

SWAP-70 regulates erythropoiesis by controlling α_4 integrin

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Supplementary Design and Methods

FACS analysis

Briefly, freshly isolated bone marrow cells or splenocytes were immunostained on ice in phosphate-buffered saline, 2 mM EDTA in the presence of either mouse IgG whole molecule (1 μ g/mL, Jackson ImmunoResearch) or 2% v/v fetal calf serum to block non-specific binding through Fc receptors. Cells were incubated with different combinations of fluorescently labeled, purified or biotin-conjugated antibodies for 25 min, followed by 20 min of incubation with second-step reagents (secondary fluorescently labeled antibodies or conjugated streptavidin) when necessary. Antibodies were purchased from BD Bioscience [CD19 (clone 1D3) bio, CD11b (clone M1/70) bio, B220 (clone RA3-6B2) bio, c-kit (clone 2B8) PE-Cy7 and APC, Sca1 (clone D7) PE-Cy7 and FITC, IL-3R α (clone 5B11) PE, CD49d (clone 9C10 (MFR4.B) PE, CD41 (clone MWR30) FITC)], eBioscience [Ter119 (clone Ter-119) PE-Cy7 and bio, CD3e (clone 145-2C11) bio, CD71 (clone R17217) PE and FITC, CD16/32 (clone 93) PE-Cy7 and Pacific Blue, CD34 (clone RAM34) FITC, IL-7R α (clone A7R34) bio, Gr-1 (clone RB6-8C5) bio], Molecular Probes (annexin V bio), Clontech (F4/80 PE), and AbD Serotec [CD71 (clone MCA1033) Alexa Fluor 647]. AnnexinV staining was performed in HEPES buffer (10 mM HEPES; Sigma) pH 7.4, 150 mM NaCl (Merk), 5 mM KCl (Carl Roth), 1 mM MgCl₂ (Sigma), and 2 mM CaCl₂ (Sigma). Intracellular staining was carried out after staining surface markers. Cells were fixed and permeabilized using a Cytotfix/Cytoperm buffer (BD Bioscience) or methanol (Merk) depending on the epitopes analyzed. Then samples were incubated with primary and subsequently with secondary antibodies at optimal concentrations that were experimentally standardized. Flow cytometry was carried out on a BD LSR II flow cytometer with FACSDiva software (BD Biosciences). Data were analyzed using the FlowJo 6.1.1 software (Tristar).

Immunofluorescence

Single cells were placed on poly-L-lysine coated slides. Samples were fixed in 3.7% paraformaldehyde in phosphate-buffered saline (15 min, room temperature), blocked with 5% v/v fetal calf serum or 2% v/v goat serum in phosphate-buffered saline and incubated with the corresponding antibodies for 30 min. For intracellular staining permeabilization was done with saponin-containing buffer (BD Bioscience). DNA was stained with DAPI (1 μ g/mL, Sigma). Immunofluorescence signals were visualized using a Leica TCS SP5 or a Zeiss LSM 510 confocal microscope.

Colony-forming assay

Nucleated bone marrow cells (1×10^5) and splenocytes (5×10^5) were placed in triplicate on methylcellulose-containing medium (Methocult) supplemented with erythropoietin (1 U/mL) only or erythropoietin (1 U/mL), stem cell factor (50 ng/mL) (R&D Systems) and interleukin-3 (10 ng/mL) (R&D Systems) to detect CFU-E or BFU-E respectively. Characteristic colonies were scored either with or without staining with 0.4% benzidine hydrochloride solution in 12% glacial acetic acid and 0.3% H₂O₂, after 2 days of incubation (for CFU-E) or 7 days of incubation (for BFU-E) at 37° C.

Colony-forming unit-erythroid adhesion assay

Briefly, 24-well fibronectin-coated plates were blocked with 2% fibronectin-free bovine serum albumin for 15 min. Bone marrow cells (5×10^4) were allowed to adhere for 2 h at 37° C. Non-attached cells were collected. Wells were washed three times with warm IMDM. Washes were pooled together with non-attached cells. The CFU-E colony-forming assay was performed on adherent cells and on the non-adherent fraction. Cell adhesion was calculated dividing the number of colonies formed by adherent cells over the total number of colonies recovered from each well. Background adhesion was determined on wells coated with 2% of fibronectin-free bovine serum albumin.

Erythroblastic island reconstitution assay

Freshly isolated bone marrow cells (5×10^5) cells were incubated for 15 min on ice in activation buffer (IMDM, 20% v/v fetal calf serum, 3.5% sodium citrate, 2 mM MnCl₂, 2 mM EGTA). Cells were transferred into eight-well chambered cover glasses (Nunc) containing 400 μ L IMDM, 20% v/v fetal calf serum and allowed to settle for 15 min. Live cells labeled with corresponding antibodies were analyzed with a Leica TCS SP5 confocal microscope.

In vivo cell proliferation assay

In vivo cell proliferation was measured using a bromodeoxyuridine (BrdU) flow kit (BD Bioscience). Mice were injected intraperitoneally with a single dose of BrdU solution provided with the kit. After predetermined times, mice were sacrificed and FACS analysis of staining was carried out as stated in the kit's protocol.

Cell adhesion to tissue

MACS-purified Ter119⁺ splenic cells were labeled with green cell tracker CFSE (Invitrogen) according to the manufacturer's

instructions. Briefly, cells were incubated in 5 μM dye solution for 30 min at 37°C followed by several washes with warm IMDM medium (Invitrogen) and left for another 30 min in medium at 37°C. Labeled cells (5×10^4) were let to adhere to frozen wild-type spleen sections in IMDM with 2 mM Mn^{++} for 30 min at room temperature. Non-attached cells were removed by gentle washing with IMDM. Coverslips with spleen sections and adhered cells were mounted on cover glasses and analyzed by confocal microscopy.

