Sin3a-associated Hdac1 and Hdac2 are essential for hematopoietic stem cell homeostasis and contribute differentially to hematopoiesis

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ABSTRACT

Class I histone deacetylases are critical regulators of gene transcription by erasing lysine acetylation. Targeting histone deacetylases using relative non-specific small molecule inhibitors is of major interest in the treatment of cancer, neurological disorders and acquired immune deficiency syndrome. Harnessing the therapeutic potential of histone deacetylase inhibitors requires full knowledge of individual histone deacetylases *in vivo*. As hematologic malignancies show increased sensitivity towards histone deacetylase inhibitors we targeted deletion of class I Hdac1 and Hdac2 to hematopoietic cell lineages. Here, we show that Hdac1 and Hdac2 together control hematopoietic stem cell homeostasis, in a cell-autonomous fashion. Simultaneous loss of Hdac1 and Hdac2 resulted in loss of hematopoietic stem cells and consequently bone marrow failure. Bone-marrow-specific deletion of Sin3a, a major Hdac1/2 co-repressor, phenocopied loss of Hdac1 and Hdac2 indicating that Sin3a-associated HDAC1/2-activity is essential for hematopoietic stem cell homeostasis. Although Hdac1 and Hdac2 show compensatory and overlapping functions in hematopoiesis, mice expressing mono-allelic Hdac1 or Hdac2 revealed that Hdac1 and Hdac2 contribute differently to the development of specific hematopoietic lineages.

Introduction

Hematopoiesis, the generation of blood cells, is a multistep differentiation process, which is controlled and maintained by epigenetic mechanisms. Differentiation of pluripotent hematopoietic stem cells (HSC) into lineage-specific progenitors and specialized hematopoietic cell lineages is initiated by external stimuli that induce chromatin modifications which drive and maintain lineage-specific expression programs.[1,2] The establishment of lineage-specific chromatin modifications is mediated by a host of chromatin-modifying enzymes including Zn^{2+}-dependent histone deacetylases (HDAC). While histones were initially identified as HDAC-substrates, many non-histone proteins have also been identified as substrates, indicating that they act beyond chromatin modifications.[3] HDAC are subdivided into three classes of which class I HDAC (HDAC1, 2, 3, 8 and 10) are homologous to yeast Rpd3.[4-6] To exert their role as transcriptional regulators class I HDAC are directed to genomic regulatory regions through interactions with multi-protein complexes harboring DNA binding proteins. HDAC1 and HDAC2 are primarily found in biochemically distinct repressor complexes, Swi-independent 3 (SIN3), Nucleosome remodeling deacetylase complex (NuRD), Co-repressor of REST (CoREST), Polycomb repressor complex 2 (PRC2) and Mitotic histone deacetylase complex (MiDAC).[4,7] Class I HDAC have been implicated in tumorigenesis as they are over-expressed in various tumor types and interact with oncogenic fusion proteins and tumor suppressors. Inhibitors of class I HDAC were shown to reduce (tumor) cell proliferation and have entered the clinic in the treatment of hematopoietic malignancies[8] and attracted interest as therapeutics in the treatment of neurological disorders[9] and acquired immune deficiency syndrome.[10] However, patients treated with HDAC inhibitors often develop hematologic side effects. To understand the mechanism of action of these inhibitors, knowledge of the *in vivo* function of individual HDAC is required. Using mouse models carrying Hdac1 and Hdac2 conditional knock-out alleles, we previously identified a redundant function for Hdac1 and Hdac2 in hematopoiesis. Dual loss of these histone deacetylases throughout the bone marrow resulted in ablation of the megakaryocyte and erythrocyte lineages accompanied by anemia and thrombocytopenia.[11] It did, however, remain unclear whether Hdac1 and Hdac2 have functions in other hematopoietic lineages and whether these functions are cell-autonomous or due to a role in non-hematopoietic cells. In addition, it is unclear which of the biochemically distinct Hdac1/2 repressor complexes are involved in epigenetic programming of hematopoietic lineages. Here, using mouse models carrying conditional knock-out alleles for *Hdac1*, *Hdac2* and *Sin3a*, we showed an intrinsic and overlapping function for Sin3a-associated Hdac1 and Hdac2 in hematopoietic stem cell homeostasis. Moreover, mice expressing only Hdac1 or Hdac2 at levels similar to those in wild-type mice revealed that Hdac1 and Hdac2 contribute differently to the total Hdac1/2 levels required for differentiation of hematopoietic lineages.

Methods

Mice

Mice with conditional *Hdac1* and *Hdac2* knock-out alleles, such as *MxCre;Hdac1*$^{L/L}$*;Hdac2*$^{L/L}$, have been described elsewhere.[11,12] To delete *Hdac1* and/or *Hdac2* in the hematopoietic system, we crossed the

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interferon-inducible *MxCre* allele[13] onto an *Hdac1*$^{L/L}$ and/or *Hdac2*$^{L/L}$ background. Cre-recombinase was induced in 6- to 8-week old mice by intra-peritoneal injection of 5 x 300 μg pI:pC (Sigma), every other day. The mice were sacrificed 4–10 days after the last injection. Thymocyte and B-cell specific deletion of *Hdac1* and *Hdac2* was obtained by intercrossing Hdac1 and/or Hdac2 conditional knock-out alleles with *Lck-Cre* or *Mb1-Cre* transgenic mice.[14,15] All cohorts were in a mixed FVB/n, C57BL/6 and 129/Sv background. All experiments were approved by a local ethical committee and performed according to national guidelines.

Flow cytometry

Bone marrow cells were collected 8 days after the last pI;pC injection. LSK cells and progenitors were stained as described previously[16] with the following labeled antibodies: IL7Rα-biotin, CD3-biotin, B220-biotin, CD11b-biotin, TER119-biotin and Ly6G-biotin (eBioscience) followed by streptavidin-PerCP-Cy5.5, CD34-FITC FcgRII/III-PE (eBioscience), ScaI-PacificBlue (Biolegend), CD34-FITC, IL7Rα-PE, and c-kit-APC (BD Biosciences). B cells were stained with CD19-APC, B220-Pacific Blue, c-Kit-PE, CD25-PE and IgM-PE (BD Biosciences), myeloid cells with Gr1-PE and CD11b-PerCP-Cy5.5, erythrocytes with CD71-FITC and TER119-Pacific Blue, megakaryocytes with CD41-PE and T cells with Thy1-PE, CD4-Pacific Blue, CD8-FITC, CD25-PerCP-Cy5.5, CD44-APC and Tcrβ-APC. All experiments were performed using a multi-color CyAn flow-cytometer (Beckman Coulter). Data were analyzed with FlowJo software (Treestar).

Western blot analysis

Cells were lysed in RIPA buffer (20 mM Tris, pH7.5, 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS) containing protease inhibitors (Roche), phosphatase inhibitors, 5 μM trichostatin and 1 mM nicotinamide. For western blots 20 μg of protein lysate were incubated with antibodies against either Hdac1 (IMG-337, Imgenex), Hdac2 (SC-7899, Santa Cruz Biotechnology), or γ-tubulin (T6557, Sigma) and horseradish peroxidase-coupled secondary antibodies (Dako). Western blots were stained with enhanced chemiluminescence (Pierce), imaged, and quantified with ChemiDoc software (BioRad).

Methylcellulose colony-forming assays

Bone marrow cells or splenocytes were isolated 8 days after pI;pC injection. To grow myeloid colonies $2x10^4$ nucleated cells were cultured with Methocult M3434 (Stem Cell Technologies). To grow B-cell colonies, $5x10^4$ nucleated bone marrow cells were cultured with Methocult M3630 (Stem Cell Technologies). Colonies were scored after 10 days.

Competitive bone marrow transfer

Donor bone marrow cells were collected from experimental and wild-type (*WT*) mice, mixed in a 1:1 ratio and injected into lethally irradiated (2 x 5.5 Gy) recipient mice (n=5 per group). After 8 weeks, bone marrow reconstitution was analyzed by flow cytometry using Ly5.1-PE and Ly5.2-FITC (BD Biosciences) antibodies. Upon successful reconstitution, *Cre*-recombinase was induced by intraperitoneal injection of 5 x 300 μg pI;pC, every other day. For 3 months, peripheral blood was monitored using Ly5.1-PE and Ly5.2-FITC antibodies. After the experiment, HSC and progenitor populations were analyzed by FACS.

RNA-sequencing of bone marrow hematopoietic stem cells/progenitors

RNA was isolated using Trizol from Lin$^-$,c-Kit$^+$/ScaI$^+$- FACS-sorted bone marrow cells. Samples were processed and sequenced (RNA-seq) on an Illumina HiSeq2000 apparatus. Bioinformatic analysis of RNA-seq reads was performed at the Genomics Core facility.

