed further below.
In accordance with our microarray data, as well as the known role of Slfn proteins in cell quiescence and proliferation and a similar phenotype previously reported for elektra T cells,[9] we found that Slfn2 deficiency leads to a lower fraction of cells in G_0 and a higher fraction in G_1 in HSPC compartments. Additionally, we found an augmented fraction of apoptotic (Annexin V$^+$) HSC and MPP in the elektra BM. Cell cycling was also indirectly investigated through assessment of hematopoietic stress response, specifically recovery following 5FU treatment. 5FU is a chemotherapeutic agent, causing death of cycling cells and a subsequent increase in cycling of quiescent cells to replenish lost cell populations.[23] Two days after 5FU treatment in WT mice Slfn2 is upregulated in HSC. At day 10-11 after 5FU treatment, two of three Slfn2-deficient mice had died whereas all WT littermates survived. Interestingly, elektra mice treated with 5FU were rescued if transplanted with fresh BM cells, showing that death in these mice is likely due to BM failure and not due to systemic effects of 5FU on elektra cells in other organ systems. A possible explanation of these observations could be that the already increased cycling and apoptosis at

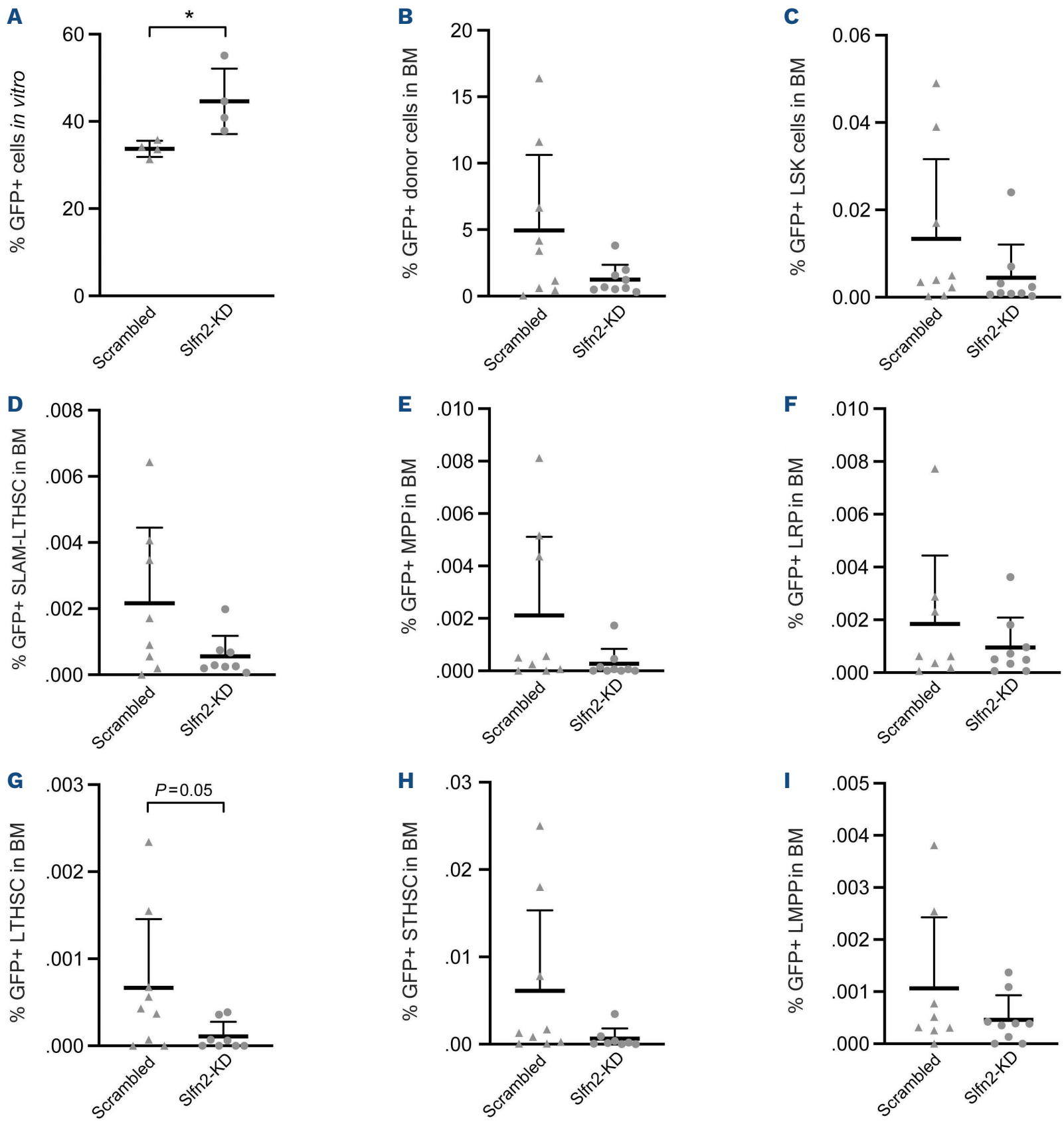


Figure 6. *Slfn2* knockdown in wild-type c-kit⁺ bone marrow cells results in a slight decrease in primitive cell engraftment. (A) Transduction efficiency at transplantation (n=4). (B and C) Engraftment of GFP+ donor cells and LSK cells in bone marrow (BM) 16-18 weeks after transplantation of transduced cells (n=8-9). (D to F) Engraftment of GFP+ donor SLAM-long-term hematopoietic stem cells (SLAM-LTHSC), multipotent progenitors (MPP), and LRP (as defined by LSK with CD150 and CD48) in BM 16-18 weeks after transplantation of transduced cells (n=8-9). (G to I) Engraftment of GFP+ donor LTHSC, short-term HSC (ST-HSC), and lymphoid-primed multipotent progenitors (LMPP) (as defined by LSK with CD34 and Flt3) in BM 16-18 weeks after transplantation of transduced cells (n=8-9).

steady state is exacerbated by 5FU treatment and leads to HSC exhaustion in elektra mice.

Our results are also in line with previous work showing how increased cycling of HSC is associated with reduced engraftment,[24,25] i.e., the cycling phenotype seen here also explains at least in part the reduced engraftment of elektra HSC.

