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# Maintenance of red blood cell integrity by AMP-activated protein kinase $\alpha 1$ catalytic subunit

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## Abstract

AMP-activated protein kinase (AMPK) plays a pivotal role in regulating cellular energy metabolism. We previously showed that AMPK $\alpha 1^{-/-}$  mice develop moderate anemia associated with splenomegaly and high reticulocytosis. Here, we report that splenectomy of AMPK $\alpha 1^{-/-}$  mice worsened anemia supporting evidence that AMPK $\alpha 1^{-/-}$  mice developed a compensatory response through extramedullary erythropoiesis in the spleen. Transplantation of bone marrow from AMPK $\alpha 1^{-/-}$  mice into wild-type recipients recapitulated the hematologic phenotype. Further, AMPK $\alpha 1^{-/-}$  red blood cells (RBC) showed less deformability in response to shear stress limiting their membrane flexibility. Thus, our results highlight the crucial role of AMPK to preserve RBC integrity.

## Introduction

AMP-activated protein kinase (AMPK) plays a key role in the maintenance of cellular energy homeostasis [1]. It exists as a heterotrimeric complex consisting of an  $\alpha$  catalytic subunit, and two regulatory subunits  $\beta$  and  $\gamma$ . There are a number of isoforms known for each subunit ( $\alpha1$ ,  $\alpha2$ ,  $\beta1$ ,  $\beta2$ ,  $\gamma1$ ,  $\gamma2$ ,  $\gamma3$ ) which are encoded by different genes, and which can give rise to a large variety of heterotrimeric combinations. Functional orthologues of AMPK are found throughout the eukaryotes, indicating that the function of this kinase complex is evolutionarily conserved. Genetic studies in lower eukaryotes suggest that activation of AMPK allows the organism to survive periods of starvation and could lead to subsequent extension of lifespan [2]. In multicellular eukaryotes, AMPK evolved as a mechanism sensitive to cellular energy status, as well as to hormones and cytokines to regulate whole-body energy balance and feeding behaviour [1]. In addition and beyond the regulation of energy homeostasis, AMPK could also exert non-metabolic functions such as maintenance of cell polarity and normal cell division, or control of cell growth and survival [3].

Mature red blood cells (RBC) have a finite lifespan and their production and recycling must be carefully balanced, or disease ensues. During their intravascular lifespan, RBC require energy to maintain a number of vital cell functions, including maintenance of the electrolyte gradient between plasma and red cell cytoplasm through the activity of ATP-driven membrane pumps [4]. On energy depletion, RBC undergo suicidal cell death or eryptosis characterized by cell swelling and phosphatidylserine exposure at their surface [5]. Recent observations have revealed a role for AMPK in the regulation of eryptosis during energy depletion. Indeed, *in vitro* studies demonstrated increased percentage of RBC exposing phosphatidylserine in AMPK $\alpha1^{-/-}$  mice and higher sensitivity to eryptosis following energy depletion [6]. Interestingly, the major phenotypic change observed in AMPK $\alpha1^{-/-}$  mice is a moderate anemia characterized by a general decline in RBC count, hemoglobin concentration,

hematocrit and a marked splenomegaly which points to abnormal erythropoiesis and/or decreased RBC survival [6-8].

In the present study, we address additional pathophysiological aspects of RBC homeostasis in AMPK $\alpha$ 1<sup>-/-</sup> mice. We report that splenectomized AMPK $\alpha$ 1<sup>-/-</sup> mice have significantly decreased RBC counts and hemoglobin concentration indicating compensatory extramedullary erythropoiesis in the spleen. In addition, AMPK $\alpha$ 1<sup>-/-</sup> bone marrow transplantation experiments confirm the hematologic phenotype in relation to blood parameters and the function of AMPK in the adaptive responses of RBC homeostasis. Finally, we demonstrate that AMPK $\alpha$ 1<sup>-/-</sup> RBC are less deformable in response to shear stress suggesting that AMPK $\alpha$ 1 may have a role in controlling structural flexibility and integrity of RBC.

