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Deletion of the *Scl* +19 enhancer increases the blood stem cell compartment without affecting the formation of mature blood lineages

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The stem cell leukemia (*Scl*)/*Tall* gene is essential for normal blood and endothelial development, and is expressed in hematopoietic stem cells (HSCs), progenitors, erythroid, megakaryocytic, and mast cells. The *Scl* +19 enhancer is active in HSCs and progenitor cells, megakaryocytes, and mast cells, but not mature erythroid cells. Here we demonstrate that in vivo deletion of the *Scl* +19 enhancer (*Scl*^{Δ19/Δ19}) results in viable mice with normal *Scl* expression in mature hematopoietic lineages. By contrast, *Scl* expression is reduced in the stem/progenitor compartment and flow cytometry analysis revealed that the HSC and megakaryocyte-erythroid progenitor populations are enlarged in *Scl*^{Δ19/Δ19} mice. The increase in HSC numbers contributed to enhanced expansion in bone marrow transplantation assays, but did not affect multilineage repopulation or stress responses. These results affirm that the *Scl* +19 enhancer plays a key role in the development of hematopoietic stem/progenitor cells, but is not necessary for mature hematopoietic lineages. Moreover, active histone marks across the *Scl* locus were significantly reduced in *Scl*^{Δ19/Δ19} fetal liver cells without major changes in steady-state messenger RNA levels, suggesting post-transcriptional compensation for loss of a regulatory element, a result that might be widely relevant given the frequent observation of mild phenotypes after deletion of regulatory elements. © 2012 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.

The stem cell leukemia (*Scl*) gene, also known as *Tall*, encodes a basic helix-loop-helix transcription factor that functions as a critical regulator of both hematopoietic and endothelial development [1]. SCL was first identified by virtue of its ectopic expression as a target of t(1;14) chromosomal translocations in T-cell acute lymphoblastic leukemia [2]. Overexpression of SCL is now recognized as one of the most common molecular abnormalities found in human T-cell acute lymphoblastic leukemia [3].

Scl is an essential regulator of the hematopoietic hierarchy at several levels. Within the hematopoietic lineage, *Scl* is expressed in hematopoietic stem cells (HSCs), progen-

itor cells, and in erythroid, megakaryocytic, and mast cells [4–6]. *Scl* null ES cells fail to differentiate in vitro and do not contribute in vivo to hematopoiesis in chimeric mice [6,7]. In addition, knockout of the *Scl* gene is embryonic lethal at E9.5, due to complete absence of hematopoiesis and major vascular defects [7–9]. More recently, the use of a conditional knockout has demonstrated that *Scl* is essential for the genesis, but not the maintenance, of HSCs [10,11]. Mice in which *Scl* was deleted in adulthood exhibited mild defects in erythropoiesis and megakaryopoiesis [11] and increased Lin[−]cKit⁺Sca⁺ stem-cell enriched population [12]. Short-term HSC (ST-HSC) function seems to be defective in *Scl* deleted cells because these cells fail to generate colony-forming unit (CFU)-S12 colonies in the spleen [10] and show reduced short-term repopulating ability [12]. Interestingly, long-term HSC (LT-HSC) function was not compromised [11] or mildly compromised [12] when the *Scl* deletion occurred post-transplantation. However, if the deletion occurred before transplantation, then a reduction in repopulating ability of the deleted cells was observed, which was not due to homing defects [12]. This defect in repopulating ability was already observed in heterozygous *Scl*

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deleted cells, indicating that haploinsufficiency is enough to affect the repopulation capacity of these cells [12]. Reduction of *Scl* expression using short hairpin RNA lentivirus in both human and mouse stem-cell enriched populations also affects the short and long-term repopulating ability of these cells [13].

A systematic survey of the promoters and chromatin structure of the murine *Scl* gene has identified several regulatory elements, functionally validated in reporter assays [14–18]. Further analysis of reporter constructs in transgenic mice identified a panel of spatially distinct enhancers, each of which directs *Scl* expression to a subdomain of the normal *Scl* expression pattern [14,16–18]. In particular, the *Scl* +19 enhancer, also known as the *Scl* +18/19 enhancer from its location 19 kb downstream of the *Scl* promoter, was shown to drive expression of *Scl* in long-term repopulating HSCs and hematopoietic progenitors, but not in mature cells [17,19]. Furthermore, expression of the *Scl* complementary DNA under the control of the *Scl* +19 enhancer rescued the formation of early hematopoietic progenitors and yolk sac angiogenesis in *Scl*^{-/-} embryos, but failed to rescue erythropoiesis and embryos still died at E9.5 [19]. These results indicate that the *Scl* +19 enhancer plays an important role in progenitors but is not sufficient to support erythroid maturation.

Transgenic mouse reporter assays are a useful tool to identify new regulatory elements; however, such approaches are unable to define nonredundant/essential roles of these elements in the context of the entire gene locus. In the case of the *Scl* gene, three hematopoietic enhancers have been described that, in combination, are responsible for the hematopoietic expression pattern of *Scl* [14,16–18]. These enhancers have evolved from common ancestral enhancers [20] and may have maintained a certain degree of redundancy.

To clarify the function of the *Scl* +19 enhancer within the context of the endogenous locus, we describe here the generation and analysis of mice lacking both copies of the *Scl* +19 enhancer (*Scl*^{Δ19/Δ19}). *Scl*^{Δ19/Δ19} mice were viable but their HSC and megakaryocyte-erythroid progenitor compartments were expanded. Analysis of *Scl* expression as well as chromatin modification status in wild-type (WT) and mutant cells suggested that post-transcriptional compensatory mechanisms contribute to the mild phenotype in addition to redundant regulatory elements within the locus.

Materials and methods

Mice, genotyping, and breeding

Mice with a +18/19 targeted stem cell enhancer (*Scl*^{Δ19/Δ19}) were generated as described [16]. Mice and tissues were routinely genotyped by polymerase chain reaction (PCR) using the following primers: WT allele, 5'-CACCTGTCCTGGGGCTAAATT-3' and 5'-GTTTTTGTACTCCCAGATGTTGAA-3'; +18/19 enhancer region deletion allele (Δ19), 5'-CTTCTATCCATCTACAGG-3'

and 5'-CACTGAATCATGCTCGTGTGG-3'. Animals were maintained in the Cambridge Central Biomedical Services in accordance with institutional guidelines.

Peripheral blood analysis, cell staining, and flow cytometry

A sample (50 μL) of freshly isolated peripheral blood from the tail vein was collected and blood parameters were measured using an ABC Vet fully automated analyzer (ABX Hematologie, Montpellier, France). For hematopoietic precursor isolation, mature bone marrow (BM) cells were depleted with a lineage depletion column (Miltenyi Biotec, GmBH, Bergisch Gladbach, Germany). For identification of common myeloid progenitors, granulocyte-macrophage progenitors, and megakaryocytic erythroid progenitors, cells were incubated with allophycocyanin anti-c-Kit (2B8; Pharmingen), phycoerythrin anti-FcγRII/III (2.4G2; Pharmingen BD Biosciences, Oxford, UK), and fluorescein isothiocyanate (FITC) anti-CD34 (RAM34; Pharmingen). Common lymphoid progenitors cells among Lin⁻ cells were enumerated after staining with allophycocyanin c-Kit, Pacific Blue Sca-1 (E13-161.7; Pharmingen), and biotin-conjugated interleukin-7 (B12-1; Pharmingen), followed by PerCP-Cy5.5 streptavidin. To further define HSC progenitors, cells were subsequently stained with FITC anti-CD34 antibodies. Stained cells were analyzed using a MoFlo cell sorter (Dako, Carpinteria, CA, USA). For identification of mast cells, a peritoneal wash was performed with 10 mL sterile phosphate-buffered saline. Collected cells were stained with anti-c-Kit (allophycocyanin) and anti-Sca-1 (Pacific Blue). Enriched HSC (Lin⁻ c-Kit⁺ Sca-1⁺) cells were sorted directly into 96% ethanol, washed extensively, and stained with propidium iodide and anti-Ki-67 (FITC) as a marker for cell cycle analysis. Whole BM erythropoietic cells were stained with CD71 (FITC) and Ter119 (phycoerythrin) antibodies.