Our study also shows that both the Slfn2 and elektra protein localize to both cytoplasm and nucleus in HT1080 cells, which is in contrast to previous studies showing exclusive cytoplasmic localization.[8] This new data opens up the possibility of nuclear transcriptional activity of the Slfn2 protein, though localization of Slfn2 is likely to be cell type specific.

Finally, we demonstrate that KD of *Slfn2* in BM cells prior to transplantation leads to an engraftment phenotype

similar to that of the elektra cells. This in combination with lack of phenotype in elektra heterozygotes supports the current standing hypothesis that elektra is a loss-of-function mutation.

Previous studies in cell lines have shown that *Slfn* gene expression is induced by IFN treatment.[5,26] Interestingly, our phenotype has several similarities to a knockout mouse model of *Irgm1*, which is an IFN-inducible GTPase. It has been shown that the protective effect of Irgm1 is exerted through negative regulation of IFN signaling.[27,28] In addition, it is known through earlier work that IFN treatment causes HSC to exit G_0 and enter an active cell cycle state.[22,29] As our data shows that dysregulation of Slfn2 leads to an increase in cycling cells, we find it unlikely that the proliferative effect of IFN on HSC is mediated by Slfn2. It would seem more probable that Slfn2, like Irgm1, is part of the negative feedback inhibition of IFN signaling, to induce re-entry into quiescence and, thus prevent HSC exhaustion following a physiological IFN response.

Earlier work has also suggested *p53* as an inducer of quiescence, in addition to its role in apoptosis initiation.[30,31] Similarly, *p57* has been implicated in the maintenance of the G_0 (quiescent) state of HSC.[32,33] Mice with a conditional knockout of *p57* also have a phenotype similar to that of the elektra mouse model investigated here. It has been shown that p57-deficiency causes a severe defect in HSC self-renewal, a reduction in HSPC populations, reduction of HSPC in G_0 phase, increased apoptosis, as well as a decrease in colony-forming activity of the HSC.[32] Though we found that neither *p53* nor *p57* were directly affected in elektra HSC, it would be of value in future work to also investigate a putative connection between these two genes and Slfn2 in HSC.