## **Materials and methods**

*Mouse studies.* AMPK $\alpha$ 1<sup>-/-</sup> mice on a 129Sv and C57BL/6 mixed background were generated and genotyped as described previously [9,10]. All procedures were performed in accordance with ethical treatment standards established by the European Convention for the Protection of Laboratory Animals (Council of Europe, ETS 123. 1991).

*Splenectomy.* Splenectomy was performed on 8 week-old female mice anesthetized with methoxyfluorane. An incision was made in the left flank, and the spleen was isolated and removed after appropriate blood vessel ligation.

*Bone marrow transplantation.* AMPK $\alpha$ 1<sup>-/-</sup> mice backcrossed to the C57BL/6 background for

5 generations were used for these experiments. Chimera were generated by irradiating CD45.1<sup>+</sup> mice with 2 doses of 600 cGy and immediately reconstituting them with 4x10<sup>6</sup> CD45.2<sup>+</sup> bone marrow cells isolated from the femur and tibia of WT, AMPK $\alpha$ 1<sup>+/-</sup> or AMPK $\alpha$ 1<sup>-/-</sup> mice. Hematological parameters of recipient mice were analyzed 10 weeks after bone marrow transfer.

*Analysis of peripheral blood and tissue iron measurements.* Complete blood cell tests were performed using an ADVIA 120 hematology analyzer (Bayer). Measurements of bilirubin were executed at the Centre d'Explorations Fonctionnelles Intégré (Institut Claude Bernard, IFR2, Paris) using standard assays. Tissue iron concentrations were determined as previously described [11].

*Scanning electron microscopy.* Washed RBC were incubated with 2% glutaraldehyde and 2% paraformaldehyde in 100 mM cacodylate buffer (320-340 mOsm, pH 7.2) for 15 min. RBC were deposited onto polylysine-coated glass cover slips, critical point-dried in CO<sub>2</sub>, sputter coated with platinum-paladium by standard techniques, and screened with Gemini Zeiss scanning electron microscope (University Paris Diderot, Paris).

*Measurements of RBC deformability and osmotic fragility.* RBC deformability was measured using a Laser Assisted Optical Rotational Cell Analyzer (LORCA, Mechatronics, The Netherlands). The change in the laserbeam diffraction pattern was detected while RBC were subjected to increasing values of applied shear rate (0.3 to 30 Pa) and a measure of the extent of cell deformation was calculated as the deformability index. To test cell fragility, RBC were incubated in hypotonic solutions for 45 min and percent lysis was calculated from the absorbance at 540 nm.

*Statistical analysis.* Results are expressed as means  $\pm$  SEM. Comparisons between groups were made by unpaired two-tailed Student's *t* test. Differences were considered statistically significant if  $P < 0.05$ .

## **Results**

*AMPK $\alpha$ 1<sup>-/-</sup> mice display a compensated anemia associated with splenomegaly.*

The AMPK $\alpha$ 1 subunit is the only AMPK catalytic isoform present in murine (Figure 1A) and human (data not shown) RBC. Absence of AMPK $\alpha$ 1 in mice results in a hemolytic disorder [6,7]. AMPK $\alpha$ 1<sup>-/-</sup> RBC exhibit prominent anisocytosis, which coincides with an increase in both the width of the RBC volume distribution (RDW) and the number of circulating reticulocytes [7]. We have confirmed here, using scanning electron microscopy that AMPK $\alpha$ 1<sup>-/-</sup> RBC population is heterogenous with no significant morphological abnormalities (Figure 1B).

Another feature of AMPK $\alpha$ 1<sup>-/-</sup> mice that may represent primary or secondary effects of the absence of AMPK $\alpha$ 1 is a severe splenomegaly [6-8]. Hence, we analyzed erythropoiesis in WT and AMPK $\alpha$ 1<sup>-/-</sup> mice by measuring the frequency of the different erythroblast subpopulations in the spleen and bone marrow. A dramatic increase in the early erythroblast

population was observed in the spleen (Suppl Figure 1A and B) and bone marrow (Suppl Figure 2A and B) from AMPK $\alpha$ 1<sup>-/-</sup> mice suggesting a rapid turnover of erythroid cells and a compensatory increase in erythropoiesis in the absence of AMPK $\alpha$ 1.