Results

Hdac1 and Hdac2 are essential for early hematopoiesis

To determine the role of Hdac1 and Hdac2 in hematopoietic development we previously developed a mouse model allowing conditional inactivation of Hdac1 and Hdac2 in the bone marrow.[11,12] In contrast to single deletions, simultaneous deletion of *Hdac1* and *Hdac2* led to rapid death due to severe anemia and thrombocytopenia, which was associated with a reduction of bone marrow cells.[11,12] To provide a rationale for the hematopoietic defects in mice lacking both Hdac1 and Hdac2 [*MxCre*$^+$;*Hdac1*$^{Δ/Δ}$;*Hdac2*$^{Δ/Δ}$ (DKO)] we analyzed their bone marrow composition. In agreement with our previous results,[11] loss of either Hdac1 or Hdac2 did not affect the number of splenocytes, thymocytes or bone marrow cells, while compound loss of both deacetylases resulted in a dramatic reduction in cellularity of these organs (Figure 1A, B). The reduction in bone-marrow cells in *DKO* mice affected all major hematopoietic lineages and was associated with an increase in apoptotic bone marrow cells (Figure 1C,D). These findings suggest that Hdac1 and Hdac2 have a critical role early during hematopoiesis. Indeed, hematopoietic progenitors and HSC were almost completely ablated in *DKO* mice, while their numbers remained normal in *MxCre*$^+$;*Hdac1*$^{Δ/Δ}$ (*Hdac1KO*) and *MxCre*$^+$;*Hdac2*$^{Δ/Δ}$ (*Hdac2KO*) mice (Figure 1E). To exclude that markers used for the identification of early hematopoietic progenitors and HSC were deregulated in cells lacking Hdac1 and Hdac2, we functionally assayed bone marrow cells from *WT* and *DKO* mice using *in vitro* colony assays in semi-solid medium. As indicated in Figure 1F, simultaneous loss of Hdac1 and Hdac2 was incompatible with the outgrowth of myeloid or pre-B-cell colonies, strongly suggesting that Hdac1 and Hdac2 have an essential and overlapping role in HSC homeostasis.

Cell-autonomous function of Hdac1 and Hdac2 in the maintenance of hematopoietic stem cells

Activation of *Mx*-promoter-driven *Cre*-recombinase results in deletion of Hdac1 and Hdac2 in hematopoietic and niche-supporting mesenchymal cells.[13] To address whether Hdac1 and Hdac2 have a cell-intrinsic function in HSC or also a function in non-hematopoietic bone marrow cells we performed competitive bone marrow transplantation assays. Non-induced *MxCre*$^+$;*Hdac1*$^{L/L}$;*Hdac2*$^{L/L}$ bone marrow cells, carrying the Ly5.2$^+$ cell surface marker, were mixed with Ly5.1$^+$ *WT* bone marrow cells in a 1:1 ratio and transplanted into lethally irradiated mice. As a control we performed the same experiment using non-induced Ly5.2$^+$ *MxCre*$^+$;*Hdac1*$^{L/L}$ or *MxCre*$^-$;*Hdac1*$^{L/L}$*Hdac2*$^{L/L}$ bone marrow cells mixed with Ly5.1$^+$ *WT* cells. Upon stable engraftment, determined through Ly5.2$^+$ and Ly5.1$^+$ flow cytometry of peripheral blood (Figure 2A,B), *Cre*-recombinase mediated deletion of *Hdac1* and *Hdac2* was induced by pI;pC injections. Long-term reconstituting abilities of *WT, Hdac1KO* and *DKO* bone marrow cells were determined by longitudinal FACS analysis of peripheral blood. This analysis revealed a progressive drop of DKO cells (Figure 2B), while single loss of Hdac1 did not

affect long-term reconstitution (Figure 2A). Furthermore, analysis of mice transplanted with *MxCre*$^{+}$;*Hdac1*$^{L/L}$;*Hdac2*$^{L/L}$ bone marrow revealed virtually no Ly5.2^{+} *DKO* progenitor cells and HSC at the end of the experiment (Figure 2C). The small remaining fraction of Ly5.2^{+} cells showed the presence of non-recombined *Hdac1* and *Hdac2* conditional knock-out alleles (Figure 2D), providing further support for an essential role for Hdac1 and Hdac2 in long-term bone marrow reconstitution.

Gene expression analysis using RNA-Seq of sorted progenitors and HSC from pI;pC-treated control and *MxCre*$^{+}$;*Hdac1*$^{L/L}$;*Hdac2*$^{L/L}$ mice revealed that only a limited set of genes were significantly (*P*-value <0.05, logFC >0.58) deregulated upon loss of Hdac1/2 (*Online Supplementary Figure S1*). Interestingly, one of the up-regulated genes, *Nuclear casein kinase and cyclin-dependent kinase substrate 1* (*Nucks1*) correlated with the levels of Bax and activated caspase-3, which are indicative of apoptosis. Moreover, Nucks1 was up-regulated very early during neuronal apoptosis *in vitro*.[17] In contrast, the survival factor *Tumor protein translationally controlled 1* (*Tpt1*) was down-regulated, further suggesting that Hdac1 and Hdac2 may control genes to allow survival of HSC. *Dermokine* (*Dmkn*) a gene which is biochemically linked to the Cripto-GRP78 regulatory signal in HSC,[18,19] was down-regulated in Hdac1/2 -deficient HSC/progenitors suggesting that Hdac1/2 regulates HSC maintenance at least in part by regulating *Dmkn* expression. In conclusion, these experiments indicate that Hdac1 and Hdac2 collectively maintain HSC in a cell-autonomous manner.

Compensatory expression of Hdac2 in Hdac1-deficient hematopoietic cells

Since simultaneous loss of Hdac1 and Hdac2, in contrast to loss of Hdac1 or Hdac2, did interfere with HSC homeostasis, our findings indicate functional redundancy