Adoptive cell transfer

The adoptive cell transfer was performed as previously described with some modifications. Wild-type recipients were lethally irradiated (8 Gy/424 sec, single dose, Röntgen beam type Yxlon Y.TU 320-D03, 200 kV) and given an intravenous injection of total bone marrow (2×10^5) either from wild-type or *Swap-70*^{-/-} donors. Blood was analyzed at predetermined time points.

Online Supplementary Table S1. Hematologic parameters of *Swap-70*^{-/-} mice.

	Wild type	<i>Swap-70</i> ^{-/-}
Red blood cells $\times 10^{12}/\text{L}$	10.27 ± 0.65	11.31 ± 0.85
Hematocrit, %	55.11 ± 2.29	55.39 ± 4.13
Mean corpuscular volume, fL	53.6 ± 1.21	50.18 ± 1.94
Mean corpuscular hemoglobin, pg	14.79 ± 0.65	14.02 ± 0.77
Hemoglobin, g/dL	14.88 ± 0.99	15.78 ± 1.69
Red cell distribution width, %	15.42 ± 0.88	18 ± 2.43

Blood was collected from wt ($n=18$) and *Swap-70*^{-/-} ($n=18$) 6-week-old mice. Hematologic measurements were performed on a Beckman Coulter Hematology Analyzer. The data represent mean \pm SD values.

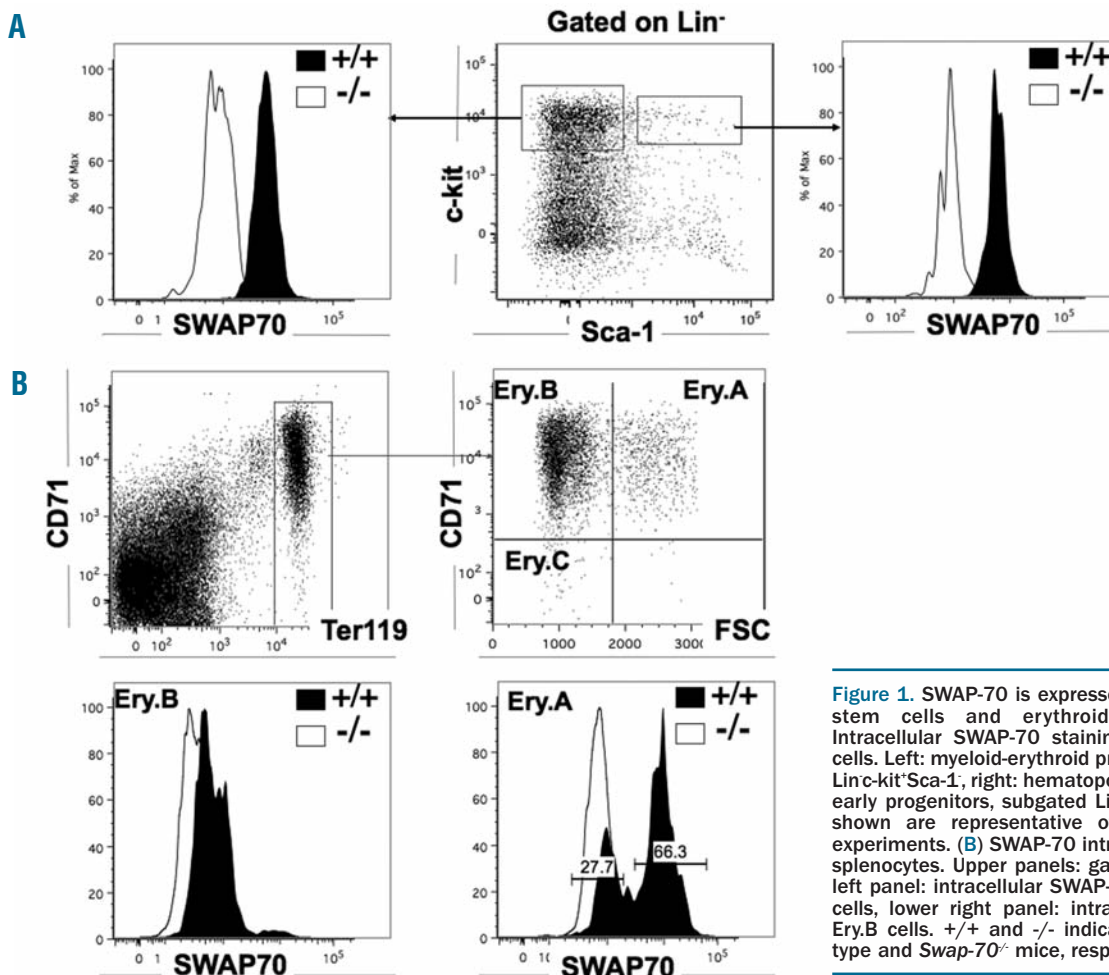
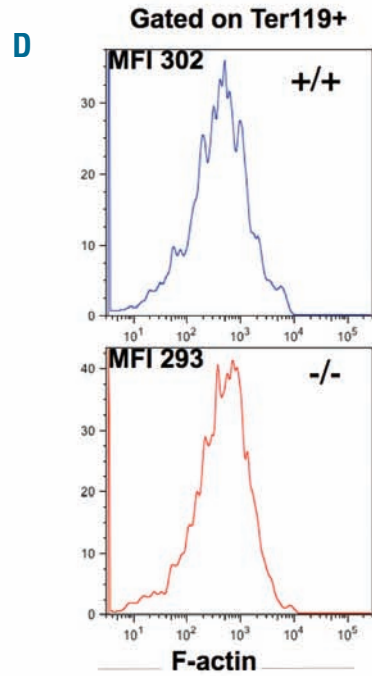
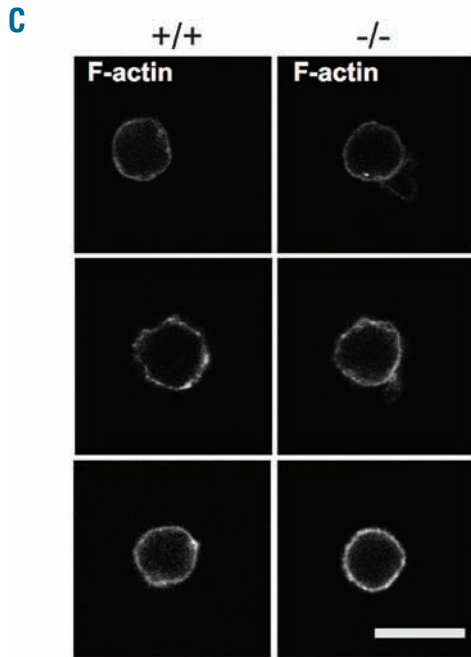
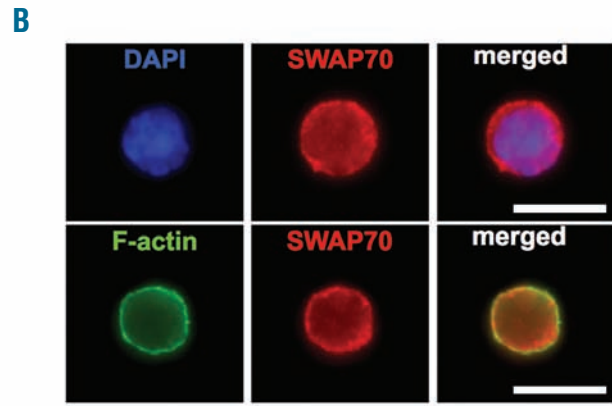
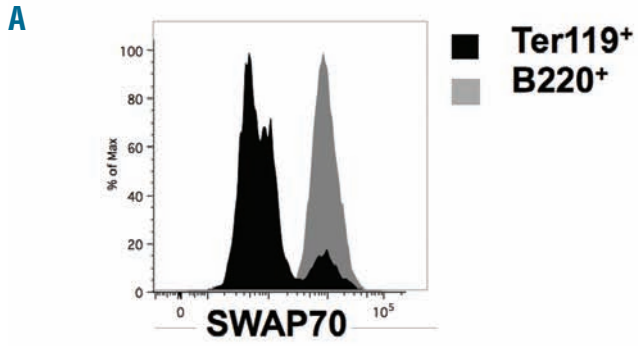
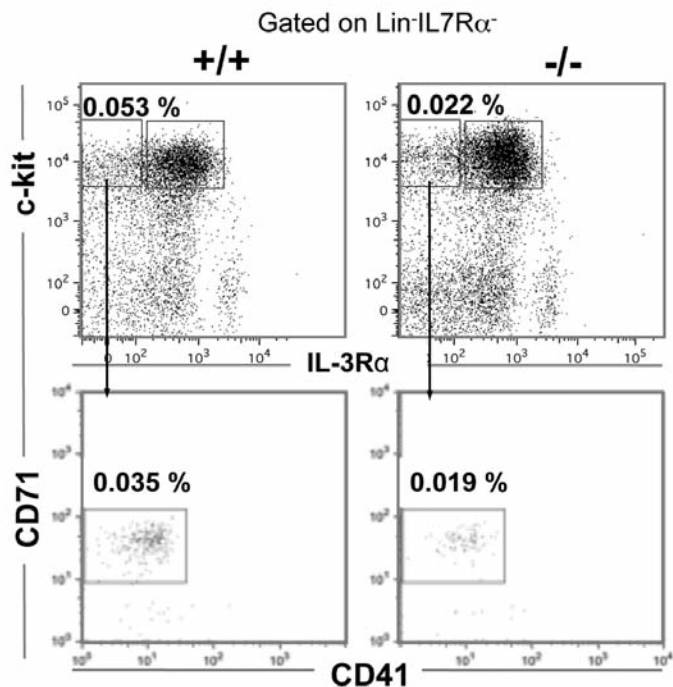
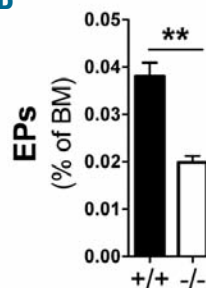