RNA isolation and quantitative PCR assays

For RNA isolation from tissues, a single-cell suspension was prepared using a tissue homogenizer. Cells were resuspended in TRI reagent (Sigma, St Louis, MO, USA) and RNA isolated as described by the manufacturer. First-strand complementary DNA synthesis was performed using the cDNA Synthesis Kit (Bioline, Taunton, MA, USA). Quantitative PCR was carried out using Stratagene Brilliant SYBR Green qPCR Master Mix (Agilent Technologies, Stockport, UK). Standard curves were obtained using serial dilutions of control sample. Data were normalized to β-actin. *Scl* messenger RNA (mRNA) primers: *Scl* Exon 5 F- catgttcaccaacaacaaccg *Scl* Exon 6 R ggtgtgaggaccatcagaaatctc; *Scl* primary transcript primers: *Scl* Exon 1 F -tatgctgtgtgctgtgtctctt; *Scl* Intron 2 R -caacactggctcccgaatacatca; β-actin primers: β-actin F -tctggcctcactgtcca; β-actin R -gtccgctagaagcacttgc.

Methylcellulose colony assays

To identify progenitor colonies, single-cell suspensions of 5 × 10⁴ BM or 2 × 10⁵ spleen cells were plated in duplicate in semisolid medium (MethoCult 3434; StemCell Technologies, Vancouver, BC, Canada). Colonies were counted and identified after 7 to 10 days in culture. To detect CFU-megakaryocyte, cells were plated in duplicate in collagen-based medium (MegaCult-C; StemCell Technologies). After 6 to 8 days in culture, slides were dehydrated, fixed, and stained with acetylthiocholiniodide (Sigma). Cultures were performed according to the manufacturer's protocol.

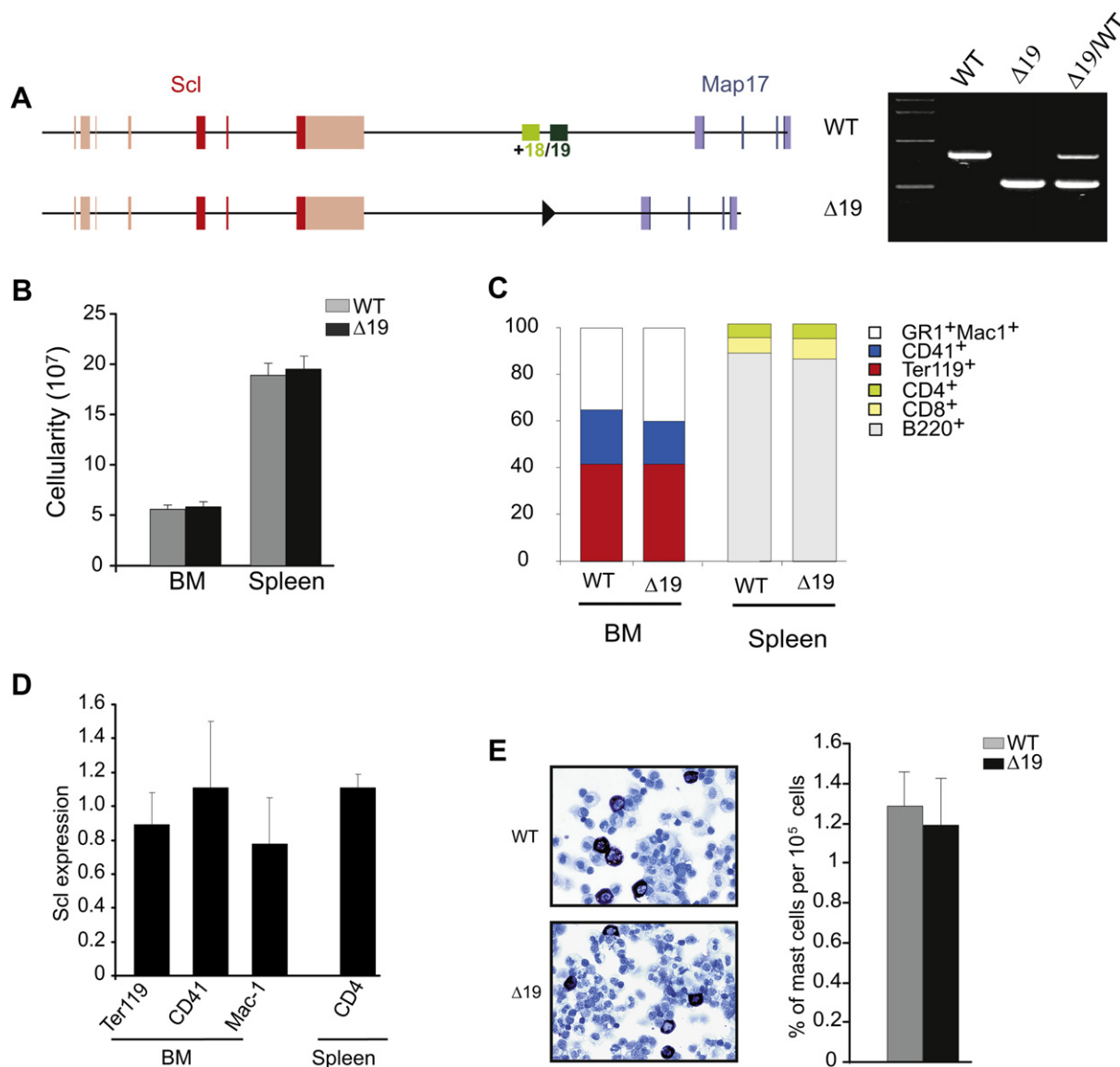


Figure 1. *Scl* ^{$\Delta 19/\Delta 19$} mice are viable and have normal mature hematopoietic lineages. (A) Left panel shows schematic representation of the *Scl* alleles used in this study: *Scl*^{WT/WT} and *Scl* ^{$\Delta 19/\Delta 19$} locus with the deletion of a 2.4-kb region containing both the +18 (light green bar) and +19 enhancers (dark green bar). Black triangle represents loxP site remaining in the genome after Cre recombination. *Scl* exons are depicted in red and the *Map17* exons in blue. Right panel shows PCR genotyping analysis of WT (*Scl*^{WT/WT}), homozygous (*Scl* ^{$\Delta 19/\Delta 19$}), and heterozygous (*Scl* ^{$\Delta 19/WT$}) knockout alleles. In the first lane is 1-kb DNA marker. (B) Analysis of total cellularity from the BM and spleen in *Scl*^{WT/WT} and *Scl* ^{$\Delta 19/\Delta 19$} adult mice. (C) Percentage of granulocytes (Gr1⁺Mac1⁺), megakaryocytes (CD41⁺), and erythrocytes (Ter119⁺) cells in BM and T cells (CD4⁺, CD8⁺) and B cells (B220⁺) in spleen of *Scl*^{WT/WT} and *Scl* ^{$\Delta 19/\Delta 19$} mice. (D) *Scl* expression in mature blood lineages of the BM and spleen in *Scl* ^{$\Delta 19/\Delta 19$} mice. Data are presented as relative expression to *Scl*^{WT/WT}. Erythroid cells were sorted using Ter119 antibody, megakaryocytes using CD41, macrophages using Mac-1 and T cells from spleen using CD4. (E) Mast cells are normal in *Scl* ^{$\Delta 19/\Delta 19$} mice. Left panel shows peritoneal cells stained with Toluidine blue and Metachromatic staining of mast cells. Right panel shows quantitative analysis of mast cells (cKit⁺ Sca1⁺) from peritoneal wash in *Scl*^{WT/WT} and *Scl* ^{$\Delta 19/\Delta 19$} mice.