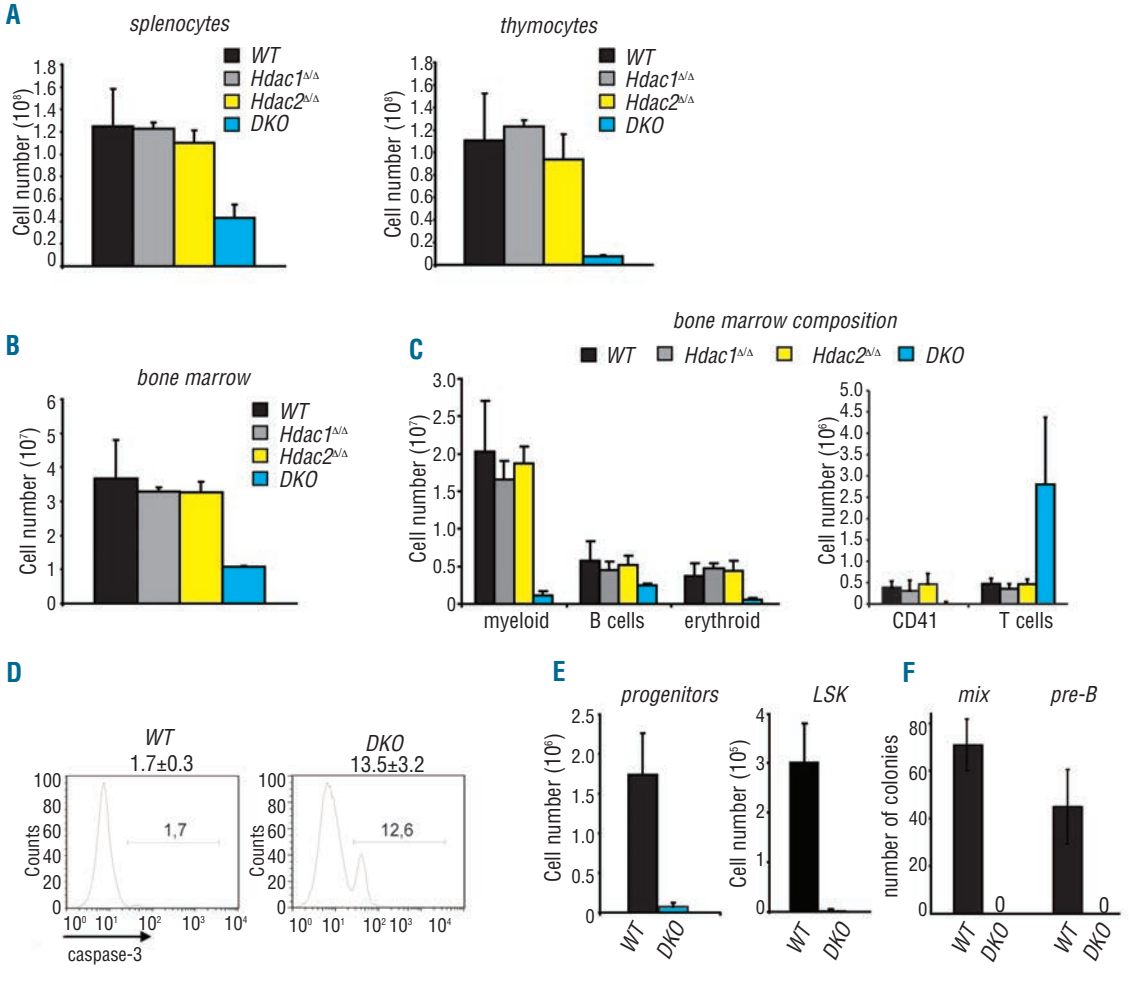

Figure 1. Hdac1 and Hdac2 are collectively essential for hematopoiesis. (A) Splenocyte and thymocyte counts of the indicated groups (n=3 per group). (B) Quantification of bone marrow cells after erythrocyte lysis. (C) The bone marrow composition of *WT*, *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$, *MxCre*$^{+}$;*Hdac2*$^{\Delta/\Delta}$ and *DKO* mice 8 days after the last pI:pC injection (n=3 per group). Bone marrow cells were isolated, quantified and stained with labeled antibodies against CD11b and Gr-1 (myeloid cells), B220 and CD19 (B cells), TER119 (erythroid cells), CD41 (megakaryocytes) and CD3 (T cells) and analyzed by flow cytometry. (D) Apoptosis in the bone marrow of control and *DKO* mice was analyzed by flow cytometry using a labeled antibody against caspase-3. Histograms are representative of three experiments per group; the average and standard deviation are presented on top. (E) Flow cytometric quantification of c-Kit^{+} cells (progenitors) and LSK (Lin^{-};c-Kit^{+};Scal^{+}) cells in the bone marrow of *WT* and *DKO* mice (n=3 per group). (F) The number of myeloid and pre-B-cell colonies from bone marrow of *WT* and *DKO* mice cultured in methylcellulose enriched with either 10 ng/mL interleukin–3, 10 ng/mL interleukin-6 and 50 ng/mL stem cell factor to induce myeloid differentiation or with 10 ng/mL interleukin-7 to induce differentiation into pre-B cells.

between Hdac1 and Hdac2 in this hematopoietic compartment. Transcription-independent up-regulation of Hdac1 or Hdac2 protein levels in cells lacking *Hdac2* or *Hdac1* was observed in many cell types and could serve as a mechanism for functional redundancy.[11] Western blot analysis revealed a significant up-regulation of Hdac2 protein levels in *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$ bone marrow, while Hdac1 levels remained unchanged in *MxCre*$^{+}$;*Hdac2*$^{\Delta/\Delta}$ bone marrow (Figure 3A). These findings indicate that loss of Hdac1 in bone marrow can be counterbalanced by a compensatory up-regulation of Hdac2 protein levels. In contrast, loss of Hdac2 was not associated with an increase in Hdac1 suggesting that Hdac1 may have a more dominant role than Hdac2 in hematopoiesis.

Hdac1/2 dosage determines normal hematopoiesis

To test this hypothesis we generated mice with mono-allelic expression of Hdac1 (*MxCre*$^{+}$;*Hdac1*$^{+/\Delta}$;*Hdac2*$^{\Delta/\Delta}$) or Hdac2 (*MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$;*Hdac2*$^{+/\Delta}$) in the bone marrow. Under these conditions Hdac1 and Hdac2 expression levels were similar to those of wild-type animals which allowed us to compare the contribution of Hdac1 or Hdac2 to independent hematopoietic lineages (Figure 3A). Previously, we showed that *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$;*Hdac2*$^{+/\Delta}$ mice died within 8-12 days following Cre-recombinase activation, which was associated with severe anemia and thrombocytopenia[11] (Figure 3B). Surprisingly, *MxCre*$^{+}$;*Hdac1*$^{+/\Delta}$;*Hdac2*$^{\Delta/\Delta}$ mice appeared macroscopically normal and showed normal cellularity of bone marrow, spleen and thymus (Figure 3C, *Online Supplementary Figure S2*), indicating that mono-allelic expression of Hdac1, without compensatory Hdac2 up-regulation, in contrast to mono-allelic Hdac2 expression, is sufficient to allow normal erythropoiesis and thrombocyte formation. In summary, these results indicate that normal hematopoiesis is controlled by total levels of Hdac1 and Hdac2 in which Hdac1 plays a more dominant role during erythropoiesis.

Differential effects of mono-allelic Hdac1 or Hdac2 expression on hematopoietic lineages

To investigate the consequences of mono-allelic expression of Hdac1 or Hdac2 in hematopoietic lineages we analyzed bone marrow cellularity and composition in *MxCre*$^{+}$;*Hdac1*$^{+/\Delta}$;*Hdac2*$^{\Delta/\Delta}$ and *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$;*Hdac2*$^{+/\Delta}$ mice. Contrasting with the situation in *DKO* mice, mono-allelic expression of Hdac2 resulted in a 30% reduction of bone marrow cells after erythrocyte lysis while mono-allelic Hdac1 expression resulted in wild-type cell numbers (Figure 3C). Interestingly, in contrast to myeloid (Gr1^{+};Mac1^{+}) cell counts, which were unaffected in both genotypes, B-lymphocyte numbers were significantly reduced by as much as 50% in both *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$;*Hdac2*$^{+/\Delta}$ and *MxCre*$^{+}$;*Hdac1*$^{+/\Delta}$;*Hdac2*$^{\Delta/\Delta}$ mice as compared to control mice (Figure 3D). Colony-formation assays confirmed these findings as the number of pre-B-cell colonies were significantly reduced in animals of both genotypes, while myeloid colony numbers appeared unchanged (Figure 3E). Finally, erythroid cell numbers were differently affected as we observed a dramatic reduction in erythropoietic cell numbers only in *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$,*Hdac2*$^{+/\Delta}$ mice (Figure 3D), suggesting that the observed anemia is caused by a defect in early erythropoiesis. Overall, these results imply a differential dependency of myeloid, lymphoid and erythroid lineages on