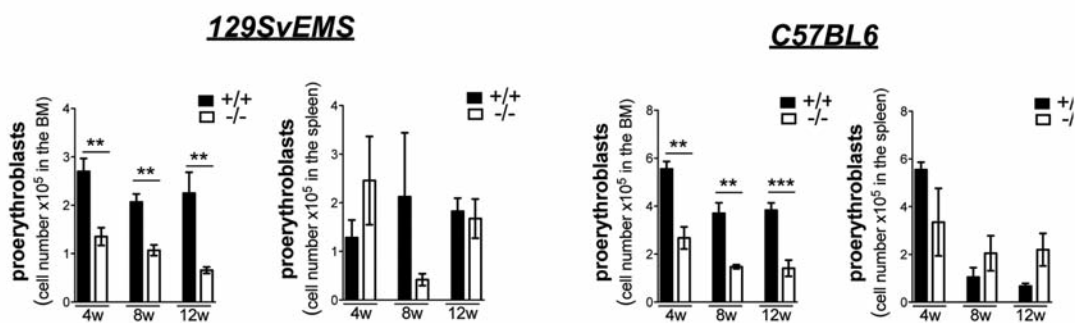
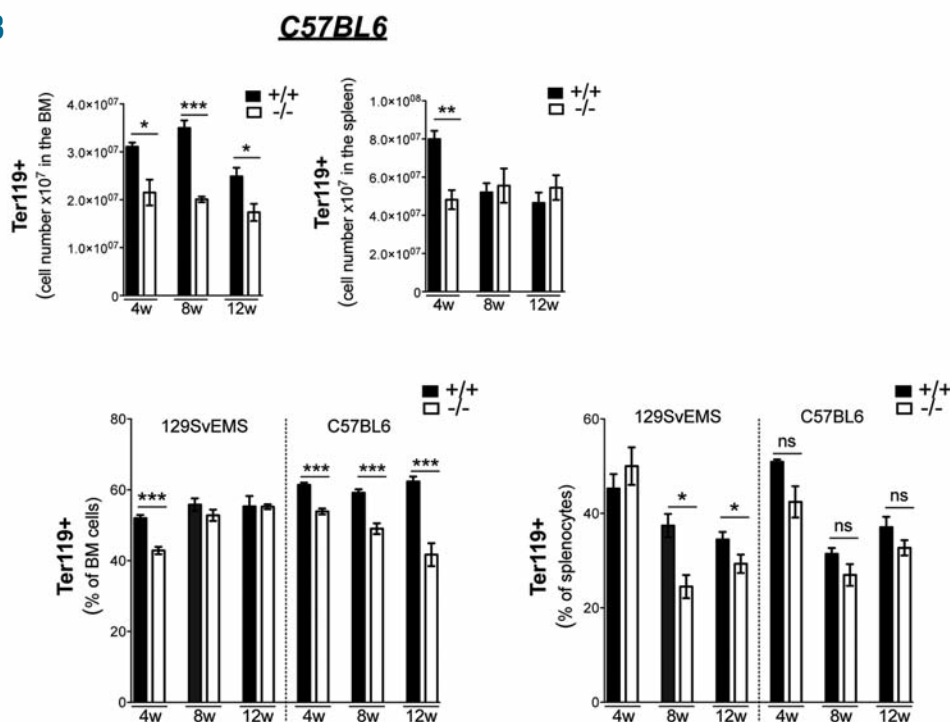
Figure 1. SWAP-70 is expressed in hematopoietic stem cells and erythroid progenitors. (A) Intracellular SWAP-70 staining in bone marrow cells. Left: myeloid-erythroid progenitors, subgated Lin⁻c-kit⁺Sca-1⁺, right: hematopoietic stem cells and early progenitors, subgated Lin⁻c-kit⁺Sca-1⁺. Data shown are representative of two independent experiments. (B) SWAP-70 intracellular staining in splenocytes. Upper panels: gating strategy. Lower left panel: intracellular SWAP-70 staining in Ery.A cells, lower right panel: intracellular staining of Ery.B cells. +/+ and -/- indicate cells from wild-type and *Swap-70*^{-/-} mice, respectively.