*Splenectomized AMPK $\alpha$ 1<sup>-/-</sup> mice exhibit a more severe anemia.*

It was previously reported that AMPK $\alpha$ 1<sup>-/-</sup> RBC have enhanced phosphatidylserine exposure and reduced half-life, suggesting that the anemia in AMPK $\alpha$ 1<sup>-/-</sup> mice may be secondary to their removal by the spleen macrophages [6,7]. To test whether AMPK $\alpha$ 1<sup>-/-</sup> deficient RBC accumulated specifically into the spleen, AMPK $\alpha$ 1<sup>-/-</sup> mice were splenectomized and hematological parameters were evaluated 40 and 90 days after. Splenectomy was followed by a dramatic decrease in RBC count, hematocrit and hemoglobin concentration in AMPK $\alpha$ 1<sup>-/-</sup> but not in control mice (Figure 2). Further, splenectomized AMPK $\alpha$ 1<sup>-/-</sup> mice sustained diminished reticulocyte numbers as compared to baseline but largely increased as compared to WT mice (Figure 2), indicating that splenic extramedullary hematopoiesis plays a compensatory role in response to chronic anemia.

*Transfer of the erythroid phenotype of AMPK $\alpha$ 1<sup>-/-</sup> mice after bone marrow transplantation.*

Lethally irradiated congenic WT recipients were transplanted with bone marrow cells from AMPK $\alpha$ 1<sup>-/-</sup>, AMPK $\alpha$ 1<sup>+/-</sup> or WT mice and resulted in a complete repopulation of the bone marrow with the donor red cells (data not shown). WT recipients mice transplanted with bone marrow cells from AMPK $\alpha$ 1<sup>-/-</sup> mice but not WT and AMPK $\alpha$ 1<sup>+/-</sup> mice, demonstrated marked splenomegaly 10 weeks post-transplantation (Figure 3A). Histologic analysis revealed that the red pulp was markedly expanded in the spleen of mice transplanted with AMPK $\alpha$ 1<sup>-/-</sup> bone marrow cells compared to that of mice transplanted with WT bone marrow cells (Figure 3B). We next examined the accumulation of iron in the spleen, which is generated by the



metabolism of heme. Prussian blue stainings of spleen sections depicted an increased iron accumulation in the spleen of mice transplanted with AMPK $\alpha$ 1<sup>-/-</sup> bone marrow cells (fourfold increase) compared to those transplanted with WT bone marrow cells (Figure 3B and 3C). This was also true of the liver, where in mice transplanted with AMPK $\alpha$ 1<sup>-/-</sup> bone marrow cells, there was a 2-fold increase in iron deposition (Figure 3D). We next analyzed the bilirubin levels in the blood plasma to determine a possible effect on RBC turnover. Indeed, unconjugated bilirubin showed higher levels in the plasma of mice transplanted with AMPK $\alpha$ 1<sup>-/-</sup> bone marrow cells ( $1.37 \pm 0.13 \mu\text{mol/l}$  in mice transplanted with AMPK $\alpha$ 1<sup>-/-</sup> bone marrow cells, versus  $0.35 \pm 0.05 \mu\text{mol/l}$  in mice transplanted with WT bone marrow cells,  $n = 3-4$ ;  $P < 0.001$ ), consistent with peripheral destruction of RBC. These results indicate that the anemia apparent in mice transplanted with AMPK $\alpha$ 1<sup>-/-</sup> bone marrow cells could be attributed, at least in part, to an increased rate of uptake and subsequent clearance of abnormal circulating RBC by the spleen.