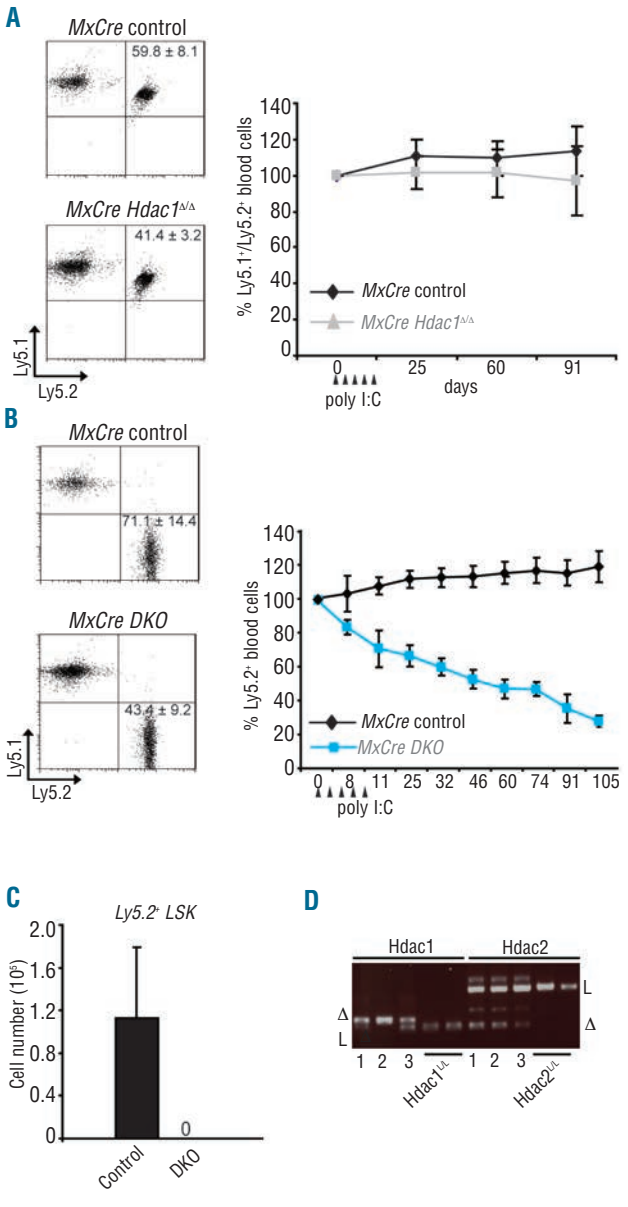

Figure 2. Hdac1 and Hdac2 have cell autonomous functions in HSC maintenance. (A) Bone marrow cells from *WT* (Ly5.1^{+}) and *MxCre*$^{+}$;*Hdac1*$^{L/L}$ (Ly5.1^{+}/Ly5.2^{+}) or *MxCre*$^{+}$;*Hdac1*$^{L/L}$ (control) mice were transplanted in a 1:1 ratio in lethally irradiated recipient mice. Bone marrow reconstitution of Ly5.1^{+} and Ly5.1^{+}/Ly5.2^{+} populations was measured 8 weeks post-transplantation (left panel). Subsequently, mice were injected five times with pI;pC (arrows right panel). Ly5.1^{+}/Ly5.2^{+} cells were monitored in the blood for 91 days (right panel). The mean value of Ly5.1^{+}Ly5.2^{+} cells was set at 100% before pI;pC injection, (n=5 mice per group). (B) Bone marrow cells from *WT* (Ly5.1+) and *MxCre*$^{+}$;*Hdac1*$^{L/L}$*Hdac2*$^{L/L}$ (Ly5.2^{+}) or *MxCre*$^{+}$;*Hdac1*$^{L/L}$*Hdac2*$^{L/L}$ (control) mice were transplanted in a 1:1 ratio in lethally irradiated recipient mice. Bone marrow reconstitution of Ly5.1^{+} and Ly5.2^{+} populations was measured 8 weeks post-transplantation (left panel). Subsequently, mice were injected five times with pI;pC (arrows right panel). Ly5.2^{+} cells were monitored in the blood for 105 days (right panel). The mean value of Ly5.2^{+} cells was set at 100% before pI;pC injection (n=5 mice per group). (C) At day 105 after the first pI:pC injection, mice were sacrificed and Ly5.2^{+} LSK in the bone marrow of the chimeras described in (B), were quantified by flow cytometry (n=3 per group). (D) After the competitive bone marrow transfer described in (C), genomic DNA was isolated from sorted Ly5.2^{+} cells and analyzed by polymerase chain reaction for the presence of floxed and Δ alleles (right panel). 1-3 represents bone marrow samples from three independent mice.

Hdac1/2 dosage with a prominent role for Hdac1 in the erythoid lineage

Critical function for Hdac1 early during erythropoiesis

Defective erythropoiesis often leads to compensatory expansion of hematopoietic tissue outside the bone marrow, a phenomenon known as extramedullary hematopoiesis.[20] Indeed, spleens in *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$,*Hdac2*$^{+/\Delta}$ mice were 2- to 3-fold larger than control spleens (Figure 4A); flow cytometry showed that this enlargement was caused by an increase in nucleated erythroid cells (Figure 4B,C). Colony assays in semi-solid media revealed a significant increase in colony numbers when using *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$,*Hdac2*$^{+/\Delta}$ splenocytes (Figure 4D) indicating extramedullary hematopoiesis. Finally, *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$,*Hdac2*$^{+/\Delta}$ liver sections revealed the presence of nucleated erythroid cells, further confirming extramedullary hematopoiesis (Figure 4E). Despite the extramedullary hematopoiesis, erythropoiesis was not rescued in *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$,*Hdac2*$^{+/\Delta}$ mice, as evidenced by the fatal anemia in these mice (Figure 3B). In order to identify the cause of ineffective erythropoiesis in *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$,*Hdac2*$^{+/\Delta}$ mice, we analyzed the different stages (I-IV) of erythropoiesis using CD71 and Ter119 surface markers (Figure 4F). In contrast to mono-allelic Hdac1 expression, mono-allelic expression of Hdac2 resulted in a profound reduction in basophilic (type II) and polychromatic (type III) erythroblasts, while pro-erythroblasts (type I) and orthochromatic erythroblasts (type IV) were only mildly affected (Figure 4F). As type IV erythroblasts in our FACS analysis also harbor long-lived enucleated erythrocytes, we conclude that Hdac1 very likely plays a critical function in the transition from type I to type II erythroblasts, the phase in which erythroblasts start to proliferate and expand. In summary, these data reveal a differential requirement for Hdac1 and Hdac2 in hematopoietic cell lineages as mono-allelic Hdac1 or Hdac2 expression is sufficient to drive myeloid differentiation while in B-lymphocytes such levels do not allow normal differentiation (Figure 3D). Our observations with mono-allelic expression of Hdac1 or Hdac2 in bone marrow indicate the importance of Hdac1 and Hdac2 dosage for hematopoiesis, with Hdac1 and Hdac2 contributing differentially in that Hdac1 plays a more dominant role than Hdac2 in the erythroid lineage.

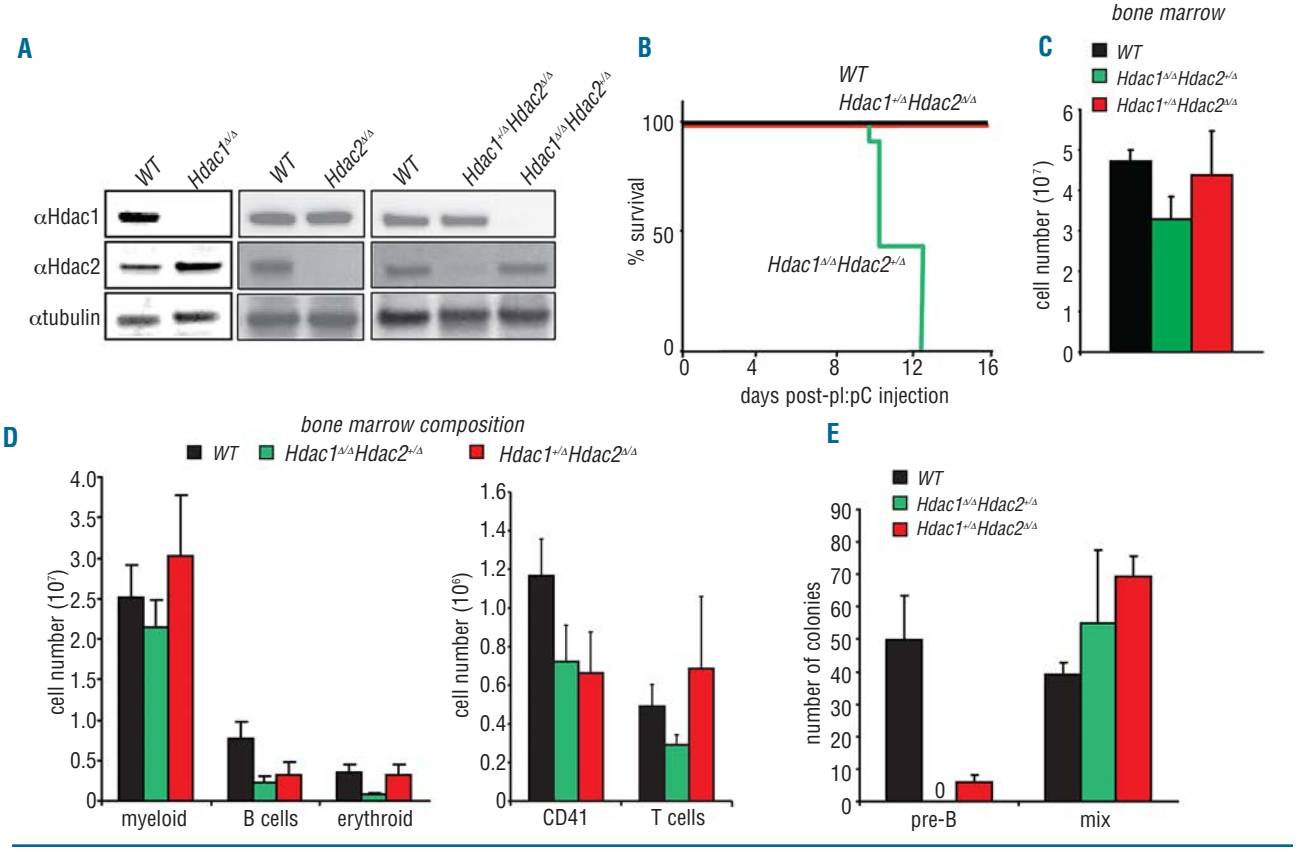

Figure 3. Hdac1 and Hdac2 do not compensate for each other equally in hematopoiesis. (A) Western blot analysis for Hdac1 and Hdac2 protein levels in the bone marrow of the mice with the indicated genotypes. Pictures are representative images of the results from three or more independent mice. Tubulin was used a loading control. (B) Kaplan-Meier survival curves demonstrating the survival of *WT*, *MxCre*$^{+}$;*Hdac1*$^{+/\Delta}$*Hdac2*$^{\Delta/\Delta}$ and *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$*Hdac2*$^{+/\Delta}$ mice after five injections of pI;pC (at least 3 mice per group). (C) Quantification of erythrolysed bone marrow of indicated genotypes (n=3 mice per group). (D) Number of bone marrow cells after erythrocyte lysis of *WT*, *MxCre*$^{+}$;*Hdac1*$^{+/\Delta}$*Hdac2*$^{\Delta/\Delta}$ and *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$*Hdac2*$^{+/\Delta}$ mice (n=3 mice per group). Bone marrow cells were isolated, quantified and stained with labeled antibodies against CD11b and Gr-1 (myeloid cells), B220 and CD19 (B cells), TER119 (erythroid cells), CD41 (megakaryocytes) and CD3 (T cells) and analyzed by flow cytometry. (E) The number of pre-B-cell and myeloid colonies from bone marrow of *WT*, *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$*Hdac2*$^{+/\Delta}$ and *MxCre*$^{+}$;*Hdac1*$^{+/\Delta}$*Hdac2*$^{\Delta/\Delta}$ mice cultured in methylcellulose enriched with either 10 ng/mL interleukin-3, 10 ng/mL interleukin-6 and 50 ng/mL stem cell factor to induce myeloid differentiation or with 10 ng/mL interleukin-7 to induce differentiation into pre-B cells.