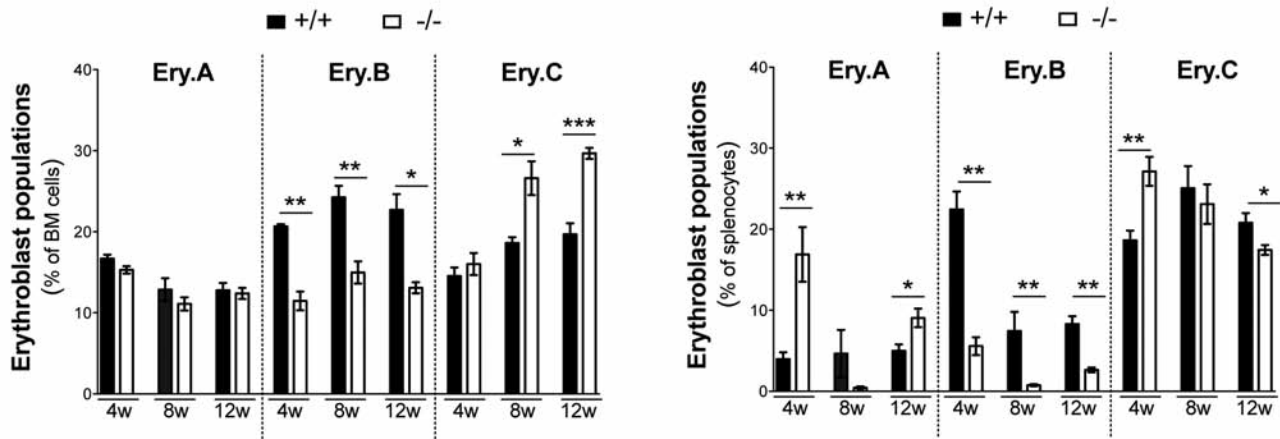
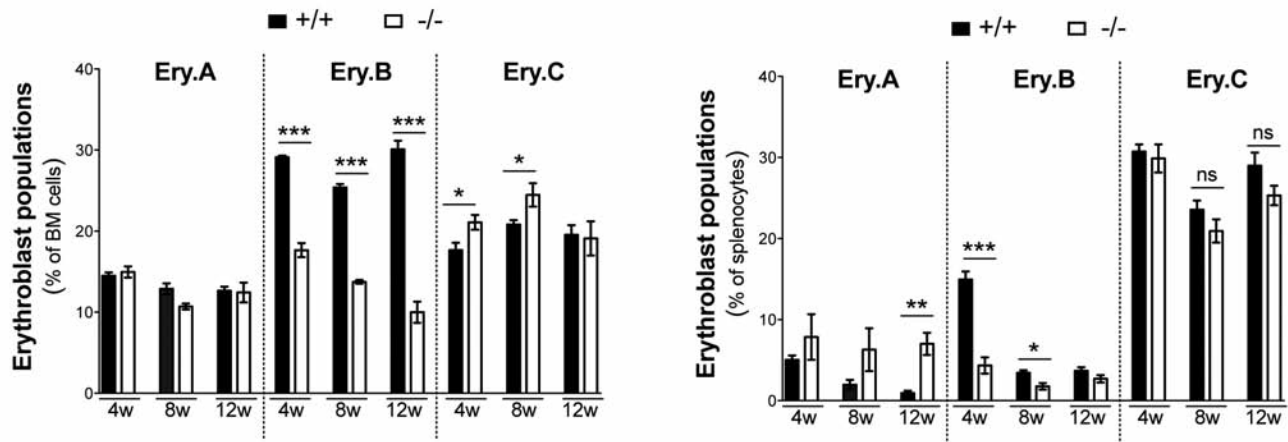
Online Supplementary Figure S2. SWAP-70 expression in erythroid progenitors in comparison to B lymphocytes. (A) FACS analysis of intracellular SWAP-70 staining in splenocytes. The histogram shows SWAP-70 expression in cells subgated on Ter119⁺ (erythroblasts, shown in black) and B220⁺ (B lymphocytes, shown in gray).

