SWAP-70 regulates erythropoiesis by controlling α4 integrin

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ABSTRACT

Background
The regulation of normal and stress-induced erythropoiesis is incompletely understood. Integrin-dependent adhesion plays important roles in erythropoiesis, but how integrins are regulated during erythropoiesis remains largely unknown.

Design and Methods
To obtain novel insights into the regulation of erythropoiesis, we used cellular and molecular approaches to analyze the role of SWAP-70 and the control of integrins through SWAP-70. In addition, mice deficient for this protein were investigated under normal and erythropoietic stress conditions.

Results
We show that SWAP-70, a protein involved in cytoskeletal F-actin rearrangements and integrin regulation in mast cells, is expressed in hematopoietic stem cells and myeloid-erythroid precursors. Although *Swap-70*$^{-/-}$ mice are not anemic, erythroblastic differentiation is perturbed, and SWAP-70 is required for an efficient erythropoietic stress response to acute anemia and for erythropoietic recovery after bone marrow transplantation in irradiated mice. SWAP-70 deficiency impairs colony-forming unit erythroid development, while burst-forming unit erythroid development is normal, and significantly affects development of late erythroblasts in the spleen and bone marrow. The α_4 integrin is constitutively hyper-activated in *Swap-70*$^{-/-}$ colony-forming unit erythroid cells, which hyper-adhere to fibronectin. Blocking α_4 and β_1 integrin chains *in vivo* restored erythroblastic differentiation and the erythropoietic stress response in *Swap-70*$^{-/-}$ mice.

Conclusions
Our study reveals that SWAP-70 is a novel regulator of integrin-mediated red blood cell development and stress-induced erythropoiesis.

Key words: erythropoiesis, α4 integrin, SWAP-70, adhesion, anemia.

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Introduction

Erythropoiesis is a complex multistep process of red blood cell generation that continues throughout the lifespan of vertebrates. Like hematopoiesis, the development of red blood cells is a hierarchical process: it proceeds from hematopoietic stem cells through several multipotent progenitor stages to committed erythroid progenitors, burst-forming unit-erythroid (BFU-E) cells and colony-forming unit-erythroid (CFU-E) cells. The latter differentiate into proerythroblasts (proEB) and subsequently to basophilic (basoEB), polychromatophilic (polyEB), and orthochromatophilic erythroblasts (orthoEB) and finally into mature erythrocytes through the reticulocyte stage.[1]

Homeostatic erythropoiesis requires tight regulation to consistently and efficiently replace senescent circulating erythrocytes. At each stage of development, it is controlled by a variety of intracellular and extracellular regulatory molecules. Surface expression of many adhesion molecules on erythrocyte progenitors and their role in erythopoiesis have been reviewed by Chasis and Mohandas.[2] A number of these adhesion molecules, including α_4, α_5, β_1, CD44, Lu, and ICAM-4, are responsible for various adhesive homotypic and heterotypic interactions within the erythropoietic niche, namely the erythroblastic island, providing survival, proliferation and differentiation signals at early stages, and supporting enucleation during terminal erythroid maturation. Moreover, homotypic and heterotypic adhesive interactions between developing erythrocyte precursors and a central macrophage, mediated by $\alpha_4\beta_1$ and ICAM-4, are required for the formation and integrity of erythroblastic islands and thus for erythroid development. Adhesion molecules modulate erythrocyte development alone or together with growth factors in a temporal manner.[3,4] In addition, growth factors modulate adhesion between hematopoietic progenitors and extracellular matrix via changes in integrin receptor expression and activation.[5,6] Interestingly, crosstalk between different integrins ($\alpha_4\beta_1$ and $\alpha_5\beta_1$) and cytokines (Kit ligand) has been shown to have opposing effects on growth and survival of erythroid progenitors.[3] Akt-, Bcl-2- and Bcl-xL-dependent survival, focal adhesion kinase (FAK) and extracellular-regulated kinase (ERK)-mediated proliferation have been described as signaling events after integrin engagement in erythroid cells.[3,4,7] However, *in vitro* and *in vivo* data have not been consistent. For instance, engagement of $\alpha_4\beta_1$ has been shown to support proliferation and to provide anti-apoptotic protection on maturing erythroblasts,[4] whereas different conclusions were made by others.[3] Blocking of $\alpha_4\beta_1$ by antibodies impairs erythrocyte development *in vitro* and *in vivo*.[8,9] which is in agreement with studies employing α_4 knockout mutants,[10] although the conditional knockout of α_4 mainly affects the stress response.[11,12] In contrast, complete deletion of β_1 does not affect erythrocyte development,[13] but β_1 is indispensable for survival during recovery from anemic stress.[12] Furthermore, very little is known about the molecules that regulate integrin function in erythroid precursors.

SWAP-70 was initially isolated from activated B-cell nuclear protein complexes.[14] It contains an F-actin-binding domain and a pleckstrin homology domain, binds PIP3, associates with RAC1, and regulates cytoskeletal F-actin rearrangements.[15] SWAP-70 is involved in integrin-mediated interactions in B cells and mast cells and its deficiency impairs *in vitro* and *in vivo* migration of these cells.[16] A conceivable function of SWAP-70 as a protein that integrates surface receptors such as integrins with signaling pathways and the F-actin cytoskeleton led us to suggest a potential role for SWAP-70 in hematopoietic cell development, particularly in erythropoiesis. Here we report SWAP-70 expression in hematopoietic stem cells and myeloid-erythroid precursors, postulate a novel function for this protein in steady-state and stress-induced erythrocyte differentiation, and lastly propose SWAP-70 as an integrin function regulator in the development of erythroid progenitor cells.

Design and Methods

Mice

Previously described wild-type (wt) and *Swap-70*$^{-/-}$ mice of two different genetic backgrounds of *M. musculus*, 129SvEMS and C57BL/6N, were used.[17] Each experiment was performed using wt and *Swap-70*$^{-/-}$ mice of the same strain, and in most cases both genetic backgrounds were used.

FACS analysis and immunostaining

Flow cytometry and immunostaining were performed as described elsewhere.[18] The details are provided in the *Online Supplementary Appendix*. To detect live erythroblastic islands, surface markers were immunostained as described previously,[19] without prior paraformaldehyde fixation, and 1 h incubation with antibodies and second-step reagents.

Colony-forming assay

The numbers of BFU-E and CFU-E were quantified in standard colony assays as previously described.[20] Details are given in the *Online Supplementary Appendix*.

Soluble ligand-binding assay

Bone marrow cells were incubated with soluble VCAM-1 or ICAM-1 Fc-chimeric molecules (20 ng/mL, R&D Systems) in either Iscove's modified Dulbecco's medium (IMDM) or IMDM with 2 mM Mn^{++}. Ligand binding was terminated by fixing cells with 3.7% paraformaldehyde in phosphate-buffered saline (PBS). Bound VCAM-1 or ICAM-1 was detected with anti-Fc fragment antibodies by FACS.