Sin3a is essential for hematopoiesis

Our results identified an essential, intrinsic and overlapping function for Hdac1 and Hdac2 in the maintenance of HSC. As Hdac1/Hdac2 heterodimers provide the deacetylase activity of multi-protein transcription repressor complexes, such as Mi-2/NuRD, CoREST and Sin3a,[4] we aimed to pinpoint the biochemical defined Hdac1/2 complex contributing to hematopoietic development and HSC maintenance. We, therefore, inactivated Sin3a in bone marrow using a mouse model carrying *Sin3a* conditional knock-out alleles[21] and the *MxCre* transgene. Upon successful deletion of Sin3a in bone marrow, mice became moribund at approximately 14 days after the *Cre*-recombinase induction (Figure 5A,B). Resembling the mice with bone marrow-specific deletion of Hdac1 and Hdac2, *MxCre*$^{+}$;*Sin3a*$^{\Delta/\Delta}$ mice displayed features of anemia and internal bleeding (*data not shown*). Indeed, peripheral blood analysis showed a 4-fold reduction in red blood cells while the number of thrombocytes was reduced 16-fold (Figure 5C). Assessment of thymus and spleen revealed a severe decrease in the cellularity of these organs (Figure 5D). Interestingly, in contrast to splenocytes, thymocyte numbers were reduced in a Sin3a dosage-dependent manner as a result of apoptosis as evidenced by immunohistochemical staining for activated caspase-3 (Figure 5D,E). Similar to the phenotype observed in *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$;*Hdac2*$^{\Delta/\Delta}$ (*DKO*) mice, histological examination of bone marrow sections as well as total bone marrow cell counts revealed a severe reduction in total cell numbers in *MxCre*$^{+}$;*Sin3a*$^{\Delta/\Delta}$ mice (Figure 6A).

Sin3a has a cell-autonomous function in hematopoietic stem cell maintenance

The overlapping phenotypes in *MxCre*$^{+}$;*Sin3a*$^{\Delta/\Delta}$ and *DKO* mice prompted us to analyze hematopoiesis in *MxCre*$^{+}$;*Sin3a*$^{\Delta/\Delta}$ mice in more detail. Indeed, like *DKO* mice, *MxCre*$^{+}$;*Sin3a*$^{\Delta/\Delta}$ mice displayed loss of all hematopoietic lineages in the bone marrow, indicating a defect early in hematopoiesis (Figure 6B). Importantly, c-Kit-positive cells, which include HSC and their immediate progenitors, were absent in Sin3a-deficient bone marrow (Figure 6C,D). Consistently, *MxCre*$^{+}$;*Sin3a*$^{\Delta/\Delta}$ bone marrow was defective in generating myeloid and pre-B cell colonies in *in vitro* colony assays, suggesting a role for Sin3a in HSC maintenance (Figure 6E). To test whether Sin3a has a cell-autonomous role in HSC we performed

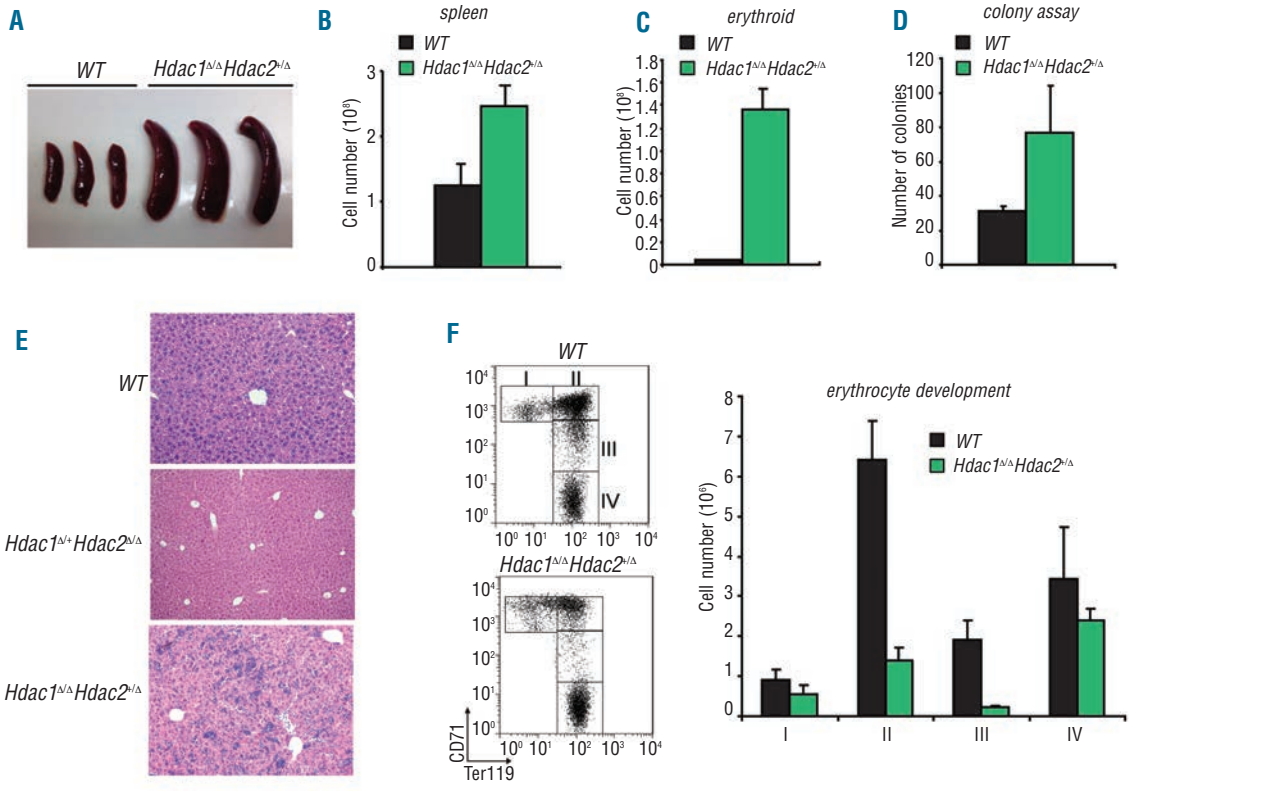

Figure 4. Erythropoiesis is severely affected only in *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$*Hdac2*$^{+/\Delta}$ mice. (A) Three *WT* and three enlarged *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$*Hdac2*$^{+/\Delta}$ spleens. (B) Splenocyte numbers in *WT* and *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$*Hdac2*$^{+/\Delta}$ mice (n=3 mice per group). (C) After erythrocyte lysis, which only affects enucleated cells, the number of nucleated erythrocytes in spleens from *WT* and *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$*Hdac2*$^{+/\Delta}$ mice was determined (n=3 mice per group). (D) The number of myeloid colonies from spleen of *WT* and *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$*Hdac2*$^{+/\Delta}$ mice cultured in methylcellulose enriched with 10 ng/mL interleukin-3, 10 ng/mL interleukin-6 and 50 ng/mL stem cell factor to induce myeloid differentiation. (E) Hematoxylin-eosin-stained histological liver sections of pI;pC-treated mice with indicated genotypes. Only *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$*Hdac2*$^{+/\Delta}$ livers showed the presence of nucleated erythroid cells (F) Erythroid development in the bone marrow of *WT*, *MxCre*$^{+}$;*Hdac1*$^{\Delta/\Delta}$*Hdac2*$^{+/\Delta}$ mice was analyzed by flow cytometry using the labeled erythroid markers CD71 and Ter119. I, II, III and IV represent pro-erythroblasts, basophilic, polychromatic and orthochromatic erythroblasts, respectively (left panel). Absolute numbers are plotted (right panel).

competitive bone marrow transplantation assays. Long-term reconstituting abilities of Ly5.2$^+$ *MxCre*$^+$;*Sin3a*$^{L/L}$ bone marrow cells were determined upon stable engraftment and subsequent *Cre*-recombinase-mediated deletion of Sin3A. Longitudinal analysis of peripheral blood revealed a progressive drop of Sin3a-deficient Ly5.2$^+$ cells (Figure 6F). Furthermore, virtually no Sin3A-deficient Ly5.2$^+$ HSC and progenitor cells were present in bone marrow at the end of the experiment (Figure 6G). These findings indicate a cell-autonomous role for Sin3a in HSC maintenance. The similarity between the phenotypes observed in *MxCre*$^+$;*Sin3a*$^{\Delta/\Delta}$ and *DKO* mice strongly suggests that Sin3a-associated Hdac1 and Hdac2 play an essential and intrinsic role in HSC homeostasis.