were performed with adult BM and spleen to assess the differentiation potential of *Scl* ^{$\Delta 19/\Delta 19$} cells (Table 2). CFU-multipotential progenitor cells, CFU-GM, burst-forming unit erythroid, and CFU-megakaryocyte colony numbers were unaffected in the adult BM. By contrast, we observed a twofold increase in CFU-GM in the spleen of *Scl* knockout mice, without any sign of spleen enlargement.

The increased number of LSK cells in *Scl* ^{$\Delta 19/\Delta 19$} mice suggested that deletion of the +19 enhancer might play an important role in immature HSCs. We purified CD34-positive and negative fractions from the LSK population and analyzed *Scl* expression (Fig. 2E). The level of *Scl* mRNA in the LSK CD34⁻ population was almost half that of the WT mice ($p = 1.7 \times 10^{-6}$), whereas that in LSK CD34⁺ population was reduced by only 20%

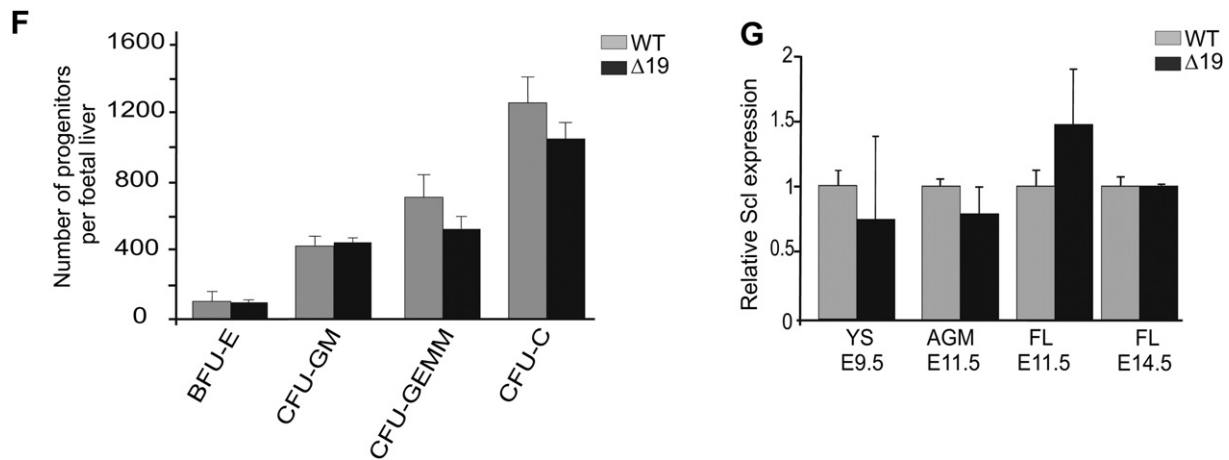


Figure 1. (Continued). (F) Progenitor analysis from E11.5 fetal liver showing the number of colonies per fetal liver in WT and $Scl^{Δ19/Δ19}$ embryos for burst-forming units erythroid (BFU-E), colony-forming units granulocyte-macrophage (CFU-GM), CFU multipotential (CFU-GEMM) progenitor cells, and CFU in culture (CFU-C). (G) Quantitative analysis of *Scl* expression (qPCR) in intact E9 yolk sacs (YS), E11.5 aortagonad-mesonephros (AGM), and fetal liver (FL) at E11.5 and E14 of $Scl^{WT/WT}$ and $Scl^{Δ19/Δ19}$ embryos. The results are presented as relative expression to $Scl^{WT/WT}$.

($p = 0.0087$). To test the possibility that the increased number of cells within the LSK population is due to cell cycle arrest, we stained the purified LSK population for the cell cycle marker Ki-67 (Fig. 2F). The $Scl^{Δ19/Δ19}$ LSK cells showed a very mild increase in G_0 phase and a slight decrease in G_1 phase compared with the WT mice; however, both changes fail to reach statistical significance. The $S/G_2/M$ phases did not differ between the two phenotypes. This showed that the +19 enhancer affects the level of *Scl* expression in HSCs, yet the changes in HSC numbers observed in $Scl^{Δ19/Δ19}$ mice are not due to major alterations in cell cycle.

In summary, $Scl^{Δ19/Δ19}$ mice exhibit an increase in short-term HSCs as assessed by flow cytometry, an increase in megakaryocytic erythroid progenitors and an increase in CFU-GM colonies in spleen. However, deletion of the *Scl* +19 element has no effect on the common myeloid progenitors, granulocyte-macrophage progenitors, and common lymphoid progenitor populations.

The +19 enhancer is not required for HSC multilineage repopulation

We showed (Fig. 2A) that LSK numbers were increased by almost twofold in $Scl^{Δ19/Δ19}$ mice. The ultimate assay for

HSC function is the long-term competitive repopulation assay. A long-term competitive repopulation assay was performed to assess the effect of the deletion on HSC function, using the CD45.1–CD45.2 system to measure blood chimerism in transplant recipients. Donor WT or $Scl^{Δ19/Δ19}$ cells were CD45.2, whereas competitor cells and recipient mice were CD45.1 (Fig. 3A). For engraftment studies we used a 1:1 ratio of donor to competitor and a total of 1×10^6 unfractionated donor and competitor BM cells were injected per recipient. Results are shown in Figure 3 as a percentage of chimerism 4 and 12 weeks post-transplantation.

Short-term engraftment was analyzed at 4 weeks post-transplantation. Two independent transplantations were performed and the results are shown in Figure 3B and C. Short-term engraftment was found to be somewhat compromised for $Scl^{Δ19/Δ19}$ cells, which is in agreement with previous phenotypic observations in *Scl* conditional knockout animals [12]. The observed reduction in engraftment by the $Scl^{Δ19/Δ19}$ is consistent between experiments, but fails to reach statistical significance.

Long-term engraftment was analyzed, at 12 weeks post-transplantation. Although no significant defect was observed with the $Scl^{Δ19/Δ19}$ donor cells, there was a bias for the $Scl^{Δ19/Δ19}$ cells to engraft better than the WT cells (Fig. 3D).

Table 1. Hematological parameters of $Scl^{Δ19/Δ19}$ and WT mice

Age (wks)	Genotype	N	RBC ($10^3/\mu\text{L}$)	Hgb (g/dL)	Hct (%)	Plt ($10^3/\mu\text{L}$)	WBC ($10^3/\mu\text{L}$)	Lympho (%)	Mono (%)	Gran (%)
6–12	WT	26	9.5 ± 1.3	17.2 ± 0.7	53.5 ± 4.0	1107 ± 234	7.7 ± 2.0	78.7 ± 5.3	4.2 ± 0.5	17.1 ± 5.1
	Δ19	26	9.6 ± 1.2	16.8 ± 0.8	53.1 ± 3.3	965 ± 209	7.4 ± 2.2	78.7 ± 5.7	4.3 ± 1.1	17 ± 4.9
78–86	WT	12	10.1 ± 1.5	14.2 ± 2.4	46.0 ± 9.1	1551 ± 254	11.3 ± 7.2	64 ± 13.4	6.8 ± 2.4	29.2 ± 11.5
	Δ19	13	10.6 ± 1.3	14.5 ± 2.1	48.3 ± 8.2	1527 ± 353	13.7 ± 5.9	64.3 ± 12.1	6.8 ± 1.8	29 ± 10.7

Gran = granulocytes; Hct = hematocrit; Hgb = hemoglobin; Lympho = lymphocytes; mono = monocytes; Plt = platelets; RBC = red blood cells; WBC = white blood cells.

