A requirement for Lim domain binding protein 1 in erythropoiesis

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During erythrocyte development, the nuclear cofactor Lim domain binding protein 1 (Ldb1) functions as a core subunit of multiprotein DNA binding complexes that include the transcription factors Scl and Gata-1 and the Lim-only adapter Lmo2. Scl, Gata-1, and Lmo2 are each required for erythropoiesis, suggesting that Ldb1-nucleated transcription complexes regulate key steps during erythropoiesis. We documented a requirement for Ldb1 in erythropoiesis in mice. Analysis of ldb1−/− embryos revealed a critical requirement for Ldb1 during primitive erythropoiesis, and conditional inactivation of ldb1 at later stages of gestation and in adult mice demonstrated that Ldb1 is continuously required for both definitive erythropoiesis and megakaryopoiesis. Down-regulation of Ldb1 in erythroblasts inhibited the expression of multiple erythroid-specific and prosurvival genes. These results represent the first unequivocal demonstration of a role for Ldb1 in erythropoiesis in vivo and establish a critical function for Ldb1-nucleated complexes in regulating the erythroid/megakaryocyte transcriptional program.

Erythropoiesis follows a stepwise differentiation process that involves the commitment of hematopoietic stem cells (HSCs) to lineage-restricted progenitors and to terminally differentiated red blood cells. The first wave of erythropoiesis, designated primitive erythropoiesis, is transient and begins around embryonic day (E) 7.5 in the mouse extraembryonic yolk sac and/or aorta-gonad-mesonephros (Palis et al., 1999; Baumann and Dragon, 2005). Primitive erythrocytes (EryPs) represent the predominant blood cells in circulation until E12, when a second wave of erythroblasts generated in the fetal liver gives rise to definitive adult-type erythrocytes.

Erythropoiesis is critically dependent on the activity of the zinc finger transcription factor Gata-1 which is required for the expression of erythroid-specific genes (Welch et al., 2004). Gata-1 binds to several different cofactors, including Fog, Runx1, PU.1, EKLF, Lmo2, and SP1, and can function as both a transcriptional activator and a transcriptional repressor (Wadman et al., 1997; Johnson et al., 2007; Kim and Bresnick, 2007; Tripic et al., 2009). Multi-subunit complexes containing Gata-1 in addition to the basic helix-loop-helix transcription factors Scl/Tal1 and E2A and the nuclear adapters Lmo2 and Lim domain binding protein 1 (Ldb1) have also been described in erythroid cell lines (Wadman et al., 1997). These multimeric complexes bind to important regulatory sites at erythroid genes, and at the β-globin locus, Ldb1 facilitates long-range promoter–enhancer interactions that are necessary for transcription (Xu et al., 2003; Song et al., 2007). Together, these results suggest that Ldb1 complexes may represent an important instrument for activating erythroid-specific gene transcription during development. Supporting this idea, Lmo2, Gata-1, and Scl are each essential for erythropoiesis suggesting that these proteins function cooperatively (Pevny et al., 1991; Warren et al., 1994; Robb et al., 1995, 1996; Shivdasani et al., 1995; Porcher et al., 1996; Schlaeger et al., 2005).

In this study, we examined the role of Ldb1 in erythroid development by evaluating the impact of ldb1 deletion on erythropoiesis using both conventional and conditional ldb1−/− deficient mice. Our results reveal an essential and continuous
role for Ldb1 in both primitive and definitive erythropoiesis as well as in adult megakaryopoiesis. Short hairpin (sh) RNA–mediated knockdown of Ldb1 resulted in the down-regulation of multiple erythroid/megakaryocyte-specific genes as well as genes important for erythocyte survival. These findings represent the first comprehensive demonstration of an in vivo role for Ldb1 in erythropoiesis and provide strong support for the idea that an Ldb1-nucleated multiprotein complex serves as a primary mechanism through which Gata-1 exerts its positive regulatory function in erythroid gene transcription.