Sin3a is essential for T- and B-lymphocyte development

Since Hdac1 and Hdac2 have overlapping functions in the regulation of committed lineages such as T- and B-lymphocytes,[22-24] it is possible that Sin3a mediates these functions, although it is unknown whether this is the case. To address this issue we specifically deleted Sin3a in thymocytes and B-lymphocytes using the *LckCre*[15] and *Mb1-Cre* transgenic mice.[14] As in a previous study,[25] analysis of *LckCre*$^+$;*Sin3a*$^{\Delta/\Delta}$ mice revealed an almost complete absence

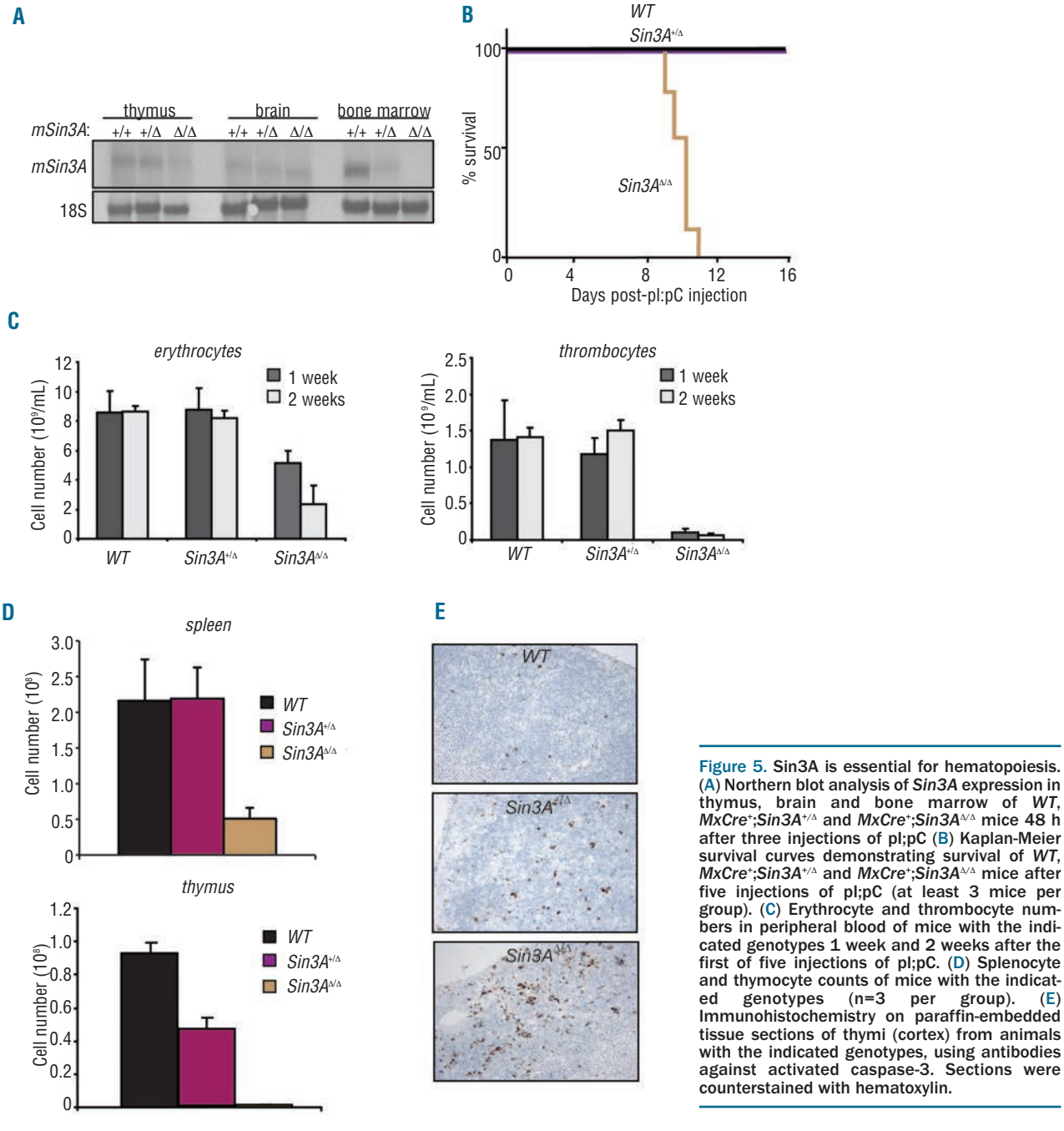

Figure 5. Sin3A is essential for hematopoiesis. (A) Northern blot analysis of *Sin3A* expression in thymus, brain and bone marrow of *WT*, *MxCre*$^+$;*Sin3A*$^{+/\Delta}$ and *MxCre*$^+$;*Sin3A*$^{\Delta/\Delta}$ mice 48 h after three injections of pI;pC (B) Kaplan-Meier survival curves demonstrating survival of *WT*, *MxCre*$^+$;*Sin3A*$^{+/\Delta}$ and *MxCre*$^+$;*Sin3A*$^{\Delta/\Delta}$ mice after five injections of pI;pC (at least 3 mice per group). (C) Erythrocyte and thrombocyte numbers in peripheral blood of mice with the indicated genotypes 1 week and 2 weeks after the first of five injections of pI;pC. (D) Splenocyte and thymocyte counts of mice with the indicated genotypes (n=3 per group). (E) Immunohistochemistry on paraffin-embedded tissue sections of thymi (cortex) from animals with the indicated genotypes, using antibodies against activated caspase-3. Sections were counterstained with hematoxylin.

of thymocytes (Figure 7A), predominantly due to a severe reduction in $CD4^+;CD8^+$ (double-positive) thymocytes (Figure 7B,C) and a significant increase in $CD25^+;CD44^-$ (DN3) thymocytes (Figure 7C,D). As these findings are identical to those resulting from deletion of both Hdac1 and Hdac2 in thymocytes[22] we conclude that Sin3a has an intrinsic role in thymocyte development which is mediated through an interaction with Hdac1 and Hdac2. B-cell-specific loss of Sin3a resulted in an almost complete absence of B-lymphocytes in the bone marrow and spleen (Figure 7E). Reduced amounts of pro-B lymphocytes were detected in *Mb1Cre;Sin3a*$^{\Delta/\Delta}$ mice, suggesting that deletion of *Sin3a* at the pro-B-lymphocyte stage resulted in apoptosis (Figure 7F). These findings completely mimic the results