A**B**

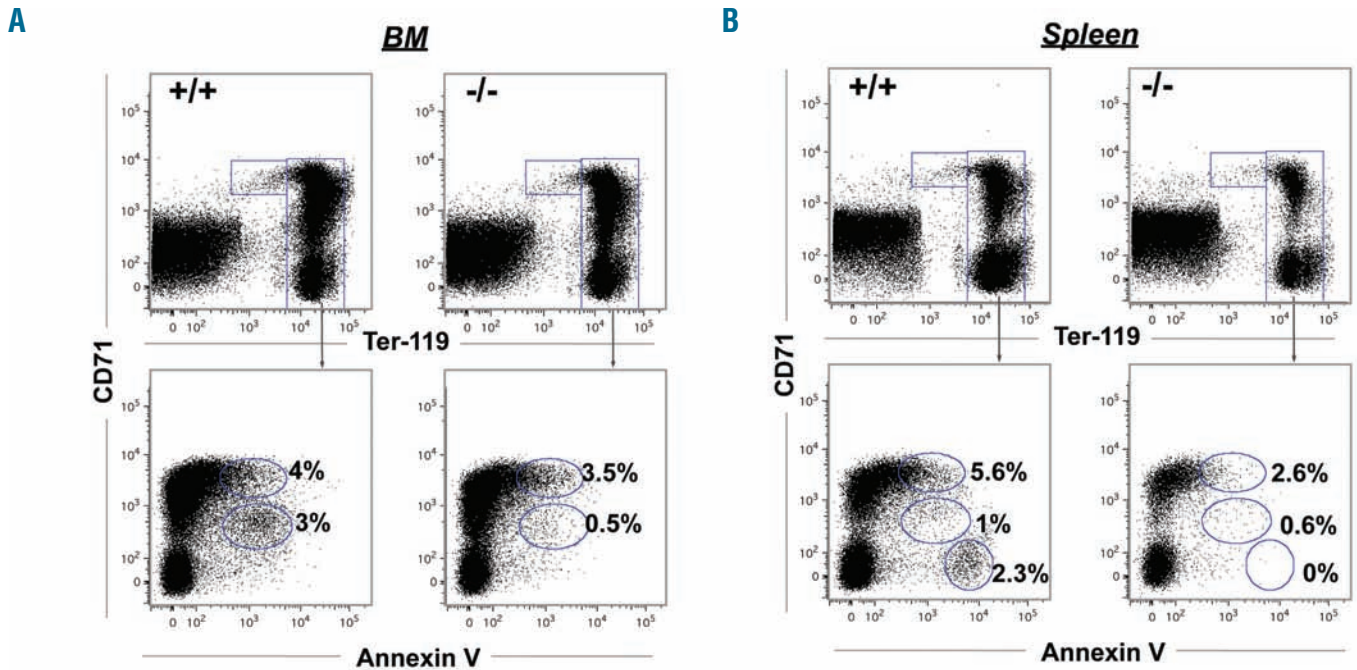
Online Supplementary Figure S3. SWAP-70 deficiency affects CFU-E development. (A) Representative dot plots obtained by FACS analysis of erythroid progenitors (CFU-E) in the bone marrow (BM) of wild-type (+/+) and *Swap-70*^{-/-} mice of both genetic backgrounds (129SvEMS and C57BL6). Upper dot plots show subgated Lin⁻IL7R α populations; lower dot plots show subgated Lin⁻IL7R α :IL3R α :c-kit⁺ cells. Percentages shown on the dot plots are of total BM. (B) Quantification of FACS data from (A) for three independent experiments (n=5 for each genotype). Data shown are mean \pm SD values. **P*<0.05, ***P*<0.01, ****P*<0.005.