Furthermore, lethally irradiated WT mice that were transplanted with hematopoietic cells derived from AMPK $\alpha$ 1<sup>-/-</sup> bone marrow showed significantly reduced RBC count, hematocrit and hemoglobin concentration, and also increased RDW and reticulocytosis compared to control recipients injected with either WT or AMPK $\alpha$ 1<sup>+/-</sup> bone marrow cells (Figure 4A). Interestingly, RBC from mice transplanted with AMPK $\alpha$ 1<sup>-/-</sup> bone marrow cells also displayed the same magnitude and spectrum of osmotic resistance (Figure 4B) as previously reported for AMPK $\alpha$ 1<sup>-/-</sup> RBC [6,7]. Hence, the hematologic phenotype of AMPK $\alpha$ 1<sup>-/-</sup> mice was clearly recapitulated in terms of blood parameters and functional properties of RBC, suggesting that AMPK $\alpha$ 1 plays an intrinsic role in RBC homeostasis.

*AMPK $\alpha$ 1 is important for red cell membrane deformability.*

We assessed RBC deformability by using a LORCA and determined the deformation index in relation to the shear stress applied on the RBC membrane surface. RBC deformability was significantly altered in AMPK $\alpha$ 1<sup>-/-</sup> mice compared to WT mice for the majority of shear stress rates studied (Figure 5). Interestingly, AMPK $\alpha$ 1 was associated to the red cell membrane as it is present in RBC ghost membrane preparations (Figure 1A), consistent with confocal microscopy analysis of human RBC stained with AMPK antibodies [6]. These findings indicate that AMPK $\alpha$ 1 could have a critical role in the control of membrane flexibility of RBC and in the microcirculation when the RBC must enter into narrow capillaries.

## **Discussion**

Our results show that anemia in AMPK $\alpha$ 1<sup>-/-</sup> mice is not the result of impaired proliferation or maturation of the erythroid lineage as erythropoiesis is increased in both the spleen and bone marrow of AMPK $\alpha$ 1<sup>-/-</sup> mice (Suppl Figures 1 and 2). This enhanced stimulation of erythropoiesis in AMPK $\alpha$ 1<sup>-/-</sup> mice is correlated with elevated concentration of plasma erythropoietin [6,7]. In line with these observations, histological analysis of AMPK $\alpha$ 1<sup>-/-</sup> spleen showed abnormal expansion of the red pulp with a significant increase in the number of erythroid precursors, indicating enhanced erythroid extramedullary erythropoiesis [7,8]. It is therefore likely that the increase in spleen size is primarily a compensatory response to the anemia of AMPK $\alpha$ 1<sup>-/-</sup> mice with a vigorous splenic erythropoiesis. Indeed, AMPK $\alpha$ 1<sup>-/-</sup> mice depend on the spleen for adequate erythropoiesis as revealed by the worsening anemia in splenectomized AMPK $\alpha$ 1<sup>-/-</sup> mice (Figure 2). Furthermore, iron accumulation in the spleen of AMPK $\alpha$ 1<sup>-/-</sup> mutant mice is markedly increased [7], indicating that enlargement of the spleen can also be attributed to an increased

rate of uptake and subsequent clearance of circulating AMPK $\alpha$ 1<sup>-/-</sup> RBC by the spleen. Previous studies demonstrated that AMPK $\alpha$ 1<sup>-/-</sup> RBC have increased phosphatidylserine exposure at their surface and must create a situation favorable to erythrophagocytosis, leading to shortened lifespan [6,7]. All together, these results suggest that the splenomegaly of AMPK $\alpha$ 1<sup>-/-</sup> mice is caused by both enhanced erythrophagocytosis clearance of AMPK $\alpha$ 1<sup>-/-</sup> RBC and expansion of extramedullary erythropoiesis in the spleen.

In order to demonstrate the cell autonomous role of AMPK $\alpha$ 1 in the RBC in the setting of anemia, bone marrow transplantation of AMPK $\alpha$ 1<sup>-/-</sup> bone marrow into WT congenic recipients was performed. Chimeric mice were found to manifest splenomegaly with decreased RBC count, hematocrit, hemoglobin concentration (Figure 4A) demonstrating an intrinsic role for AMPK $\alpha$ 1 in RBC. This was further confirmed by osmotic resistance observed with RBC from WT recipients mice transplanted with bone marrow cells from AMPK $\alpha$ 1<sup>-/-</sup> mice (Figure 4B).