Colony-forming unit-erythroid adhesion assay and erythroblastic island reconstitution assay

The CFU-E adhesion assay and the erythroblastic island reconstitution assay were performed as previously described.[19,21] Details are given in the *Online Supplementary Appendix*.

In vivo *integrin-blocking assay*

Mice were injected i.v. with 30 μg of blocking antibodies (eBioscience , CD49d (clone CR1-2) and CD29 (clone eBioHMb1-1) diluted in PBS. Control mice received 30 μg of isotype-matched control antibodies in PBS or PBS alone. Hematopoietic organs were analyzed by FACS.

Induction of anemia

Hemolytic anemia was induced by phenylhydrazine injection. Mice received sterile phenylhydrazine solution (Sigma) intraperitoneally to achieve a desired dose (40 mg/kg or 80 mg/kg) at indicated time points. Control mice were injected with PBS only. Blood was analyzed at days 0, 3, 6 and 10 on a hematological analyzer: ADVIA 120 (Bayer Diagnostic, Rockefeller University, NY, USA) or XE-2100 (Sysmex, Medical Clinik I, Hematology/Oncology, TU, Dresden, Germany).

Statistic analysis

Data were analyzed using unpaired two-tailed t-tests. *P<0.05, **P<0.01, ***P<0.005.

Results

SWAP-70 is expressed early in erythropoiesis and is required for colony-forming unit-erythroid development

SWAP-70 is expressed in cells of the hematopoietic system,[22] including B lymphocytes,[14] mast cells[23] and dendritic cells.[24] However, a detailed analysis of the different hematopoietic lineages and cell types has not yet been performed. We, therefore, aimed to investigate the expression of SWAP-70 during early hematopoiesis. As shown in *Online Supplementary Figure S1A*, SWAP-70 was found in the primitive stem cell and progenitor containing fraction (Lin$^-$c-kit$^+$Sca-1$^+$) and myeloid-erythroid progenitors (Lin$^-$c-kit$^+$Sca-1$^-$). SWAP-70 levels in terminally differentiating erythroblasts were analyzed as proposed by Liu *et al.*[25] (*Online Supplementary Figure S1B*). According to the scheme, Ery.A, Ery.B and Ery.C erythroblast subpopulations form a developmental sequence with earlier progenitors (basophilic erythroblasts) residing in the Ery.A gate, followed by late basophilic and polychromatic erythroblasts (Ery.B), and later erythroid progenitors (orthochromatic erythroblasts) and reticulocytes found in the Ery.C gate. High levels of SWAP-70 were detected in the majority of Ery.A cells (*Online Supplementary Figure S1B*, lower right), which, comparable to splenic B220+ cells, was used as a positive control (*Online Supplementary Figure S2A*). Some of the Ery.A and Ery.B cells were SWAP-70^{low} (*Online Supplementary Figure S1B*, histograms). Ery.C did not withstand the fixation and permeabilization procedure and were not, therefore, analyzed. SWAP-70 was not detected by immunoblotting of lysates from mature erythrocytes circulating in the bloodstream (*data not shown*). As assessed by immunofluorescence analysis of MACS-purified Ter119$^+$ erythroblasts, SWAP-70 is distributed throughout the cytoplasm with partial overlap of F-actin structures at the cell membrane (*Online Supplementary Figure S2B*). Since SWAP-70 regulates F-actin dynamics in several cell types, we stained F-actin for microscopic analysis and for FACS analysis of F-actin content in wt and *Swap-70*$^{-/-}$ erythroblasts. No significant difference was observed (*Online Supplementary Figure S2C and 2D*). On Giemsa stained cytospins (*data not shown*) *Swap-70*$^{-/-}$ erythroblasts looked similar to wt ones suggesting that erythroblast morphology is not grossly affected by the absence of SWAP-70.

As SWAP-70 expression was detected in early erythroid progenitors, we sought to investigate whether SWAP-70 deficiency affects erythrocyte development. Megakaryocyte-erythocyte progenitors were identified by the surface expression of FcγR (CD16/32) and CD34 within lineage-Sca-1$^-$c-kit$^+$ bone marrow cells (Figure 1A) as proposed by Akashi *et al.*[26] A significant reduction in the percentage of megakaryocyte-erythocyte progenitors was observed in the bone marrow of young 129SvEMS *Swap70*$^{-/-}$ mice but not in older animals (Figure 1B), while in *Swap-70*$^{-/-}$ mice of C57BL/6 background reduced numbers of megakaryocyte-erythocyte progenitors were detected only in older (more than 14 weeks) animals.

The development of BFU-E and CFU-E was analyzed by a clonogenic assay. The numbers of BFU-E colonies derived from either bone marrow or splenocytes were similar between wt and *Swap-70*$^{-/-}$ mice (Figure 1C). In contrast, *Swap-70*$^{-/-}$ bone marrow cells and splenocytes generated significantly fewer CFU-E colonies (Figure 1D). These results were confirmed by FACS analysis of CFU-E defined as Lin$^-$IL7Rα$^-$Il3Rα$^-$CD41$^-$c-kit$^+$CD71$^+$ (*Online Supplementary Figure S3*) as described previously.[20] Together, these data show that the hematopoietic tissues (bone marrow and spleen) of *Swap-70*$^{-/-}$ mice do not support efficient development of CFU-E.