RESULTS AND DISCUSSION

Ldb1 expression in the hematopoietic lineage

To examine Ldb1 expression in the hematopoietic lineage, adult bone marrow and fetal liver cells were stained intracellularly with affinity-purified polyclonal Ldb1 antisera. FACS analysis revealed that Ldb1 levels are highest in lineage+/-Sca-1+ c-kit+ (LSK) cells (Fig. S1 A), which are known to contain hematopoietic progenitors including HSCs (Ikuta and Weissman, 1992; Morrison et al., 1995). Indeed, in a separate study we found that Ldb1 is critical and continuously required for HSC maintenance (unpublished data). Slightly lower levels of Ldb1 were detected in lineage-committed progenitor populations (common myeloid progenitors [CMPs], megakaryocyte-erythroid progenitors [MEPs], and granulocyte-macrophage progenitors [GMPs]; Fig. S1 A). Interestingly, Ldb1 was also expressed at all stages of erythroid development but was strongly down-regulated in mature myeloid (Gr1+) and lymphoid (B and T) cells (Fig. S1 B and C), suggesting that Ldb1 may also have an important and specific function in erythropoiesis (Fig. S1 C).

Defective primitive erythropoiesis in the absence of Ldb1

It was reported previously that benzidine-positive cells are undetectable in ldb1−/− yolk sacs, suggesting absent or defective primitive erythropoiesis (Mukhopadhyay et al., 2003). In agreement with this observation, in vitro–cultured embryoid bodies derived from ldb1−/− ES cells did not generate erythroid lineage cells, although myeloid lineage cells could be generated in the absence of Ldb1 (Fig. S2 A). Mesoderm/hemangioblast marker genes and genes involved in Ldb1 complex formation (lmo2, scl, gata2, and gata1) were expressed in day 5 ldb1−/− embryoid bodies, but no expression of erythroid-specific genes was detectable at any time point analyzed (Fig. S2 B and C; and not depicted). Consistent with these data, there was no evidence of blood formation in E9 ldb1−/− embryos (Fig. 1 A, left). E9 ldb1−/− yolk sac blood islands contained extremely low numbers of cells (Fig. 1 A, middle) that included rare myeloid blasts but no erythroblasts (Fig. 1 A, right). To evaluate the differentiation potential of progenitor cells in ldb1−/− yolk sacs, single cell suspensions from E9 yolk sacs were cultured in vitro in hematopoietic differentiation medium. Cells from E9 ldb1−/− yolk sacs generated ~10-fold fewer total colonies than cells from control yolk sacs (Fig. 1 B and not depicted). In addition, although control yolk sac cells gave rise to both erythroid and nonerythroid colonies that were apparent by day 6 of culture, ldb1−/− yolk sac cells generated only nonerythroid colonies consisting of myeloid blasts or mature macrophages that were

Figure 1. Ldb1 is required for primitive erythropoiesis. (A) E9.0 embryos were photographed, paraffin embedded, and sectioned for H&E staining. Left, E9.0 ldb1+/+ and ldb1−/− embryos from an ldb1+/− × ldb1+/− mating showing yolk sacs and blood vessels. Bars, 500 µm. Center, H&E staining of E9.0 embryos showing yolk sac blood islands. Arrows designate hematopoietic cells in yolk sac blood islands. Bars, 50 µm. Original magnification, 400×. Right, Giemsa staining of cytopsins prepared from E9.0 yolk sacs. Bars, 50 µm. Original magnification, 400×. Data are representative of 3 ldb1−/− embryos and 11 littermate controls. Controls consisted of ldb1+/+ and ldb1−/+ embryos, which were phenotypically indistinguishable (not depicted). (B) Single cell suspensions from yolk sacs were prepared for in vitro methylcellulose culture. Erythroid (E) and nonerythroid (non-E) CFCs in E9.0 ldb1+/+ and ldb1−/- embryos were prepared for in vitro methylcellulose culture. Erythroid (E) and nonerythroid (non-E) CFCs in E9.0 ldb1+/+ and ldb1−/- yolk sacs. Colonies were counted on days 6 and 14 after the initiation of the in vitro culture. One representative of two experiments is shown. Values are expressed as means ± SD. (C) Giemsa staining of cytopsins prepared from day 14 methylcellulose cultures derived from E9.0 ldb1+/+ or ldb1−/- YS. Bars, 50 µm. Original magnification 400×. Images are representative of two experiments.
apparent only after 10–14 d of culture (Fig. 1, B and C and not depicted). Together, these results demonstrate that hematopoietic specification is impaired and primitive erythropoiesis is abrogated in the absence of ldb1.