Peripheral blood parameters were measured from age- and sex-matched young (6–12 weeks) and old (78–86 weeks) mice.

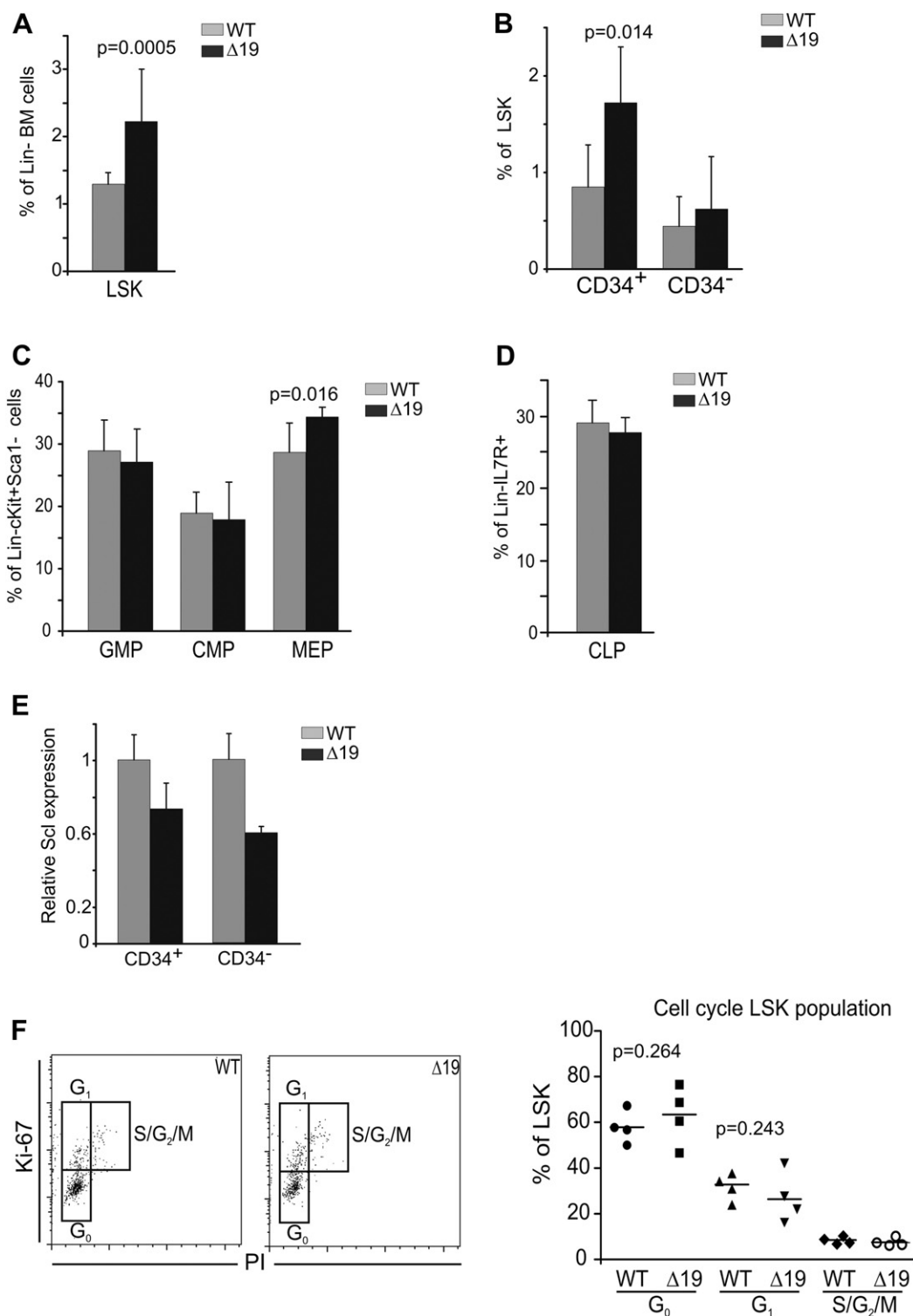


Figure 2. Phenotypic analysis of progenitor cells in adult BM of WT and $Scl^{\Delta 19/\Delta 19}$ mice. (A) $Scl^{\Delta 19/\Delta 19}$ animals have an increased number of Lin⁻Sca1⁺Kit⁺ cells compared with the $Scl^{WT/WT}$. Histogram shows percentage of Sca1⁺Kit⁺ cells within the Lin⁻ BM cells of $Scl^{WT/WT}$ and $Scl^{\Delta 19/\Delta 19}$ mice. (B) No significant difference is observed in the number of long-term CD34⁻ stem cells in the two genotypes; however, there is a 2-fold increase in short-term CD34⁺ stem cells in $Scl^{\Delta 19/\Delta 19}$ mice. Histogram shows percentage of CD34⁺ and CD34⁻ within the LSK population in BM. (C) Analysis of BM progenitor population reveals a normal population of granulocyte-macrophage progenitors (GMPs) and common myeloid progenitors but an increase in the megakaryocytic erythroid progenitor (MEP) population in $Scl^{\Delta 19/\Delta 19}$. (D) No difference was observed in the number of common lymphoid progenitors (CLPs) between WT and $Scl^{\Delta 19/\Delta 19}$ animals. (E) Expression level of *Scl* in short-term (LSK CD34⁺) and long-term (LSK CD34⁻) stem cells. (F) Cell cycle fluorescence-activated cell sorting analysis on sorted LSK population stained for the Ki-67 and propidium iodide (PI) in $Scl^{WT/WT}$ and $Scl^{\Delta 19/\Delta 19}$ mice. Left panel shows representative plots and right panel shows quantification results. Analysis was performed on six animals from each genotype.

Table 2. Hematopoietic progenitors are normal in *Scl*^{Δ19/Δ19} mice

Tissue	Genotype	BFU-e	CFU-GM	CFU-GEMM	CFU-MK
BM	WT	2 ± 1	28 ± 16	4 ± 2	13 ± 4
	Δ19	2 ± 1	26 ± 12	3 ± 3	15 ± 1
Spleen	WT	3 ± 1	17 ± 2	5 ± 2	9 ± 6
	Δ19	4 ± 1	34 ± 6	5 ± 2	11 ± 9

Methylcellulose-based colony assays were performed in whole BM and spleen from WT and Δ19 mice. The numbers shown are per 5×10^4 BM and 2×10^5 spleen cells for burst-forming units-erythroid (BFU-e), colony-forming units-granulocyte macrophage (CFU-GM), CFU-multipotential progenitors (CFU-GEMM), and CFU-megakaryocytes (CFU-MK). Results represent six age- and sex-matched mice of each genotype. Values are expressed as mean ± standard deviation.

However, when the percentage of donors for each lineage was calculated (Fig. 3E), we did not observe any significant differences for any lineage engraftment contribution.