In the circulation, RBC are highly pleomorphic and adopt a broad spectrum of shapes, an important characteristic for maintaining the survival of RBC under high shear conditions in the vascular system. Remarkably, AMPK $\alpha$ 1<sup>-/-</sup> RBC are less deformable in response to shear stress than control RBC indicating limited structural flexibility in the absence of AMPK $\alpha$ 1 (Figure 5). These findings are suggestive of hemolytic anemia phenotype caused by the absence of AMPK $\alpha$ 1 activity in RBC leading to a rigidification of the cell membrane that possibly affects diffusion into narrow capillaries and consequently, RBC homeostasis. Putative AMPK substrate proteins in RBC were recently identified [12], including the band 3 protein from the red cell membrane and enzymes involved in the oxidative stress response, which may contribute to the molecular defects associated with the increased membrane rigidity of AMPK  $\alpha$ 1<sup>-/-</sup> RBC [7].

In conclusion, impaired function of AMPK $\alpha$ 1 induces sufficient alterations in RBC structural flexibility to affect their lifespan and to incurably provoke their elimination from circulation.

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### Figure legends

**Figure 1:** RBC from AMPK  $\alpha 1^{-/-}$  mice lack AMPK  $\alpha$ -subunit expression and show normal morphology. (A) Expression of AMPK $\alpha 1$  and  $\alpha 2$  catalytic subunits in murine skeletal muscle, liver, RBC and RBC ghost membrane preparations. (B) Scanning electron microscopy of RBC from WT (left) and AMPK $\alpha 1^{-/-}$  mice (right). The scale bar represents 4  $\mu\text{m}$ .

**Figure 2:** Consequence of splenectomy in WT and AMPK $\alpha 1^{-/-}$  mice. RBC count, Hb concentration, hematocrit and reticulocyte numbers at baseline (b) and 40 and 90 days after splenectomy in WT and AMPK $\alpha 1^{-/-}$  mice. Results are expressed as means  $\pm$  SEM (n=7-8); \* p< 0.05; \*\* p< 0.01; # p< 0.001 compared to AMPK $\alpha 1^{-/-}$  baseline and \$ p<0.0001 compared to WT mice on the respective day after splenectomy.

**Figure 3:** Transfer of the erythroid phenotype of AMPK $\alpha 1^{-/-}$  mice after bone marrow cells transplantation from mutant mice into lethally irradiated WT recipients. (A) Comparison of

spleen size in representative WT recipients transplanted with bone marrow cells derived from WT (left), AMPK $\alpha$ 1+/- (middle) or AMPK $\alpha$ 1-/- (right) mice. (B) Perls' iron stained section of spleen from representative WT recipients transplanted with bone marrow cells derived from WT (left), AMPK $\alpha$ 1+/- (middle) or AMPK $\alpha$ 1-/- (right) mice. Original magnification, x100. (C) Iron content in the spleen of WT recipients transplanted with bone marrow cells derived from WT (black bar), AMPK $\alpha$ 1+/- (dashed bar) or AMPK $\alpha$ 1-/- (empty bar) mice. Results are expressed as means  $\pm$  SEM (n=5-6); # p<0.001 compared to WT mice; § p<0.001 compared to AMPK $\alpha$ 1+/- mice. (D) Iron content in the liver of WT recipients transplanted with bone marrow cells derived from WT (black bar), AMPK $\alpha$ 1+/- (dashed bar) or AMPK $\alpha$ 1-/- (empty bar) mice. Results are expressed as means  $\pm$  SEM (n=5-6); # p<0.001 compared to WT; § p<0.001 compared to AMPK $\alpha$ 1+/- mice.

