Live cell surface-staining (as described in [1])

Cells from freshly isolated tissues were gently strained through 40-μm strainer in the presence of cold phosphate-buffered saline and 5% fetal calf serum (PBS/5% FCS) or 0.2% bovine-serum albumin (BSA). Cells were immunostained for 20–45 minutes on ice in the presence of blocking rabbit IgG (Jackson ImmunoResearch, West Grove, PA), 1 μg/mL fluorochrome-conjugated anti-Ter119 (BD Biosciences, San Diego, CA), and 1 μg/mL fluorochrome-conjugated anti-CD71 (BD Biosciences). 7-AAD (BD Biosciences) or DAPI (Roche, Indianapolis, IN) was used to exclude dead cells. Staining for Fas or FasL was for 1 hour on ice with 5 μg/mL biotin-conjugated anti-Fas (Jo2 clone, BD Biosciences) or 5 μg/mL biotin-conjugated anti-FasL (MFL3 clone, BD Biosciences) and APC-conjugated streptavidin (Invitrogen). Annexin V staining was carried out according to the manufacturer's instructions (BD Biosciences).

Intracellular protein staining

To detect intracellular Bcl-x_L and Bim, cells were first stained with LIVE/DEAD fixable viability stain (Invitrogen), followed by surface-labeling for Ter119 and CD71 in the presence of blocking IgG. Next, cells were fixed with PBS solution containing 3% paraformaldehyde and 2% sucrose, and permeabilized with BD Cytofix/Cytoperm™ Perm/Wash reagents, and stained in the Perm/Wash solution with anti-Ter119 (to maintain Ter119 signal in fixed cells) and with anti–Bcl-x_L antiserum (BD Biosciences 556361) or anti-Bim antibody (Cell Signaling, #2819), or isotype control. Isotype control for Bcl-x_L was Normal Rabbit Serum (Jackson Immunoresearch). Isotype control for Bim was Rabbit IgG (Jackson Immunoresearch). Primary Bcl-x_L or Bim staining was detected using secondary antibody (anti–Rabbit-APC, Invitrogen A-10931). Biological sample used for determining the isotype control's background fluorescence consisted of a cell mix pooled from all mouse samples in a given experiment (isotype signal was similar across individual biological samples regardless of genotype or treatment).

Phospho-Stat5 staining

Cells were first stained with LIVE/DEAD viability dye, then resuspended in phosphowash (PBS, 1mM sodium orthovanadate, 1mM β-glycerol phosphate, 1μg/mL microcystin), fixed in 1.6% paraformaldehyde, permeabilized in 80% acetone and stored at −80°C. Thawed cells were stained in PBS/3% milk with AF647-conjugated anti–phospho-Stat5 antibody (BD Biosciences, 612599) and for Ter119 and CD71 as described previously. For all flow cytometry experiments, cells were analyzed on LSR II flow cytometer (BD Biosciences). Cell sorting was done on a DakoCytomation MoFlo (Fort Collins, CO).

FACS data analysis

Data were analyzed with FlowJo software (Tree Star, Ashland, OR) as described.[1–3] Singlets and live cells (based on DAPI, 7-AAD, or LIVE/DEAD stains) were selected, and subsets were gated based on Ter119, CD71 expression and forward scatter (FSC). Gating strategy for fetal liver and adult erythroblasts was performed as described.[1] For Fas surface stain, Fas-positive gate was drawn based on secondary-only control, as described.[4] For Annexin V, positive gate was drawn based on the sample where Annexin V stain was omitted, as described.[4] For each subset in each biological sample, non-specific isotype control's mean fluorescent intensity (MFI) signal was subtracted from the MFI of Bcl-x_L or Bim prior to plotting the data.

Quantitative real-time PCR
Total RNA was prepared from freshly sorted erythroblasts using the AllPrep DNA/RNA Micro Kit (Qiagen) with on column DNase treatment. Reverse-transcription was conducted using Superscript II (Invitrogen) with random hexamer primers. The ABI 7300 sequence detection system, TaqMan reagents and TagMan MGB probes (Applied Biosystems) were used and several dilutions of each template were used to ensure detection in the linear range of the assay. A 'no template' and 'no reverse-transcriptase' controls were included. The threshold cycle (Ct) for housekeeping genes GAPDH or β-actin were subtracted from the Ct for genes of interest to yield a relative expression value. The relative expression for each gene of interest in each sample was then normalized to expression in the S0 subset. QRT-PCR probes used: β-actin (Mm02619580_g1). Bcl-x_L primers were CTGGGACACTTTTGTGGATCTCT and GAAGCGCTCCTGGCCTTT. Bim_{EL} primers were TCTTTTGACACAGACAGGAGC and AATCATTTGCAAACACCCTCC. Bim_L primers detected both Bim_{EL} and Bim_L isoforms, and were CTCAGTGCAATGGCTTCCATA and AATCATTTGCAAACACCCTCC. In Fig. 3D, we used an additional forward primer to specifically measure Bim_L: ACAGAACCGCAAGACAGGAGCCCG.