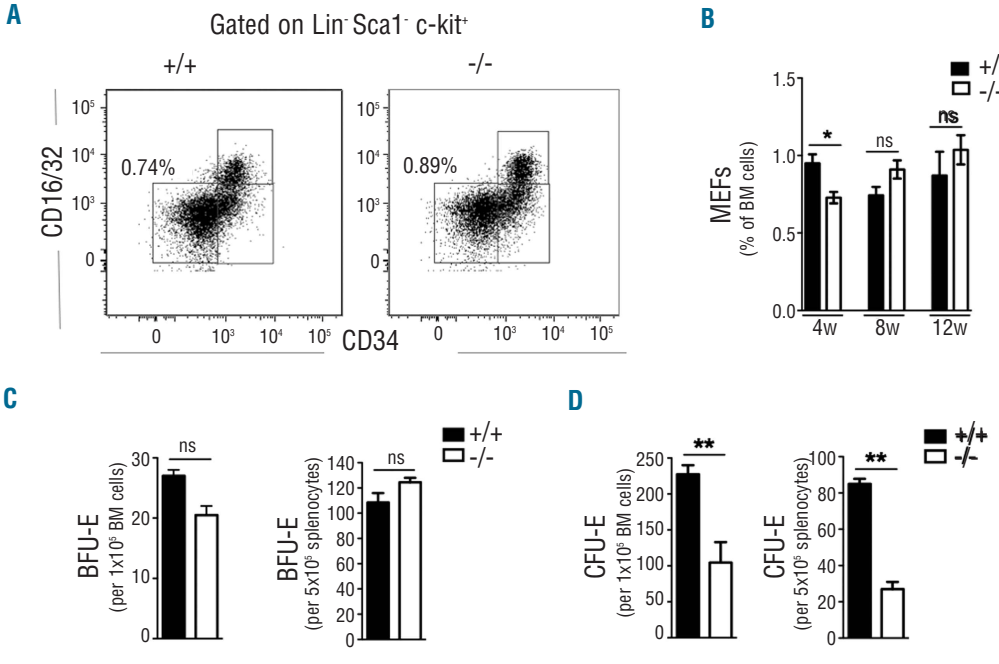


Figure 1 SWAP-70 deficiency affects CFU-E development. (A) Representative FACS plots of megakaryocyte-erythrocyte progenitors (MEP) analysis in the bone marrow of 8-week old mice. Percentages shown are of total BM cells. (B) Quantified FACS data from (A) for wild-type or *Swap-70*$^{-/-}$ mice of different ages. At least four mice of each genotype per group on the 129SvEMS background were analyzed. (C) and (D) BFU-E and CFU-E colony-forming assay of nucleated BM cells (1x10^5) or splenocytes (5x10^5). Data shown represent the mean of three independent experiments (n=5 for each group) ± SD. +/+ and -/- indicate wild-type and *Swap-70*$^{-/-}$ cells, respectively.

Disturbed erythroblastic differentiation in Swap-70$^{-/-}$ erythropoietic tissues

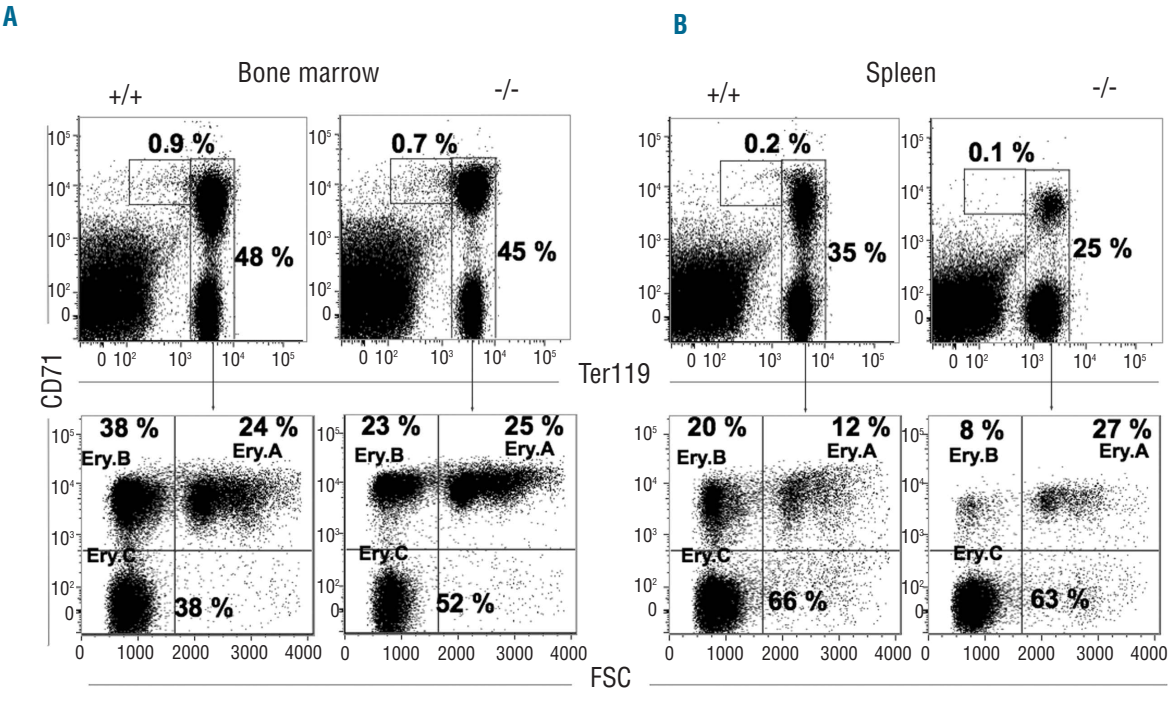
We hypothesized that SWAP-70 may be important for the development, homeostasis and/or survival of CFU-E in the bone marrow and spleen. To evaluate whether the reduced number of CFU-E affects erythroid homeostasis, we performed detailed analyses of progenitors following the CFU-E stage. The 2-fold reduction of CFU-E in *Swap-70$^{-/-}$* mice did not lead to a dramatic decrease in the percentage of proEB and Ter119^{+} erythroblasts in the bone marrow of adult mice (Figure 2A). However, the absolute numbers of these cells were strongly reduced (Figure 2C, left; *Online Supplementary Figure S4A and S4B*, left).

The number of splenic Ter119^{+} cells was severely affected in *Swap-70$^{-/-}$* mice (Figure 2B and 2C, right). In the 129SvEMS background the deficiency was acquired later in development. Seemingly reduced erythroblast numbers in the spleen of C57BL5 *Swap70$^{-/-}$* animals were not statistically significant (*Online Supplementary Figure S4B*, right).

Regardless of the age or the erythropoietic site – bone marrow or spleen – the aberration of Ery.A, Ery.B and Ery.C populations is obvious in *Swap-70$^{-/-}$* mice (Figure 2A). When the relative amount of each erythroblastic subpopulation was quantified as percentage of total bone marrow cells or splenocytes, it became apparent that SWAP-70 is required for the developmental transition from Ery.A to Ery.C (*Online Supplementary Figure S5A and S5B*). Generation of Ery.B cells is reduced by nearly 2-fold in the absence of SWAP-70. It is noteworthy that the most mature erythroblasts, Ery.C, were present even at higher frequency, suggesting that maturation is compensated for and proceeds and that the Ery.C pool of progenitors either accumulates or is generated in higher relative numbers despite the fact that Ery.B development is impaired.

We tested whether increased apoptosis of late erythroblasts and/or abnormal proliferation of early erythroblasts might account for the altered erythroblastic profile in *Swap-70$^{-/-}$* hematopoietic tissues. In contrast, lower numbers of annexinV^{+}Ter119^{+} erythroblasts were detected in the bone marrow and spleen of *Swap-70$^{-/-}$* mice compared to wt mice (*Online Supplementary Figure S6*). The *in vivo* proliferation rate was slower only in *Swap-70$^{-/-}$* splenic erythroblasts as assessed by BrdU labeling (*Online Supplementary Figure S7*).

In summary, SWAP-70 deficiency affects erythropoiesis, resulting in lower numbers of Ter119^{+} in the bone marrow and spleen, and influences the developmental transition of erythroblasts from Ery.A to Ery.B and Ery.C.