**Ldb1 is required for definitive fetal erythropoiesis**

To examine the role of Ldb1 in definitive erythropoiesis, we used a conditional deletion allele of ldb1 (ldb1fl) generated in ES cells by homologous recombination (Suleiman et al., 2007). Although ldb1−/− mice die between E9 and E10, Ldb1fl/β mice were viable and fertile and exhibited no hematopoietic defects (unpublished data). Cre-recombinase–mediated deletion of the ldb1 allele in oocytes recapitulated the early embryonic lethality observed in ldb1−/− mice (unpublished data). Because our results indicated that Ldb1 was required for normal hematopoietic specification and primitive erythropoiesis, we generated mice in which conditional deletion of ldb1 was mediated by the tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 2 (Tie2) Cre transgene (Kisanuki et al., 2001). In a previous study, it was shown that HSCs are generated in normal numbers and primitive erythropoiesis is partially rescued in Tie2Cre sclfl/β embryos but that Tie2Cre-mediated gene deletion occurs in fetal liver hematopoietic progenitor cells that give rise to definitive erythrocytes (Schlaeger et al., 2005).

To increase the probability that ldb1 would be completely deleted after Cre expression, one Ldb1 allele was deleted in the germline to generate ldb1−/Δ mice. Tie2Cre ldb1−/Δ mice were then crossed with Ldb1fl/β mice to generate Tie2Cre ldb1fl/Δ embryos. Tie2Cre-mediated deletion of the ldb1 allele in fetal liver cells was confirmed by PCR of genomic DNA (Fig. S3 C). Tie2Cre ldb1fl/Δ embryos developed normally through E12. However, by E12.5, all Tie2Cre ldb1fl/β embryos exhibited widespread hemorrhage and edema (Fig. S3 A). Fetal liver size and cellularity were markedly decreased in E12.5 Tie2Cre ldb1fl/Δ mice (Fig. 2, B and C). Immature CD71hiTer119low–med proerythroblasts and total Ter119+ cells were also strongly reduced in Tie2Cre ldb1fl/Δ embryos (Fig. 2, B and C).

To evaluate the developmental potential of E12.5 Tie2Cre ldb1fl/Δ fetal liver cells, we next performed in vitro methylcellulose progenitor assays. After 8 d of culture, colonies were identified by visual inspection and enumerated. The number of erythroid colonies originating from Tie2Cre Ldb1fl/Δ fetal liver cells was strongly reduced relative to controls (Fig. 2, D and E). In addition, erythroid colonies from Tie2Cre ldb1fl/Δ mice were derived almost exclusively from EryP–colony forming cells (CFCs; Fig. 2 E). In contrast, most erythroid colonies from control fetal livers were derived from definitive burst forming unit–erythroid (BFU-E) progenitors (Fig. 2, D and E; and not depicted). The near absence of definitive BFU-E colonies in Tie2Cre Ldb1fl/Δ fetal liver cultures is consistent with the reduced number of fetal liver erythroid progenitors (MEPs; Fig. 2, B and C). In contrast, the number of nonerythroid CFU–macrophage (M) and CFU–GM progenitors were not significantly reduced in Tie2Cre ldb1fl/Δ fetal livers, identifying a selective requirement for ldb1 in erythroid development (Fig. 2, D and E).

