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# *Zeb1* maintains long-term adult hematopoietic stem cell function and extramedullary hematopoiesis

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Emerging evidence implicates the epithelial-mesenchymal transition transcription factor *Zeb1* as a critical regulator of hematopoietic stem cell (HSC) differentiation. Whether *Zeb1* regulates long-term maintenance of HSC function remains an open question. Using an inducible *Mx-1-Cre* mouse model that deletes conditional *Zeb1* alleles in the adult hematopoietic system, we found that mice engineered to be deficient in *Zeb1* for 32 weeks displayed expanded immunophenotypically defined adult HSCs and multipotent progenitors associated with increased abundance of lineage-biased/balanced HSC subsets and augmented cell survival characteristics. During hematopoietic differentiation, persistent *Zeb1* loss increased B cells in the bone marrow and spleen and decreased monocyte generation in the peripheral blood. In competitive transplantation experiments, we found that HSCs from adult mice with long-term *Zeb1* deletion displayed a cell autonomous defect in multilineage differentiation capacity. Long-term *Zeb1* loss perturbed extramedullary hematopoiesis characterized by increased splenic weight and a paradoxical reduction in splenic cellularity that was accompanied by HSC exhaustion, lineage-specific defects, and an accumulation of aberrant, preleukemic like c-kit$^+$CD16/32$^+$ progenitors. Loss of *Zeb1* for up to 42 weeks can lead to progressive splenomegaly and an accumulation of Gr-1$^+$Mac-1$^+$ cells, further supporting the notion that long-term expression of *Zeb1* suppresses preleukemic activity. Thus, sustained *Zeb1* deletion disrupts HSC functionality in vivo and impairs regulation of extramedullary hematopoiesis with potential implications for tumor suppressor functions of *Zeb1* in myeloid neoplasms. 

## HIGHLIGHTS

- *Zeb1* loss increases lineage-biased HSC subsets and increases HSC survival potential.
- *Zeb1* loss induces a cell-intrinsic HSC differentiation defect after transplantation.
- *Zeb1* loss disrupts extramedullary hematopoiesis with preleukemic potential.

Epithelial to mesenchymal transition (EMT) is a mechanism that is utilized in the fundamental physiologic processes of embryogenesis, wound healing, and tissue development where epithelial cells reduce their cell polarity and cell adhesion properties while acquiring mesenchymal-like cell characteristics and enhanced migratory potential [1,2]. Deregulated EMT has been observed in multiple pathologic settings, including organ fibrosis that can cause organ failure [3], and cancer, where increased EMT-mediated migratory capacity of cancer cells promotes metastasis and, therefore, therapy resistance [4]. Understanding the transcriptional mechanisms regulating EMT in embryogenesis, tissue maintenance, and disease contexts are therefore of considerable biological and clinical interest.

EMT is specifically regulated by transcription factors (TFs) in the ZEB, SNAI and TWIST families [2,5]. Zinc finger E-box binding homeobox TF *Zeb1* functions as an archetypal EMT TF in postgastrulation embryogenesis and tissue maintenance and development processes, including myogenesis, neurogenesis, chondrocyte development, and skeletal development [6–12]. In the context of cancer, deregulated *Zeb1* function drives EMT in solid tumors, enhancing metastasis and, ultimately, conferring drug resistance [13–15].

EMT TFs are more recently emerging as critical regulators in nonepithelial tissues, with prominent roles identified for ZEB, SNAI, and TWIST family members in the hematopoietic system [16–22]. A

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0301-472X/

https://doi.org/10.1016/j.exphem.2024.104177

case in point is *Zeb1*, which acts as a crucial modulator of hematopoietic stem cell (HSC) fate decisions, including self-renewal. Beyond a well-established role in T-cell differentiation [17,18], *Zeb1* functions as an essential regulator of multilineage differentiation in hematopoiesis [18–20]. Whether *Zeb1* regulates long-term maintenance of adult HSCs, however, remains ill-defined. Studies performed to date to elucidate the role of *Zeb1* in HSCs have largely been carried out in mouse models where *Zeb1* was conditionally deleted in the hematopoietic system [18,20]. In our previous study, *Zeb1* deletion was induced in "floxed" *Zeb1;Mx1-Cre*+ mice by the administration of the interferon mimic polyinosinic:polycytidylic acid (poly I:C), and HSCs in adult $Zeb1^{-/-}$ mice were analyzed at 14 days after the last injection of poly I:C [18]. However, poly I:C is known to temporarily induce changes in HSC proliferation and frequency consistent with the impact of interferon type I responses on HSCs that may confound analysis of adult HSC function in the setting of *Zeb1* deletion [23]. In principle, this issue can be circumvented by breeding *Zeb1* "floxed" mice to constitutively active, hematopoietic-specific *Vav-Cre* + mice, a strategy effectively used by another laboratory together with other *Cre* recombinase mouse strains to confirm that *Zeb1* acts as a critical mediator of HSC differentiation in synergy with the ZEB family member, *Zeb2* [20]. However, *Vav-Cre* conditionally ablates gene function in utero when HSCs emerge at approximately embryonic day 11 [24,25], and the *Zeb1* phenotype observed may merely reflect the functional impact of *Zeb1* loss during HSC development in the embryo rather than adult HSC maintenance.

Here, we revisit the genetically engineered mouse model that contains "floxed" alleles of *Zeb1* ($Zeb1^{fl/fl}$ mice) and an inducible *Mx1-Cre* ($Mx1\text{-}Cre^{+/-}$) to explore whether *Zeb1* is required for the long-term maintenance of adult HSCs. Reasoning that the proinflammatory milieu induced by poly I:C administration would subside with time, we waited for 32 weeks after induction of *Zeb1* deletion to analyze the hematopoietic potential of $Zeb1^{-/-}$ mice and identified that *Zeb1* regulates long-term cell-autonomous HSC function, including multilineage differentiation, and extramedullary hematopoiesis in the spleen.

## MATERIALS AND METHODS

### Mice

We utilized $Zeb1^{fl/fl}$ mice that were bred with $Mx1\text{-}Cre^{+/-}$ mice to generate an experimental cohort of $Zeb1^{fl/fl}$;$Mx1\text{-}Cre^{-/-}$ (control) and $Zeb1^{fl/fl}$;$Mx1\text{-}Cre^{+/-}$ ($Zeb1^{-/-}$) mice [26]. *Zeb1* was deleted after intraperitoneal administration of poly I:C (6 doses every alternate day, 0.3 mg per dose, GE Healthcare). All experiments were performed under the regulations of the UK Home Office.

### Genotyping

To confirm *Zeb1* deletion, genomic DNA was isolated from peripheral blood (PB), PB T cells, C-KIT$^+$ bone marrow (BM) cells and total BM cells using Isolate II Genomic DNA Kit (Bioline) according to the manufacture instructions. Polymerase chain reaction (PCR) was performed on a T100$^{TM}$ Thermal Cycler (Bio-Rad) to amplify the genomic DNA. The PCR reaction mix included the following in a final volume of 25 $\mu$L: 12.5 $\mu$L of the Mango Mix (Bioline), 0.10 $\mu$L of each primer either *Zeb1* or *Cre* (the stock concentration was prepared at 100 uM), 8.30 $\mu$L of nuclease-free water and 4 $\mu$L of each DNA sample (final DNA concentration ranges from 20 to 80 ng/$\mu$L). The T100$^{TM}$ Thermal Cycler was set to the following conditions for *Zeb1*: volume 25 $\mu$L; 95°C for 5 min; 95°C for 30 sec, 64°C for 45 sec, and 72°C for 1 min 39x; 72°C for 5 min; and 10°C for ∞. The following conditions were used for *Cre*: volume 25 $\mu$L; 95°C for 3 min; 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min 30x; 72°C for 5 min; and 10°C for ∞. The amplified products of the PCR reaction were run on a 2% agarose gel containing a 1:30,000 dilution of SafeView (BioLegend). Gel bands were detected using a Bio-Rad Gel Doc XR and viewed and annotated using ImageLab Software. The PCR primer sequences are as follows:

*Zeb1*fl forward 5′–CGTGATGGAGCCAGAATCTGACCCC–3′,
*Zeb1*fl reverse 5′–GCCCTGTCTTTCTCAGCAGTGTGG-3′,
*Zeb1* excised reverse 5′–GCCATCTCACCAGCCCTTACTGTGC-3′,
Generic Cre forward 5′–TGACCGTACACCAAAATTTG–3′, and
Generic Cre reverse 5′–ATTGGCCCTGTTTCACTATC–3′.

### Flow Cytometry Analysis

Bones (femurs, tibias, iliac bones) were crushed using a pestle and mortar in phosphate-buffered saline (PBS) supplemented with 2% fetal bovine serum (FBS), and the BM cell suspension was filtered through a 70 $\mu$m cell strainer (Miltenyi Biotec). Spleen and thymi were minced through a 70-$\mu$m cell strainer to obtain a homogeneous cell suspension. PB was obtained from the tail vein in ethylenediaminetetraacetic acid-treated tubes (Starstedt). Red blood cells were lysed using an ammonium chloride solution (StemCellTechnologies). For the immunophenotypic analysis, cells were stained as follows. For hematopoietic stem and progenitor cells (HSPCs; LSK and SLAM), a lineage cocktail was prepared from a pool of biotinylated antibodies of differentiated cell markers in PBS with 2% FBS (MAC1 and GR1 for myeloid cells; TER119 for erythroid lineage; B220 for B cells; and CD3e, CD4, CD8a for T cells). Here, SCA1-APCCy7, CKIT-APC, CD150-PECy7, and CD48-FITC were used to study HSCs, multipotent progenitors (MPPs), HPC1, and HPC2, respectively. For the committed progenitors (LK), the same lineage cocktail as described for LSK SLAM was used, and SCA1-APCCy7, CKIT-APC, CD34-FITC, CD16/32-PECy7, CD135-PE, and CD127-BV650 were used to study CMP, GMP, MEP, CLP, and innate lymphoid cells (ILC2) defined as (Lin$^-$ SCA-1$^+$ CKIT$^-$). The lineage cocktail was detected by adding streptavidin as a secondary antibody. Lineage-positive cells from the BM and spleen were stained for GR1-PECy7 and MAC1-APC (myeloid cells), CD4-PE and CD8-APCCy7 (T cells), and B220-FITC (B cells). For the preleukemic-like population in spleen, cells were stained for CD127-BV650, CKIT-APC, and CD16/32-PECy7 and analyzed as CD127$^-$ CKIT$^+$ CD16/32$^+$. For the apoptosis assay, after staining the cells for cell surface markers, they were stained with Annexin V-PE antibody (BioLegend) for 30 min in the dark at room temperature, and diamidino-2-phenylindole (DAPI, 1 $\mu$g/mL) (Molecular Probes) was added before running the samples. Cell cycle analysis in HSCs and HPC1 was performed using intracellular staining of Ki67. After extracellular staining, the cells were fixed in 1% paraformaldehyde (PFA) (ThermoFisher) for 20 min at 4°C, permeabilized using PBS containing 0.1% saponin (Sigma) for 30 min at 4°C, and then stained with Ki67 antibody for 30 min at 4°C in dark. Cells were incubated with DAPI at a final concentration of 5 $\mu$g/mL in the dark for 5 min before running the samples. All antibodies were purchased from BioLegend except CD34, which

was obtained from eBioscience. Samples were analyzed using BD LSRFortessa™ (BD Biosciences). Data were analyzed using FlowJo 10.0.8 (Tree Star, Inc.).

