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

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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 \( \text{Slfn2} \) 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, 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 (G0) state. Several factors regulate entry into the proliferating G phase (followed by S/G2/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. It has been suggested that HSC quiescence correlates with repopulation potential and self-renewal capacity. 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. \( \text{Slfn2} \) belongs to subgroup I, the shortest of the Slfn proteins, and has in cell lines been shown to localize exclusively in the cytoplasm. 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 \( \text{Slfn2} \) 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 homzygous T cells proliferated excessively and underwent apoptosis upon cytokine-me-
Additionally, small interfering RNA (siRNA)-mediated knockdown (KD) of Slfn2 in Sca1+ mouse BM cells enhances proliferation in colony formation assays. Slfn2 is also known to be expressed in a primitive hematopoietic cell line, and its expression is downregulated when these cells are differentiated to various lineages.

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. 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 fluorescent-activated cell sorting
Flow cytometry analyses were performed on a Beckton Dickinson Cantoll or custom order LSR II at the Lund University FACS core. Sorting was performed on a custom order Ariall or Arialli. 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. Progenitor analysis was done as previously described. Apoptotic cells were defined using Annexin-V 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 CD9highCD48−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 polymerase 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−CD105−) 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. 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 CD9hiCD150 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
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 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 S5A and B). 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 G1 in LSK, MPP, and LRP (LSK CD150 CD48) compartments, as well as a higher fraction of G1 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 HSPC 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. 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](image-url) **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 CD9hiCD48-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      | GO:0071241~cellular response to inorganic substance                  | 3.00              | <0.001    |
| Annotation Cluster 1      | GO:0035068~micro-ribonucleoprotein complex                            |                   | 0.009     |
| Annotation Cluster 2      | GO:0017241~in utero embryonic development                            | 2.63              | <0.001    |
| Annotation Cluster 2      | mmu05206:MicroRNA in cancer                                           |                   | <0.001    |
| Annotation Cluster 2      | GO:0002329~pre-B-cell differentiation                                 |                   | 0.001     |
| Annotation Cluster 2      | GO:0001783~B-cell apoptotic process                                   |                   | 0.001     |
| Annotation Cluster 2      | GO:0021522~spinal cord motor neuron differentiation                    |                   | 0.006     |
| Annotation Cluster 2      | GO:0060412~ventricular septum morphogenesis                          |                   | 0.011     |
| Annotation Cluster 3      | GO:0051301~cell division                                             |                   | 0.004     |
| Annotation Cluster 3      | GO:0007049~cell cycle                                                |                   | 0.013     |
| Annotation Cluster 3      | GO:0007067~mitotic nuclear division                                   |                   | 0.027     |
| Annotation Cluster 4      | GO:0007049~cell cycle                                                |                   | 0.013     |
| **Downregulated**         |                                                                      |                   |           |
| Annotation Cluster 1      | mmu05737~cytoplasm                                                   | 2.26              | 0.043     |
| Annotation Cluster 2      |                                                                      |                   |           |
| Annotation Cluster 2      | mmu03040:Spliceosome                                                 | 1.84              | 0.001     |
| Annotation Cluster 2      | mmu04010:MAPK signaling pathway                                      |                   | 0.001     |
| Annotation Cluster 2      | mmu05162:Measles                                                      |                   | 0.010     |
| Annotation Cluster 2      | mmu05134:Legionellosis                                               |                   | 0.016     |
| Annotation Cluster 2      | mmu04141:Protein processing in endoplasmic reticulum                 |                   | 0.018     |
| Annotation Cluster 2      | mmu05164:Influenza A                                                 |                   | 0.019     |
| Annotation Cluster 2      | mmu04612:Antigen processing and presentation                          |                   | 0.031     |
| Annotation Cluster 2      | mmu04915:Estrogen signaling pathway                                  |                   | 0.043     |
| Annotation Cluster 2      | mmu05145:Toxoplasmosis                                               |                   | 0.049     |
| Annotation Cluster 4      | mmu03040:Spliceosome                                                 | 1.29              |           |
| Annotation Cluster 4      | GO:0031012~extracellular matrix                                       |                   | <0.001    |
| Annotation Cluster 4      | mmu04010:MAPK signaling pathway                                      |                   |           |
| Annotation Cluster 4      | mmu05162:Measles                                                      |                   |           |
| Annotation Cluster 4      | GO:0005515~protein binding                                           |                   | 0.008     |
| Annotation Cluster 4      | GO:0044822~poly(A) RNA binding                                       |                   | 0.009     |
| Annotation Cluster 4      | GO:0045444~fat cell differentiation                                   |                   | 0.013     |
| Annotation Cluster 4      | GO:0005681~spliceosomal complex                                       |                   | 0.034     |
| Annotation Cluster 4      | GO:0045893~positive regulation of transcription, DNA-templated       |                   | 0.038     |
| Annotation Cluster 4      | GO:0008134~transcription factor binding                               |                   | 0.038     |
| Annotation Cluster 4      | GO:007062~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 CD9CD48 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 shows that residue 135 is localized in a \( \beta \) 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 G0, and an increased G1 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.
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causes increased proliferation and apoptosis in T cells. It has been shown that the dysregulation of Slfn2 gene.7 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+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;10 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 CycclinD1,16,15 which would fit with our observations. However, we found that CycclinD1 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 G1/S transition and regulated by CyclinD proteins), Ccnb2 (involved in S/G2/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 Ifi27t1 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 G0, and a higher fraction in G1 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

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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.8 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+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;10 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 CycclinD1,16,15 which would fit with our observations. However, we found that CycclinD1 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 G1/S transition and regulated by CyclinD proteins), Ccnb2 (involved in S/G2/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 Ifi27t1 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 G0, and a higher fraction in G1 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
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. 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. In addition, it is known through earlier work that IFN treatment causes HSC to exit G0 and enter an active cell cycle. Since we found that neither p57 nor Slfn2 were directly affected in 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 G0 phase, increased apoptosis, as well as a decrease in colony-forming activity of the HSC. Though we found that neither p57 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 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.

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**Data-sharing statement**

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

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