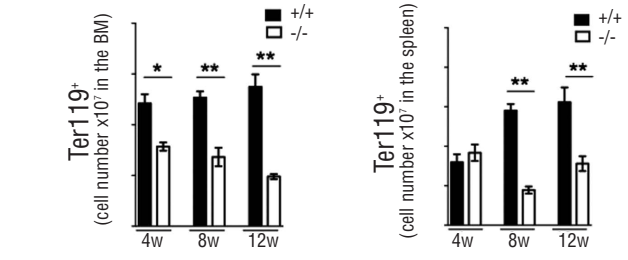


Figure 2. SWAP-70-deficient mice have lower erythropoietic reserves. Representative FACS plots of freshly isolated bone marrow (BM) cells (A) and splenocytes (B) from wt (+/+) and *Swap-70$^{-/-}$* (-/-) 8-week old mice. Upper plots: Ter119^{low}CD71^{high} comprise proerythroblasts and Ter119^{+} comprise total erythroblasts. Percentages shown are of total BM and spleen cells. Lower plots: erythroblasts, subgated Ter119^{high}. Percentages shown are of parent populations. (C) Automated-quantified (MACSQuant, Miltenyi) total numbers of Ter119^{+} cells in the BM (per 1 femur and 1 tibia) and in the spleen of wt (+/+) and Swap-70-/- (-/-) mice of different ages. At least four mice of each genotype per group on the 129SvEMS background were analyzed. Mean ± SD. *P<0.05, **P<0.01, ***P<0.005.

SWAP-70 controls the activation state of α_4 integrins

Integrin-dependent homotypic hyper-aggregation and hyper-adhesion of *Swap-70*$^{-/-}$ B lymphocytes and mast cells has been previously shown.[16,18] To gain insights into the mechanisms by which SWAP-70 affects erythropoiesis, its integrin regulatory function was analyzed in erythroid precursors. We speculated that SWAP-70 might control integrin activity during erythropoiesis and, therefore, decided to focus on the $\alpha_4\beta_1$ integrin, known to be important in erythroblast development.[4,9]

The adhesive properties of wt and *Swap-70*$^{-/-}$ bone marrow CFU-E, the first stage of erythropoiesis that depends on SWAP-70, were compared. No significant difference was observed (*data not shown*) in the surface expression of $\alpha_4\beta_1$ as measured by FACS of wt and *Swap-70*$^{-/-}$ Lin$^-$c-kit$^+$CD71$^+$ bone marrow progenitors. The activation status of $\alpha_4\beta_1$ in wt and *Swap-70*$^{-/-}$ CFU-E was tested in a soluble ligand (VCAM-1) binding assay. Without prior activation, a significant fraction of *Swap-70*$^{-/-}$ CFU-E bound higher amounts of VCAM-1 (Figure 3A; middle histogram), generating a distinct VCAM-1^{high} population. The VCAM-1^{low} populations of *Swap-70*$^{-/-}$ and wt largely overlapped, and the wt showed only a VCAM^{med} population in addition. After stimulation of $\alpha_4\beta_1$ by adding Mn^{++}, a non-specific integrin activator, the mean fluorescence intensity values increased for both wt and *Swap-70*$^{-/-}$, giving rise to rather homogenous VCAM-1^{high} populations (Figure 3A, bottom histogram), suggesting a high affinity VCAM-1-$\alpha_4\beta_1$ interaction. No VCAM-1^{low} cells remained and the difference between wt and *Swap-70*$^{-/-}$ progenitors largely vanished. These data suggest pre-activation of $\alpha_4\beta_1$ integrin on *Swap-70*$^{-/-}$ erythroid progenitors. Similarly, early erythroid progenitors have been reported to express α_L integrin.[27] However, we confirmed the effect of SWAP-70 on α_4 regulation to be specific by performing similar assays using soluble ICAM-1, which is the ligand for α_L (*Online Supplementary Figure S8A*).

The aberrant $\alpha_4\beta_1$ pre-activation in *Swap-70*$^{-/-}$ CFU-E was further analyzed in a functional adhesion assay in which CFU-E adhesion to fibronectin was evaluated.[21] Adherent and non-adherent bone marrow cells were tested for colony formation (*Online Supplementary Figure S9A*). In total, fewer colonies were recovered from either adherent or non-adherent *Swap-70*$^{-/-}$ bone marrow cells, which corroborates our finding that CFU-E development is SWAP-70 dependent. Approximately half of wt bone marrow CFU-E were attached to fibronectin (Figure 3C). A similar number of colonies was recovered from the non-attached wt cell fraction. However, a significantly higher fraction of *Swap-70*$^{-/-}$ bone marrow CFU-E adhered to fibronectin. Thus, the ratio of adherent to non-adherent CFU-E was higher for *Swap-70*$^{-/-}$ cells (Figure 3D). These results demonstrate that *Swap-70*$^{-/-}$ CFU-E hyper-adhere to fibronectin, possibly due to constitutive pre-activation of the α_4 integrin, suggesting a role for SWAP-70 as a regulator of α_4 integrin function.

Erythroblastic island architecture is affected by a lack of SWAP-70

The above results suggest that SWAP-70 regulates α_4 integrin function in early erythroid precursors. Later in ery-