**Ldb1 is continuously required for adult erythropoiesis and megakaryopoiesis**

To determine if Ldb1 is also required for adult erythropoiesis, we generated Mx1Cre ldb1fl/β mice so that ldb1 deletion could be induced by injection of polyinosinic–polycytidylic acid (pl–pl; Kühn et al., 1995). As shown in Fig. 3 A, most Mx1Cre ldb1fl/β mice, but not control ldb1fl/β mice, died within 20 d of pl–pl injection. As reported previously, injection of pl–pl causes a transient anemia (Hall et al., 2003), and this was observed in both control and Mx1Cre ldb1fl/β mice (Fig. 3 B). However, although the hematocrit normalized 14 d after pl–pl injection in control mice, the hematocrit continued to decline in Mx1Cre ldb1fl/β mice (Fig. 3 B). The total number of bone marrow cells was also dramatically decreased in Mx1Cre ldb1fl/β mice after pl–pl–mediated ldb1 deletion (unpublished data). Similar to Tie2Cre ldb1fl/Δ mice, Linhi– Sca1–c–kit–CD34+FoxcY+β MEPI populations, as well as CD71+ Ter119+ erythroblasts, were significantly reduced in pl–pl–treated Mx1Cre ldb1fl/β mice (Fig. S4 C and Fig. S4 A). BFU-E and mixed (erythroid/myeloid) colonies were virtually absent in day 8 methylcellulose cultures of bone marrow cells from pl–pl–treated Mx1Cre ldb1fl/β mice (Fig. S4, B and C). Similar to the results obtained with Tie2Cre ldb1fl/Δ fetal liver cells, granulocyte/macrophage colony formation was much less affected by inactivation of ldb1 in adult bone marrow cells (Fig. S4 C).

FACS analysis of the few CD71+ Ter119+ erythroblasts present in Mx1Cre ldb1fl/β mice after pl–pl–induced deletion of ldb1 revealed an increase in the percentage of apoptotic (annexin V+) cells as well as a reduced percentage of cycling cells, indicating a requirement for Ldb1 in cell survival (Fig. 3 C and
Figure 2. *Ldb1* is required for definitive fetal erythropoiesis. (A) Giemsa-stained peripheral blood from E12.5 Tie2Cre *ldb*+/fl and Tie2Cre *ldb*fl/embryos. Bars, 50 µm. Original magnification, 400×. One representative of three experiments is shown. (B) Number of total fetal liver cells, LSK cells, LK cells, CMPs + GMPs, MEPs, and Ter119+ cells in E12.5 *Ldb1*fl/cre and control embryos (controls consisted of littermate *ldb*+/fl and *ldb*fl/embryos). NS, not significant; **, P < 0.01. MEP, CMP, and GMP populations were designated as shown in C. Ter119+ cell counts were obtained from 39 control embryos and 9 Tie2Cre *ldb*fl/cre embryos. LSK, LK, CMP+GMP, and MEP cell counts were obtained from 32 control embryos and 12 Tie2Cre *ldb*fl/cre embryos. Values are expressed as means ± SD. (C) Lin^lo^/Sca1^lo^/c-kit^+ /FcγR^lo/^CD34^lo/^MEPs (left), total (Ter119+) erythroid cells (center), and CD71^hi^/Ter119^lo^ proerythroblasts (right) in E12.5 Tie2Cre *ldb*fl/cre and Tie2Cre *ldb*+/fl mice. Numbers are the percentage of cells in the indicated gate. (D) Numbers of erythroid (E), non-erythroid (non-E), and total colonies in day 8 methylcellulose cultures from E12.5 control and Tie2Cre *ldb*fl/cre fetal livers. NS, not significant; *, P < 0.05; **, P < 0.01. One representative of three experiments is shown. Values are expressed as means ± SD. (E) Representative primitive erythroid (EryP), definitive erythroid (BFU-E), and myeloid (CFU-GM) colonies from Tie2Cre *ldb*+/fl and Tie2Cre *ldb*fl/cre fetal livers at day 8 of culture. Bars, 200 µm. Original magnification, 50×. Images are representative of duplicate cultures from three litters.
the peripheral blood of Mx1Cre\textsuperscript{Ldb1\textsuperscript{-/-}} mice were markedly reduced relative to control \textit{ldb1\textsuperscript{fl/fl}} mice 8 d after pI:pC injection (Fig. 4 B).