For HSC sorting, BM cell suspension was obtained, and red blood cells were lysed using ammonium chloride solution (StemCellTechnologies). Cells were enriched for CKIT by magnetic-activated cell sorting (MACS) (MACS®, Miltenyi Biotec) using anti-CKIT magnetic beads (Miltenyi Biotec). $CKIT^+$ cells were stained as described earlier, and HSCs were sorted using a BD FACSAria™ Fusion (BD Biosciences).

### Transplantation Experiments

For primary transplantation, 150 HSCs from *Zeb1*$^{-/-}$ and control cells mixed with $2 \times 10^5$ whole BM (CD45.1) (supporting cells) were intravenously transplanted into lethally irradiated mice (CD45.1). To monitor the engraftment, tail vein bleeding was performed at different time points post-transplant.

For cell autonomous transplantation, *Zeb1* was deleted specifically in hematopoietic cells (but not in BM niche cells) after transplanting $5 \times 10^5$ whole BM (CD45.2) from *Zeb1*$^{fl/fl}$ *Mx1-Cre*$^+$ and *Zeb1*$^{fl/fl}$ *Mx1-Cre*$^-$ along with $5 \times 10^5$ whole BM (CD45.1) (supporting cells) into lethally irradiated recipients (CD45.1). Six weeks later, six doses of poly I:C (every alternate day, 0.3 mg per dose) were intraperitoneally injected to delete *Zeb1*. Mice were dissected at week 32 after the last dose of poly I:C and analyzed. For the cell autonomous secondary transplantation, 200 CD45.2 donor HSCs were sorted from control and *Zeb1*$^{-/-}$ primary recipients, mixed with competitor cells, and retransplanted into lethally irradiated recipients.

### Statistical Analysis

Figures were prepared using Prism (GraphPad Software, Inc.). Statistical analyses were done using the Mann-Whitney *U* test to calculate significance as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Outliers were detected using Prism.

## RESULTS

### *Zeb1* Maintains Long-term Multilineage Hematopoietic Differentiation

In this report, we used a conditional inducible *Zeb1* knockout (KO) mouse model by crossing *Zeb1*$^{fl/fl}$ mice [26] with mice expressing interferon-inducible *Mx1-Cre* recombinase [27]. This generated offspring that were either *Zeb1*$^{fl/fl}$*;Mx1-Cre*$^{-/-}$ (control) or *Zeb1*$^{fl/fl}$*;Mx1-Cre*$^{+/-}$ mice. *Zeb1*$^{fl/fl}$*;Mx1-Cre*$^{+/-}$ mice or control mice were injected intraperitoneally with poly I:C, which activates the *Mx1* promoter to stimulate Cre recombinase expression that excises the "floxed" *Zeb1* alleles in hematopoietic tissues of *Zeb1*$^{fl/fl}$*;Mx1-Cre*$^{+/-}$ mice [27] and generates *Zeb1*$^{-/-}$ mice. To study the requirement of *Zeb1* in long-term hematopoiesis, control or *Zeb1*$^{-/-}$ mice were left for 32 weeks after poly I:C treatment, and PB was monitored every 4 weeks to assess changes in hematopoiesis after *Zeb1* loss during this time (Figure 1A). A significant, sustained reduction in the proportion of $Mac1^+$ $Gr1^-$ cells that comprise monocytes was observed in PB from *Zeb1*$^{-/-}$ mice over 32 weeks, whereas $Mac1^+$ $Gr1^+$ cells that contain granulocytes remained unchanged during the same time (Figure 1B and C) [28,29]. Over 32 weeks, no significant change was observed in B cells in PB from *Zeb1*$^{-/-}$ mice (Figure 1D). We observed that T-cell frequency was unchanged at week 4 after *Zeb1* deletion, significantly reduced by week 16, and subsequently restored at 32 weeks (Figure 1E). Given the established critical role of *Zeb1* in T-cell development demonstrated by our laboratory and others [9,17,18], this unanticipated result was likely attributable to incomplete *Zeb1* deletion observed in T cells (Figure 1F). This caveat aside, our data demonstrate the requirement for *Zeb1* in the long-term maintenance of HSC-mediated differentiation to $Mac\text{-}1^+$ $Gr1^-$ monocytes in PB.

The hematopoietic potential in BM of *Zeb1*$^{-/-}$ mice was evaluated at 32 weeks following induction of *Zeb1* deletion. We first assessed the total cellularity of BM from *Zeb1*$^{-/-}$ mice, which was marginally reduced (Figure 2A). Next, we immunophenotyped differentiated hematopoietic cells from BM. The frequency of B cells significantly increased in *Zeb1*$^{-/-}$ mice, yet myeloid, erythroid, and T cells were unaltered (Figure 2B). When we quantified the absolute number of these populations to account for the observed changes in cellularity after *Zeb1* deletion, we found a comparable number of B cells in the BM, a reduction in T cell number, and no significant alterations in myeloid cell compartments of *Zeb1*$^{-/-}$ mice (Figure 2C). Incomplete deletion of *Zeb1* was observed in total BM of poly I:C-treated *Zeb1*$^{fl/fl}$*;Mx1-Cre*$^{+/-}$ mice, suggesting that some differentiated hematopoietic cells partially escaped *Zeb1* inactivation in the BM (Supplementary Figure E1A).

### Persistent *Zeb1* Loss Expands HSPC Numbers in Adult Hematopoiesis

To examine the requirement of *Zeb1* for long-term maintenance of HSPCs, we immunophenotyped stem and progenitor cell numbers in the BM at 32 weeks after *Zeb1* deletion. Using the SLAM markers CD150 and CD48, we analyzed four populations within the LSK ($Lin^-$ $Sca\text{-}1^+$ $c\text{-}Kit^+$) population: LSK $CD150^+$ $CD48^-$ (HSCs), LSK $CD150^-$ $CD48^-$ (MPPs), LSK $CD150^-$ $CD48^+$ (HPC1), and LSK $CD150^+$ $CD48^+$ (HPC2) (Figure 2D). *Zeb1*$^{-/-}$ mice displayed an expansion in frequency and absolute numbers of HSCs and MPPs (Figure 2E and F). However, HPC1 frequency was reduced in *Zeb1*$^{-/-}$ mice, whereas there was no change in the frequency of HPC2 (Figure 2E). The absolute numbers of HPC1 and HPC2 were significantly reduced in *Zeb1*$^{-/-}$ mice compared with control mice (Figure 2F). Complete deletion of *Zeb1* was observed in HSPCs containing c-Kit+ cells from the BM of poly I:C-treated *Zeb1*$^{fl/fl}$*;Mx1-Cre*$^{+/-}$ mice (Supplementary Figure E1A). These data indicate that *Zeb1* is crucially required to maintain the abundance of HSPCs during long-term, steady-state adult hematopoiesis.

### Long-term *Zeb1* Loss Confers an Expansion of Lineage-biased HSC subsets and a Cell Survival Advantage for HSPCs Without Changes in Cell Cycle Status

Next, we explored the potential cellular mechanisms underpinning HSC and MPP expansion following long-term *Zeb1* deletion. Genetic and epigenetic heterogeneity contributes to the differential long-term clonal expansion of HSCs with ramifications for myeloid or lymphoid bias or balanced myeloid/lymphoid potential [30]. After long-term *Zeb1* deletion, in the HSC compartment we found evidence for increased frequency and absolute number of $CD150^{low}$ (lymphoid-biased HSCs), $CD150^{med}$ (lineage-balanced HSCs), and $CD150^{high}$ (myeloid-biased HSCs) (Supplementary Figure E1B and C). Notably, myeloid-biased HSCs from *Zeb1*$^{-/-}$ mice were the most profoundly increased HSC population (Supplementary Figure E1B and C). These

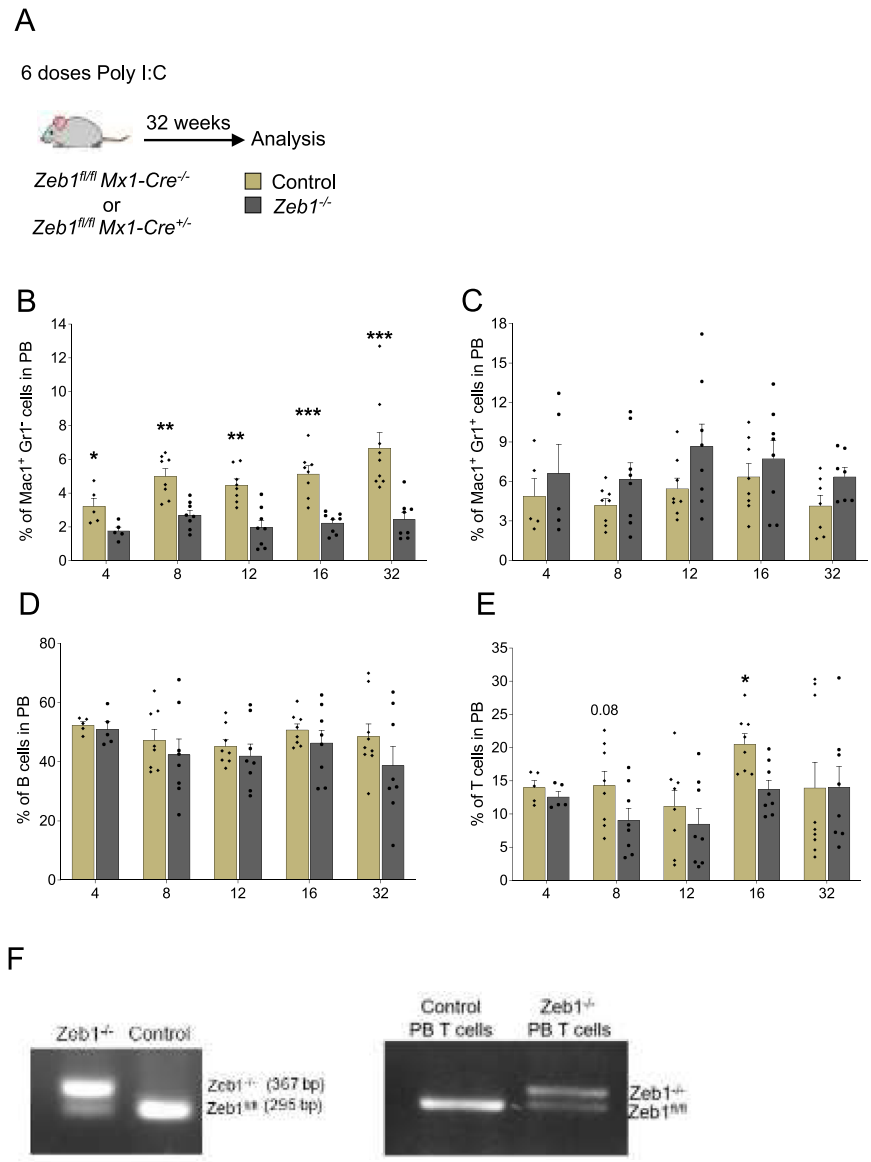


**Figure 1** Persistent loss of *Zeb1* leads to a reduction in Mac1$^+$ myeloid cells in PB. **(A)** A scheme of poly I:C treatment of *Zeb1*$^{fl/fl}$ *Mx1-Cre*$^-$ (control) and *Zeb1*$^{fl/fl}$ *Mx1-Cre*$^+$ (*Zeb1*$^{-/-}$) mice and analysis at week 32 after the last dose of poly I:C. **(B-E)** The frequency of differentiated cells in PB from control and *Zeb1*$^{-/-}$ mice at different time points after the last dose of poly I:C from 2 to 4 independent experiments (n = 5 at week 4, n = 8 at weeks 8–16, n = 8–9 at week 32). **(F)** Representative gel electrophoresis analysis confirming *Zeb1* deletion in total PB and PB T cells. Error bars show mean ± SEM. Mann-Whitney *U* test was used to calculate significance as follows: **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

data highlight deregulated lineage bias of HSCs in the context of persistent *Zeb1* deletion.