CFU-e colony assay
Freshly isolated fetal liver cells were plated in M3231 Methocult® methylcellulose (StemCell Technologies, Vancouver, Canada) with the indicated dose of rh-Epo (Amgen). On day 3, plates were stained with 3,3'-Diaminobenzidine (Sigma-Aldrich) and scored for erythroid colonies.

Enzyme-Linked Immunosorbent Assay (ELISA)
ELISA for endogenous mouse Epo or injected recombinant human Epo were performed according to the manufacturer's instructions (Quantikine ELISA, R&D Systems, Minneapolis, MN). EnVision 2102 Multilabel Reader (Perkin Elmer, Waltham, MA) was used to quantify fluorescence. Data were converted into mU/ml by multiplying pg/ml value by 129,000 IU/mg (International Standard for fully glycosylated Epo protein).[5]

Data analysis
Data analysis, including all statistical tests, was performed using Microsoft Excel Software (Redmond, WA), or GraphPad Prism Software (La Jolla, CA) where indicated.

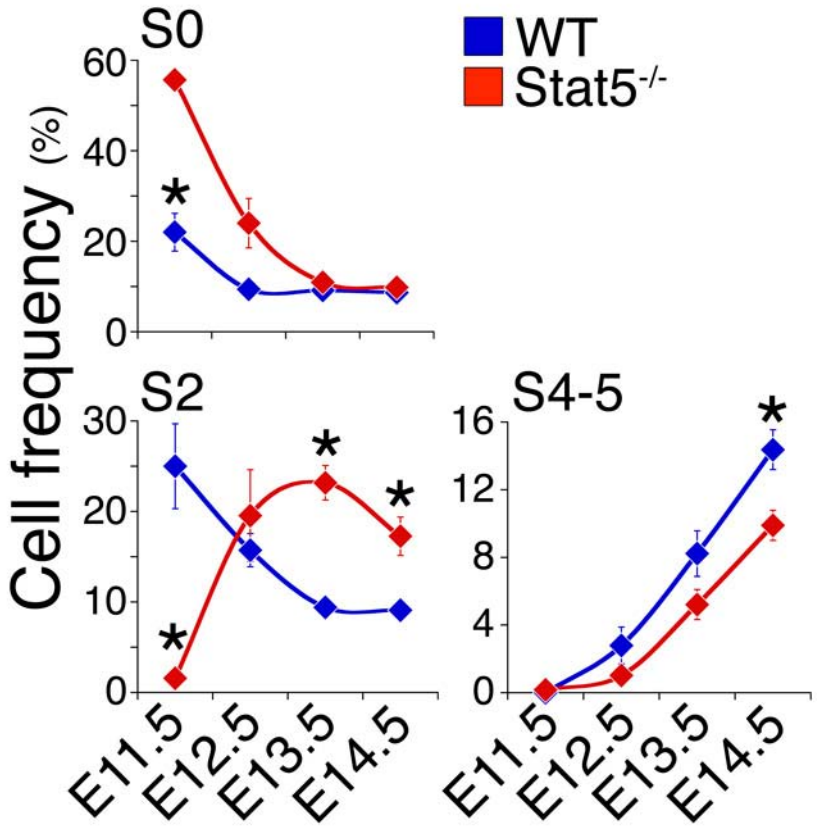

Figure S1. Delayed erythroid maturation in the Stat5$^{-/-}$ fetal liver
Summary statistics for the analysis shown in Fig. 1A,B for fetal liver subsets S0, S2 and S4/5. Stars indicate statistically significant differences between wild-type and Stat5$^{-/-}$. The S0 subsets differ significantly on E11.5 ($p=0.006$); the S2 subsets differ significantly on E11.5 ($p=0.037$), E13.5 ($p=0.0002$) and E14.5 ($p=0.015$); and S4/5 subsets differ significantly on E14.5 ($p=0.015$, two-tailed t-test, unequal variance).