A**B**

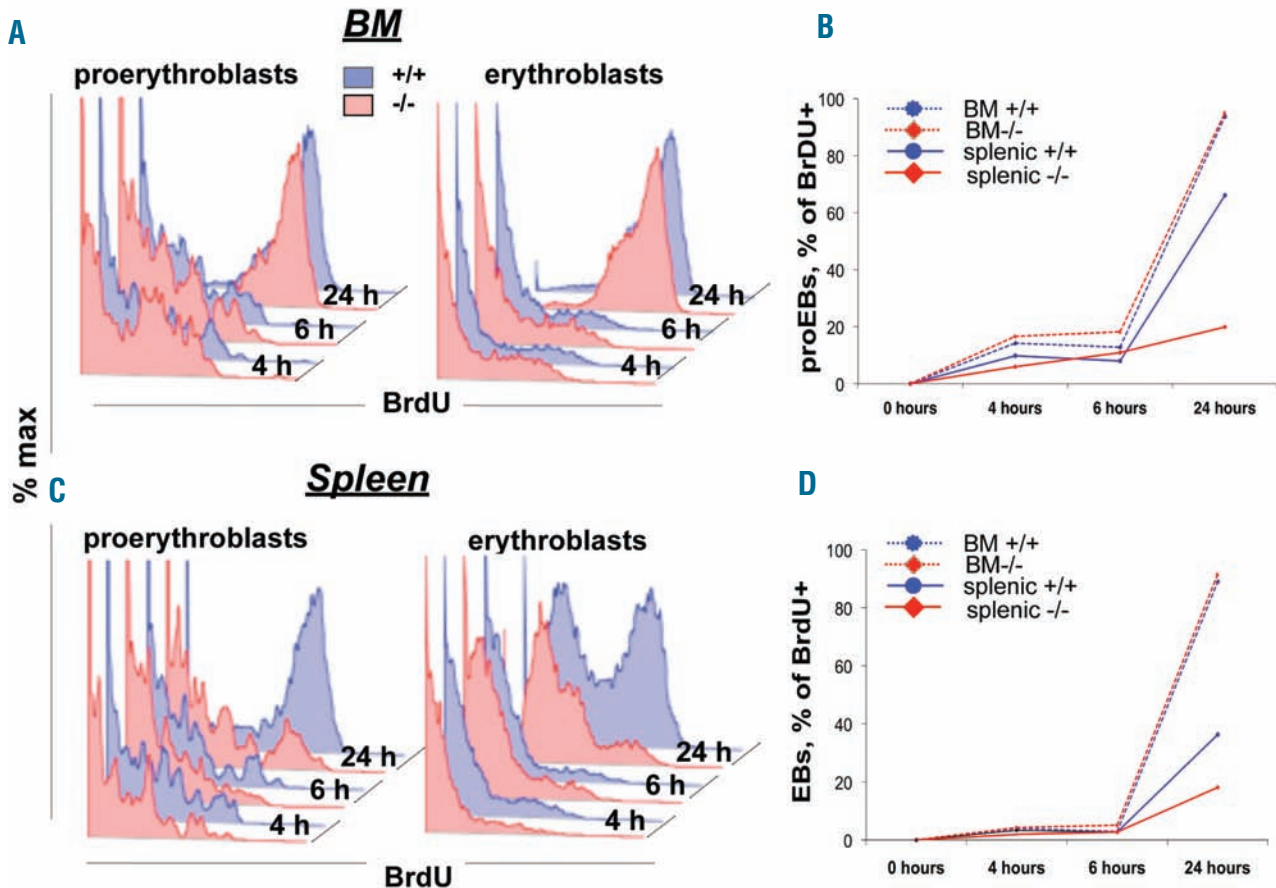
Online Supplementary Figure S4. FACS analysis of bone marrow (BM) and splenic Ter119⁺ erythroblasts. Automated-quantified (MACSQuant, Miltenyi) total numbers of proerythroblasts (A) and total Ter119⁺ erythroblasts (B) in the BM (per 1 femur and 1 tibia) and in the spleen of wild type (+/+) and *Swap-70*^{-/-} mice of different ages. At least five mice of each genotype per group were analyzed. (C) Relative numbers of Ter119⁺ erythroblasts in wt (+/+) and *Swap-70*^{-/-} mice of two genetic backgrounds at different ages (from 4 to 12 weeks) shown as percentage of total BM cells (left) or splenocytes (right) respectively.

A**129SvEMS****B****C57BL6**

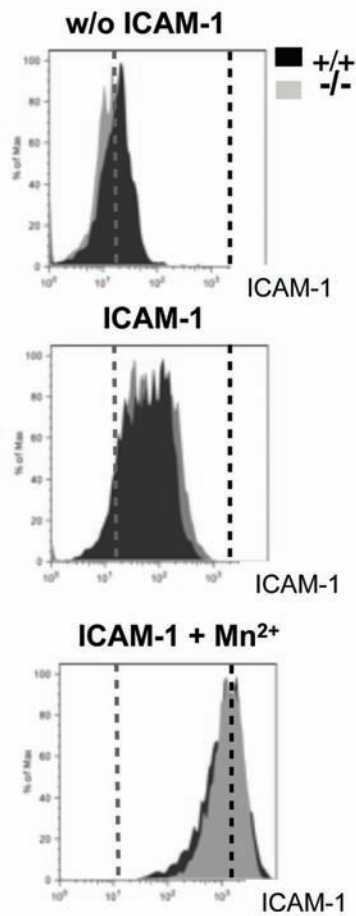
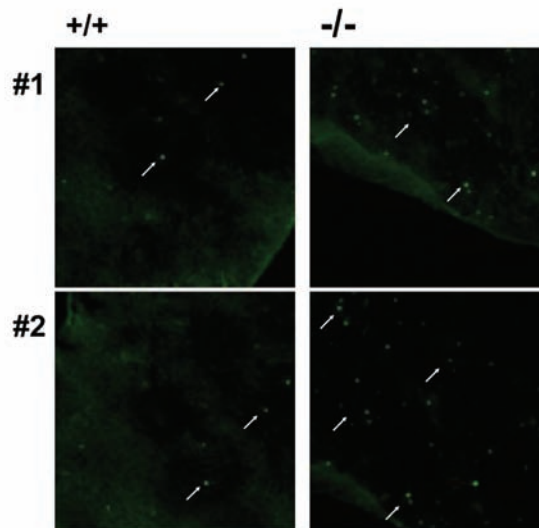
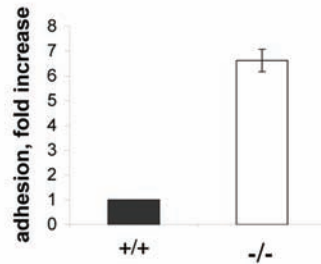
Online Supplementary Figure S5. Analysis of bone marrow (BM) and splenic Ter119⁺ erythroblastic populations. (A) and (B) Relative numbers of erythroblastic populations in wt (+/+) and *Swap-70*^{-/-} mice of two genetic backgrounds at different ages (from 4 to 12 weeks) shown as percentage of total BM cells (left) or splenocytes (right) respectively. At least five mice of each genotype per group were analyzed. Mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.