We then asked if the expansion of HSCs and MPPs in *Zeb1*$^{-/-}$ mice was caused by enhanced cell survival. Immunophenotypic expansion of HSCs and MPPs in *Zeb1*$^{-/-}$ mice was associated with a reduction in apoptotic levels, whereas the reduction in HPC1 and HPC2 numbers was not associated with deregulated apoptosis, as judged by the Annexin V assay (Figure 2G). We also questioned whether expansion of HSCs in *Zeb1*$^{-/-}$ mice was associated with changes in HSC proliferation. Intracellular staining of Ki-67, a nuclear proliferation antigen [31], and DAPI allows analysis of the quiescent phase of the cell cycle, G0, where cells do not express Ki-67, to be distinguished from actively cycling counterparts in G1, S, and G2-M phases of the cell cycle [31–33]. No alterations in the G0, G1, and S-G2-M cycling status of HSCs and MPPs from *Zeb1*$^{-/-}$ mice were observed (Supplementary Figure E1D and E). Together, these data imply that *Zeb1* acts as a regulator of HSPC apoptosis independently of cell cycle regulation.

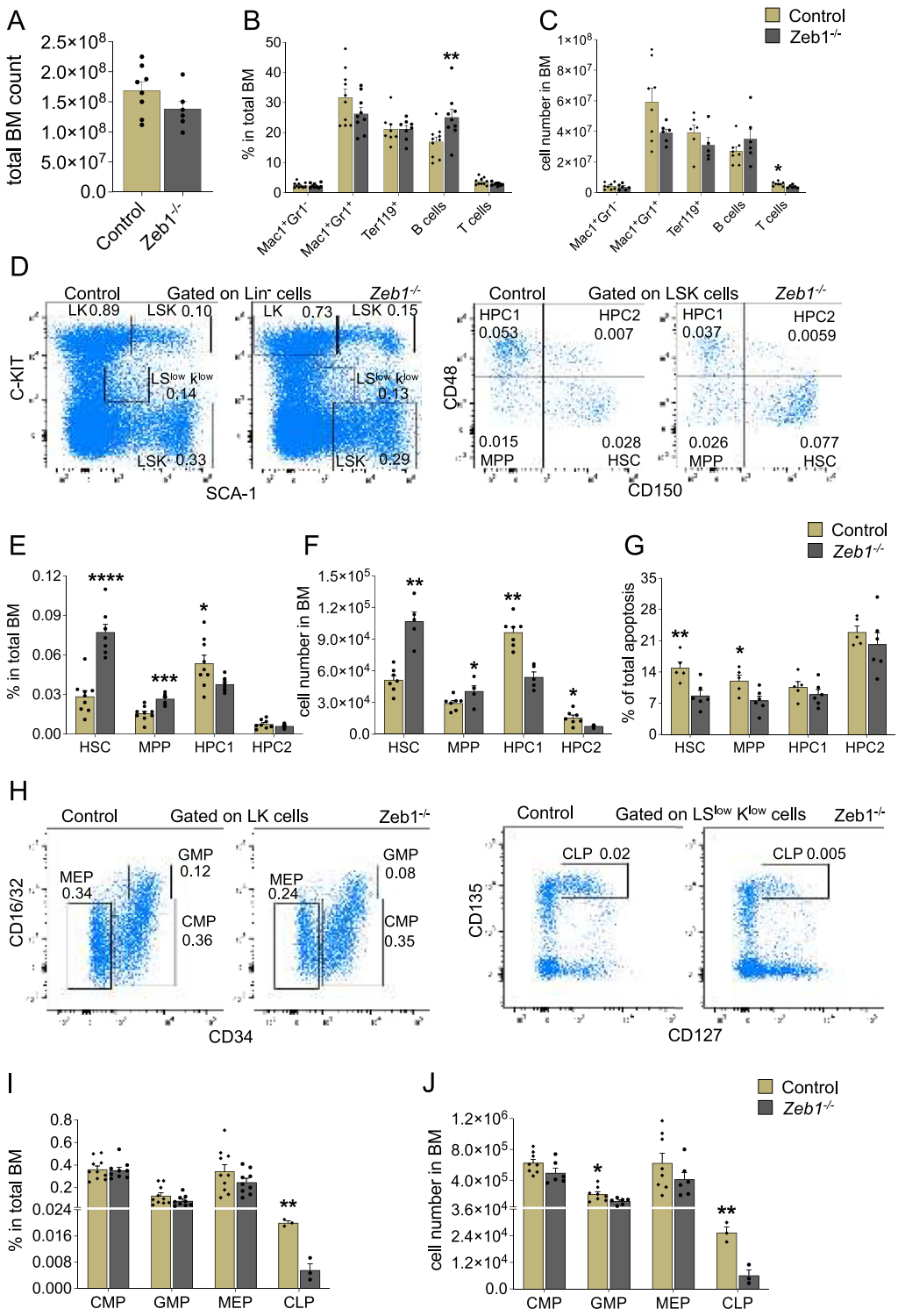


Figure 2 Prolonged loss of *Zeb1* results in an expansion of BM HSCs associated with enhanced cell survival. **(A)** Analysis of total cells of BM from control (n = 8) and *Zeb1*$^{-/-}$ mice (n = 6) 32 weeks after the last dose of poly I:C from three independent experiments. **(B)** Analysis of the frequency of differentiated cells in BM at week 32 after *Zeb1* deletion from 3–5 independent experiments (n = 9–10, except Ter119$^{+}$ with n = 8). **(C)** Analysis of the absolute cell count of the differentiated cells in BM at week 32 after *Zeb1* deletion from 3–4 independent experiments (n = 6-8, except Ter119$^{+}$ with n= 5–6). **(D)** Representative FACS plots of the analysis of HSPCs, including HSC, MPP, HPC1, and HPC2, according to the expression of SLAM markers CD150 and CD48 from control and *Zeb1*$^{-/-}$ 32 weeks after the last dose of poly I:C. **(E)** Analysis of the frequency of HSPCs in the BM from control (n = 9) and *Zeb1*$^{-/-}$

### *Zeb1* is Required for Common Lymphoid Progenitors in Long-term Hematopoiesis

We next asked if long-term loss of *Zeb1* expression affected committed myeloid and lymphoid progenitors downstream of HSCs and MPPs. Within the LK population using CD34 and CD16/32, we analyzed three populations: LK CD34$^+$ CD16/32$^-$ (CMP), LK CD34$^+$ CD16/32$^+$ (GMP), and LK CD34$^-$ CD16/32$^-$ (MEP) (Figure 2H). These populations give rise to myeloid, megakaryocytic, and erythroid lineages. By analyzing both the frequency and absolute numbers of these cells, we did not find significant differences between the two genotypes, except for the GMP compartment, which was reduced in absolute number (Figure 2I and J). In striking contrast, common lymphoid progenitor (CLP) cells, which are defined as Lin$^-$ c-Kit$^{low}$ Sca-1$^{low}$ CD127$^+$ (IL7r$\alpha$) CD135$^+$, were reduced in frequency and absolute number in *Zeb1*$^{-/-}$ mice (Figure 2H, I and J). These data indicate that *Zeb1* is required for maintenance of CLPs during long-term, steady-state adult hematopoiesis.

### HSCs Lacking Long-term *Zeb1* Expression Show a Multilineage Hematopoietic Differentiation Defect After Transplantation

To test the functionality of HSCs from *Zeb1*$^{-/-}$ mice, we isolated 150 HSCs (CD45.2) using fluorescence-activated cell sorting (FACS) from either control or *Zeb1*$^{-/-}$ mice at 32 weeks following deletion, mixed them with 2 $\times$ 10$^5$ BM competitor cells (CD45.1) and transplanted this cell mixture into lethally irradiated recipients (CD45.1) (Figure 3A). Engraftment capacity was monitored in PB until week 16. Significant engraftment failure was observed in *Zeb1*-deficient HSCs from week 8 and continued to decrease progressively until week 16 (Figure 3B). To test the donor contribution to specific hematopoietic lineages in PB, we analyzed PB for CD45.2 (donor) and CD45.1 (competitor) in conjunction with Mac1$^+$ myeloid, Mac1$^+$ Gr1$^+$ myeloid, B220$^+$ B cells, and CD4$^+$/CD8$^+$ T cells. The most profound engraftment defect to PB was noted within lymphoid lineages where no engrafted T cells were derived from recipients transplanted with *Zeb1*$^{-/-}$ HSCs and a substantial reduction in donor contribution to B cells and Mac1$^+$ was observed in recipients of *Zeb1*$^{-/-}$ HSCs (Figure 3C). Furthermore, we noted a reduction in donor contribution to Mac1$^+$ Gr1$^+$ myeloid cells (Figure 3C). Thus, long-term *Zeb1* expression is required for the multilineage differentiation function of HSCs.

At 16 weeks after primary HSC transplantation, we evaluated control or *Zeb1*$^{-/-}$ donor engraftment in the BM and spleen. Consistent with PB data, we found reduced *Zeb1*$^{-/-}$ donor contribution to total BM (Figure 3D). In the BM of recipients, we found no donor T cell engraftment from *Zeb1*$^{-/-}$ HSCs, and donor contribution to B cells and Mac1$^+$ and Mac1$^+$ Gr1$^+$ myeloid cells were dramatically reduced from recipients receiving *Zeb1*$^{-/-}$ HSCs (Figure 3D). Furthermore, a similar pattern of splenic engraftment defect was observed in recipients of *Zeb1*$^{-/-}$ HSCs (Figure 3E).