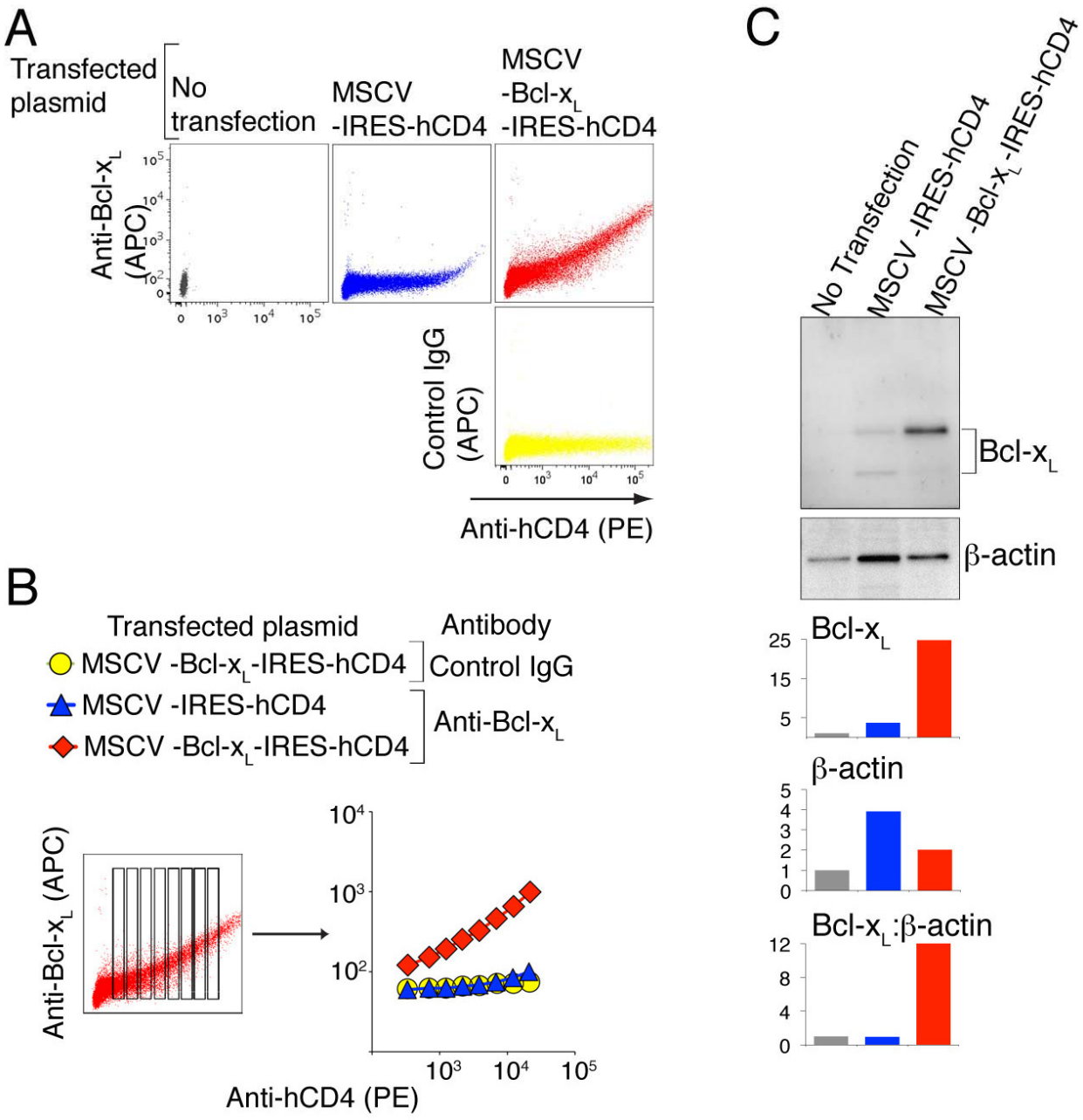

Figure S2. Validation of Bcl-x_L measurement by flow cytometry
(A–B) The anti–Bcl-x_L antiserum (BD Biosciences 556361) accurately measures Bcl-x_L protein levels over a wide expression range. 293T-derived phoenix cells were transfected with bicistronic retroviral vectors, either 'empty' vector expressing hCD4 only (MSCV-IRES-hCD4), or both Bcl-x_L and hCD4 connected via an internal-ribosomal entry site (MSCV-Bcl-x_L-IRES-hCD4). Cells were simultaneously stained with a PE-conjugated anti-hCD4 antibody (indicating hCD4 expression) and with the rabbit anti–Bcl-x_L antiserum, followed by an APC-conjugated

anti-rabbit IgG antibody (indicating Bcl-x_L expression). It was previously shown that expression of two genes encoded by the same bicistronic transcript is highly linearly correlated. [6] Therefore, expression of hCD4, detected with PE-conjugated anti-hCD4 antibodies, is a reliable indicator of the expression of Bcl-x_L in cells transduced with a bicistronic vector encoding both. Here we show that hCD4 expression (PE fluorescence), which varied by >100 fold in the transduced cells, was strongly correlated with expression of the Bcl-x_L protein as measured by the Bcl-x_L antiserum. No Bcl-x_L signal was seen in control cells that were either not transduced (grey), or transduced with 'empty' vector expressing hCD4 only (blue). Further, control cells transduced with the bicistronic vector expressing both Bcl-x_L and hCD4, but stained with non-immune rabbit serum in place of the anti–Bcl-x_L antiserum, were negative for the APC-Bcl-x_L signal (yellow). (A) shows the raw flow-cytometric data. (B) shows quantitation of the flow-cytometric data in (A). Briefly, hCD4 (PE fluorescence) and Bcl-x_L (APC fluorescence) were measured in narrow vertical gates (see left panel for illustration). The two were found to be highly correlated (Right panel, data points are median fluorescence; correlation between PE and APC fluorescence in MSCV-Bcl-xL-IRES-hCD4 cells was R^2=0.991). (C) Western blot analysis of the phoenix cells described in the experiment in (A–B), with quantitation of the signal in each lane shown immediately below. The membrane was sequentially blotted with the same anti–Bcl-x_L antiserum used for flow-cytometric measurements in (A), and with an anti–β-actin antibody. The blot was viewed and quantitated with the Bio-Rad Molecular Imager ChemiDoc XRS+ with Image Lab Software.