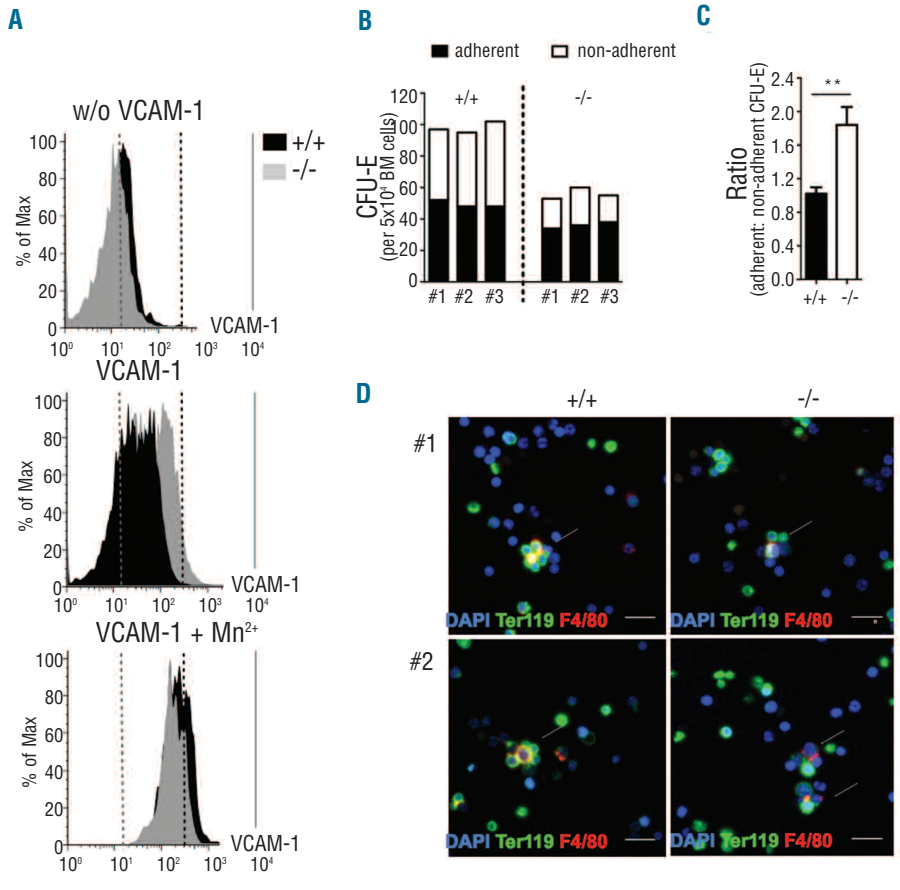


Figure 3. Alpha-4 integrins are pre-activated in *Swap-70*$^{-/-}$ bone marrow progenitors. (A) FACS analysis of soluble VCAM-1 Fc binding by bone marrow (BM) early erythroid progenitors (subgated Lin$^-$Sca-1$^-$IL-7Rα^-IL-3Rα^-CD41$^-$c-kit$^+$CD71$^+$). +/+ (shown in black) and -/- (shown in gray) indicate cells from wt and *Swap-70*$^{-/-}$ mice respectively. Fluorescence intensity histogram of cells incubated for 20 min without VCAM-1 Fc as background control (top), with soluble VCAM-1 Fc (20 μg/mL) (center), or with soluble VCAM-1 Fc (20 μg/mL) in the presence of 2 mM Mn^{++} (bottom) are shown. Bound VCAM-1 was detected by labeled anti-Fc antibodies. (B) CFU-E adhesion to fibronectin. The results are expressed as the number of CFU-E colonies obtained from adherent (black bars) and non-adherent (white bars) cells or as a ratio of adherent to non-adherent CFU-E (C). (D) Immunofluorescence images of *in vitro* reconstituted erythroblastic islands from wt (+/+) or *Swap-70*$^{-/-}$(-/-) BM cells in the presence of 2 mM Mn^{++}. Erythroblasts and macrophages are labeled with anti-Ter119 or anti-F4/80 antibodies respectively. DAPI staining is shown in blue. White arrows indicate erythroblastic islands. Pictures were taken with a Leica confocal microscope. Scale bars, 15 μm.

throid development, α_4-dependent interactions are necessary for erythroblasts to associate via VCAM-1 with a central macrophage, which supports erythroblastic island formation and integrity, thereby creating a specialized niche for erythroid development.[28] We, therefore, investigated whether SWAP-70-mediated α_4 integrin regulation is involved in erythroblastic island formation and/or maintenance.

First the α_4 expression on erythroblasts was analyzed (*Online Supplementary Figure S9B*). In agreement with published data,[4] $\alpha_4\beta_1$ down-regulation was observed from proEB to Ery.C. The main difference between wt and *Swap-70*$^{-/-}$ mice was observed in splenic Ery.A and Ery.B populations where *Swap-70*$^{-/-}$ erythroblasts were mainly $\alpha_4\beta_1^{high}$ and $\alpha_4\beta_1^{med}$.

Next we looked at whether a SWAP-70 deficiency affects the erythroblastic niche, potentially altering normal erythroblast development. To investigate the structure of erythroblastic islands, we performed an *in vitro* reconstitution assay as described previously.[19] Figure 3F shows examples of reconstituted erythroblastic islands. We did not detect a significant difference in the number of erythroblastic islands formed from either wt or *Swap-70*$^{-/-}$ bone marrow cells under non-activating or activating (Mn^{++}) conditions (*data not shown*). However, a detailed analysis of *Swap-70*$^{-/-}$ erythroblastic islands revealed higher numbers of irregular structures and aggregates, such as macrophages surrounded by fewer erythroblasts or homotypic (erythroblast-erythroblast) aggregates (Figure 3D). Therefore, the architecture of erythroblastic islands rather than their formation seems to be affected by a lack of SWAP-70.

In vivo *inhibition of* α_4 *and* β_1 *restores SWAP-70 deficiency defects*

Our results strongly suggest a role for SWAP-70 in controlling the proper functioning of $\alpha_4\beta_1$ integrins in erythroid cells. *Swap-70*$^{-/-}$ early progenitors (CFU-E) aberrantly pre-activate $\alpha_4\beta_1$ (Figure 3). In the absence of SWAP-70, adhesion of Ery.A cells to splenic sections is higher (*Online Supplementary Figure S8B and S8C*), and erythroblastic island reconstitution *in vitro* is irregular (Figure 3D). Consequently, we asked whether interfering with the α_4 integrin function in *Swap-70*$^{-/-}$ mice could have a positive effect and revert the erythroblastic developmental abnormality.

To test our hypothesis, we blocked α_4 and β_1 integrin chains by a single intravenous injection of anti-α_4, anti-β_1 or a combination of both antibodies. No changes in erythroblastic populations in wt and *Swap-70*$^{-/-}$ bone marrow were observed after 12 h (*data not shown*). However, the effect of blocking antibodies on bone marrow erythropoiesis of *Swap-70*$^{-/-}$ mice was significant after 24 h. Neither relative nor absolute numbers of Ter119$^+$ cells changed significantly after injection (Figure 4B). Interestingly, the Ery.A-to-Ery.B transition was rescued by blocking either the α_4, the β_1 or both chains (Figure 4A and 4C, *Online Supplementary Figure S10*).