We asked whether the multilineage differentiation defects observed in *Zeb1*$^{-/-}$ HSCs originate from engraftment defects in HSCs or committed progenitors. Within LSK compartments, the donor contribution to HSC, MPP, and HPC2 was equal between recipients of control or *Zeb1*$^{-/-}$ HSCs (Figure 3F). However, there was a significant reduction in the donor contribution to HPC1 in the *Zeb1*$^{-/-}$ genotype compared with control (Figure 3F). We also analyzed committed progenitors downstream of HSPCs and found a dramatic reduction in donor contribution to GMP, CLP, and ILC2 populations and near significant reductions in CMP and MEP populations in recipients of *Zeb1*$^{-/-}$ HSCs (Figure 3G). Overall, these data demonstrate that although long-term *Zeb1* loss expands the immunophenotypically defined HSC compartment (Figure 2E and F), *Zeb1*$^{-/-}$ HSCs are functionally compromised, as evidenced by the multilineage hematopoietic differentiation defects observed after transplantation.

### Long-term *Zeb1* Expression is Required for Cell-autonomous HSC Function

The *Mx1-Cre* conditional gene KO system can delete genes in nonhematopoietic tissues that regulate hematopoietic function, such as BM niche cells [27,34]. To obviate this issue, we used competitive transplantation experiments to evaluate the cell-intrinsic requirement for *Zeb1* in the long-term maintenance of HSC function. We transplanted 5 $\times$ 10$^5$ BM cells from *Zeb1*$^{fl/fl}$*;Mx1-Cre*$^-$ and *Zeb1*$^{fl/fl}$*;Mx1-Cre*$^+$ (CD45.2) admixed with an equal number of competitor cells (CD45.1) into lethally irradiated recipients (CD45.1). Eight weeks later, *Zeb1* deletion was induced by administering recipients with poly I:C (Figure 4A). Mice were monitored by PB bleeding for 32 weeks following *Zeb1* deletion (Figure 4A). Before treating mice with poly I:C, donor cells were comparable between both genotypes (Figure 4B). However, we observed a gradual reduction in *Zeb1*$^{-/-}$ donor cells in PB (Figure 4B) and Mac1$^+$ and Mac1$^+$ Gr1$^+$ myeloid cells from 4 weeks to 32 weeks after *Zeb1* deletion along with more marked changes in B cell, and T cell engraftment (Figure 4C–F). These data demonstrate that long-term *Zeb1* expression modulates HSC multilineage differentiation in a cell autonomous manner.

At 32 weeks after the last dose of poly I:C, we evaluated whether donor contributions to HSPCs and progenitor compartments in the BM of transplant recipients were perturbed in the context of long-term *Zeb1* deletion. No significant change was noted in donor contributions of HSCs and MPPs between the two genotypes; however, a moderate reduction in HPC2 and a more substantial reduction in HPC1 was observed in the *Zeb1*$^{-/-}$ genotype (Figure 4G). Within the committed

(n = 7) mice from four independent experiments at week 32 after *Zeb1* deletion. **(F)** Analysis of the absolute cell count of HSPCs in the BM from control (n = 7) and *Zeb1*$^{-/-}$ (n = 5) mice from three independent experiments at week 32 after *Zeb1* deletion. **(G)** Apoptosis analysis using Annexin V in BM HSPCs from control (n = 5) and *Zeb1*$^{-/-}$ (n = 6) mice from three independent experiments at week 32 after *Zeb1* deletion. **(H)** Representative FACS plots of the analysis of the committed myeloid and lymphoid progenitors in the BM from control and *Zeb1*$^{-/-}$ 32 weeks after the last dose of poly I:C. **(I)** Analysis of the frequency of the committed myeloid and lymphoid progenitors in the BM from control (n = 9–10, CLP n = 3) and *Zeb1*$^{-/-}$ (n = 9, CLP n = 3) mice from five independent experiments at week 32 after *Zeb1* deletion. Two samples of CLP were analyzed 42 weeks after *Zeb1* deletion. **(J)** Analysis of the absolute cell count of the committed myeloid and lymphoid progenitors in the BM from control (n = 8, CLP n = 3) and *Zeb1*$^{-/-}$ (n = 6, CLP n = 3) mice from four independent experiments at week 32 after *Zeb1* deletion. Two samples of CLP were analyzed 42 weeks after *Zeb1* deletion. Error bars show mean $\pm$ SEM. Unpaired *t* test was used in **(I)** and **(J)**. Mann-Whitney *U* test was used to calculate significance as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

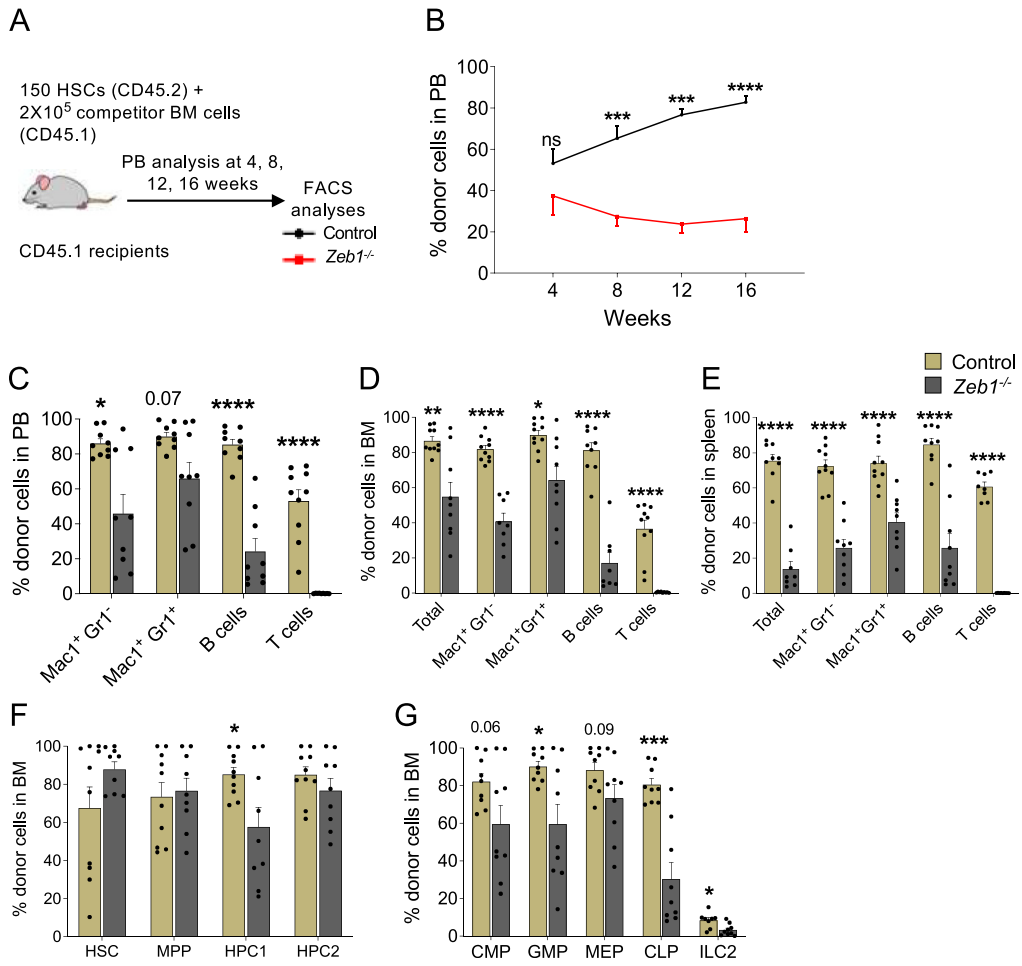


**Figure 3** Persistent *Zeb1* loss results in a multilineage hematopoietic differentiation defect after HSC transplantation. **(A)** A scheme of the competitive HSC transplantation. In total, 150 HSCs from control or *Zeb1*$^{-/-}$ mice (donor CD45.2) 32 weeks after *Zeb1* deletion mixed with 2 × 10$^5$ BM competitor cells (CD45.1) were transplanted into lethally irradiated recipients (CD45.1), and the mice were monitored by bleeding the tail vein at different time points until week 16. The percentage of total donor cells and donor contribution to the differentiated cells in the PB **(B and C)**, BM **(D)**, and spleen **(E)** 16 weeks after HSC transplantation from control (n = 9-10) and *Zeb1*$^{-/-}$ (n = 8–9) mice from two independent experiments. Donor contribution to BM HSPCs **(F)** and the committed myeloid and lymphoid progenitors **(G)** from control (n = 9-10) and *Zeb1*$^{-/-}$ (n = 9) from two independent experiments. Error bars show mean ± SEM. Mann-Whitney *U* test was used to calculate significance as follows: **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

progenitor compartment, there was also a moderate reduction in GMP and CLP and a significant reduction in ILC2 lymphoid progenitors from the *Zeb1*$^{-/-}$ genotype (Figure 4H) [35]. No significant changes were observed in CMP and MEP populations between the two genotypes (Figure 4H). In concert, these data indicate that cell-autonomous regulation of *Zeb1* is required for functional differentiation of HSCs toward multipotent and committed progenitors.

## Extended *Zeb1* Loss Results in a Cell-autonomous Multilineage Hematopoietic Differentiation Defect After Transplantation

To specifically test the cell autonomous functionality of HSCs after long-term deletion of *Zeb1* in primary recipients (Figure 4A), we sorted 200 HSCs from control and *Zeb1*$^{-/-}$ genotypic groups at 32 weeks after *Zeb1* deletion and transplanted them with BM competitor cells into lethally irradiated recipients (Figure 5A). Analysis of engraftment in PB of transplant recipients revealed a rapid engraftment defect at week 4 (Figure 5B). A near complete loss of *Zeb1*$^{-/-}$ donor cells in PB was observed at weeks 12 and 17 (Figure 5B). This was associated with a loss of donor-derived T, B, and Mac1$^+$ cells in PB and a substantial reduction in Mac1$^+$ Gr1$^+$ myeloid donor cells from *Zeb1*$^{-/-}$ recipients (Figure 5C). Consistent with PB data, a near complete loss of donor cells in BM and spleen and a complete loss of donor cells in thymus were observed in transplant recipients receiving *Zeb1*$^{-/-}$ HSCs (Figure 5D). Thus, prolonged loss of *Zeb1* severely perturbs the cell-intrinsic differentiation capacity of HSCs after transplantation.