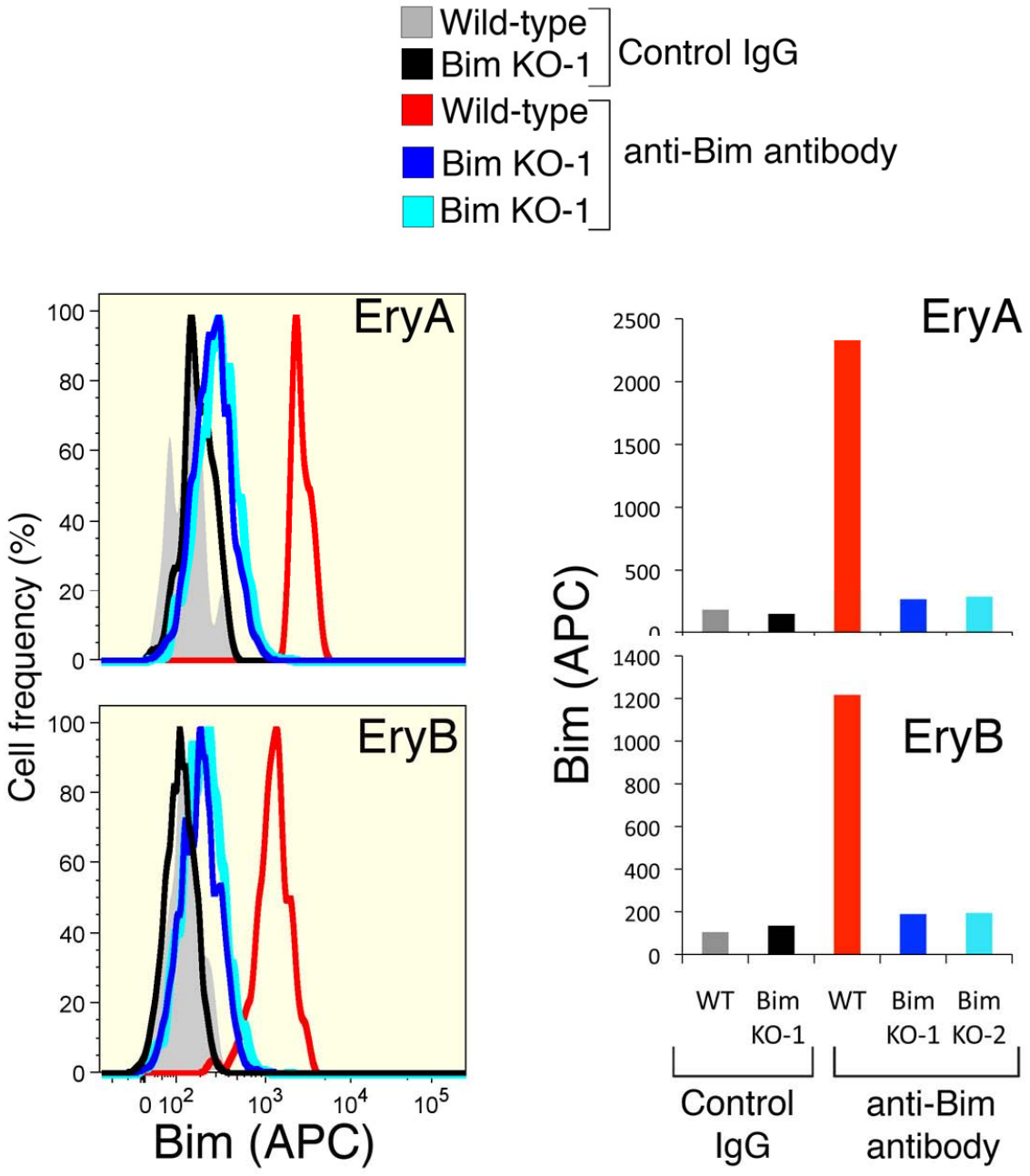

Figure S3. Validation of Bim measurement by flow cytometry.
Freshly harvested spleen from either a wild-type (red) or two $Bim^{-/-}$ mice (='Bim KO', two shades of blue) were stained with Ter119 and CD71, fixed, permeabilized and stained intracellularly with anti-Bim antibody (Cell Signaling, #2819). Alternatively they were stained intracellularly with control, polyclonal purified rabbit IgG (grey or black for wild-type and $Bim^{-/-}$ cells, respectively). All cells were stained with a secondary, APC-conjugated anti-Rabbit IgG. Flow-cytometry histograms for EryA and EryB are shown on the left, and the median

fluorescence of each histogram is shown in the right panels. Median fluorescence is similar for wild-type cells stained with control IgG and and $Bim^{-/-}$ cells stained with either control IgG or the anti-Bim antibody. Only wild-type cells stained with the anti-Bim antibody show increased fluorescence, of 10 to 20 fold that of the various control samples.