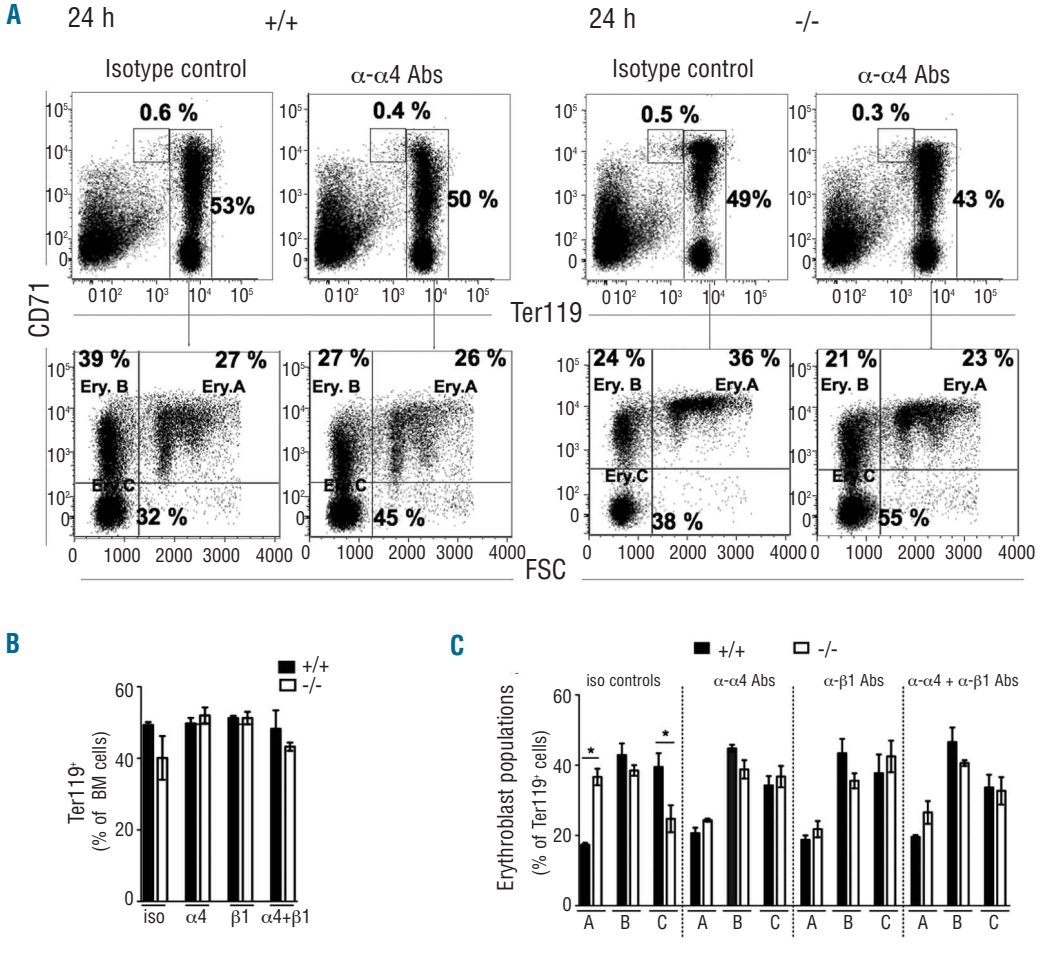


Figure 4. Alpha4 and beta1 integrin activity regulation is required for erythroblast developmental transition. (**A**) Representative FACS plots of bone marrow (BM) Ter119$^+$ erythroblasts 24 h after treatment with anti-α4 (α-α4) blocking antibodies (Abs) (30 μg, single injection) or with isotype-matched Abs. Percentages shown are of parent populations. (**B**) and (**C**) Histograms showing the mean ± SD of two independent experiments (at least 4 mice of the 129SvEMS background for each group) for mice treated for 24 h with anti-α4, anti-β1, their combination (30 μg, single injection) or with isotype-matched Abs. +/+ and -/- indicate cells isolated from wt and *Swap-70*$^{-/-}$ mice respectively. A, B and C stand for Ery.A, Ery.B and Ery.C respectively. **P*<0.05, ***P*<0.01, ****P*<0.005.

Thus, *in vivo* blocking of α_4 and/or β_1 restores the defects caused by the lack of SWAP-70.

Recovery from acute anemia is delayed in Swap-70$^{-/-}$ mice

To assess the overall biological impact of SWAP-70 on steady-state erythropoiesis, we analyzed the peripheral blood of SWAP-70-deficient mice and did not detect any abnormalities (*Online Supplementary Table S1*). However, would *Swap-70*$^{-/-}$ mice recover normally from anemic stress? Some mouse mutants such as α_4 and β_1 conditional "knockouts" fail to respond properly to acute anemia despite having nearly normal blood parameters.[12,13] Wild-type and *Swap-70*$^{-/-}$ mice were subjected to phenylhydrazine-induced hemolytic anemia. A high dose of phenylhydrazine led to severe anemia with a drop in hematocrit to 10% as measured by day 3 (*data not shown*). Wild-type mice mounted an efficient response and only one out of five mice died at day 5, corresponding to a survival rate of 80% (Figure 5A). However, all *Swap-70*$^{-/-}$ mice died during the recovery period.

To assess the kinetics of recovery, mice were subjected to a sublethal dose of phenylhydrazine. Treatment with phenylhydrazine led to a drop in hematocrit to about 20% in both wt and *Swap-70*$^{-/-}$ mice at day 3 (Figure 5B, left). However, the hematocrit of *Swap-70*$^{-/-}$ mice was restored significantly less efficiently than that of wt mice; by day 6, when the maximum response is expected,[29] wt mice had re-established nearly normal hematocrit levels, whereas the hematocrit had reached only 35% in *Swap-70*$^{-/-}$ mice (Figure 5B). Another indication of an inefficient or delayed response was the lower reticulocyte count in the blood of *Swap-70*$^{-/-}$ mice at day 6 (Figure 5B, right).

To assess whether the pretreatment of *Swap-70*$^{-/-}$ mice with α_4- or β_1-blocking antibodies would support an efficient recovery comparable to that of wt, we injected mice with a single dose of blocking antibodies 24 h prior to induction of anemia and monitored recovery over 10 days (Figure 5C). In these experiments, the kinetics of the response was different from that observed in previous experiments due to less efficient induction of anemia (drop of hematocrit to 30%) at day 3 (*data not shown*). At day 6, all mice already had a normal hematocrit and reticulocyte counts, similar to day 10 of previous experiments (i.e. at the end of the recovery period). Nevertheless, a significant difference in reticulocyte numbers of mice from different groups was observed (Figure 5C). *Swap-70*$^{-/-}$ mice injected with α_4- or a combination of α_4- and β_1-blocking antibodies had similar reticulocyte counts to wt mice. Non-treated *Swap-70*$^{-/-}$ mice had significantly higher numbers of circulating reticulocytes compared to wt and anti-α_4-treated *Swap-70*$^{-/-}$ mice. Interestingly, the blocking of β_1 had a very pronounced effect in *Swap-70*$^{-/-}$ mice: the number of reticulocytes was two times higher than that in wt or *Swap-70*$^{-/-}$ mice of any group. This effect might be due to interference not only with VLA-4 ($\alpha_4\beta_1$) but also with other β_1-contain-