## Long-term Loss of *Zeb1* Expression Impairs Extramedullary Hematopoiesis

Given that *Zeb1* has been implicated as a crucial regulator of cell migration in other tissues [36,37], we hypothesized that prolonged

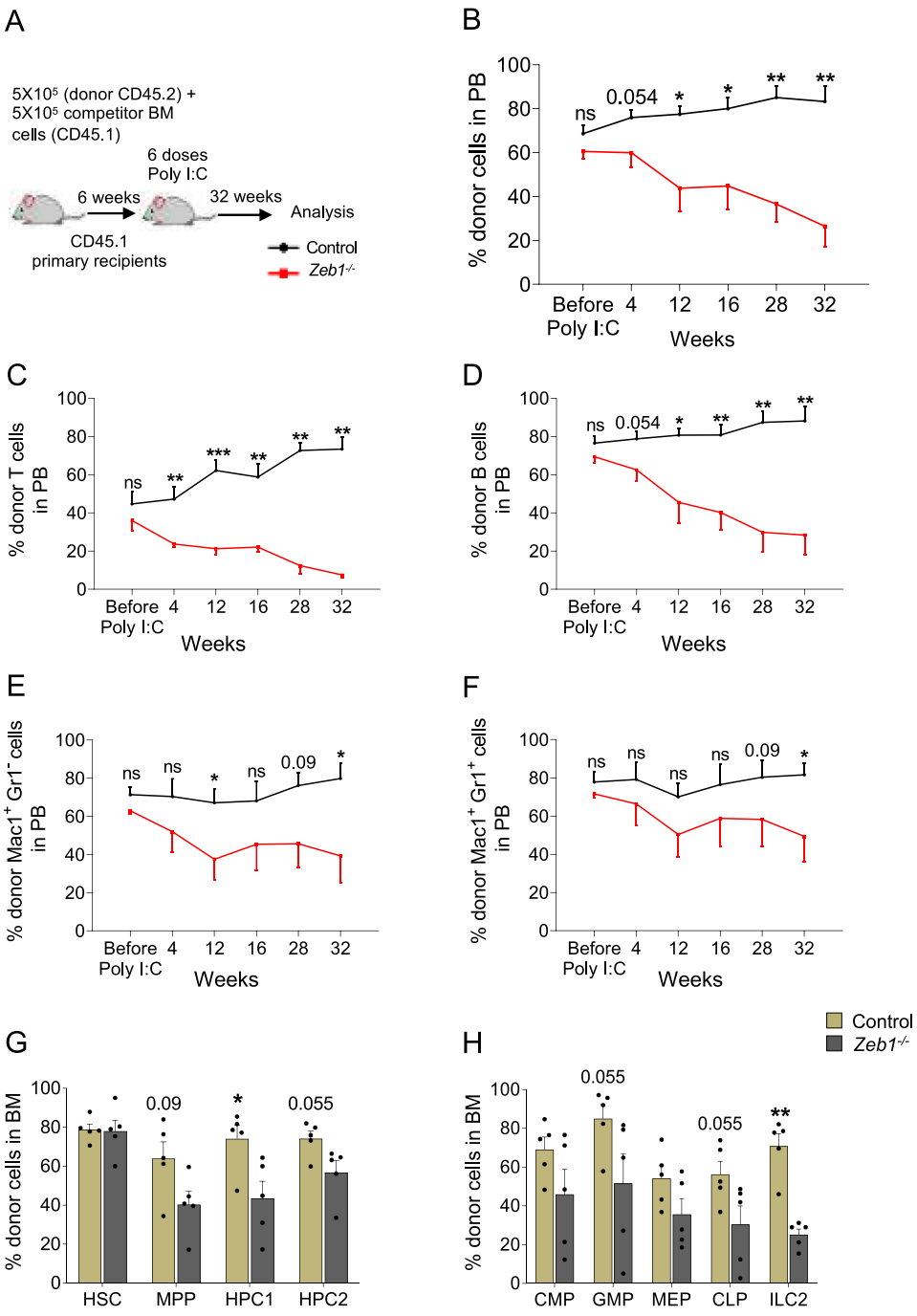


Figure 4 Persistent *Zeb1* loss results in a cell autonomous multilineage hematopoietic differentiation defect. **(A)** A scheme of cell autonomous transplant to assess the effect of *Zeb1* loss in hematopoietic cells but not in niche cells. Here, $5 \times 10^5$ BM cells from *Zeb1*$^{fl/fl}$ *Mx1-Cre*$^{-/-}$ or *Zeb1*$^{fl/fl}$ *Mx1-Cre*$^{+/-}$ (CD45.2) + $5 \times 10^5$ competitor BM cells (CD45.1) were transplanted into lethally irradiated recipients (CD45.1). Then, 6 weeks later, mice were injected with poly I:C to delete *Zeb1* and analyzed at different time points until week 32 after the last dose of poly I:C. **(B)** The percentage of donor cells in PB before and after poly I:C injection from control (n = 6 before poly I:C injection, n = 9 at week 4, n = 8 at week 12, and n = 5 at weeks 16–32) and *Zeb1*$^{-/-}$ (n = 5 before poly I:C injection, n = 7 at weeks 4 and 12, and n = 5 at weeks 16–32) mice from 2–3 independent experiments. Analysis of PB donor contribution to **(C)** T cells (CD4$^+$ CD8$^+$), (D) B cells (B220$^+$), **(E)** Mac1$^+$ Gr1$^-$ myeloid cells, and **(F)** Mac1$^+$ Gr1$^+$ myeloid cells from control (n = 6 before poly I:C injection, n = 5–9 at week 4, n = 8 at week 12, and n = 5 at weeks 16–32) and *Zeb1*$^{-/-}$ (n = 5 before poly I:C injection, n = 5–7 at weeks 4–32) mice from 2–3 independent experiments. Donor contribution to BM HSPCs **(G)** and the committed myeloid and lymphoid progenitors (CLP here is defined as Lin$^-$ SCA-1$^{low}$ C-KIT $^{low}$ CD127$^+$) **(H)** from control (n = 5) and *Zeb1*$^{-/-}$ (n = 5) from two independent experiments. Error bars show mean ± SEM. Mann-Whitney *U* test was used to calculate significance as follows: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

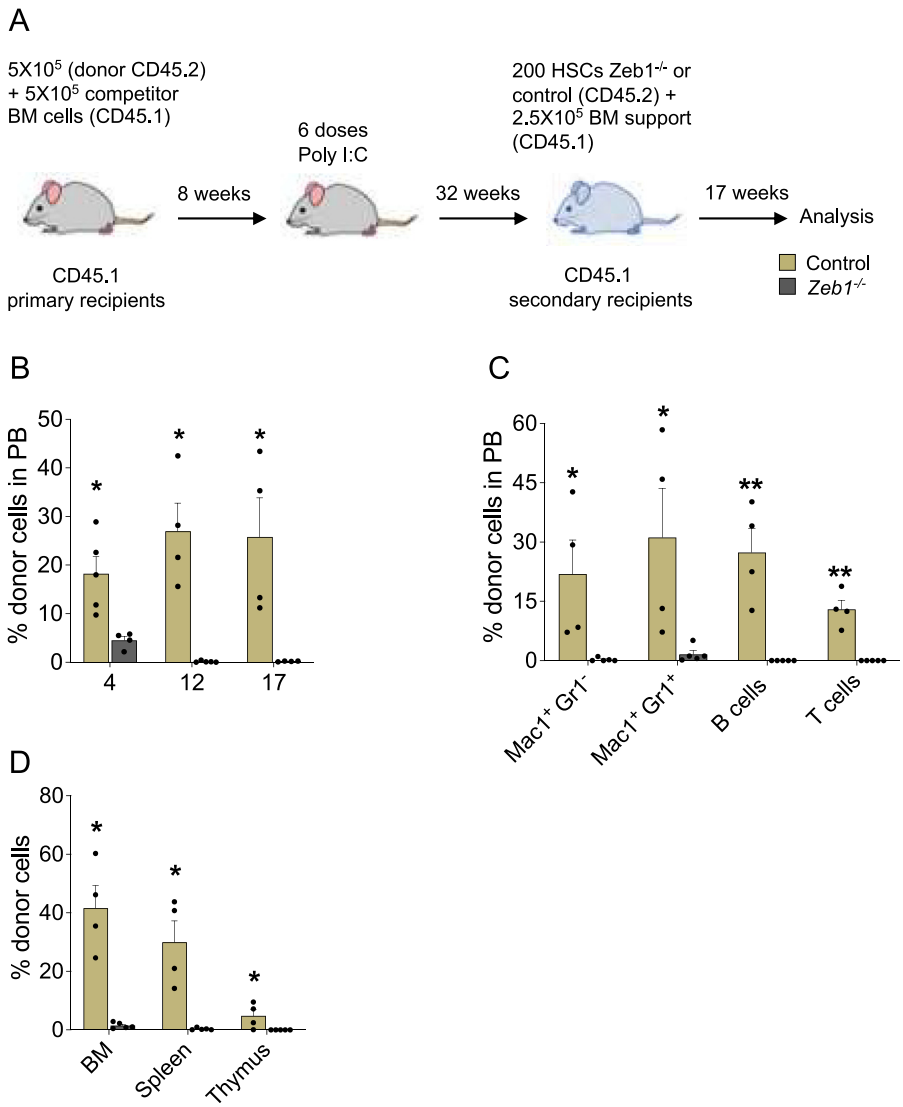


**Figure 5** *Zeb1* regulates HSC differentiation in a cell autonomous manner after transplantation. **(A)** Schema of HSC transplantation in cell autonomous manner. In total, 200 HSCs from primary recipients 32 weeks after the last poly I:C dose from control or $Zeb1^{-/-}$ mice mixed with 2.5 × $10^5$ BM competitor cells (CD45.1) were transplanted into lethally irradiated recipients (CD45.1), and the mice were analyzed at different time points until week 17. **(B)** The percentage of donor cells in PB at different time points postsecondary cell autonomous HSC transplantation from control (n = 4) and $Zeb1^{-/-}$ (n = 4–5) mice. **(C)** Donor contribution to PB $Mac1^+$ $Gr1^-$ myeloid cells, $Mac1^+$ $Gr1^+$ myeloid cells, $B220^+$ B cells, and $CD4^+/CD8^+$ T cells at week 17 postsecondary cell autonomous HSC transplantation from control (n = 4) and $Zeb1^{-/-}$ (n = 5) mice. **(D)** The percentage of donor cells in BM, spleen, and thymus at week 17 postsecondary cell autonomous HSC transplantation from control (n = 4) and $Zeb1^{-/-}$ (n = 5) mice. Error bars show mean ± SEM. Mann-Whitney *U* test was used to calculate significance as follows: $^*p < 0.05$, $^{**}p < 0.01$.