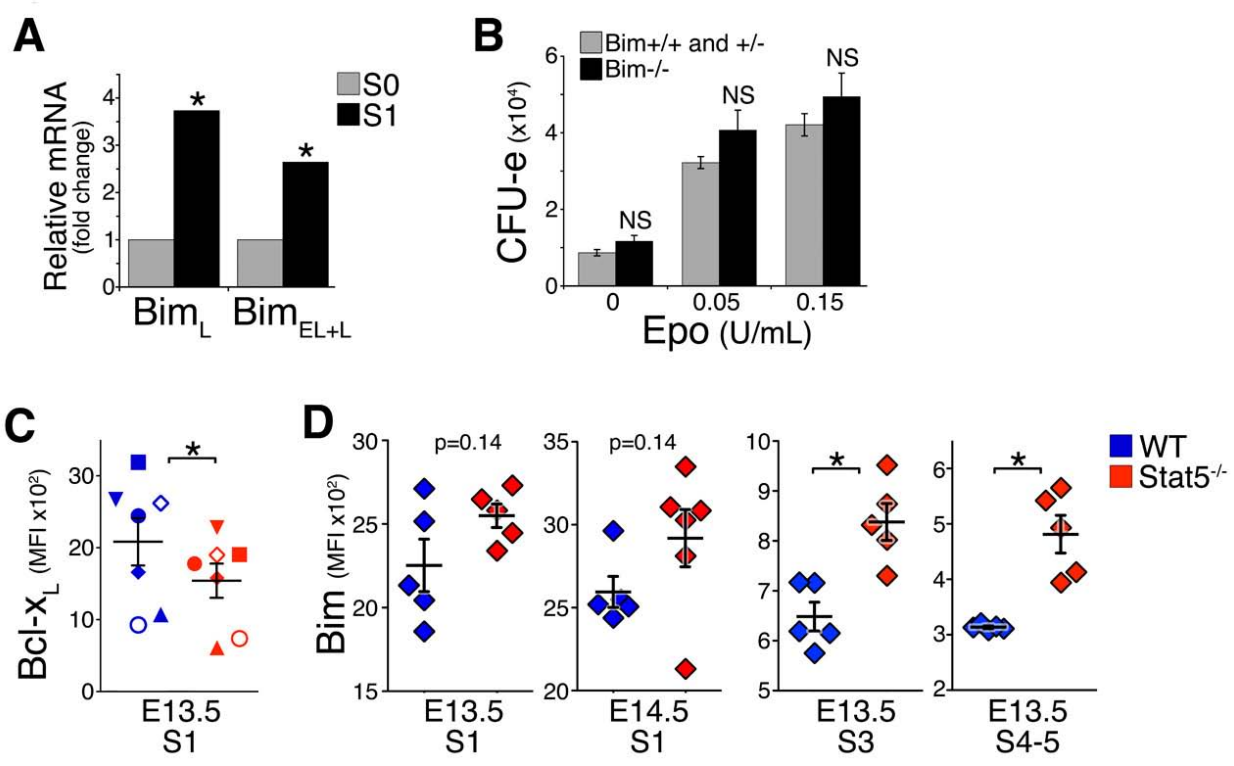

Figure S4. Bim and Bcl-x$_L$ in wild-type and Stat5$^{-/-}$ fetal liver

(A) Quantitative real-time PCR for the Bim isoforms Bim$_{EL}$ and Bim$_L$ in sorted S0 and S1 subsets from freshly harvested wild-type fetal liver. Data were expressed as a ratio to β-actin mRNA in each sample and normalized to the levels measured in S0 cells. Differences between S0 and S1 are statistically significant, Bim$_{EL+L}$, *p=0.0003; Bim$_L$, *p=0.0000004 (2-tailed t-test, unequal variance). (B) CFU-e assay performed on wild-type and Bim$^{-/-}$ fetal livers. CFU-e colonies per whole fetal liver are shown for each genotype. Data are mean ±SEM of 6 individual fetal livers per genotype from the same E13.5 litter. Grey bars are a pool containing one Bim wild-type embryo and five Bim$^{+/-}$ embryos. No significant (NS) difference in CFU-e numbers between the genotypes was observed (p>0.05, two-tailed t-test, unequal variance). Similar results were obtained when data were expressed as CFU-e colonies per 1 × 10^6 plated cells. Representative of two similar experiments. (C) Lower Bcl-x$_L$ and higher Bim protein expression in Stat5$^{-/-}$ embryos compared with wild-type littermate controls, at the indicated embryonic day and differentiation subset. n=11 to 21 embryos per genotype, with each symbol type representing median expression for one litter. Means ±SEM for the population are indicated. Statistical significance values: E13.5 S1, *p=0.012 (paired t-test). See also Fig 1E. (D) Higher Bim protein expression in Stat5$^{-/-}$ embryos compared with wild-type littermate controls: data points are individual embryos. Mean ±SEM for the population is shown. Statistical significance values: E13.5 S3 and S4–5, *p<0.01, two-tailed t-test, unequal variance. See also Fig 1E.