**Figure 4:** Hematologic parameters and functional properties of RBC after bone marrow cells transplantation from AMPK $\alpha$ 1-/- mice into lethally irradiated WT recipients. (A) Hematologic parameters in WT mice transplanted with bone marrow cells derived from WT (black bar), AMPK $\alpha$ 1+/- (dashed bar) or AMPK $\alpha$ 1-/- (empty bar) mice. Results are expressed as means  $\pm$  SEM (n=7-8); \*\* p< 0.01; # p<0.001 compared to WT mice; °° p< 0.01; § p<0.001 compared to AMPK $\alpha$ 1+/- mice (n=5-6). (B) Osmotic resistance of RBC from WT recipients transplanted with bone marrow cells derived from WT, AMPK $\alpha$ 1+/- or AMPK $\alpha$ 1-/- mice. Peripheral blood was exposed to different concentration of NaCl (%) and hemolysis was assessed by spectrometry of supernatant. Results are expressed as means  $\pm$  SEM (n=5-6); \* p< 0.05; \*\* p<0.01; # p<0.001 compared to WT mice; ° p<0.05; °° p< 0.01; § p<0.001 compared to AMPK $\alpha$ 1+/- mice.

**Figure 5:** Reduced deformability of AMPK $\alpha$ 1<sup>-/-</sup> RBC. Deformability profile of RBC from WT (black bar) and AMPK $\alpha$ 1<sup>-/-</sup> (empty bar) mice in relation to the shear stress (0.3 to 50 Pa). Results are expressed as means  $\pm$  SEM (n=6); \* p< 0.05; \*\* p<0.01; # p<0.001 compared to WT mice.

### Supplemental figure legends

**Supplemental Figure 1:** Splenic erythropoiesis in AMPK $\alpha$ 1<sup>-/-</sup> mice. (A) Flow cytometry analysis of the erythroid surface markers CD71 and Ter119 expression in the spleen from the WT (top) and AMPK $\alpha$ 1<sup>-/-</sup> (bottom) mice. (B) Percentage of spleen cells sorted from the ProE, Ter119, Ery.A, B and C subsets in WT (black bar) and AMPK $\alpha$ 1<sup>-/-</sup> (empty bar) mice. Results are expressed as means  $\pm$  SEM (n=6-8); \*\* p<0.01; # p<0.001 compared to control mice.

**Supplemental Figure 2:** Bone marrow erythropoiesis in AMPK $\alpha$ 1<sup>-/-</sup> mice. (A) Flow cytometry analysis of the erythroid surface markers CD71 and Ter119 expression in the bone marrow from the WT (top) and AMPK $\alpha$ 1<sup>-/-</sup> (bottom) mice. (B) Percentage of bone marrow cells sorted from the ProE, Ter119, Ery.A, B and C subsets in WT (black bar) and AMPK $\alpha$ 1<sup>-/-</sup> (empty bar) mice. Results are expressed as means  $\pm$  SEM (n=6-8); \* p<0.05 compared to control mice.

Figure 1

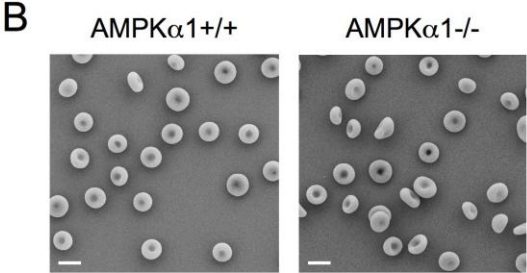
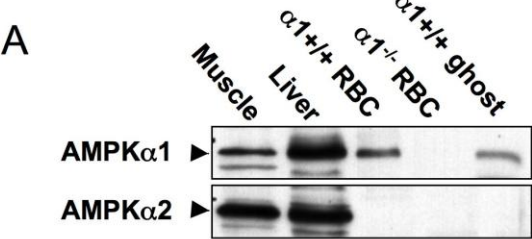