loss of *Zeb1* would impact extramedullary hematopoiesis in the spleen, which may rely on migration of hematopoietic cells from the BM [38]. In support of this hypothesis, we observed splenomegaly in $Zeb1^{-/-}$ mice at 32 weeks after induction of gene deletion, which caused an increase in splenic weight and an unexpected reduction in spleen cellularity (Figure 6A–C). By immunophenotyping fully mature hematopoietic cells in the spleen, we found a reduction in the frequency of $Mac1^+$ myeloid cells, $Ter119^+$ erythroid cells, and T cells in $Zeb1^{-/-}$ mice (Figure 6D and E). As observed in the BM (Figure 2B), the frequency of splenic B cells significantly increased in $Zeb1^{-/-}$ mice; however, no significant change was found in their absolute numbers (Figure 6E and G). We also found a decrease in absolute numbers of $Mac1^+$, $Mac1^+$ $Gr1^+$, $Ter119^+$ and T cells in the spleen at 32 weeks after *Zeb1* deletion (Figure 6F and G). In stark contrast to the HSC expansion observed in the BM of $Zeb1^{-/-}$ mice (Figure 2E and F), we found a dramatic reduction of splenic HSC numbers in $Zeb1^{-/-}$ mice, with no significant changes in MPP and HPC2 (Figure 6H and I). Contrasting with the reduction of HPC1

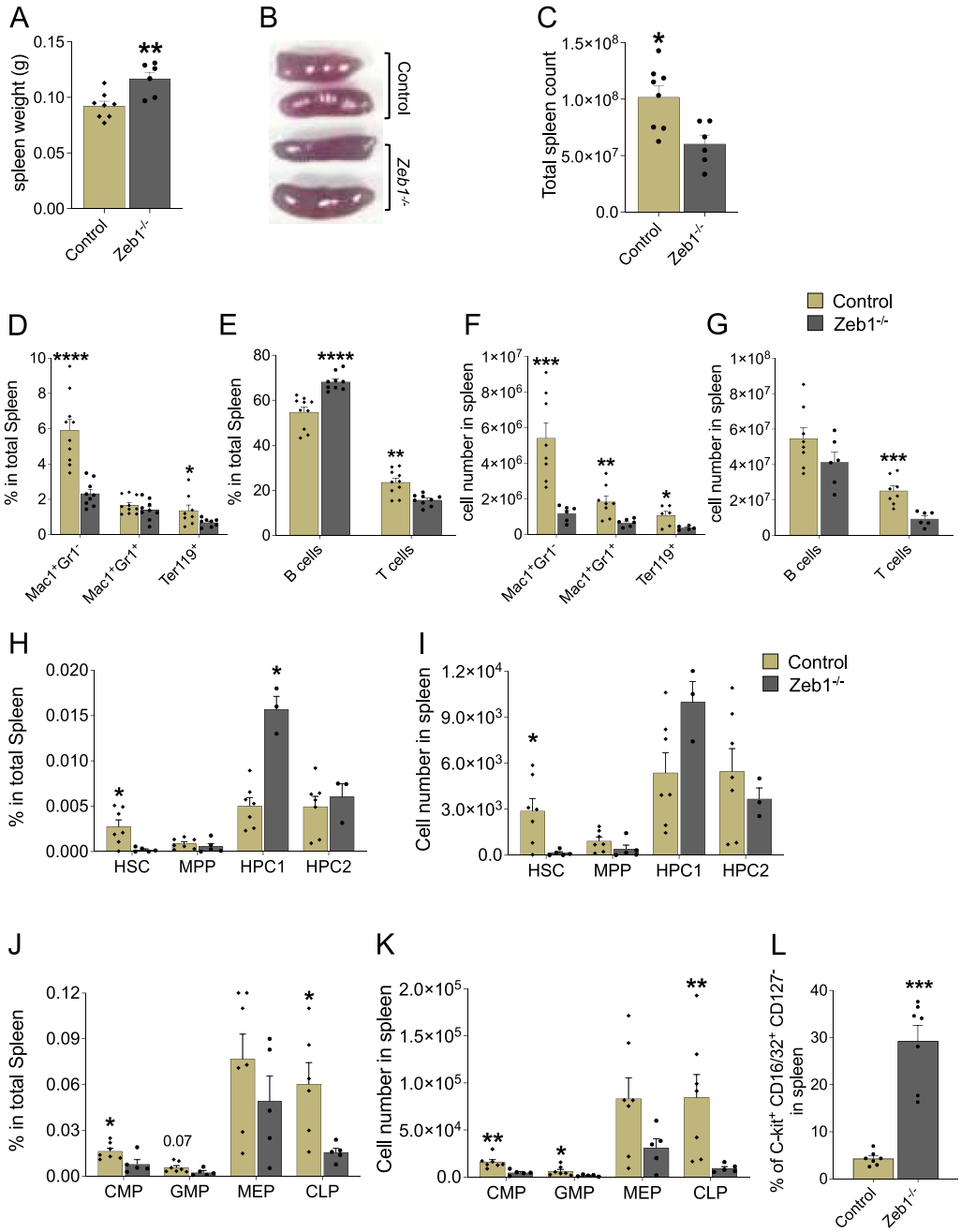


Figure 6 Persistent loss of *Zeb1* results in impaired extramedullary hematopoiesis. **(A)** Spleen weight (n = 6–8) and a representative picture **(B)** and analysis of total spleen cells **(C)** (n = 6–8) from control and *Zeb1*$^{-/-}$ mice 32 weeks after the last dose of poly I:C from 3–4 independent experiments. **(D and E)** Analysis of the frequency of the differentiated cells in spleen at week 32 after *Zeb1* from 3–4 independent experiments (n= 9–10, except with Ter119$^{+}$ n = 8). **(F and G)** Analysis of the absolute count of the differentiated cells in spleen at week 32 after *Zeb1* deletion from 3–4 independent experiments (n = 6–8, except Ter119$^{+}$ with n=6-5). **(H)** Analysis of the Frequency of HSPCs in spleen from control (n = 7) and *Zeb1*$^{-/-}$ (n = 5, except HPC1 and HPC2 with n = 7 for control and n = 3 for *Zeb1*$^{-/-}$) mice from three independent experiments at week 32 after *Zeb1* deletion. **(I)** Analysis of the absolute count of HSPCs in spleen from control (n = 7) and *Zeb1*$^{-/-}$ (n = 5, except HPC1 and HPC2 with n=7 for control and n = 3 for *Zeb1*$^{-/-}$) mice from three independent experiments at week 32 after *Zeb1* deletion. **(J)** Analysis of the Frequency of committed progenitors in spleen from control (n = 7) and *Zeb1*$^{-/-}$ (n = 5) mice from three independent experiments at week 32 after *Zeb1* deletion. **(K)** Analysis of the absolute count of the committed progenitors in spleen at week 32 after *Zeb1* deletion from control (n = 7) and *Zeb1*$^{-/-}$ (n = 5) mice from three independent experiments. **(L)** Analysis of C-kit$^{+}$ CD16/32$^{+}$ CD127$^{-}$ population in spleen from control (n = 7) and *Zeb1*$^{-/-}$ (n = 7) mice from three independent experiments at week 32 after *Zeb1* deletion. Error bars show mean ± SEM. Mann-Whitney *U* test was used to calculate significance as follows: **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

abundance observed in the BM of *Zeb1*$^{-/-}$ mice (Figures 2E and F), HPC1 frequency was expanded in the spleens of *Zeb1*$^{-/-}$ mice (Figure 6H). Within the myeloid LK compartment, the frequency and total numbers of CMPs and GMPs, but not MEPs, were significantly decreased in the spleen after prolonged *Zeb1* loss (Figure 6J and K). In the lymphoid progenitor compartment, a profound decrease in CLPs was observed at 32 weeks after *Zeb1* deletion (Figure 6J and K). Notably, we observed an expansion of preleukemic c-kit$^{+}$CD16/32$^{+}$ progenitors [39] in the spleens of *Zeb1*$^{-/-}$ mice and in cell-autonomous transplantation experiments (Figure 6L and Supplementary Figure E2). Finally, we assessed the impact of *Zeb1* in extramedullary hematopoiesis during aging in a small experimental cohort (n = 2 for each genotype). At 42 weeks after *Zeb1* deletion, one *Zeb1*$^{-/-}$ mouse became moribund. Two control animals and the other *Zeb1*$^{-/-}$ mouse in the cohort at 42 weeks after *Zeb1* deletion were healthy and exhibited similar hematologic behaviors, including extramedullary hematopoiesis, compared with mice analyzed at 32 weeks after *Zeb1* deletion (Figures 2 and 6). Postmortem analysis of the moribund *Zeb1*$^{-/-}$ mouse revealed progressive splenomegaly, and the spleen was noticeably less red in hue than control mice or the healthy *Zeb1*$^{-/-}$ mouse at 42 weeks (Supplementary Figure E3A). Immunophenotypic features unique to the moribund *Zeb1*$^{-/-}$ mouse included an expansion of most splenic HSPC populations, a differentiation block in splenic myeloid cells evidenced by an accumulation of Mac1$^{+}$Gr-1$^{+}$ cells, an expansion of BM HPC1/HPC2 populations, and significant depletion of B cells from PB (Supplemental Figure E3B, C, D, and E). These preliminary data support the idea that long-term expression of *Zeb1* in HSCs is required to suppress preleukemic activity [18]. Overall, we show that long-term *Zeb1* loss impairs extramedullary hematopoiesis in the spleen.

## DISCUSSION

To meet the physiologic demands of blood supply in the body, a rare pool of self-renewing HSCs must differentiate into specialized myeloid and lymphoid cells with diverse functions providing immune defense, clotting, and oxygen supply. Critically, HSCs must provide this cover during a lifetime, and deregulation of the molecular mechanisms sustaining their genetic, epigenetic, and functional integrity can lead to the development of both benign and malignant hematologic conditions. However, our understanding of the transcriptional control of long-term adult HSC maintenance is still rudimentary. In this report, we exploited the unique ability of the poly I:C-inducible *Mx1-Cre* recombinase system to conditionally ablate the EMT transcription factor *Zeb1* exclusively within the adult hematopoietic system to pose the outstanding question of whether *Zeb1* is required for the long-term maintenance of adult HSCs. It should be noted that we cannot exclude the possibility that proinflammatory, interferon-like damage to HSC integrity induced by poly I:C administration in this model is irreversible and may endure after long-term *Zeb1* deletion or that simultaneous poly I:C administration and *Zeb1* deletion may generate a heightened hematologic phenotype [40,41]. These limitations aside, we found that adult HSC functionality in transplantation was disrupted, and regulation of extramedullary hematopoiesis in the spleen was impaired in mice engineered to be deficient in *Zeb1* for 32 weeks, demonstrating the requirement of *Zeb1* in the long-term maintenance of HSCs in adult hematopoiesis.

Importantly, our results sit in broad agreement with our previous work and that of others that found a cell-intrinsic role for *Zeb1* in HSC self-renewal and differentiation using the *Mx1-Cre* and *Vav-Cre* recombinase systems (Supplementary Table E1) [18,20]. For example, mirroring our results here, in the acute deletion setting using *Mx1-Cre*, where we measured the consequences of *Zeb1* deletion shortly after poly I:C administration, we observed that Mac-1$^{+}$ myeloid cells, HPC1, and CLPs were reduced in *Zeb1*$^{-/-}$ mice [18]. Barring the unchanged PB T-cell numbers observed in the current study due to incomplete *Zeb1* deletion, atrophy of the thymus and severe disruption of T-cell development were observed in both the acute and extended *Zeb1* deletion settings (unpublished data)[18].