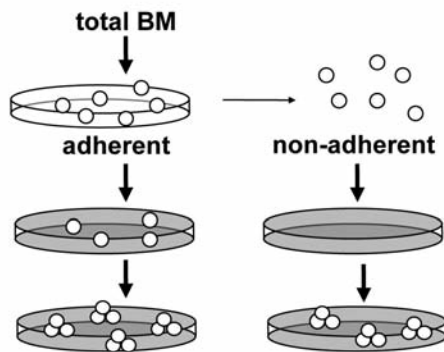
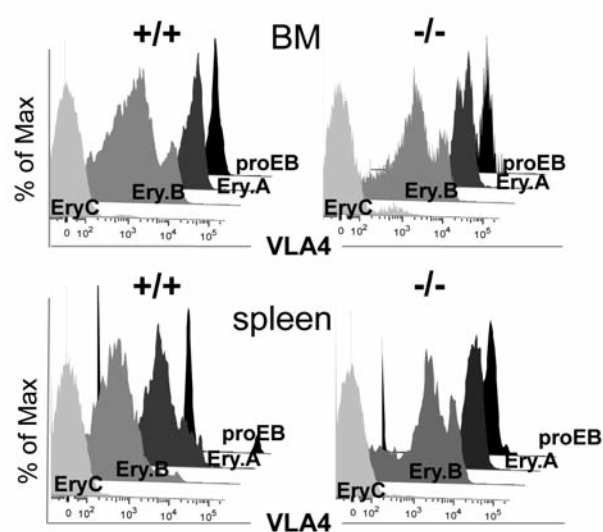
Online Supplementary Figure S6. Analysis of apoptotic rate among erythroid progenitors. FACS analysis of annexin V-positive erythroblasts in the bone marrow (BM) (A) and spleen (B). +/+ and -/- indicate cells from wild-type and *Swap-70*^{-/-} mice respectively. Upper plots: gating of total BM and splenic cells according to the expression of the CD71 and Ter119 surface markers. Lower plots: erythroblasts subgated on Ter119⁺ according to the annexin V staining. Percentages shown are of parent populations.



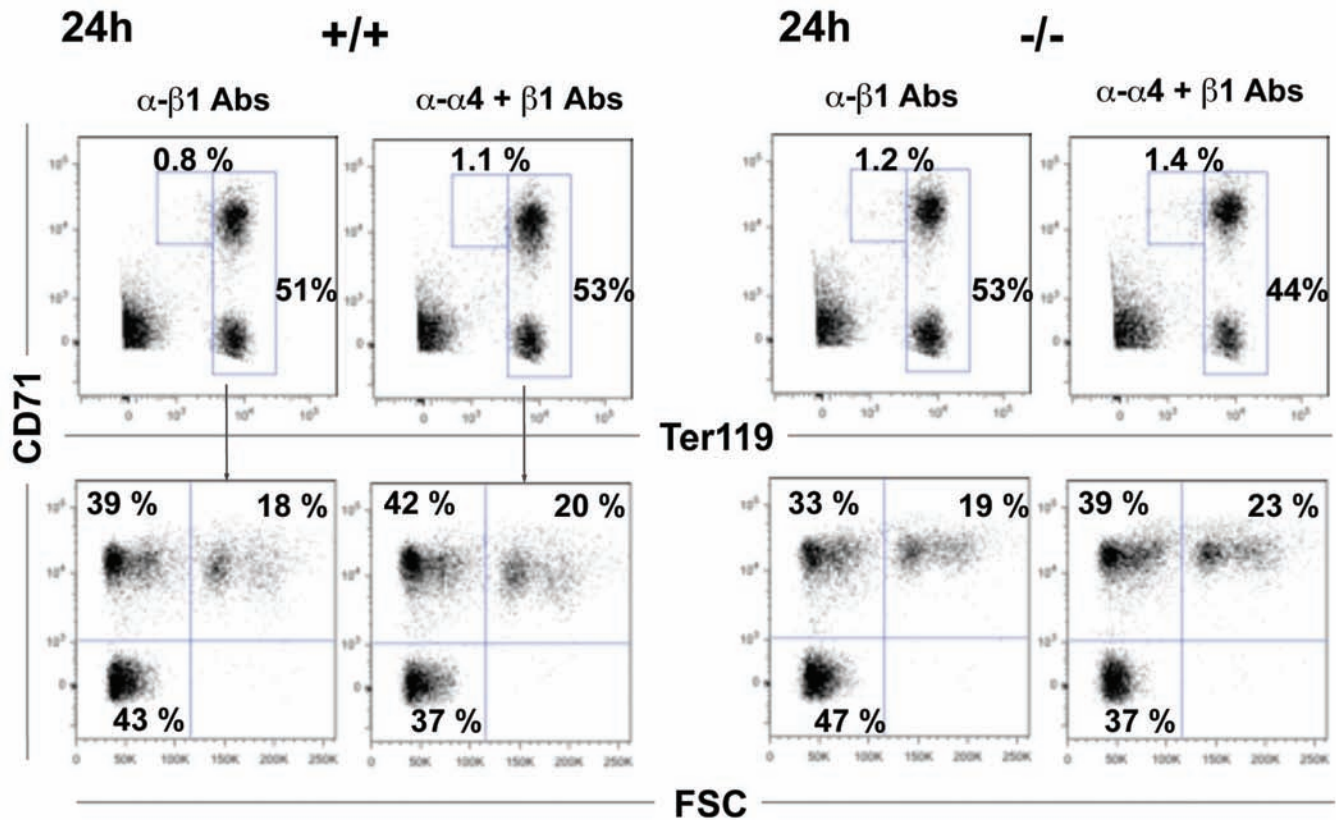
Online Supplementary Figure S7. *In vivo* BrdU incorporation assay. (A) and (C) Histograms showing the intracellular BrdU staining subgated on bone marrow (BM) and splenic proerythroblasts (left) and Ter119⁺ erythroblasts (right) respectively within the time course (4h to 24 h) upon single intraperitoneal administration of a BrdU solution. Data presented as an overlay of 4 h, 6 h and 24 h time points. +/+ (blue) and -/- (red) correspond to cells from wild-type and *Swap-70*^{-/-} mice respectively. (B) and (D) Quantification of FACS data from (A) and (C) combining BrdU^{high} and BrdU^{low} populations.