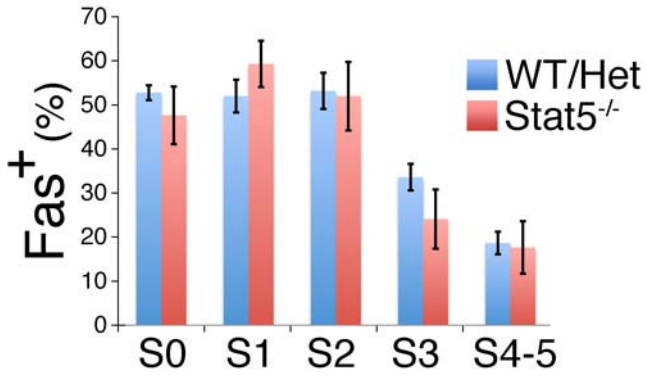

Figure S5. Normal expression and downregulation of Fas in Stat5$^{-/-}$ fetal liver
Cell surface Fas was measured on freshly explanted fetal liver from wild-type or Stat5$^{+/-}$ heterozygous embryos (n=6) and from Stat5$^{-/-}$ embryos (n=3). No significant differences were detected between the genotypes.

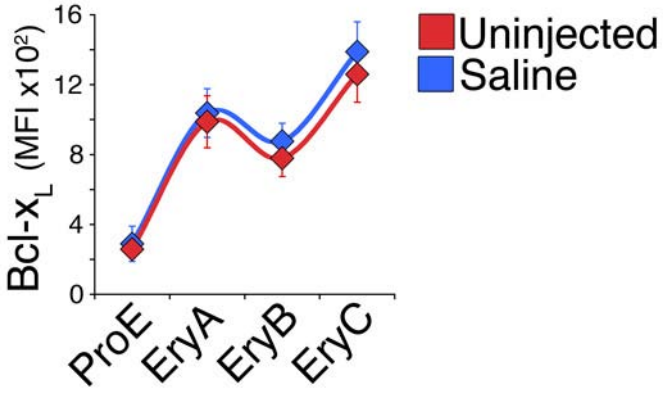

Figure S6. Saline injection has no effect on the expression of Bcl-x_L in erythroblasts
Mice were either left uninjected, or were injected with subcutaneous saline (a total volume of 150 µl). Spleen cells were harvested and stained for Bcl-x_L, Ter119 and CD71. No significant difference was found between uninjected mice and saline-injected mice in Bcl-x_L expression in any of the erythroblast subsets. Saline injection was used as a control for Epo injection in most of the experiments in the main Figures. Data are mean± sem of five saline-injected mice and 3 uninjected mice.

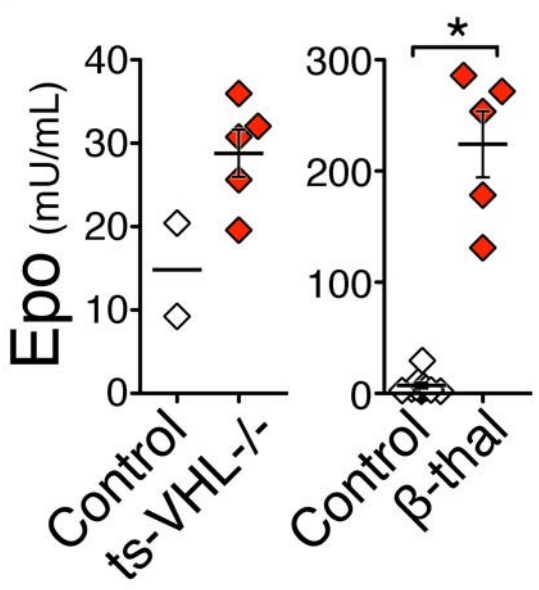

Figure S7. Plasma Epo measurments in mice with β-thalassemia and in ts-VHL$^{-/-}$ mice
For each mouse model, two independent ELISAs, each with similar results, were pooled together. Individual mouse data as well as mean ±SEM are shown. Epo increase in β-thalassemia was significant at p=0.002 (two-tailed t-test, unequal variance).