Redundancy of the *Scl* regulatory elements

We previously identified several different regulatory elements that control the spatial and temporal expression of the *Scl* gene in vitro and in vivo [16,17,23], which are summarized in Figure 4A. The *Scl* +19 element is controlled by a multiprotein complex that includes GATA-2, Fli-1, and Elf-1 [16]. To identify possible redundancy between the +19 enhancer and other *Scl* regulatory elements, we performed chromatin immunoprecipitation (ChIP) assays on E14.5 fetal liver cells with markers for repressive (H3K9me3 and H3K9me2) and active (H3K4me3 and H3K9Ac) chromatin. Using the two markers for repressive chromatin, we did not observe any differences between the *Scl*^{Δ19/Δ19} and *Scl*^{WT/WT} fetal liver cells at different elements of the *Scl* locus (Fig. 4B, bottom panels). By contrast, ChIP performed with active euchromatin marks revealed changes at *Scl* promoter region 1b (Fig. 4B, top panels). Interestingly, both active euchromatin marks are reduced in *Scl*^{Δ19/Δ19} fetal liver cells, indicating that the *Scl* +19 element is required for enhanced activity of the *Scl* promoter. Another region that was significantly decreased by the *Scl* +19 deletion was the *Scl*-4 enhancer located 4 kb upstream of the start site of the *Scl* coding sequence. Chromatin analysis of the endogenous gene locus therefore demonstrates direct functional consequences of the deletion of a distal enhancer on the status of the *Scl* promoter region.

Surprisingly, the expression of *Scl* mRNA is unaltered in *Scl*^{Δ19/Δ19} E14.5 fetal livers (Fig. 1G and Fig. 4C), suggesting post-transcriptional compensation. To test this hypothesis, we quantified the amount of *Scl* primary transcript present in WT and mutant fetal liver cells from the same litters by qPCR using primers spanning the exon 1b:intron 1 boundary (Fig. 4C). This analysis demonstrated a 50% reduction of primary transcript in mutant cells, consistent with the ChIP data over the *Scl* promoter 1B. Taken together therefore, our results reveal post-transcriptional compensatory mechanisms as the likely cause for the

similar levels of steady-state mRNA in wild type and enhancer-deleted cells.

Discussion

The murine *Scl* locus has emerged, over the past decade or so, as a paradigm gene locus for studying transcriptional control mechanisms in blood stem and progenitor cells [14–18,20,23–25]. Concerted biochemical, comparative genomics, and transgenic studies led to the identification of three distinct regions (–4, +19, and +40) active in progenitor cells, with the +19 enhancer being the most specific HSC enhancer element [14,16,17,19,23]. Importantly, none of the previous studies of *Scl* regulation had performed extensive analysis of steady-state hematopoiesis after deletion of regulatory elements from the endogenous locus. Here we report a comprehensive phenotypic analysis of mice lacking the *Scl* +19 enhancer. *Scl*^{Δ19/Δ19} are viable with specific defects in the hematopoietic stem/progenitor cell compartment. However, *Scl*^{Δ19/Δ19} HSCs did not display any significant imbalance in the production of mature cells, and were able to function in transplantation assay. Rather than being accompanied by compensatory increases in active chromatin marks elsewhere in the locus, deletion of the +19 enhancer causes a reduction of active histone marks at the –4 enhancer and *Scl* promoters.

The observation of relatively mild phenotypes after the deletion of regulatory elements is a common finding. Early studies on β-globin enhancers showed only moderate reductions in expression [26–28] and similar results were also obtained after the deletion of the α-globin upstream enhancer [29]. Perhaps the most publicized failure to elicit strong phenotypes after deletion of enhancer elements comes with the demonstration that germline deletion of four ultra-conserved elements did not cause any major phenotype [30]. Given that the complete knockout of genes such as *Scl* or globins results in major phenotypes, the minor phenotypes after enhancer knockouts have commonly been attributed to compensation by additional regulatory elements with overlapping activity, within the same gene locus. Most recently, Snow and colleagues [31] have shown that deletion of a *Gata2* enhancer, predicted to be responsible for the *Gata2*-dependent positive feedback loop in HSCs, had no major phenotypical consequences despite a significant reduction in *Gata2* expression and the lack of phenotype was again attributed to redundancy with another cis-regulatory element.

We have previously demonstrated that the *Scl* +19 element is controlled by a multiprotein complex that includes GATA-2, Fli-1, and Elf-1 [16]. Using whole-genome transcription factor binding mapping by ChIP-Seq, we have recently identified other important hematopoietic regulators that also bind to the *Scl* +19 enhancer, including the Ets factors Pu.1 and Erg [32,33]. Like GATA-2 and Fli1, Pu.1 and Erg were also bound to other *Scl* enhancers (–4 and/or