To evaluate the impact of Ldb1 down-regulation on the expression of erythroid- and megakaryocytic-specific genes, Fig. S4 A). To determine if Ldb1 is also important for megakaryocyte survival, sternal sections were obtained from pI:pC-treated Mx1Cre\textsuperscript{Ldb1\textsuperscript{-/-}} and control \textit{ldb1\textsuperscript{fl/fl}} mice and stained with hematoxylin and eosin (H&E). Strikingly, as early as 8 d after pI:pC injection, no megakaryocytes were detectable in sternal sections from pI:pC-treated Mx1Cre\textsuperscript{Ldb1\textsuperscript{-/-}} mice, pointing to a critical role for Ldb1 in megakaryocyte survival (Fig. 3 D). In agreement with this finding, platelet counts in

**Ldb1 is required for the expression of erythroid, megakaryocyte, and prosurvival genes**

To evaluate the impact of Ldb1 down-regulation on the expression of erythroid- and megakaryocytic-specific genes,
knockdown of ldb1 strongly inhibited the induced expression of several erythroid genes including α- and β-globin, epb4.2, slc4a1, and alas2 (Fig. 4 A). In addition, expression of the megakaryocyte-specific transcript itga2b was also markedly decreased (Fig. 4 A). These results confirm and extend previous studies supporting a critical requirement for Ldb1 in erythroid gene expression (Xu et al., 2003; Anguita et al., 2004; Lahlil et al., 2004; Song et al., 2007). The dramatic increase in apoptotic erythroblasts after in vivo deletion of ldb1 (Fig. 3 C) also prompted us to examine the effect of ldb1 knockdown on the expression of erythroid prosurvival factors. As shown in Fig. 4 B, expression of three prosurvival genes, epor, bcl-xl, and sox6, was also significantly decreased in ldb1 shRNA MEL cells, especially at later time points in cell culture when these genes were highly induced in control cells (Fig. 4 B). These results indicate that Ldb1 protein complexes regulate the expression of prosurvival as well as erythroid- and megakaryocyte-specific genes.

In the present study, we relate data demonstrating a critical, continuous, and specific role for Ldb1 in both erythropoiesis and megakaryopoiesis. These results are consistent with the high expression of Ldb1 in erythroid progenitors (Fig. S1) and with the observation that deletion of ldb1 leads to a severe developmental block at the common megakaryocyte/erythroid progenitor stage (Fig. 2 C and Fig. 3 C). Deletion of ldb1 severely impacted the erythroid/megakaryocyte lineages but had little effect on the development of myeloid cells. Using B cell– or T cell–specific Cre transgenes, we also found no evidence of a requirement for ldb1 in lymphocyte development (unpublished data). A similar nonmandatory role for lmo2 in T cell development was also demonstrated (McCormack et al., 2003).

Our results indicate that an important function of Ldb1 during erythropoiesis is to induce the expression of lineage-specific and prosurvival genes, presumably via its documented role as a key subunit of Ldb1/Lmo2/Scl/E2A/Gata-1 multimeric complexes (Fig. 4). Ldb1 nucleated complexes have been shown to regulate the expression of several erythroid genes including α- and β-globin, epb4.2, and gypA (Xu et al., 2003; Anguita et al., 2004; Lahlil et al., 2004; Song et al., 2007). In addition, genome-wide profiling studies have detected Ldb1 at most Gata-1–bound DNA elements in erythroid cell lines, suggesting that Ldb1 complexes represent a major instrument for Gata-1–mediated gene activation (Tripic et al., 2009; Soler et al., 2010). In this paper, we provide evidence that down-regulation of Ldb1 severely impairs the induced expression of multiple erythroid- and/or megakaryocyte-specific genes in erythroleukemia cells. Interestingly, the expression of genes encoding other Ldb1 complex subunits, including lmo2, gata-1, and scl, was not as severely affected by down-regulation of ldb1, indicating that failure to assemble functional Ldb1 complexes, rather than reduced expression of Ldb1 complex subunits, is responsible for the reduction in erythroid gene expression (Fig. 4). In summary, these results establish an essential function for Ldb1 during erythrocyte and megakaryocyte development and survival.

Figure 4. Ldb1 complexes regulate expression of erythroid, megakaryocytic, and prosurvival genes. Stable clones of MEL cells expressing ldb1 shRNA or control shRNA were treated with 1.5% DMSO to induce erythroid differentiation. Total RNA was isolated at the indicated times and gene expression was quantified by real-time RT-PCR with β-actin as control. (A) Expression of genes encoding Ldb1 complex subunits and erythroid/megakaryocytic lineage proteins are shown. (B) Expression of prosurvival genes (bcl-xl, epor, and sox6) in MEL cells expressing ldb1 shRNA or control shRNA after induction with DMSO. Results shown are representative of two independent induction experiments. Values are expressed as means ± SD.
and suggest that Ldb1-nucleated complexes represent an important mechanism by which Gata-1 activates gene transcription in these lineages.