A**B****C**

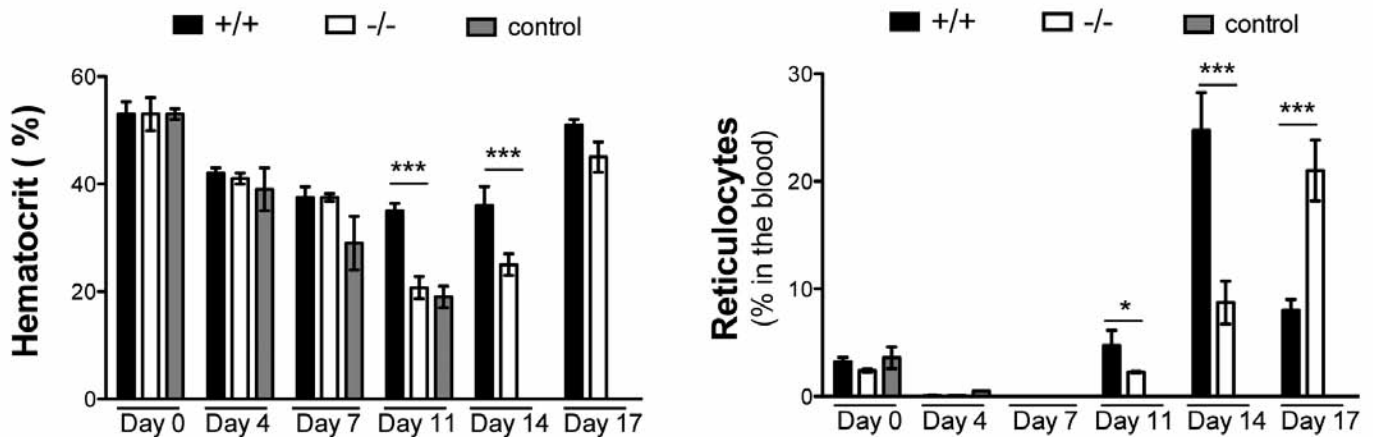
Online Supplementary Figure S8. Integrin-mediated interactions in Swap-70^{-/-} erythroid progenitors. **(A)** FACS analysis of soluble ICAM-1 binding by bone marrow early erythroid progenitors (subgated Lin⁻Sca-1⁺IL-7R α 1L-3R α CD41⁺c-kit⁺CD71⁺). +/+ (black) and -/- (gray) indicate cells from wild-type and Swap-70^{-/-} mice respectively. Fluorescence intensity histogram of cells incubated for 20 min without ICAM-1 Fc as background control (top), with soluble ICAM-1 Fc (20 μ g/mL) (center), or with soluble ICAM-1 Fc (20 μ g/mL) in the presence of 2 mM Mn²⁺ (bottom). Bound ICAM-1 was detected by labeled anti-Fc antibody **(B)** Erythroblast adhesion to frozen wild-type spleen sections. CFSE-labeled erythroblasts were left 20 min to adhere. +/+ and -/- correspond to wild-type and Swap-70^{-/-} erythroblasts respectively. **(C)** The bar chart shows the relative increase in adhesion of Swap-70^{-/-} erythroblasts (Swap-70^{-/-} green cells on spleen sections were counted) normalized to adhesion of wt erythroblasts (wt green cells on spleen sections).

A**B**

Online Supplementary Figure S9. Analysis of integrin function and expression on bone marrow (BM) erythroid progenitors. **(A)** Experimental procedure for the CFU-E-fibronectin adhesion assay. BM cells (5x10⁴) were incubated in fibronectin-coated wells in duplicate. After 2 h, both unattached and attached cells were assayed separately for CFU-E colony formation on methylcellulose-Epo plates. Background adhesion to BSA-coated wells was subtracted. **(B)** FACS analysis of $\alpha_4\beta_1$ (VLA-4) expression on bone marrow (upper histograms) and splenic (lower histograms) erythroid progenitors of wild-type (+/+) or Swap-70^{-/-} (-/-) mice. $\alpha_4\beta_1$ expression on proerythroblasts (proEB), Ery.A, Ery.B and Ery.C are shown as overlaid histograms. One representative experiment of three independent experiments is shown.



Online Supplementary Figure S10. Alpha4 and beta1 integrin activity regulation is required for erythroblast developmental transition. Representative dot plot obtained by FACS analysis of bone marrow Ter119⁺ erythroblasts 24 h after treatment with anti-β1 (α-β1) blocking antibodies (Abs) (30 μg, single injection) on the left and the combination of anti-α4 and anti-β1 (α-α4 + α-β1) blocking Abs (30 μg, single injection) on the right. +/+ and -/- indicate cells isolated from wild-type and *Swap-70*^{-/-} mice respectively. Upper plots: gating of total bone marrow cells according to the expression of the CD71 and Ter119 surface markers. Ter119^{high}CD71^{high} cells are proerythroblasts and Ter119^{high} cells are total erythroblasts. Percentages shown are of total bone marrow and spleen cells. Lower plots: erythroblasts subgated Ter119^{high}. Percentages shown are of parent populations.



Online Supplementary Figure S11. *Swap-70*^{-/-} mice exhibit a delayed response during acute anemia. Hematocrit recovery and reticulocyte counts in the blood of lethally irradiated (9 Gy) wild-type recipient mice transplanted with total bone marrow cells (2x10⁵) isolated from wild-type (+/+) or *Swap-70*^{-/-} (-/-) donors. Control mice did not receive any bone marrow cells. Representative of three independent experiments, n=4 per group. For all experiments, the data shown are the mean ± SD values. *P<0.05, **P<0.01, ***P<0.005.