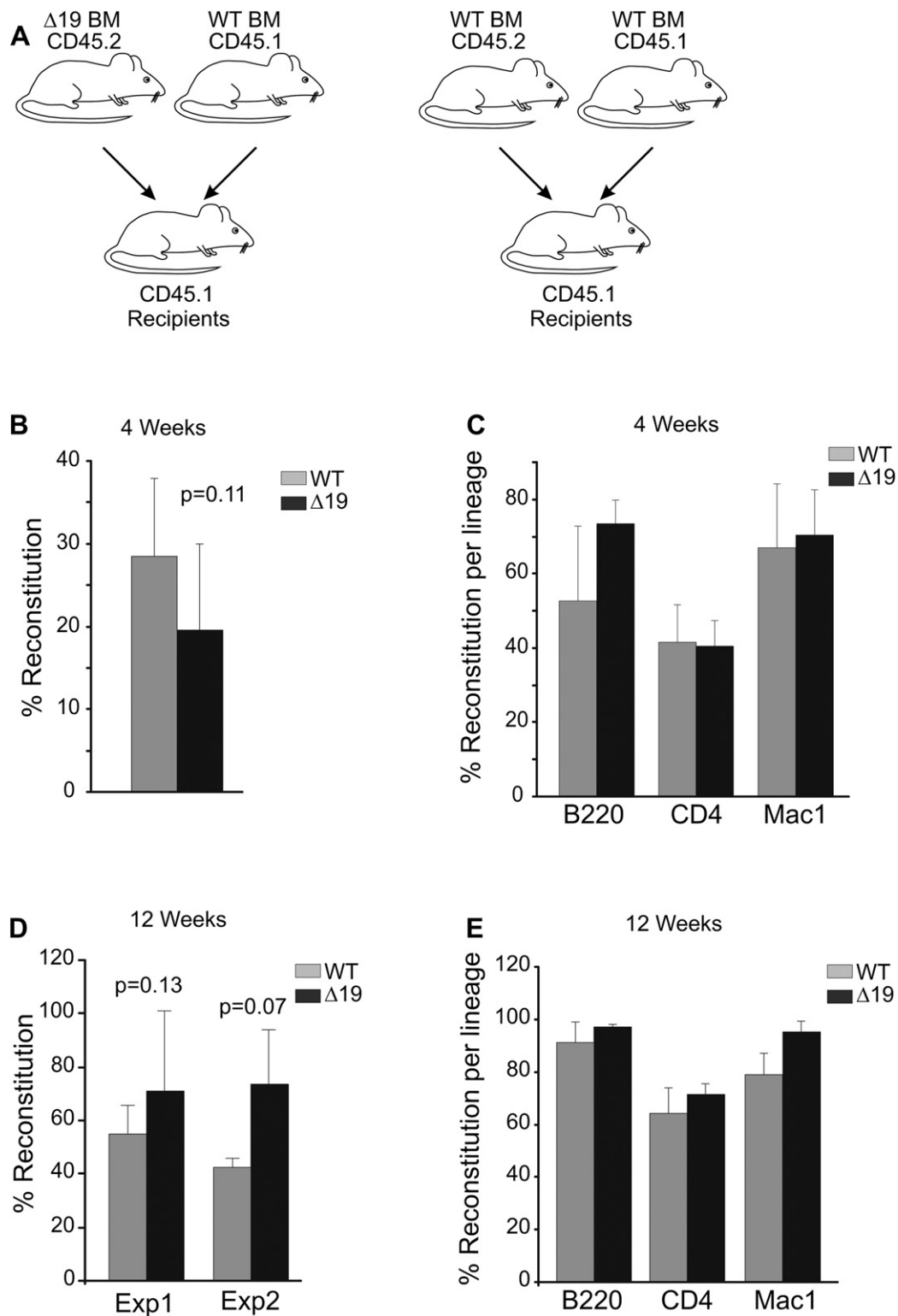


Figure 3. Enhanced long-term repopulation in $Scl^{\Delta 19/\Delta 19}$ mice. (A) Outline of transplantation assay. CD45.2 $Scl^{WT/WT}$ or $Scl^{\Delta 19/\Delta 19}$ unfractionated BM was mixed with CD45.1 unfractionated BM at a ratio of 1:1. Five to six lethally irradiated CD45.1 recipients were injected with 1×10^6 cells per group ($Scl^{\Delta 19/\Delta 19}$ or $Scl^{WT/WT}$ donor CD45.2). (B) Percentage reconstitution (CD45.2⁺ cells) measured 4 weeks post-transplantation by fluorescence-activated cell sorting (FACS) analysis of peripheral blood with CD45.1 and CD45.2 antibodies. (C) Recipient peripheral blood FACS analysis for different lineage markers: B cells (B220), macrophages (Mac-1), and T cells (CD4). CD45.2 donor reconstitution for each lineage is expressed as a percentage. (D) Percentage reconstitution measured 12 weeks post-transplantation by FACS analysis of peripheral blood with CD45.1 and CD45.2 antibodies. Two independent transplantations are shown. (E) Recipient peripheral blood FACS analysis for different lineage markers: B cells (B220), macrophages (Mac-1), and T cells (CD4). CD45.2 donor reconstitution for each lineage is expressed as a percentage.

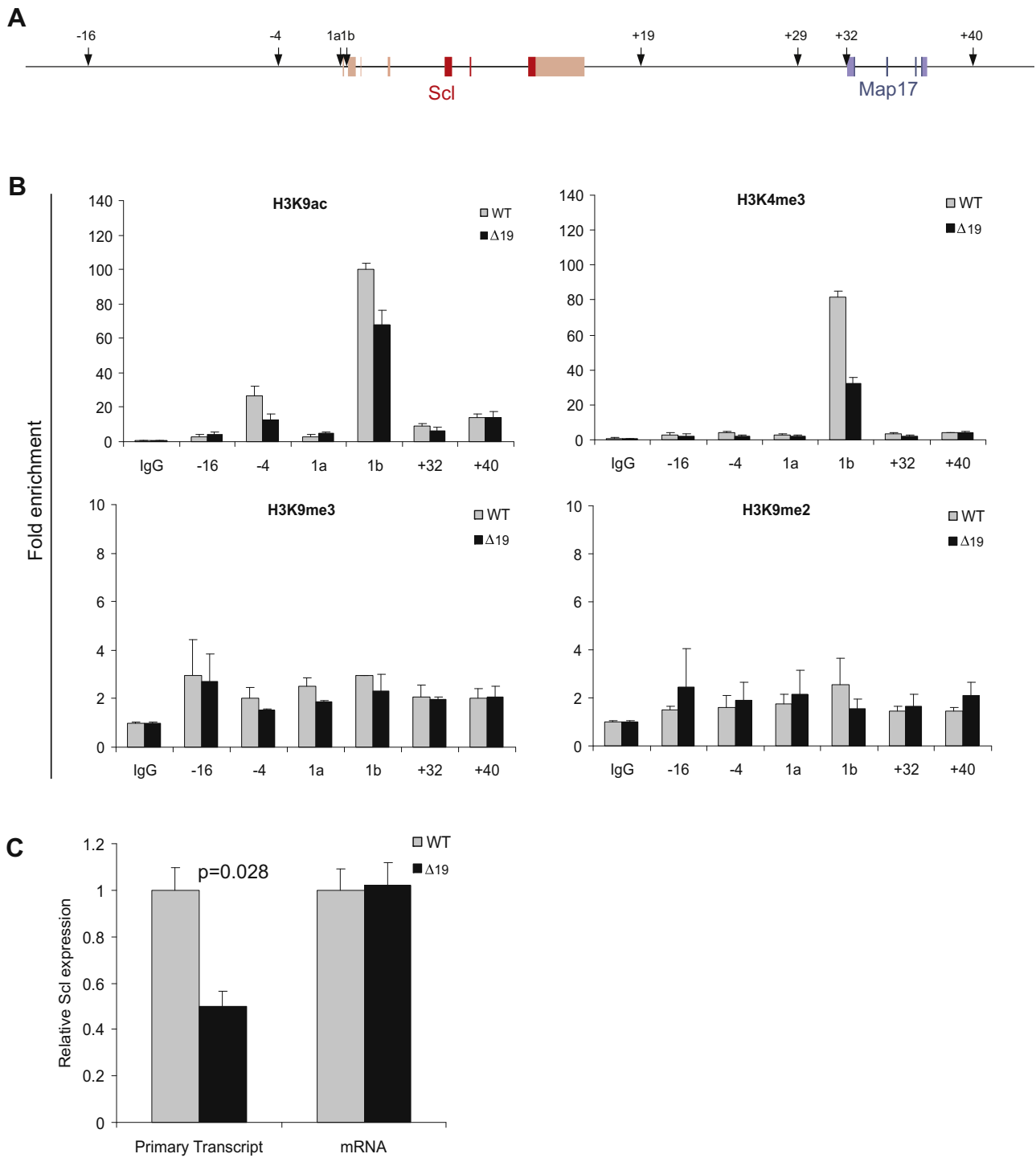


Figure 4. Decreased activity at *Scl* promoters in *Scl*^{Δ19/Δ19} mice. (A) The mouse *Scl* and neighboring *Map17* gene loci. *Scl* exons are depicted in red and the *Map17* exons in blue. Arrows indicate functionally characterized *Scl* regulatory elements. The number above each arrow indicates the position, in kb, of the enhancer in relation to the *Scl* promoter 1a. 1a- *Scl* Promoter 1a; 1b- *Scl* Promoter 1b. (B) ChIP assay for active (H3K4me3 and H3K9Ac) and repressive (H3K9me3 and H3K9me2) chromatin marks. Fold enrichment was compared with that of the IgG control. (C) qRT-PCR analysis of *Scl* primary transcript and mRNA expression in E14.5 fetal liver cells shows a reduction of primary transcript levels in enhancer deleted cells.

+40 regions), suggesting that the entire *Scl* transcriptional domain serves as a target for these upstream regulators. Of note, the recurrent presence of overlapping sets of transcription factors at several enhancers of a particular gene locus

may be one of the reasons that deletion of individual enhancer elements often causes only mild phenotypes.

HSCs have the ability of self-renewal, extensive proliferation, and to contribute to all lineages of hematopoiesis.