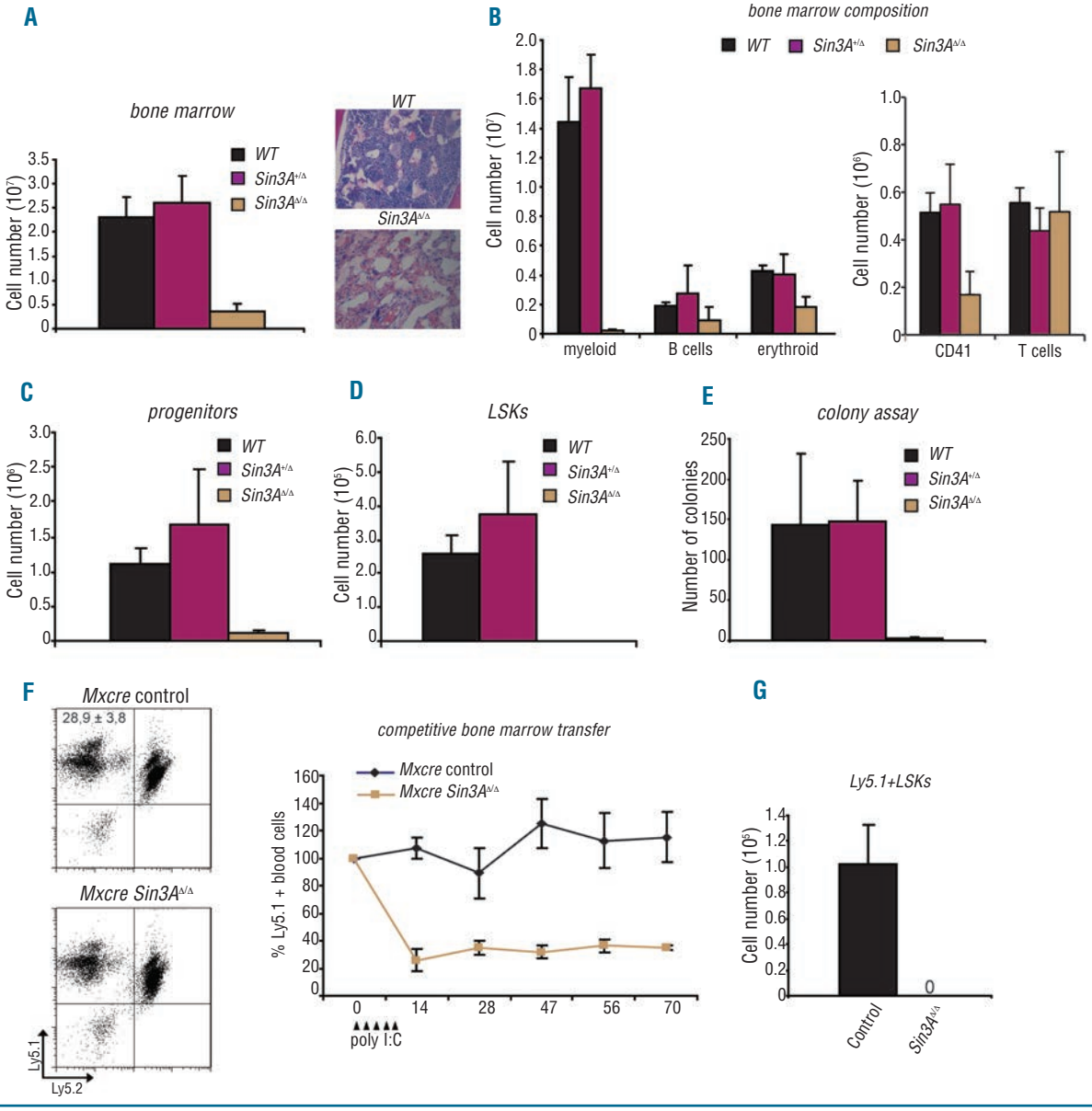

Figure 6. Sin3A has a cell-autonomous function in HSC maintenance. (A) Quantification of bone marrow cells from *WT*, *MxCre*$^+$;*Sin3A*$^{+/\Delta}$ and *MxCre*$^+$;*Sin3A*$^{\Delta/\Delta}$ mice (n=3 mice per group) (left panel). Representative hematoxylin–eosin-stained paraffin-embedded tissue sections from bone marrow of mice with the indicated genotypes showing loss of cellularity in Sin3A-deficient bone marrow. (B) The bone marrow composition of the indicated genotypes (n=3 mice per group). Bone marrow cells were isolated, quantified and stained with labeled antibodies against CD11b and Gr-1 (myeloid cells), B220 and CD19 (B cells), TER119 (erythroid cells), CD41 (megakaryocytes) and CD3 (T cells) and analyzed by flow cytometry. (C) Flow cytometric quantification of c-kit$^+$ cells (progenitors) in the bone marrow of animals with the indicated genotypes (n=3 per group). (D) Flow cytometric quantification of LSK cells ($lin^-c\text{-}Kit^+Scal^+$) in the bone marrow of animals with the indicated genotypes (n=3 per group). (E) The number of myeloid colonies from bone marrow of animals with the indicated genotypes cultured in methylcellulose enriched with 10 ng/mL interleukin-3, 10 ng/mL interleukin-6 and 50 ng/ml stem cell factor to induce myeloid differentiation. (F) Bone marrow cells from *WT* ($Ly5.2^+$) and *MxCre*$^+$;*Sin3A*$^{L/L}$ ($Ly5.1^+$) or *MxCre*$^-$;*Sin3A*$^{L/L}$ (control) mice were transplanted in a 1:1 ratio in lethally irradiated recipient mice. Bone marrow reconstitution of $Ly5.1^+$ and $Ly5.2^+$ populations was measured 8 weeks post-transplantation (left panel). Subsequently, mice were injected five times with pI;pC (arrows right panel). $Ly5.2^+$ cells were monitored in the blood for 70 days (right panel). The mean value of $Ly5.2^+$ cells was set at 100% before pI;pC injection, (n=5 mice per group). (G) Flow cytometric quantification of $Ly5.1^+$ LSK in the bone marrow of the chimeras described in (E) (n=3 per group).

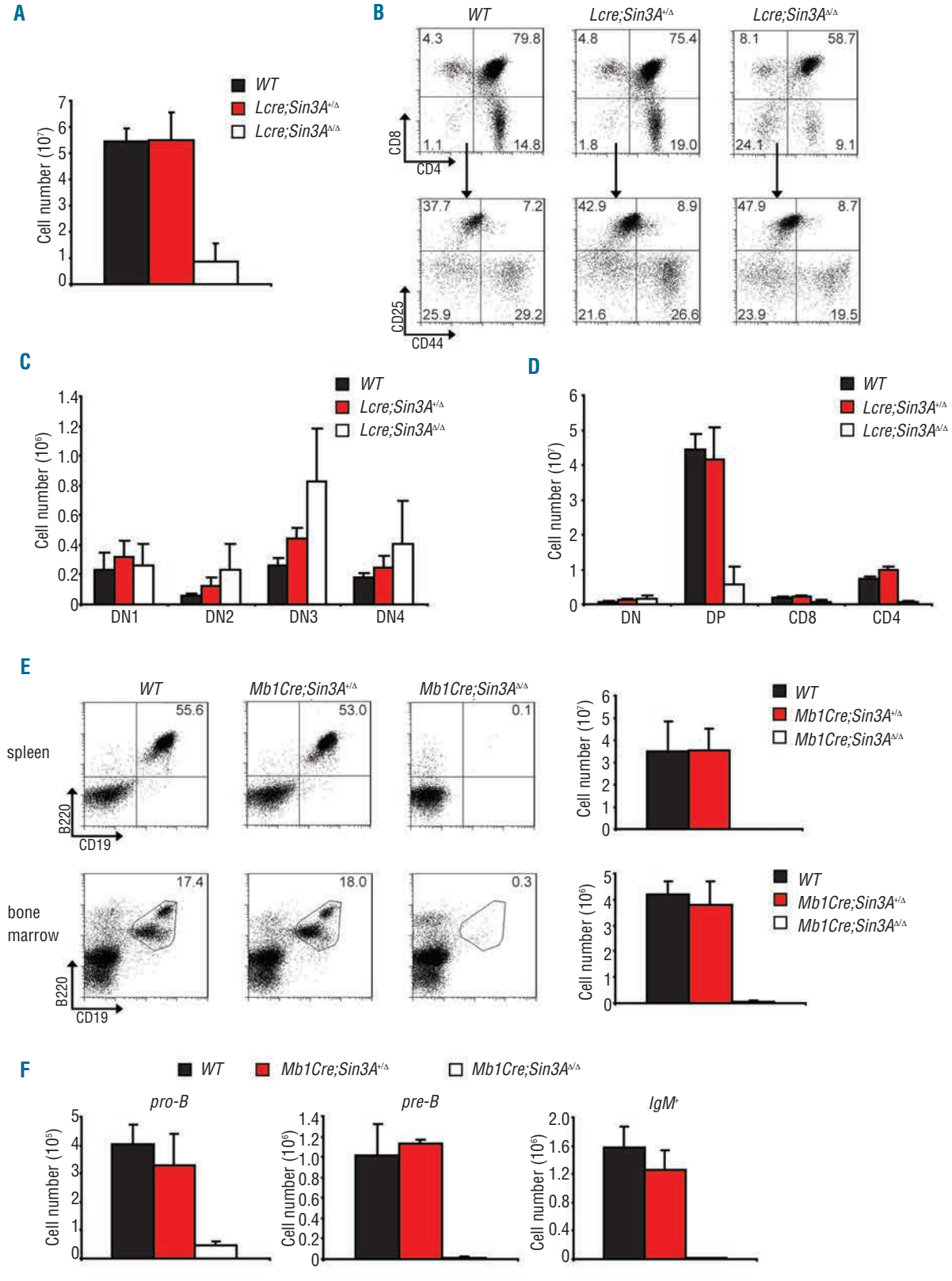

Figure 7. Sin3A is essential for lymphocyte development. (A) Quantification of cells in the thymus from *WT*, *LCre*$^{+}$;*Sin3A*$^{+/\Delta}$ and *LCre*$^{+}$;*Sin3A*$^{\Delta/\Delta}$ mice (n=3 mice per group). (B) Representative dot plots of flow cytometric analyses of thymocyte development in 6-week old mice with the indicated genotypes. (C) Quantification of thymic subsets of 6-week old mice with the indicated genotypes (n=3 mice per group). CD25^{-}CD44^{+} = DN1; CD25^{+}CD44^{+} = DN2; CD25^{+}CD44^{-} = DN3; CD25^{-}CD44^{-} = DN4. (D) Quantification of thymic subsets of 6-week old mice with the indicated genotypes (n=3 mice per group). DN = CD4^{-}CD8^{-}, DP = CD4^{+}CD8^{+}, CD4 SP = CD4^{+}CD8^{-}, CD8 SP = CD4^{-}CD8^{+}. (E) Representative flow cytometry plots of B lymphocytes in the spleen and bone marrow of *WT*, *Mb1Cre*;*Sin3A*$^{+/\Delta}$ and *Mb1Cre*;*Sin3A*$^{\Delta/\Delta}$ mice, using the labeled B-cell markers CD19 and B220 (left panel). Quantification of B lymphocytes in spleen and bone marrow (n=3 mice per group) (right panel). (F) Quantification of pro-B, pre-B and IgM^{+} B lymphocytes in the bone marrow of mice with the indicated genotypes (n=3 mice per group).