Figure 2

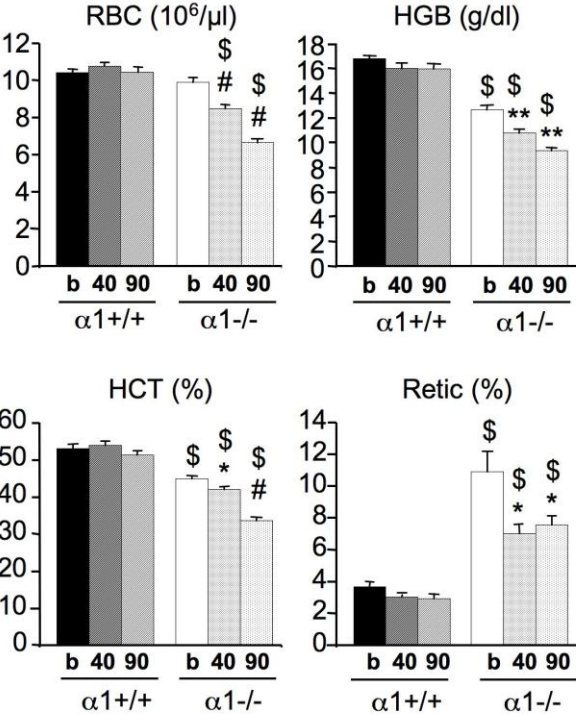


Figure 3

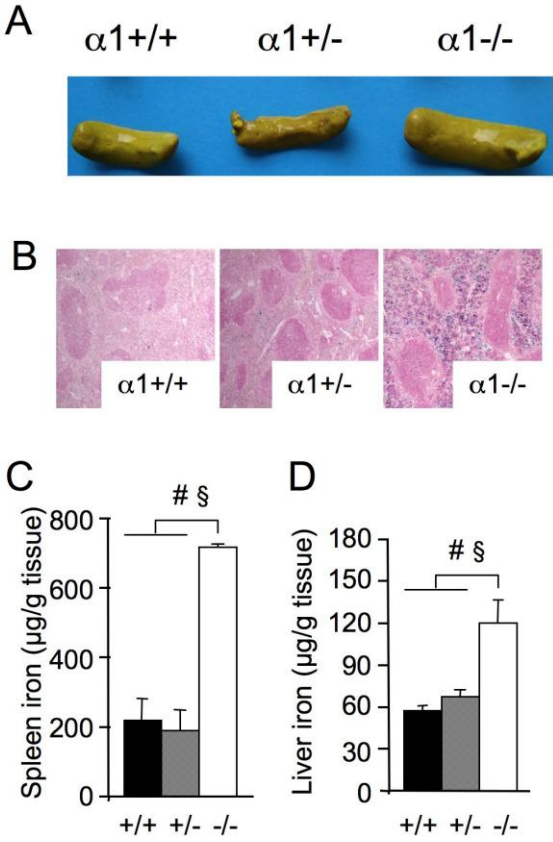


Figure 4

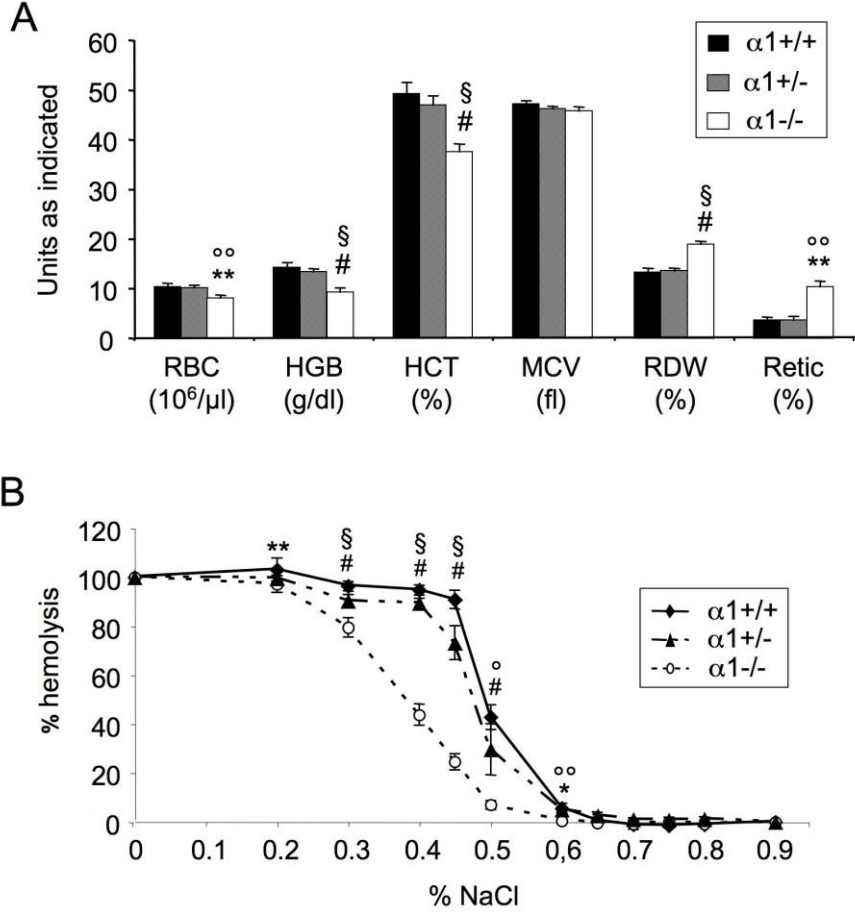