Adult HSCs can be divided into LT-HSC and ST-HSC populations. We observed a significant increase in the HSC-enriched LSK population in *Scl*^{Δ19/Δ19} mice. Of note, a similar phenotype has been described previously using conditional deletion of *Scl* in adult HSCs [12], demonstrating the specific function of the +19 element in HSC expression of *Scl*. Furthermore, even though the ST-HSCs and LT-HSCs in our *Scl*^{Δ19/Δ19} mouse model showed a reduced level of *Scl* expression, there was no major defect in the long-term competitive repopulation assay, which again is consistent with analysis of adult HSC function in conditional *Scl* knockout mice [10–12]. However, unlike the conditional *Scl* knockout, the *Scl*^{Δ19/Δ19} mutation is constitutive and, therefore, present at the early developmental stages where *Scl* function is absolutely critical [6–8]. Importantly, the data presented here suggests that the main compensatory mechanism for loss of the *Scl* +19 enhancer may not be through alternative regulatory elements. This notion is based on the observation that, despite the lack of change in steady-state mRNA levels, active histone marks over the *Scl* promoter as well as the levels of *Scl* primary transcripts were reduced significantly after deletion of the +19 enhancer from the endogenous *Scl* locus. It has been shown previously that *Scl* mRNA stability is enhanced during terminal erythroid maturation [34], thus providing a potential mechanism for post-transcriptional control of *Scl* mRNA steady-state levels.

It is, of course, not unexpected that the activity of important regulators such as *Scl* is controlled at multiple levels from transcription [14–19,23,24,35] to post-transcriptional [34,36] to translational [37] to post-translational [38] control. Compensation for loss of a transcriptional regulatory element can occur at any of those levels. The studies reported here have therefore not only demonstrated a remarkable tissue-specific function for the *Scl* +19 stem cell enhancer, but also highlight the need to consider nontranscriptional compensatory mechanisms when interpreting enhancer knockout phenotypes.

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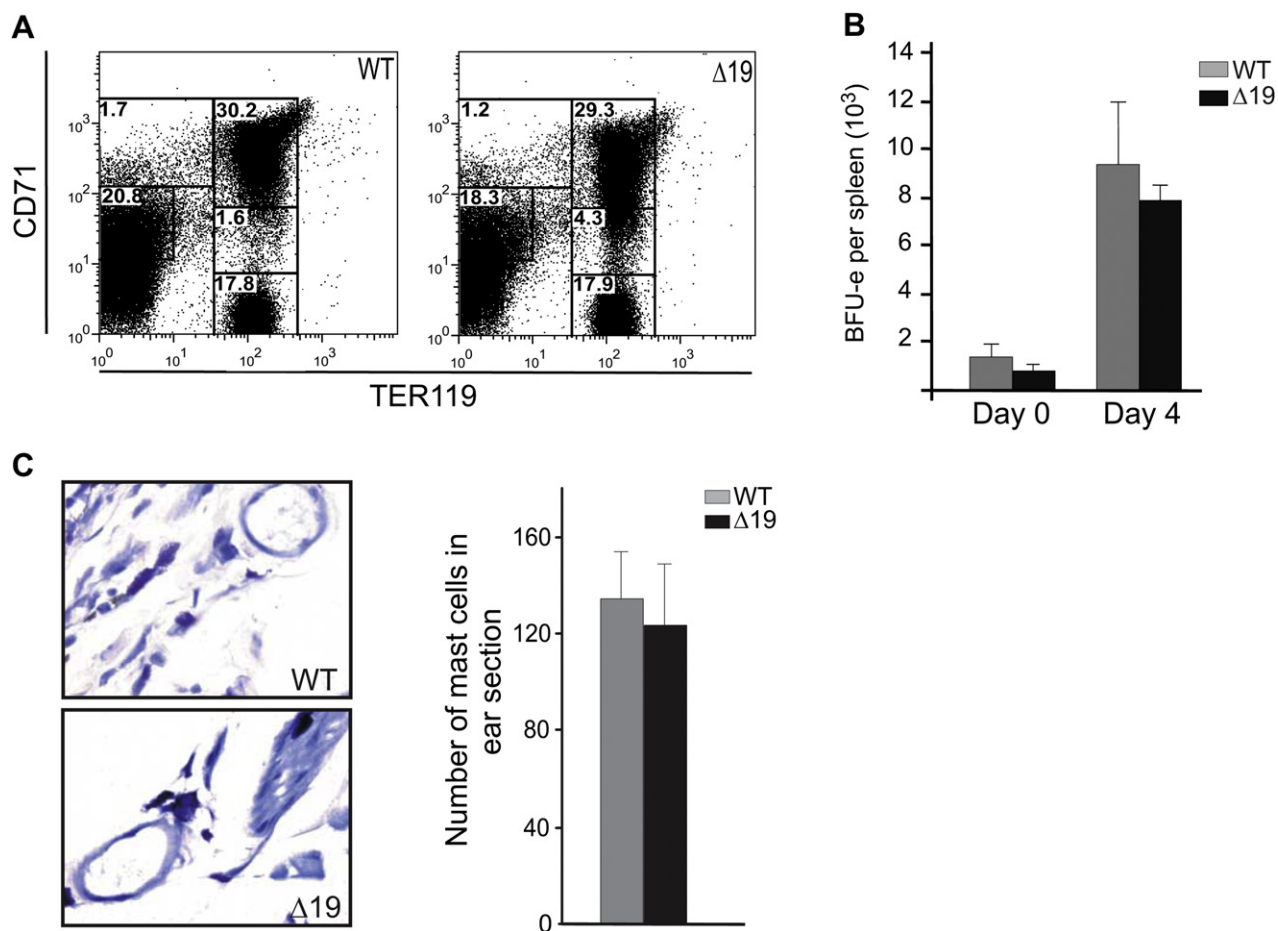
Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