MATERIALS AND METHODS

Mice. Tie2Cre and Mx1Cre transgenic mice were purchased from The Jackson Laboratory. All mice were bred and maintained in a National Institutes of Health (NIH) Research Animal Facility in accordance with the specifications of the Association for Assessment and Accreditation of Laboratory Animal Care. Mouse protocols were approved by the NIH Animal Care and Use Committee.

Ldb1 antibody and Ldb1 intracellular staining. Polyclonal rabbit antisera were raised against Ldb1 peptide MLDRDVQPTMPYPTYLEPC-amide, affinity purified, and conjugated with Alexa Fluor 647 (A20186, Invitrogen). Purified rabbit IgG (AB-105-C; R&D Systems) conjugated in the same way served as background control. Freshly isolated single cell suspensions were surface stained, fixed with 2% formaldehyde, permeabilized with 0.5% Triton X-100 (Bio-Rad Laboratories) in PBS, stained with Alexa Fluor 647–conjugated rabbit anti-Ldb1 or rabbit IgG, and analyzed by flow cytometry.

Embryonic stem cell (ESC) lines. The generation of ldb1+/ΔESCs (Mukhopadhyay et al., 2003), ldb1+/- ESCs (Hwang et al., 2008), and ldb1+/- ESCs (Suleiman et al., 2007) has been described previously.

ESC culture and embryoid body formation. Undifferentiated ESCs were cultured in modified DME (Invitrogen) containing 15% FCS and LIF on mitomycin-treated embryonic fibroblast feeders. To generate embryoid bodies, ESC cultures were trypsinized until 5–10 cell clusters were formed. Trypsinization was stopped by addition of 10 ml IMDM medium containing 10% fetal calf serum and cells were incubated for 45 min at 37°C to allow reattachment of embryonic fibroblasts. ESCs were transferred to 10-cm bacterial Petri dishes and incubated in IMDM plus 15% FCS for 3–6 d in the presence of 40 ng/ml SCF and observed for the formation of embryoid bodies. Embryoid bodies generated in the primary culture were harvested by gravity sedimentation and plated in secondary methylcellulose cultures containing 50 ng/ml of recombinant mouse (rm) SCF, 10 ng/ml rm IL-3, and 10 ng/ml rh IL-6 or 50 ng/ml rm SCF and 3 U/ml rh Epo (STEMCELL Technologies Inc.).

CFC assays. Assays for hematopoietic progenitors in fetal liver or adult bone marrow were performed by culturing enumerated cells in methylcellulose-based IMDM medium containing 3 U/ml of recombinant human (rh) Epo, 50 ng/ml rm SCF, 10 ng/ml rm IL-3, and 10 ng/ml rh IL-6 (STEMCELL Technologies Inc.). For cultures of fetal liver cells and bone marrow cells, 2.5 × 105–105 cells were plated in duplicate and scored for colony formation at day 8. For yolk sac cultures, single cell suspensions of E9 yolk sacs were cultured in methylcellulose to analyze their colony forming potential.

Flow cytometry and cell cycle analysis. Conjugated antibodies, including mouse and hamster IgG isotype controls, were purchased from BD or e Bioscience. The lineage marker (Lin) mixture for fetal liver cells included the following biotinylated antibodies: CD3− (145-2C11), CD4 (GK1.5), CD8a (53–6), CD8β (S3–5.8), TCR-β (H57–597), TCR-γδ (GL3), CD19 (1D3), B220 (RA3-6B2), Gr1 (RB6-8C5), Ter119, CD49d (DX5), and NK1.1 (PK136). Lin mixture for adult bone marrow cells included all of these plus Mac-1 (M1/70). Other conjugated antibodies used for surface staining included: CD34 (RAM34), CD43 (S7), CD71 (C2), c-kit (2B8), Sca1 (D7), and FcγIII/IR (2.4G2). Biotinylated primary antibodies were detected by incubation of antibody-coated cells with streptavidin-PerCP-Cy5.5 or APC-Cy7 in a two step staining procedure. Percentage of apoptotic cells was determined by annexin V (BD) staining according to the manufacturer’s instructions. For cell cycle analysis, cells were surface stained, fixed in 2% formaldehyde, permeabilized in 0.1% NP-40, and then stained with DAPI (Invitrogen). Data were acquired with a FACSCalibur or LSR II flow cytometer and analyzed with FlowJo software (Tree Star, Inc.).