In summary, this study identifies Slfn2 as a novel and critical regulator of adult murine HSC, through regulation of their quiescence status. We show that loss of Slfn2 function, via homozygosity for the elektra mutation in the *Slfn2* gene, leads to abnormal cell cycle status and apoptosis, which results in a drastic reduction in reconstitution following HSC transplantation and a poor response to hematopoietic stress induced by 5FU. Further studies are needed to map the effect of Slfn2 activation in the maintenance of the stem cell state. Additionally, it is known that HSC cycling and quiescence is altered in aging and hematopoietic malignancies[2] and Slfn2, as a new cell cycle regulator involved in HSC function, may therefore be of value for future work in these research areas.

Disclosures

No conflicts of interest to disclose.

Contributions

SW, EM, VS, UB, GK, and SK designed experiments and analyzed data; SW, MD, ES, AR, EM, VS, JS, ER, KS, THMG, YL, UB and GK performed experiments; SS aided in design of the microarray, processed its data, and helped writing the corresponding methods section; SW, GK, and SK wrote the paper; GK and SK supervised the study.

Acknowledgments

The authors would like to thank the Lund Stem Cell Center Vector Core facility, FACS Core facility, and animal housing facility for their supportive work.

Funding

This work was supported by the European Commission (Stemexpand); Hemato-Linné and Stemtherapy program project grants from the Swedish Research Council; a project grant to SK from the Swedish Research Council; Swedish Cancer Society; Swedish Children Cancer Foundation; Clinical Research Award from Lund University Hospital; a grant to SK from The Tobias Foundation awarded by the Royal Academy of Sciences; and project grants to SW from The Olle Engkvist Foundation and The Royal Physiographic Society in Lund.

Data-sharing statement

Original data and protocols are available to other investigators upon request by contacting the corresponding author or last author.

References

1. Zon LI. Intrinsic and extrinsic control of haematopoietic stem-cell self-renewal. Nature. 2008;453(7193):306-313.
2. Pietras EM, Warr MR, Passegue E. Cell cycle regulation in hematopoietic stem cells. J Cell Biol. 2011;195(5):709-720.
3. Nakamura-Ishizu A, Takizawa H, Suda T. The analysis, roles and regulation of quiescence in hematopoietic stem cells. Development. 2014;141(24):4656-4666.
4. Mavrommatis E, Fish EN, Platanias LC. The schlafen family of proteins and their regulation by interferons. J Interferon Cytokine Res. 2013;33(4):206-210.
5. Katsoulidis E, Carayol N, Woodard J, et al. Role of Schlafen 2 (SLFN2) in the generation of interferon alpha-induced growth inhibitory responses. J Biol Chem. 2009;284(37):25051-25064.
6. Lee NK, Choi HK, Yoo HJ, Shin J, Lee SY. RANKL-induced schlafen2 is a positive regulator of osteoclastogenesis. Cell Signal. 2008;20(12):2302-2308.
7. Schwarz DA, Katayama CD, Hedrick SM. Schlafen, a new family of growth regulatory genes that affect thymocyte development. Immunity. 1998;9(5):657-668.
8. Neumann B, Zhao L, Murphy K, Gonda TJ. Subcellular localization of the Schlafen protein family. Biochem Biophys Res Commun. 2008;370(1):62-66.
9. Berger M, Krebs P, Crozat K, et al. An Slfn2 mutation causes lymphoid and myeloid immunodeficiency due to loss of