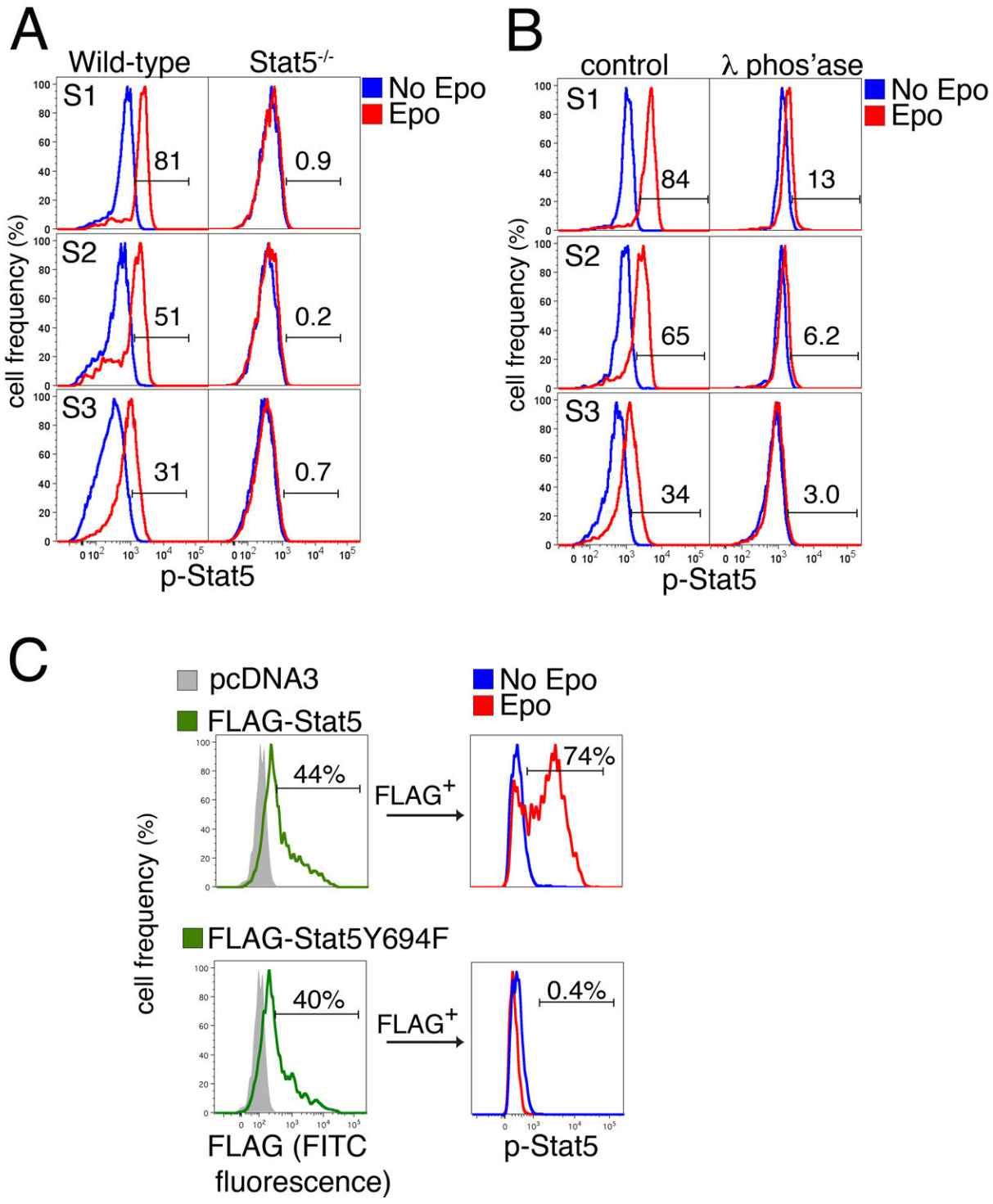

Figure S8. Validation of phosphorylated Stat5 measurement by flow cytometry
(A) No phospho-Stat5 signal in Stat5$^{-/-}$ fetal liver. Freshly–harvested fetal liver cells from either wild-type or Stat5$^{-/-}$ littermate embryos were stimulated *in vitro* with 2U/ml Epo for 15 minutes.

The cells were immediately stained for cell-surface CD71 and Ter119, and for intracellular phospho-Stat5 with an anti–phospho-Stat5 antibody (BD Biosciences, 612599). There was increased fluorescence in Epo-stimulated cells in wild-type fetal liver S1 to S3 subsets (compare red histograms corresponding to Epo-stimulated cells with blue histograms corresponding to non-stimulated cells), but no change in fluorescence with Epo stimulation of $Stat5^{-/-}$ fetal liver cells. (B) Loss of the phospho-Stat5 signal following treatment with λ phosphatase. Freshly isolated wild-type fetal liver cells were stimulated with Epo as described in (A). Following fixation and permeabilization, the cells were treated for 15 minutes with λ phosphatase, prior to staining for intracellular phospho-Stat5. The phosphatase treatment caused a loss in the phospho-Stat5 signal compared with control cells that were not treated with phosphatase following Epo stimulation. (C) Freshly harvested fetal liver cells were electroporated with either 'empty' vector (pcDNA3) or with vectors encoding either FLAG-tagged Stat5 (C-terminal tag, kind gift of Dr. Aki Yoshimura), or with FLAG-tagged Stat5Y694F, in which the C-terminal tyrosine residue that becomes phosphorylated upon Epo stimulation is mutated. Cells were incubated overnight in Epo (0.2U/ml) to allow expression of the FLAG-Stat5 constructs. They were then starved of Epo for 3 hours, stimulated with 2U/ml Epo for 15 minutes and stained for both intracellular phospho-Stat5 and intracellular FLAG. Both FLAG-Stat5 and FLAG-Stat5Y694F were well expressed (left panels, see increased FLAG fluorescence compared with cells transfected with 'empty' vector). However, only cells expressing FLAG-Stat5, and not cells expressing the point mutant FLAG-Stat5Y694, showed Epo-dependent phospho-Stat5 fluorescence (right panels).