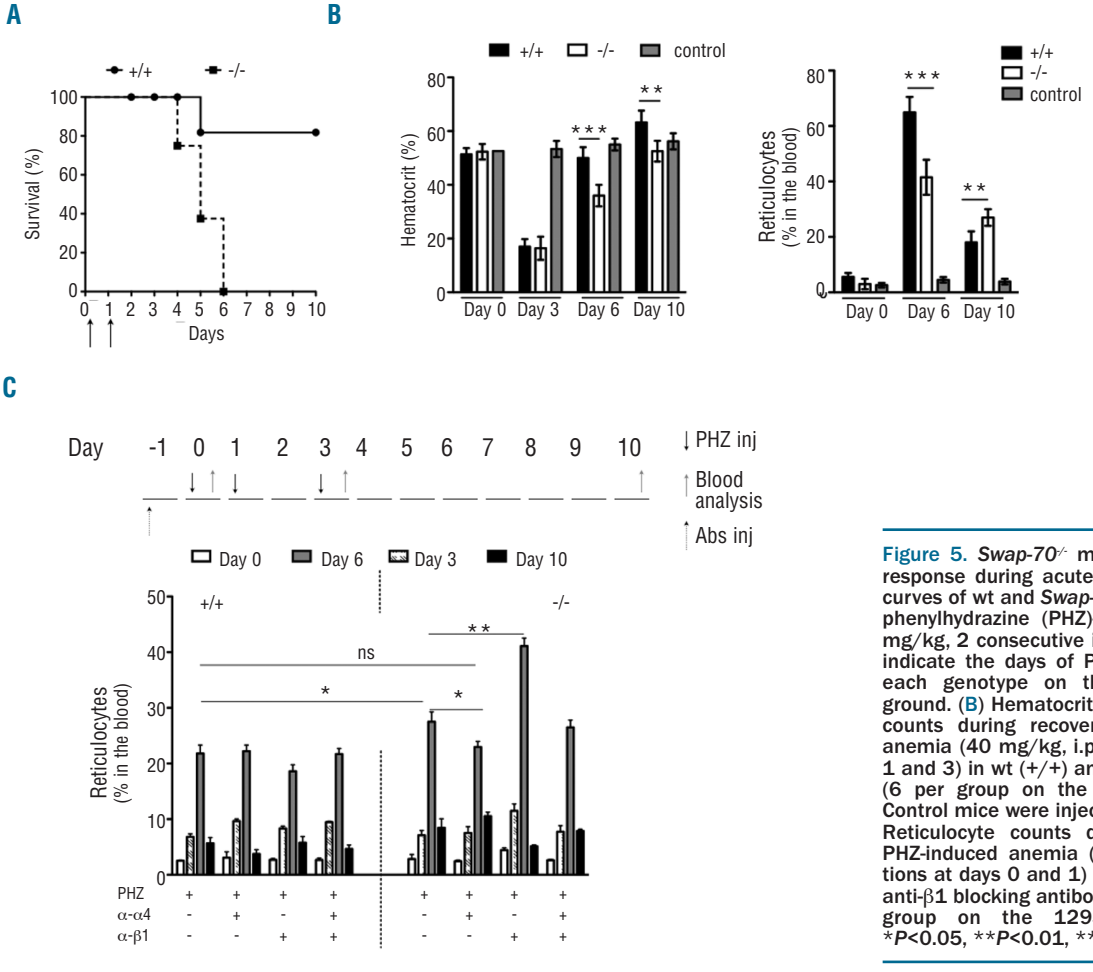


Figure 5. ***Swap-70***$^{-/-}$ **mice exhibit a delayed response during acute anemia. (A) Survival curves of wt and *Swap-70*$^{-/-}$ mice after severe phenylhydrazine (PHZ)-induced anemia (80 mg/kg, 2 consecutive i.p. injections). Arrows indicate the days of PHZ injection. n=5 for each genotype on the 129SvEMS background. (B) Hematocrit level and reticulocyte counts during recovery from PHZ-induced anemia (40 mg/kg, i.p. injections at days 0, 1 and 3) in wt (+/+) and *Swap-70*$^{-/-}$ (-/-) mice (6 per group on the C57B6 background). Control mice were injected with PBS only. (C) Reticulocyte counts during recovery from PHZ-induced anemia (40 mg/kg, i.p. injections at days 0 and 1) with prior anti-α4 and anti-β1 blocking antibody treatment. n=4 per group on the 129SvEMS background. *P<0.05, **P<0.01, ***P<0.005.**

ing integrin pairs (e.g. $\alpha_v\beta_1$, expressed on macrophages or $\alpha_5\beta_1$, expressed on erythroblasts).[27]

We also tested the necessity for SWAP-70 for proper stress-induced erythropoiesis in other settings (after lethal irradiation) and showed that it is required for efficient recovery and re-establishment of *in vivo* erythopoiesis (*Online Supplementary Figure S11*).

Discussion

Functions of SWAP-70 have so far only been investigated in cells of the immune system. In this study, we report a novel role of SWAP-70 as a regulator of integrin-mediated erythroblast transition during terminal maturation and stress-induced erythropoiesis. We demonstrate the expression of SWAP-70 in a primitive stem cell and progenitor containing fraction and myeloid-erythroid precursors (*Online Supplementary Figure S1*). Early in erythropoiesis, SWAP-70 is required at the CFU-E stage and its deficiency in *Swap-70*$^{-/-}$ mice primarily affects CFU-E in the bone marrow and spleen (Figure 1). Comparable numbers of BFU-E in wt and *Swap-70*$^{-/-}$ hematopoietic tissues may indicate a requirement for SWAP-70 either for the differentiation of BFU-E into CFU-E or, alternatively, for CFU-E survival and maintenance. Among others, c-kit and Epo signaling pathways control the development of erythroid cells at the BFU-E/CFU-E stage.[30,31] Our data show no evidence for a requirement of SWAP-70 in Epo-dependent CFU-E development when sorted wt and *Swap-70*$^{-/-}$ CFU-E were plated onto Epo-containing methylcellulose, giving rise to the same number of colonies (*data not shown*). Moreover, the differentiation of Epo-dependent early erythroblasts is not impaired in *Swap-70*$^{-/-}$ mice. However, the crosstalk between different cytokine signaling pathways (c-kit and Epo) and, for example, elements of the extracellular matrix, such as fibronectin, should be studied in more detail in future experiments. *Swap-70*$^{-/-}$ mice show reduced CFU-E but normal BFU-E frequencies similar to c-kitW/W mice [c-kit receptor natural white-spotted (W)], which fail to express the c-kit receptor on the cell surface,[32] or to kitL$^{SL/SL}$ mice, which lack the kit ligand.[33] We previously reported an involvement of SWAP-70 in c-kit-mediated and c-kit-independent adhesion and migration of mast cells on fibronectin.[18] Taking into account our mast cell data, we hypothesize that SWAP-70 might act at this stage of erythroid development by integrating and/or modulating c-kit and integrin signaling pathways, although other mechanisms should also be considered. The results of *in vitro* adhesion of CFU-E to fibronectin presented in this study strongly suggest a function of SWAP-70 as a negative regulator of integrin activity in these cells. Whether the impaired development of *Swap-70*$^{-/-}$ CFU-Es *in vivo* is caused by loss of that integrin control function remains a hypothesis, which future experiments need to test.

The reduced absolute number of Ter119$^+$ cells in the bone marrow of *Swap-70*$^{-/-}$ mice probably reflects a general decline in hematopoiesis since the relative number of these cells was not significantly affected (Figure 2). However, SWAP-70 appears to be more important for the erythroblastic development in the spleen. This could be due to different environmental conditions encountered by developing erythrocytes in their respective niches and localizations. The murine spleen features a unique microenvironment that supports extensive erythropoiesis in stress conditions[34] but suppresses steady-state erythropoiesis. An enhanced rate of apoptosis of splenic erythroblasts in comparison to bone marrow erythroblasts mediated through Fas-FasL homotypic interactions has been described[25] but is not yet fully understood. In *Swap-70*$^{-/-}$ mice, the decreased number of splenic Ter119$^+$ erythroblasts is unlikely to be due to increased apoptosis, but rather to correlate with moderately reduced proliferation as assayed by BrdU incorporation (*Online Supplementary Figures S6 and S7*). Interestingly, annexin V staining revealed even lower numbers of positive erythroblasts in the *Swap-70*$^{-/-}$ spleen. As has been described, Fas-mediated apoptosis provides a negative autoregulatory mechanism to maintain the correct number of erythroblasts in steady state.[35,36] One explanation could, therefore, be that as *Swap-70*$^{-/-}$ erythroblasts are found in lower numbers, they do not exceed homeostatic numbers and thus do not undergo as much apoptosis as wt cells. In both erythropoietic organs, the erythroblastic developmental transition is affected by SWAP-70 deficiency. In the bone marrow and spleen of *Swap-70*$^{-/-}$ mice, similar numbers of Ery.A, i.e. basophilic erythroblasts, are generated but the frequency of the following progenitor, Ery.B, is strongly reduced. However, these mice compensate for this deficiency and produce similar numbers of orthochromatic erythroblasts, reticulocytes and mature erythrocytes, thereby maintaining blood homeostasis. Is SWAP-70 required for Ery. A to Ery.B progression or does it regulate the rate of the Ery.B to Ery.C transition? Ery.B erythroblasts start to enucleate and SWAP-70 might interfere with this process, for example, by regulating F-actin dynamics and/or RhoGTPases, which are required for efficient enucleation,[37] similar to its function in mast cells.[18] However according to our data the expression of SWAP-70 terminates earlier and is very low at the Ery.B stage (*Online Supplementary Figure 1B*). The prominent function of SWAP-70 may, therefore, be during the Ery.A to Ery.B transition. The increase of Ery.C cells probably reflects a compensatory mechanism.