Although there are clear commonalities in *Zeb1*-mediated regulation of adult HSCs in the acute and extended deletion settings using the *Mx1-Cre* model, we also observed fundamental differences after extended *Zeb1* deletion, which may have implications for the role of *Zeb1* in the long-term maintenance of HSCs (Supplementary Table E1). Immunophenotypically defined HSCs and their immediate downstream progeny, multipotent progenitors, expanded significantly after prolonged *Zeb1* deletion, which was mechanistically linked to the expansion of specific lineage-biased/balanced HSC populations. This differs to acute deletion of *Zeb1* in HSCs, where an unperturbed HSC compartment and equivalent proportions of HSC lineage-biased/balanced populations were observed [18,42]. HSC expansion after long-term *Zeb1* deletion also appears to be linked to cell survival and not altered proliferative status. Although we did not see gross changes in apoptosis in the acute *Zeb1* deletion setting, we observed that *EpCAM*+ expressing HSPCs in *Zeb1*$^{-/-}$ mice confer a cell survival signal through the EpCAM-BCL-XL axis [18]. Given the normally intimate association between apoptotic and cell cycle regulation, which in concert stabilize genomic integrity against the backdrop of genomic insults [43], it will be of interest to characterize the cell cycle-independent apoptotic gene networks operating in the context of long-term maintenance of *Zeb1*$^{-/-}$ HSCs.

Despite observing HSC expansion after 32 weeks of *Zeb1* deletion, when *Zeb1*$^{-/-}$ HSCs were transplanted they displayed a panlineage differentiation defect reminiscent of the HSC repopulation defect observed in the setting of acute *Zeb1* deletion [18]. Engraftment of HSCs from the *Zeb1*$^{-/-}$ genotype was equivalent to their control counterparts in competitive transplantation experiments, suggesting that long-term *Zeb1* expression in HSCs is not required for homing. Notably, only HPC1 and committed progenitor cell descendants of *Zeb1*$^{-/-}$ HSCs displayed reduced engraftment potential. However, in the acute deletion setting, although *Zeb1*$^{-/-}$ HSCs engrafted no differently to controls, almost all multipotent progenitor and committed progenitor cell subpopulations were more drastically reduced after competitive transplantation. These data collectively underscore the critical role of *Zeb1* in mediating the differentiation of the progenitor compartment under the stress conditions of transplantation, which can be partially compensated for by *Zeb1* independent mechanisms in the setting of long-term *Zeb1* deficiency. Future work should therefore be aimed at deciphering which additional multipotent progenitor and committed progenitor subsets exhibit long-term *Zeb1* dependency in transplantation [44].

We also identified a potential role for *Zeb1* in the long-term maintenance of B-cell differentiation from adult HSCs, as evidenced by expansion of B cells in the BM and spleen, both key sites of B-cell development in mice [45]. This result was unexpected given the observed reduction in CLPs from *Zeb1*$^{-/-}$ mice yet concurs with the

ability of *Zeb1* to repress B cell promoting gene networks and/or activate B-cell growth suppressive networks [46,47]. Also of relevance, *cellophane* mice, which encode a mutated 901 amino acid protein lacking the C-terminal zinc finger of *Zeb1*, display a nonstatistically significant expansion during specific stages of B-cell development, as assessed by a combination of B220, IgM, IgD, CD21/35, and CD23 immunophenotypic markers in the BM or spleen [48]. Thus, as our analysis in this study was restricted solely to $B220^+$ population immunophenotyping, it is possible that the B-cell expansion observed in the BM and spleen in the setting of prolonged *Zeb1* deletion may mask accumulation or blockade in specific developing B-cell compartments. In contrast, it should also be noted that $Zeb1^{-/-}$ HSC-derived B cells demonstrated reduced engraftment capacity, which likely reflects a key difference between *Zeb1*-mediated control of B-cell maturation/differentiation in the transplantation setting and steady-state hematopoiesis.

Given that long-term absence of *Zeb1* resulted in HSC expansion in the BM, the main site of hematopoiesis in adults, and as *Zeb1* regulates migration in other tissue settings [36,49], we posited that expanded HSCs in the BM may result in alterations in HSCs and other progenitors in the spleen, a site of extramedullary hematopoiesis that becomes particularly active in pathologic settings like leukemic development in both mice and humans [50]. Consistent with the development of a pathologic state in mice after long-term *Zeb1* loss, we observed splenomegaly with an atypical reduction in cellularity, HSC exhaustion, defects in maturation of myeloid and lymphoid lineages, and, most relevantly, the expansion of aberrant, preleukemic-like c-$kit^+CD16/32^+$cells. There are multiple explanations for long-term *Zeb1*-mediated defects in extramedullary hematopoiesis in the spleen. First, as a known EMT inducer, *Zeb1* is involved in mediating repression of adhesion genes (e.g., EpCAM and CDH1) [13,18,51,52]. Thus, loss of *Zeb1* in HSCs in the BM may lead to increased expression of cell adhesion molecules and associated polarity genes, suggesting that long-term expression of *Zeb1* is required to modulate the motility of HSC ingress to the spleen from the BM. Furthermore, exhaustion of HSC numbers in the spleen of $Zeb1^{-/-}$ mice brings to prominence the expansion of HSCs observed in the BM of $Zeb1^{-/-}$ mice, which may be caused by *Zeb1*-mediated disruption of HSC egress from the spleen. Second, *Zeb1* has been shown to be an important regulator of the CXCR4 signaling axis in other settings [53,54], and it is possible the CXCR4 axis, a key regulator of hematopoietic cell migration [55,56], acts as a long-term regulator of *Zeb1* mediated trafficking of hematopoietic cells to and from the BM and spleen. Given the established association of *Zeb1* to cell adhesion molecules alluded to above and the requirement for cell adhesion molecules in hematopoietic cell migration [57,58], future work should be directed at exploring the association among *Zeb1*, ZEB1 cell adhesion target genes, and CXCR4 in the regulation of migration of hematopoietic cells between the BM and spleen and vice versa. Third, HSC exhaustion and progenitor defects in the spleen of $Zeb1^{-/-}$ mice may be caused by enhanced apoptosis. Finally, the splenic phenotype in $Zeb1^{-/-}$mice, including expansion of preleukemic-like c-$kit^+CD16/32^+$cells, together with mild changes to BM cellularity, myeloid-biased BM HSC expansion, and multilineage differentiation defects are features consistent with *Zeb1* dependency during aging [59,60]. Most of the $Zeb1^{-/-}$ mice in our study were analyzed at 32 weeks after *Zeb1* deletion, which equates to middle age in humans [61]. However, we also presented evidence of progression of the preleukemic phenotype at 42 weeks after *Zeb1* deletion, typified by further splenomegaly that leads to the accumulation of Gr-$1^+$Mac-$1^+$ cells, a myeloid differentiation block reminiscent of that observed in acute myeloid leukemia [39]. As there is a well-established relationship between aging and the development of hematopoietic neoplasms [62,63], it would be of interest to perform in-depth studies focused on whether long-term *Zeb1* deficiency during aging elicits the development of myeloid malignancies with high prevalence, congruent with emerging evidence that *Zeb1* acts as tumor suppressor in acute myeloid leukemia [18].

### Conflict of Interest Disclosure

The authors declare no conflicts of interest.

### Acknowledgments

The Rodrigues laboratory at Cardiff University, Cardiff, UK is supported by the Saudi Arabia Cultural Bureau, Blood Cancer UK, Leukaemia Cancer Society, and Leukaemia and Myeloma Research UK. We would like to thank the Deanship of Scientific Research at Shaqra University, Shaqra, Saudi Arabia for supporting the work of A. Almotiri and The Hashemite University, Zarqa, Jordan for supporting the work of A. Abdelfattah. This work was also supported by grants to T Brabletz, S Brabletz, and MP Stemmler from the German Research Foundation (SPP2306 project 461704629, BR 1399/15-1 and BR 4145/3-1; FOR243/P04; TRR305/A03, A04, B01, B07; BR1399/9-1, BR1399/10-1, BR4145/1-1 and BR4145/2-1).

### Author Contributions

A. Almotiri designed and performed experiments, analyzed and interpreted data, prepared the figures, and contributed to writing the manuscript. A. Abdelfattah performed experiments, analyzed data, and reviewed the manuscript. ES performed experiments and analyzed data. MPS, SB, and TB contributed to experimental design and analysis and reviewed the manuscript. NPR conceived and supervised the project, designed experiments, analyzed and interpreted the data, and wrote the manuscript.

## SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.exphem.2024.104177.

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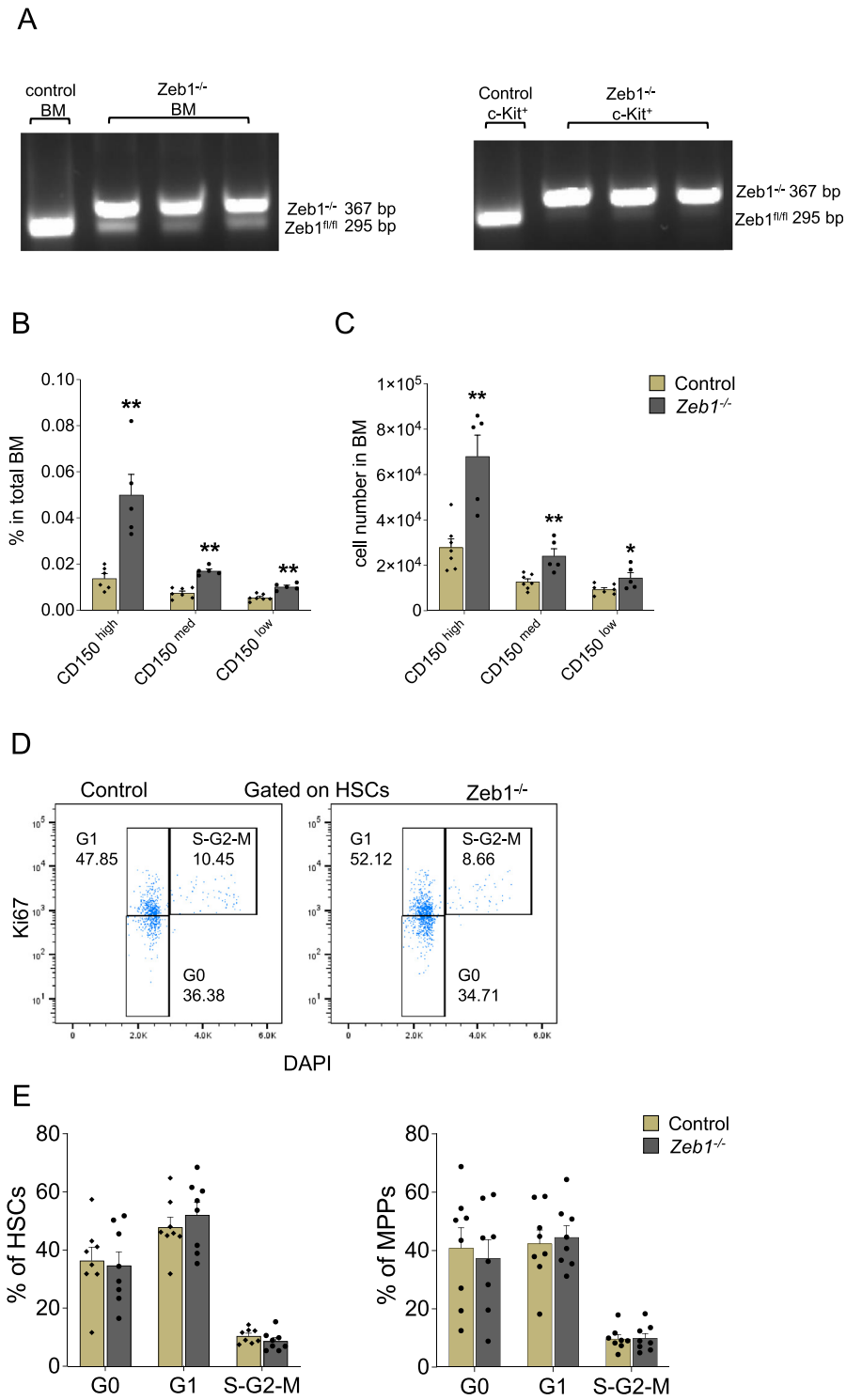
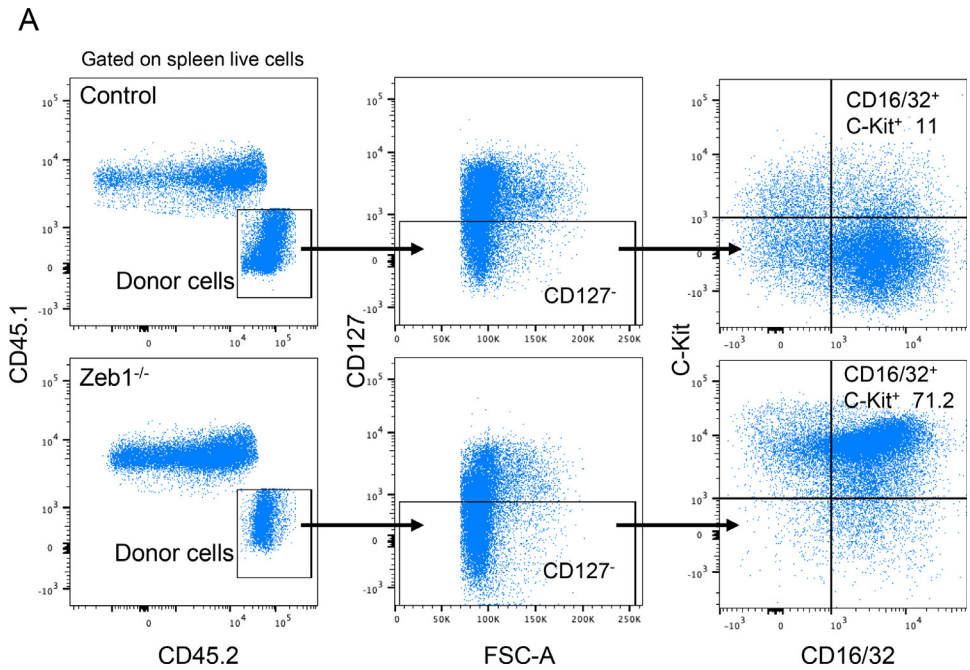



**Supplementary Figure 1** **(A)** Representative gel electrophoresis analysis assessing *Zeb1* deletion in total BM and BM C-KIT$^+$ cells 32 weeks after *Zeb1* deletion. **(B and C)** Analysis of lineage biased HSCs populations based on CD150 expression in the BM from control (n=6-7) and *Zeb1*$^{-/-}$ (n=5) mice from 3 independent experiments at week 32 after *Zeb1* deletion. **(D and E)** Analysis of cell cycle profile of HSC and MPP populations in BM using Ki67 and DAPI 32 weeks after *Zeb1* ablation from control (n=8) and *Zeb1*$^{-/-}$ (n=8) mice from 3 independent experiments. Error bars show mean ± SEM. Mann-Whitney U test was used to calculate significance as follows: *P < .05, **P < .01.

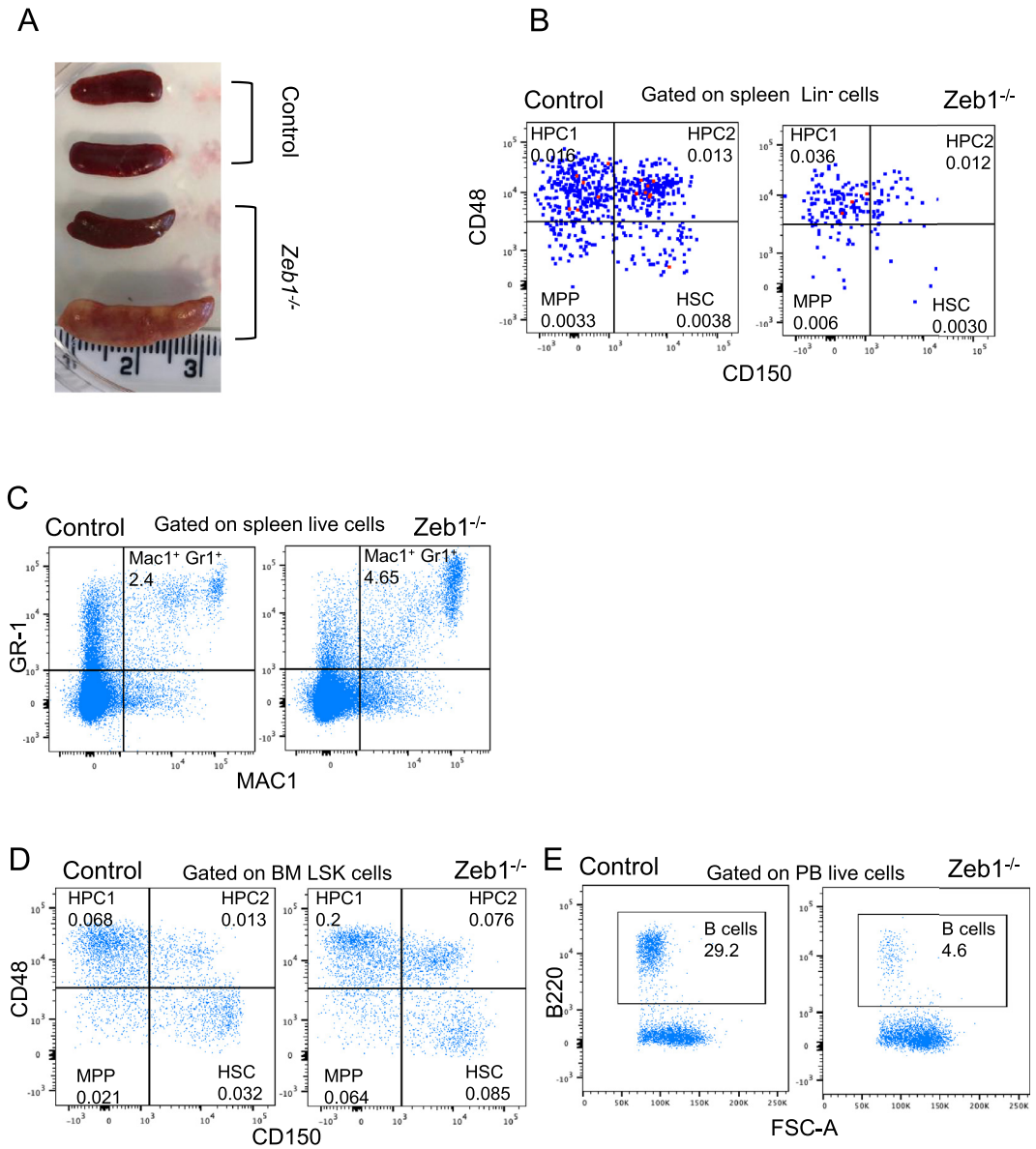



**Supplementary Figure 2** **(A)** Representative flow cytometry plots of CD16/32$^{+}$ C-Kit$^{+}$ CD127$^{-}$ cells within the spleen donor cells at week 32 after Zeb1 deletion in the cell autonomous transplant setting (n=3 for each genotype).

**Supplementary Table E1** Comparison between the phenotypes of long-term (32 weeks) and acute (14 days) deletion of *Zeb1* in the murine hematopoietic system

| Cell type | site | Long-term deletion | Acute deletion |
|---|---|---|---|
| HSPCs | BM | - Increased HSC, MPP associated with reduced apoptotic levels.<br>- Decreased HPC1, HPC2 | - No change in HSC, MPP, HPC1<br>- Decreased HPC2 |
| | Spleen | - Decreased HSC<br>- Increased HPC1<br>- No change in MPP, HPC2 | [a] No change in HSC, MPP, HPC1, HPC2 |
| Committed Progenitors | BM | - Decreased CLP CD135$^{+}$, GMP<br>- No change in CMP, MEP | - Decreased CLP CD135$^{+}$<br>- No change in CMP, GMP, MEP |
| | Spleen | - Decreased CMP. GMP, CLP<br>- No significant change in MEP | - No change in CMP, GMP, MEP, CLP |
| Mature cells | BM | - Decreased T cells<br>- Increased frequency of B cells<br>- No change in myeloid cells | [a] No change in myeloid, B cells, T cells |
| | PB | - Decreased Mac1$^{+}$ Gr1$^{-}$ cells | - Decreased Mac1$^{+}$ Gr1$^{-}$ cells |
| | Spleen | - Decreased Mac1$^{+}$ Gr1$^{-}$, Mac1$^{+}$ Gr1$^{+}$, T cells, Ter119$^{+}$ cells<br>- Increased frequency of B cells<br>- Perturbed extramedullary hematopoiesis with increased spleen weight, reduced cellularity, and expanded C-Kit$^{+}$ CD16/32$^{+}$ CD127$^{-}$ population | [a] Normal extramedullary hematopoiesis |

Zeb1-deficient HSCs showed multilineage differentiation defects in transplantation experiments in a cell autonomus manner in both acute and long-term deletion models.
Long-term deletion data are based on results in this publication, and acute deletion data are based on Almotiri et al. [18] and unpublished observations (denoted with [a]).




**Supplementary Figure 3** **(A)** Pictures of spleen from control (n=2) and *Zeb1*$^{-/-}$ mice (n=2) at 42 weeks after the last dose of Poly I:C. Representative flow cytometry plots from a control and moribund *Zeb1*$^{-/-}$ mouse at 42 weeks after the last dose of Poly I:C of splenic HSPCs **(B)** splenic Mac1$^{+}$ Gr1$^{+}$ **(C)** HSPCs in BM **(D)** and B cells in PB **(E).**