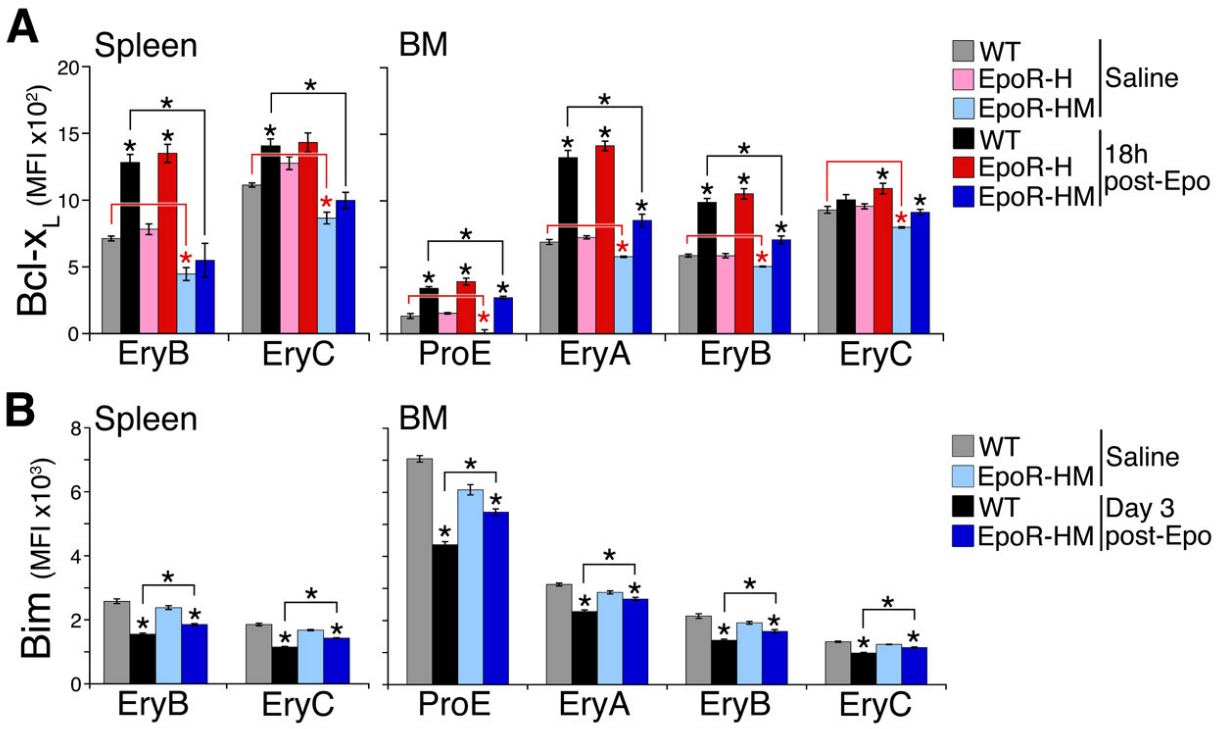

Figure S9. The Bcl-x_L and Bim Epo responses in the EpoR-H and EpoR-HM mice
Experiment and same mouse dataset as in Fig. 6C, 6E. (A) Bcl-x_L expression in spleen (left) and bone-marrow (right) in erythroid subsets, 18 hours post-Epo or saline injection in wild-type, EpoR-H, and EpoR-HM mice. Experiment and same mouse dataset as in Fig. 6C. *Left panel*: Black stars without brackets indicate significant Bcl-x_L induction in spleen EryB and EryC subsets in Epo treated mice (*p<0.005). No significant Bcl-x_L induction was observed in EpoR-HM spleens (light blue vs. dark blue bars). EpoR-HM mice failed to increase Bcl-x_L to the level of EpoR-H or wild-type mice (black stars with brackets, *p<0.02). Basal Bcl-x_L levels in EpoR-HM EryB and EryC were also lower than in basal wild-type mice (red stars with red brackets, *p<0.05). *Right panel*: Bcl-x_L induction in the bone-marrow erythroid subsets was observed for all three genotypes. Stars indicate significant differences. Wild-type Epo response vs. control: ProE, EryA and EryB, *p<0.0002. EpoR-H Epo response vs. control: ProE, EryA and EryB, *p<0.0001; EryC, *p=0.025. EpoR-HM Epo response vs. control: ProE, *p=0.003; EryA, EryB and EryC, *p<0.05. Magnitude of Bcl-x_L induction in EpoR-HM subsets was lower compared with wild-type induction (black stars with brackets, *p<0.006). Basal Bcl-x_L levels in EpoR-HM bone-marrow subsets were lower than in basal wild-type mice (red stars with red brackets, *p<0.02). (B) Bim expression in spleen (left) and bone-marrow (right) in erythroid subsets, 3 days following Epo or saline injection in wild-type, EpoR-H, and EpoR-HM mice. Experiment and same mouse dataset as in Fig. 6E. Data are mean ±SEM of n=4 to 5 mice per bar. In both wild-type and EpoR-HM spleen EryB and EryC subsets, Bim was significantly suppressed below their respective basal levels with Epo (black stars, no brackets, *p<0.001). Similar findings were observed in the bone-marrow subsets (wild-type subsets: *p<0.0005; EpoR-HM subsets:

*p<0.03). Magnitude of Bim suppression in EpoR-HM mice was lower than in the wild-type mice (black stars with brackets, *p<0.005). Some differences in basal Bim levels were observed between wild-type and EpoR-HM mice (gray bars vs. light blue bars, *not* indicated in plots: spleen EryB, p=NS; spleen EryC, *p=0.02; BM ProE, *p=0.004; BM EryA, *p=0.01; BM EryB, p=NS; BM EryC, *p=0.03).

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