RT-PCR and quantitative RT-PCR. The MEL cell line expressing ldb1 shRNA has been described previously (Song et al., 2007). For gene expression studies, total cell RNA was isolated using PicoPure RNA isolation kit (Arcturus), and 100 ng of each RNA sample was reverse transcribed using SuperScript first-strand synthesis system (Invitrogen) and assayed by RT-PCR. Transcript quantification was performed with a LightCycler 480 (Roche). Duplicates were run for each sample in a 96-well plate. β-Actin was used as the endogenous reference gene. All quantitative RT-PCR reactions were run in three independent experiments. The relative quantification method was used, with the ratio of the mRNAs level for the gene of interest normalized to the level of β-actin and the mean of control bone marrow samples as the calibrator value. The specificity of the products was confirmed based on melting curves and electrophoresis.

Genotyping and deletion analysis of Tie2Cre ldb1+/- mice and Mx1Cre ldb1Δ/Δ mice. PCR genotyping of Tie2Cre ldb1+/- mice and Mx1Cre ldb1Δ/Δ mice was performed on tail DNA. Primers used for detection of the Cre transgene were 5′-CGATGCAACGAGTATGAGG-3′ and 5′-GACCTTGCTGTCACCTGGCTG-3′. For Tie2Cre ldb1Δ/Δ mice, a combination of the following three oligonucleotide primers were used to detect the wild-type ldb1, the ldb1Δ, and the Cre-mediated deleted (ldb1Δ) alleles: Ldb1S 5′-CAGCAACGGAGAAGAAGATGATCAG-3′, Ldb1AS 5′-CTTATTGTGACACAGCAGCTGATGATG-3′, and Ldb1A 5′-TCAAGGCC-TGGCCCTTTAACCACCA-3′. Ldb1S-Ldb1AS amplified a 320-bp wild-type allele fragment and 445-bps flanked allele fragment. Ldb1A-Ldb1AS amplified a 390-bps ldb1 Cre-mediated recombination allele fragment. For Mx1Cre ldb1Δ/Δ mice, a combination of the following three oligonucleotide primers were used to detect the wild-type ldb1, the ldb1Δ, and the Cre-mediated deleted (ldb1Δ) alleles: Ldb1A 5′-TCAGGGCTGCCCCTAAAACCTAA-3′, Ldb1B 5′-TGGGACCTACAAGGCTGAGACA-3′, and Ldb1C 5′-TGGGCTGACCTATGTCAGCAA-3′. Ldb1B-Ldb1C amplified a 488-bp wild-type allele fragment and a 534-bp flanked allele fragment. Ldb1A-Ldb1C amplified a 342-bp ldb1 deleted allele fragment.

Conditional inactivation of Ldb1 in adult mice. To inactivate ldb1 in adult hematopoietic cells, 250 μl pLpc (1 mg/ml; GE Healthcare) was injected intraperitoneally into each mouse on days 1, 3, and 5 (the day of the first injection was designated day 1). Mice were sacrificed on day 8 unless otherwise noted. Cells obtained from bone marrow or spleen were either analyzed directly or cultured in methylcellulose to analyze their colony forming potential.

Online supplemental material. Fig. S1 shows ldb1 expression in hematopoietic cells. Fig. S2 shows impaired erythroid developmental potential of ldb1+/- ESCs. Fig. S3 shows defective erythropoiesis in Tie2Cre ldb1−/− embryos. Fig. S4 shows that Ldb1 is continuously required for survival and expansion of erythroblasts in adults. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100504/DC1.

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