immune cell quiescence. Nat Immunol. 2010;11(4):335-343.
10. Bruno L, Hoffmann R, McBlane F, et al. Molecular signatures of self-renewal, differentiation, and lineage choice in multipotential hemopoietic progenitor cells in vitro. Mol Cell Biol. 2004;24(2):741-756.
11. Karlsson G, Rorby E, Pina C, et al. The tetraspanin CD9 affords high-purity capture of all murine hematopoietic stem cells. Cell Rep. 2013;4(4):642-648.
12. Pronk CJ, Rossi DJ, Mansson R, et al. Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy. Cell Stem Cell. 2007;1(4):428-442.
13. Bagger FO, Kinalis S, Rapin N. BloodSpot: a database of healthy and malignant haematopoiesis updated with purified and single cell mRNA sequencing profiles. Nucleic Acids Res. 2019;47(D1):D881-D885.
14. Brady G, Boggan L, Bowie A, O'Neill LA. Schlafen-1 causes a cell cycle arrest by inhibiting induction of cyclin D1. J Biol Chem. 2005;280(35):30723-30734.
15. Zhao L, Neumann B, Murphy K, Silke J, Gonda TJ. Lack of reproducible growth inhibition by Schlafen1 and Schlafen2 in vitro. Blood Cells Mol Dis. 2008;41(2):188-193.
16. Jumper J, Evans R, Pritzel A, et al. Highly accurate protein structure prediction with AlphaFold. Nature. 2021;596(7873):583-589.
17. 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.
18. Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. Nat Methods. 2010;7(4):248-249.
19. Benveniste P, Frelin C, Janmohamed S, et al. Intermediate-term hematopoietic stem cells with extended but time-limited reconstitution potential. Cell Stem Cell. 2010;6(1):48-58.
20. Venkatraman A, He XC, Thorvaldsen JL, et al. Maternal imprinting at the H19-Igf2 locus maintains adult haematopoietic stem cell quiescence. Nature. 2013;500(7462):345-349.
21. Suo M, Rommelfanger MK, Chen Y, et al. Age-dependent effects of Igf2bp2 on gene regulation, function, and aging of hematopoietic stem cells in mice. Blood. 2022;139(17):2653-2665.
22. Essers MA, Offner S, Blanco-Bose WE, et al. IFNalpha activates dormant haematopoietic stem cells in vivo. Nature. 2009;458(7240):904-908.
23. Harrison DE, Lerner CP. Most primitive hematopoietic stem cells are stimulated to cycle rapidly after treatment with 5-fluorouracil. Blood. 1991;78(5):1237-1240.
24. Foudi A, Hochedlinger K, Van Buren D, et al. Analysis of histone 2B-GFP retention reveals slowly cycling hematopoietic stem cells. Nat Biotechnol. 2009;27(1):84-90.
25. Passegué E, Wagers AJ, Giuriato S, Anderson WC, Weissman IL. Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. J Exp Med. 2005;202(11):1599-1611.
26. Mavrommatis E, Arslan AD, Sassano A, Hua Y, Kroczynska B, Platanias LC. Expression and regulatory effects of murine Schlafen (Slfn) genes in malignant melanoma and renal cell carcinoma. J Biol Chem. 2013;288(46):33006-33015.
27. Feng CG, Weksberg DC, Taylor GA, Sher A, Goodell MA. The p47 GTPase Lrg-47 (Irgm1) links host defense and hematopoietic stem cell proliferation. Cell Stem Cell. 2008;2(1):83-89.
28. King KY, Baldridge MT, Weksberg DC, et al. Irgm1 protects hematopoietic stem cells by negative regulation of IFN signaling. Blood. 2011;118(6):1525-1533.
29. Sato T, Onai N, Yoshihara H, Arai F, Suda T, Ohteki T. Interferon regulatory factor-2 protects quiescent hematopoietic stem cells from type I interferon-dependent exhaustion. Nat Med. 2009;15(6):696-700.
30. Asai T, Liu Y, Bae N, Nimer SD. The p53 tumor suppressor protein regulates hematopoietic stem cell fate. J Cell Physiol. 2011;226(9):2215-2221.
31. Liu Y, Elf SE, Asai T, et al. The p53 tumor suppressor protein is a critical regulator of hematopoietic stem cell behavior. Cell Cycle. 2009;8(19):3120-3124.
32. Matsumoto A, Takeishi S, Kanie T, et al. p57 Is required for quiescence and maintenance of adult hematopoietic stem cells. Cell Stem Cell. 2011;9(3):262-271.
33. Zou P, Yoshihara H, Hosokawa K, et al. p57(Kip2) and p27(Kip1) cooperate to maintain hematopoietic stem cell quiescence through interactions with Hsc70. Cell Stem Cell. 2011;9(3):247-261.