Eshghi *et al.* proposed a two-phase model for growth factor and extracellular matrix regulation of erythropoiesis, with an Epo-dependent, integrin-independent phase followed by an Epo-independent, $\alpha_4\beta_1$-integrin-dependent phase.[4] According to their published data, bone marrow and splenic Ery.B cells would phenotypically correspond to 2-day *in vitro*-cultured fetal liver erythroblasts that are in the $\alpha_4\beta_1$-integrin-dependent phase. Similar to CFU-E that feature preactivated $\alpha_4\beta_1$ (Figure 3), *Swap-70*$^{-/-}$ erythroblasts showed a strong increase in adhesion to frozen spleen sections, indicating more active integrins (*Online Supplementary Figure S8*). We hypothesized that perturbed adhesive interactions of developing *Swap-70*$^{-/-}$ erythroblasts might impair the developmental transition to Ery.B. We analyzed $\alpha_4\beta_1$ expression on erythroblasts, but interpret the contrasting $\alpha_4\beta_1$ pattern of bone marrow and splenic erythroblasts as a reflection of the altered erythroblastic subpopulations in *Swap-70*$^{-/-}$ mice.

Next, we interfered with the α_4 and β_1 integrin function *in vivo*. For this purpose, we treated mice with blocking antibodies and analyzed bone marrow after 24 h (Figure 4 and *Online Supplementary Figure S10*). Single antibody injection did not cause any notable change in wt, but could clearly rescue the impaired transition from Ery.B to Ery.C in *Swap-70*$^{-/-}$ bone marrow when either α_4 or β_1 chains were blocked. Of note, no significant changes in Ter119$^+$

absolute or relative cell numbers were observed. We cannot exclude, however, the possibility of side effects such as targeting cells other than erythroblasts. In our study, we tested two doses of blocking antibodies, 30 μg and 50 μg per mouse, both of them having similar effects on *Swap-70*$^{-/-}$ mice and no effect on wt mice.

We further analyzed the structure of erythroblastic islands in *Swap-70*$^{-/-}$ mice assuming that SWAP-70 might be important for their formation and integrity, which also require integrin-mediated interactions. Similar numbers of erythroblastic islands were assembled from wt and *Swap-70*$^{-/-}$ bone marrow single cell suspensions. However, their structure was compromised when *Swap-70*$^{-/-}$ cells were used, i.e. they contained fewer cells around the central macrophage and were disorganized in contrast to the flower-like structures observed with wt cells. The increased erythroblastic homotypic aggregation of *Swap-70*$^{-/-}$ cells was notable (Figure 3). These results might also reflect the altered erythroblastic subset distribution in *Swap-70*$^{-/-}$ mice, since the irregular erythroblastic islands with fewer cells might be formed with the shortage of *Swap-70*$^{-/-}$ Ery.B cells.

The requirement for SWAP-70 in steady-state conditions is not as crucial as for the quick stress response to acute anemia. Although a SWAP-70 deficiency affects early erythroid progenitors at the CFU-E stage, the erythroblastic profile and the erythroblastic island architecture, it does not influence blood homeostasis (*Online Supplementary Table S1*). Thus, the activity of a fraction of the CFU-E may suffice in steady-state conditions for the normal turnover of circulating erythrocytes. The excess CFU-E in the wt could serve as a reserve for stress conditions. In fact, BFU-E and CFU-E numbers increase dramatically 10- to 100-fold during stress erythropoiesis.[39] Notwithstanding, it is not known whether all the CFU-E progenitors are in an active differentiation state *in vivo* and contribute fully to erythropoietic homeostasis. During the response to acute anemia, a tight cooperation of cytokines and integrins is a prerequisite for an efficient response. It was previously shown that convergence of Sonic hedgehog (Shh), bone morphogenic protein 4 (BMP4), and stem cell factor-dependent signaling is necessary for the development and expansion of BMP4 responsive stress erythroid progenitors.[34] Recent studies reported combinatorial and distinct roles of α_4 and α_5 integrins in stress erythropoiesis.[11,12] We hypothesized that the reduced splenic erythroid reserve of *Swap-70*$^{-/-}$ mice and the lack of the integrin-regulatory function of SWAP-70 in erythroid progenitors might affect efficient stress responses (Figure 5). Upon severe phenylhydrazine-induced anemia with a concomitant drop of hematocrit to 10%, SWAP-70 was essential for survival. In subsequent experiments in which lower doses of phenylhydrazine were used or in which bone marrow transplantation after myeloablation was performed, a delay in the recovery of *Swap-70*$^{-/-}$ mice compared to wt mice was evident (Figure 5 and *Online Supplementary Figure 11*). This failure of Swap-70$^{-/-}$ mice to mount an efficient stress response is probably due to delayed erythrocyte production since *Swap-70*$^{-/-}$ mice ultimately recover after stress. Regardless of the level of induced anemia, the kinetics of hematocrit restoration or reticulocyte production differed significantly between wt and *Swap-70*$^{-/-}$ mice.

Similar to other "knockout" mouse models for integrin-related proteins, such as the focal adhesion kinase Fak$^{-/-}$,[7] or the conditional integrin β_1 deficient mouse,[12] the data obtained from *Swap-70*$^{-/-}$ mice suggest that SWAP-70 is required for splenic and stress-induced erythropoiesis. In addition, a recent report lists SWAP-70 among proteins present in focal adhesions, structures involved in integrin signaling modulating cell migration, growth and differentiation.[40] The findings presented here, together with the reported capability of SWAP-70 to associate with the cell membrane, its interaction with RAC1 and RHOA and its role in the modulation of F-actin cytoskeletal rearrangements, support the notion of hitherto unknown functions of SWAP-70 in regulating α_4 integrin-dependent maturation and/or development of erythroid progenitors under homeostatic or stress conditions.

Authorship and Disclosures

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