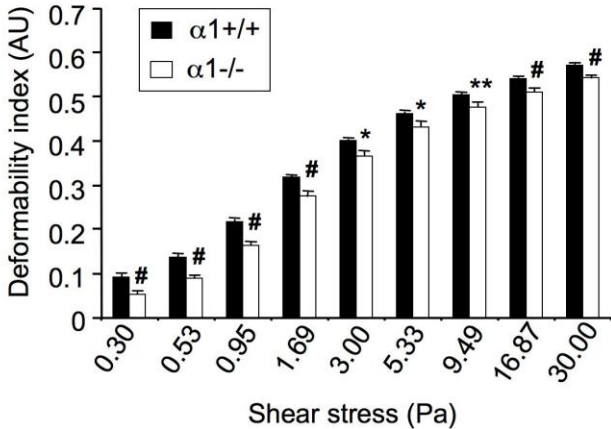
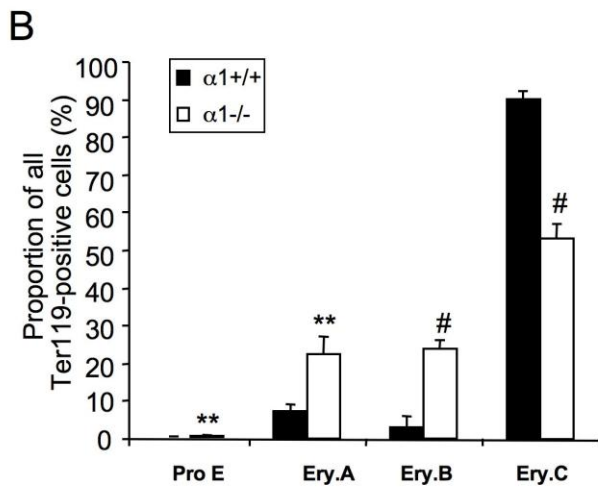
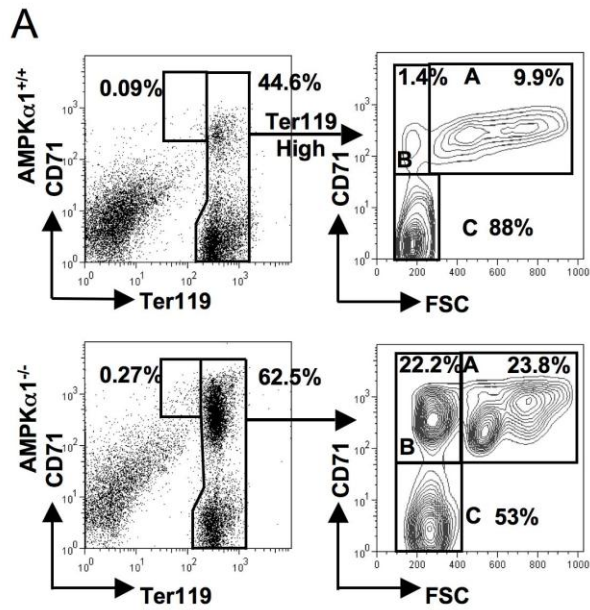


Figure 5



# Supplemental Figure 1



## Supplemental Figure 2