obtained with simultaneous deletion of Hdac1 and Hdac2 in the B-cell compartment,[24] suggesting that the association of Hdac1 and Hdac2 with the Sin3a complex has an intrinsic place in B-lymphocyte differentiation. Overall, the data presented here indicate an essential role for Sin3a-associated Hdac1 and Hdac2 in HSC homeostasis and lymphocyte development which is consistent with the biochemical data supporting the existence of a Sin3a/Hdac1/2 complex.

Discussion

Using an *in vivo* mouse model carrying *Hdac1* and *Hdac2* conditional knock-out alleles we identified a critical, cell-autonomous role for Hdac1 and Hdac2 in HSC homeostasis. Simultaneous loss of Hdac1 and Hdac2 resulted in depletion of HSC and early hematopoietic progenitors. Likewise, bone marrow-specific inactivation of *Sin3a*, a major component of the Sin3a/HDAC1/2-repressor complex, resulted in a phenotype identical to that resulting from loss of Hdac1 and Hdac2, identifying the Sin3a/Hdac1/2 complex as a major cell-autonomous regulator of HSC maintenance. The rapid loss of HSC and progenitors upon ablation of Hdac1/2 or Sin3a may suggest that these cell populations were lost due to induction of apoptosis rather than because of slow exhaustion of the HSC compartment. This is in agreement with previous findings that deletion of Hdac1/2 or Sin3a results in cell cycle arrest and apoptosis.[11,21-27] Deregulated expression of genes linked to cell survival and stem cell maintenance, such as *Dmkn*, *Nurcks1* and *Tpt1*, warrants further investigation to determine whether these genes are direct Hdac1/2 targets.

Previously, other researchers investigated the role of Hdac1/Hdac2-containing NuRD and CoREST complexes in HSC maintenance and hematopoiesis. *MxCre*-mediated deletion of Chromodomain protein 4 (Chd4), a major component of the NuRD complex, resulted in an acute and rapid expansion of HSC and erythroid progenitors, suggesting that Chd4 is critical for maintaining HSC quiescence.[28] In addition, progenitors of the myeloid and lymphoid lineages were lost upon Chd4 deletion, suggesting that the NuRD complex is critical for the myeloid and lymphoid lineages. Inhibition of lysine demethylase Lsd1 or Rcor1, two major components of the CoREST complex, enhanced the proliferation of granulomonocytic, erythroid and megakaryocytic progenitors but blocked the terminal differentiation of these lineages.[29,30] These findings illustrate a differential role for the NuRD and CoREST complexes compared to Sin3a repressor complexes in HSC homeostasis. The fact that loss of HSC in the absence of Sin3a is not compensated by NuRD or CoREST complexes provides further support for a critical role for Sin3a/HDAC1/2 in HSC homeostasis. However, these results do not exclude the possibility that loss of Sin3a affects the activity or the composition of NuRD and CoREST complexes.

Consistent with an essential role for Sin3a/Hdac1/2 in HSC homeostasis, members of this complex have been identified as integral components of a network that regulates "stem-cellness" in embryonic stem cells. Hdac1, Hdac2 and Sin3a were found in complex with Nanog, Sox2 and Oct4 in a transcription regulatory complex known as Nanog and Oct4 associated deacetylase (NODE), which regulated Nanog expression.[31-33] In line with this it was shown that HDAC inhibitors can enhance the formation of induced pluripotent stem cells, probably by derepression of critical pluripotency regulators, including Nanog, Oct4 and Klf4.[34] Alternatively, since Hdac1 was found at predominantly active genes in embryonic stem cells and early thymocytes it is also possible that Hdac1 is required for activation of regulators of self-renewal.[35,36] Moreover, genetic experiments showed that Sin3a or Sin3a interacting factors, such as Ronin,[37] are essential for the survival of inner cell mass in blastocysts while loss of Sin3a in embryonic stem cells resulted in unresolved DNA damage and apoptosis, suggesting a critical role for Sin3A in embryonic stem cell maintenance.[21,38]

Functional compensation between Hdac1 and Hdac2, as seen in the bone marrow, suggests equality between Hdac1 and Hdac2 function. However, using mouse models with mono-allelic expression of Hdac1 or Hdac2, we showed an inequality between the contributions of Hdac1 and Hdac2 to different hematopoietic lineages. Analysis of the myeloid lineage revealed that mono-allelic loss of Hdac1 or Hdac2 did not affect either myeloid cell numbers or myeloid colony formation, suggesting that in this lineage Hdac1 and Hdac2 fully compensate for each other and therefore contribute equally. In contrast, mono-allelic expression of Hdac1 resulted in impaired erythrocyte development, which was not observed in the presence of mono-allelic Hdac2 expression. These observations suggest a dosage-dependent function for Hdac1/Hdac2 complexes in erythrocyte development in which Hdac1 plays a dominant role. Consistent with this hypothesis, HDAC1 was found to play a critical role in converting the NuRD complex from a repressor to an activator during GATA-1-directed erythroid differentiation.[39] Our analysis suggests that Hdac1 plays a prominent role during early hematopoiesis at the transition of stage II to stage III erythroblasts, which is characterized by a massive proliferative expansion of erythroblasts. Since loss of Chd4, a core component of the NuRD complex, resulted in an identical block during erythrocyte development it is possible that Hdac1 associated with the NuRD complex regulates erythrocyte development.[28]

Together these data suggest a model in which Hdac1 and Hdac2 determine hematopoietic lineage formation by acting in a dosage-dependent manner. Which lineage is formed may be dependent on the presence of a particular HDAC complex, which critically regulates expression of lineage "identity" genes in a manner similar to that shown for Dnmt1, an HDAC-interacting DNA methyltransferase.[40,41] The similarity in dosage-dependent tumor suppression by Dnmt1 and Hdac1/2[22,42] as well as the observation that HDAC1 deacetylated Dnmt1, strongly suggests a close relation between DNA methylation and histone acetylation in hematopoietic cells.[43]

Similar to a dominant role for Hdac1 in erythropoiesis, we recently showed a prominent role for Hdac1 in thymocyte development and tumor suppression.[22] In other cell types such as neurons and oocytes, Hdac2 seemed to play a more dominant role.[12,44] Since Hdac1 and Hdac2 are simultaneously expressed in most cell types it is very likely that regulation of Hdac1/2 is an important mechanism in controlling the cell-type specific functions of these enzymes. Post-translational modifications are known to regulate Hdac1 and Hdac2 activity and complex formation.[45-48] In addition, endogenous molecules such as sphingosine-1-phosphate and intermediates of nitrogen and carbon

metabolism were shown to regulate HDAC activity.[49,50] Intriguingly, β-hydroxybutyrate, a major source of energy for mammals during prolonged exercise or starvation and a compound structurally related to HDAC inhibitors, was shown to inhibit class I HDAC and conferred protection against oxidative stress.[51] These observations portray an intricate relationship between cellular metabolism, Hdac1/Hdac2 activity and epigenetic changes. Consequently, cellular metabolism may be an important regulator of cell fate and lineage differentiation. Finally, inositol tetraphosphate [Ins(1,4,5,6)P4] was shown to control HDAC3 complex formation. As the residues involved in binding Ins(1,4,5,6)P4 are conserved in Hdac1 and Hdac2, it was suggested that these enzymes are controlled by the PTEN tumor suppressor.[52] The similarity in PTEN and HDAC1/2 function in dosage-dependent tumor suppression and control of HSC homeostasis could be considered to support this hypothesis.[22,53,54] As our data support a model in which Hdac1/2 dosage plays a critical, cell-autonomous role in HSC homeostasis and hematopoietic differentiation it will be interesting to identify the signals that regulate the activity of these ubiquitously expressed nuclear deacetylases and thereby determine cellular identity.

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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