References

1. Bloor AJ, Sanchez MJ, Green AR, Gottgens B. The role of the stem cell leukemia (SCL) gene in hematopoietic and endothelial lineage specification. *J Hematother Stem Cell Res.* 2002;11:195–206.
2. Begley CG, Aplan PD, Davey MP, et al. Chromosomal translocation in a human leukemic stem-cell line disrupts the T-cell antigen receptor delta-chain diversity region and results in a previously unreported fusion transcript. *Proc Natl Acad Sci U S A.* 1989;86:2031–2035.
3. Begley CG, Green AR. The SCL gene: from case report to critical hematopoietic regulator. *Blood.* 1999;93:2760–2770.
4. Elefanty AG, Begley CG, Metcalf D, Barnett L, Kontgen F, Robb L. Characterization of hematopoietic progenitor cells that express the transcription factor SCL, using a lacZ “knock-in” strategy. *Proc Natl Acad Sci U S A.* 1998;95:11897–11902.
5. Elefanty AG, Begley CG, Hartley L, Papaevangelou B, Robb L. SCL expression in the mouse embryo detected with a targeted lacZ reporter gene demonstrates its localization to hematopoietic, vascular, and neural tissues. *Blood.* 1999;94:3754–3763.
6. Robb L, Elwood NJ, Elefanty AG, et al. The *scl* gene product is required for the generation of all hematopoietic lineages in the adult mouse. *EMBO J.* 1996;15:4123–4129.
7. Porcher C, Swat W, Rockwell K, Fujiwara Y, Alt FW, Orkin SH. The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. *Cell.* 1996;86:47–57.
8. Robb L, Lyons I, Li R, et al. Absence of yolk sac hematopoiesis from mice with a targeted disruption of the *scl* gene. *Proc Natl Acad Sci U S A.* 1995;92:7075–7079.
9. Shivdasani RA, Mayer EL, Orkin SH. Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature.* 1995;373:432–434.
10. Hall MA, Curtis DJ, Metcalf D, et al. The critical regulator of embryonic hematopoiesis, SCL, is vital in the adult for megakaryopoiesis, erythropoiesis, and lineage choice in CFU-S12. *Proc Natl Acad Sci U S A.* 2003;100:992–997.
11. Mikkola HK, Klintman J, Yang H, et al. Haematopoietic stem cells retain long-term repopulating activity and multipotency in the absence of stem-cell leukaemia SCL/tal-1 gene. *Nature.* 2003;421:547–551.
12. Curtis DJ, Hall MA, Van Stekelenburg LJ, Robb L, Jane SM, Begley CG. SCL is required for normal function of short-term repopulating hematopoietic stem cells. *Blood.* 2004;103:3342–3348.
13. Brunet de la Grange P, Armstrong F, Duval V, et al. Low SCL/TAL1 expression reveals its major role in adult hematopoietic myeloid progenitors and stem cells. *Blood.* 2006;108:2998–3004.
14. Delabesse E, Ogilvy S, Chapman MA, Piltz SG, Gottgens B, Green AR. Transcriptional regulation of the SCL locus: identification of an enhancer that targets the primitive erythroid lineage in vivo. *Mol Cell Biol.* 2005;25:5215–5225.
15. Gottgens B, Barton LM, Gilbert JG, et al. Analysis of vertebrate SCL loci identifies conserved enhancers. *Nat Biotechnol.* 2000;18:181–186.
16. Gottgens B, Broccardo C, Sanchez MJ, et al. The *scl* +18/19 stem cell enhancer is not required for hematopoiesis: identification of a 5' bifunctional hematopoietic-endothelial enhancer bound by Fli-1 and Elf-1. *Mol Cell Biol.* 2004;24:1870–1883.
17. Sanchez M, Gottgens B, Sinclair AM, et al. An SCL 3' enhancer targets developing endothelium together with embryonic and adult haematopoietic progenitors. *Development.* 1999;126:3891–3904.
18. Sinclair AM, Gottgens B, Barton LM, et al. Distinct 5' SCL enhancers direct transcription to developing brain, spinal cord, and endothelium: neural expression is mediated by GATA factor binding sites. *Dev Biol.* 1999;209:128–142.
19. Sanchez MJ, Bockamp EO, Miller J, Gambardella L, Green AR. Selective rescue of early haematopoietic progenitors in *Scl*(-/-) mice by expressing *Scl* under the control of a stem cell enhancer. *Development.* 2001;128:4815–4827.
20. Gottgens B, Ferreira R, Sanchez MJ, et al. cis-Regulatory remodeling of the SCL locus during vertebrate evolution. *Mol Cell Biol.* 2010;30:5741–5751.

21. Salmon JM, Slater NJ, Hall MA, et al. Aberrant mast-cell differentiation in mice lacking the stem-cell leukemia gene. *Blood*. 2007;110:3573–3581.
22. Yang L, Bryder D, Adolfsson J, et al. Identification of Lin(-)Sca1(+) kit(+)CD34(+)Flt3- short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. *Blood*. 2005;105:2717–2723.
23. Ogilvy S, Ferreira R, Piltz SG, Bowen JM, Gottgens B, Green AR. The SCL +40 enhancer targets the midbrain together with primitive and definitive hematopoiesis and is regulated by SCL and GATA proteins. *Mol Cell Biol*. 2007;27:7206–7219.
24. Gottgens B, Nastos A, Kinston S, et al. Establishing the transcriptional programme for blood: the SCL stem cell enhancer is regulated by a multiprotein complex containing Ets and GATA factors. *EMBO J*. 2002;21:3039–3050.
25. Silberstein L, Sanchez MJ, Socolovsky M, et al. Transgenic analysis of the stem cell leukemia +19 stem cell enhancer in adult and embryonic hematopoietic and endothelial cells. *Stem Cells*. 2005;23:1378–1388.
26. Bender MA, Byron R, Ragoczy T, Telling A, Bulger M, Groudine M. Flanking HS-62.5 and 3' HS1, and regions upstream of the LCR, are not required for beta-globin transcription. *Blood*. 2006;108:1395–1401.
27. Bender MA, Roach JN, Halow J, et al. Targeted deletion of 5'HS1 and 5'HS4 of the beta-globin locus control region reveals additive activity of the DNaseI hypersensitive sites. *Blood*. 2001;98:2022–2027.
28. Hug BA, Wesselschmidt RL, Fiering S, et al. Analysis of mice containing a targeted deletion of beta-globin locus control region 5' hypersensitive site 3. *Mol Cell Biol*. 1996;16:2906–2912.
29. Anguita E, Sharpe JA, Sloane-Stanley JA, Tufarelli C, Higgs DR, Wood WG. Deletion of the mouse alpha-globin regulatory element (HS -26) has an unexpectedly mild phenotype. *Blood*. 2002;100:3450–3456.
30. Ahituv N, Zhu Y, Visel A, et al. Deletion of ultraconserved elements yields viable mice. *PLoS Biol*. 2007;5:e234.
31. Snow JW, Trowbridge JJ, Johnson KD, et al. Context-dependent function of "GATA switch" sites in vivo. *Blood*. 2011;117:4769–4772.
32. Tijssen MR, Cvejic A, Joshi A, et al. Genome-wide analysis of simultaneous GATA1/2, RUNX1, FLI1, and SCL binding in megakaryocytes identifies hematopoietic regulators. *Dev Cell*. 2011;20:597–609.
33. Wilson NK, Foster SD, Wang X, et al. Combinatorial transcriptional control in blood stem/progenitor cells: genome-wide analysis of ten major transcriptional regulators. *Cell Stem Cell*. 2010;7:532–544.
34. Murrell AM, Bockamp EO, Gottgens B, et al. Discordant regulation of SCL/TAL-1 mRNA and protein during erythroid differentiation. *Oncogene*. 1995;11:131–139.
35. Bockamp EO, McLaughlin F, Murrell AM, et al. Lineage-restricted regulation of the murine SCL/TAL-1 promoter. *Blood*. 1995;86:1502–1514.
36. Begley CG. The SCL transcription factor and differential regulation of macrophage differentiation by LIF, OSM and IL-6. *Stem Cells*. 1994; 12(suppl 1):143–149. discussion 149–151.
37. Calkhoven CF, Muller C, Martin R, et al. Translational control of SCL-isoform expression in hematopoietic lineage choice. *Genes Dev*. 2003;17:959–964.
38. Tang T, Arbiser JL, Brandt SJ. Phosphorylation by mitogen-activated protein kinase mediates the hypoxia-induced turnover of the TAL1/SCL transcription factor in endothelial cells. *J Biol Chem*. 2002;277:18365–18372.



Supplementary Figure E1. *Scf*^{Δ19/Δ19} mice hematopoietic phenotype. (A) Representative fluorescence-activated cell sorting analysis of CD71 and Ter119 staining of adult BM shows a normal erythroid differentiation profile for WT and *Scf*^{Δ19/Δ19} mice. (B) Erythropoiesis of 6- to 8-week-old sex- and age-matched *Scf*^{WT/WT} and *Scf*^{Δ19/Δ19} mice was stressed with phenylhydrazine (PHZ). Burst-forming erythroid (BFU-e) colonies were increased equally in the spleens of *Scf*^{WT/WT} and *Scf*^{Δ19/Δ19} mice 4 days after the first injection. (C) Left panel shows representative ear sections of *Scf*^{WT/WT} and *Scf*^{Δ19/Δ19} stained with Toluidine blue and quantification of mast cells is summarized in histogram.