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Citation for published version:
Cambridge, EL, McIntyre, Z, Clare, S, Isherwood, C, Arends, M, Goulding, D, Caetano, S, Ballesteros Revirego, C, Kane, L, Harcourt, K, Sanger Mouse Genetics Project, T, Adams, DJ, White, JK & Speak, AO 2016, 'The AMP-activated protein kinase beta 1 subunit modulates murine erythrocyte development' Experimental Hematology. DOI: 10.1016/j.exphem.2016.09.006

Digital Object Identifier (DOI):
10.1016/j.exphem.2016.09.006

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Experimental Hematology

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Download date: 05. Apr. 2019

The AMP-activated protein kinase beta 1 subunit modulates erythrocyte integrity

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Running title: Prkab1 modulates erythrocyte integrity

Keywords: Anemia, RBC membrane, knockouts, osmotic fragility

Introduction

Erythrocytes are enucleated, terminally differentiated cells with a finite lifespan and an estimated turnover of 1% every day. In order to deal with stress, haemolysis and/or hypoxia, the production of erythrocytes can be substantially modulated. *In vivo* control of erythrocyte survival is affected by many factors including energy balance, maintenance of electrolyte gradients and control of reactive oxygen species. Alterations to erythrocyte membrane deformability has a major role in regulating cellular function and intravascular survival with reduced deformability resulting in splenic sequestration of abnormal cells, shortened half-life and the clinical presentation of hemolytic anemia [1].

The evolutionary conserved serine/threonine kinase AMP-activated protein kinase (AMPK) is a critical regulator of energy balance [2, 3]. AMPK is a heterotrimeric complex containing a catalytic alpha subunit paired with beta and gamma regulatory subunits. There are several isoforms for each subunit encoded by separate genes, two alpha (*Prkaa1* and *Prkaa2*), two beta (*Prkab1* and *Prkab2*) and three gamma (*Prkag1, Prkag2* and *Prkag3*). *Prkaa1* and *Prkag1* can control oxidative stress, erythrocyte-intrinsic cellular metabolic stress and membrane elasticity, making them critical regulators of erythrocyte integrity and lifespan [4-7]. However, the specific role of beta subunit isoforms in the context of erythrocyte development has not been studied.

Here we report that *Prkab1* deficient mice present with splenomegaly, increased splenic iron deposits, microcytic anemia, compensatory extramedullary hematopoiesis, altered erythrocyte morphology and increased erythrocyte osmotic resistance.

Materials and method

Mice

Generation of *Prkab1*$^{tm1b(KOMP)Wtsi}$ (hereafter referred to as *Prkab1*^{tm1b}) mice was performed using ES cell clone EPD0033_3_C09. Genotyping carried out according to [8] with cre conversion as reported [9]. All experiments were performed in accordance with the UK Home Office regulations, UK Animals (Scientific Procedures) Act 1986 and approved by the Wellcome Trust Sanger Institute animal welfare and ethical review body.

Gene expression analysis

RNA was extracted from spleens using Purelink® RNA mini kit (Ambion). Gene expression was assessed using FAM-conjugated TaqMan® assays as listed in the supplementary methods. Template RNA was added in duplex reactions in triplicate using *B2m* VIC primer limited probe (Mm00437762_m1) as the endogenous control using the EXPRESS One-Step Superscript® qRT-PCR Kit (Thermo Scientific) and an Applied Biosystems 7900HT analyser. Relative gene expression between endogenous control and target gene was analysed using the ΔΔCT method [10] with RQ manager (Life Technologies) applying automatic thresholds.

Western blot analysis

Protein lysates were prepared from spleens with protein quantification, electrophoresis, transfer and antibody incubations performed according to standard protocols. Blots were visualised using HRP-conjugated secondary antibodies and ECL reagents then imaged with a LAS 4000 (GE Healthcare). Primary antibodies used: AMPK beta 1 (1/1000, #12063), AMPK beta 2 (1/1000, #4148), AMPK pan alpha (all Cell Signalling Technology, 1/1000, F6 #2793) and vinculin (Sigma, 1/5000, V284).

Blood collection and analysis

Retro-orbital or tail vein blood was collected into EDTA-coated tubes for haematology or heparinised tubes for plasma preparation. Complete blood counts were determined using a Scil Vetabc system. Plasma was analysed for bilirubin, iron,

and ferritin using an Olympus AU400 analyser (Beckman Coulter Ltd) with reagents supplied by Beckman Coulter or Randox. Erythropoietin was determined using a Meso Scale Discovery array.

Histological analysis

Spleen, liver and leg bones were fixed in formalin, embedded in paraffin and sections stained with haematoxylin and eosin or Perls' Prussian blue according to standard methods. These were assessed in a blinded manner for any pathological abnormalities. Scanning electron microscopy (SEM) was performed as previously described [11] with erythrocytes adhered to poly-l-lysine coated coverslips.

Erythropoiesis analysis

Staining of single cell suspensions of spleen, bone marrow and whole blood with CD71, Ter119, CD45, Syto® 16 and Sytox® blue was performed as previously described [12] and analysed on a BD™ LSRII instrument (full details in supplementary methods).

In vivo clearance of erythrocytes

This was performed as described previously [4] with the exception that samples were labelled with either 10 μM Vybrant® CFDA (*Prkab1*$^{+/+}$) or 1 μM CellTracker™ Deep red (*Prkab1*$^{tm1b/tm1b}$, both Molecular Probes). Erythrocytes were counted and adjusted to $2x10^6$ RBC/μl and the two genotypes pooled and injected via the tail vein into recipient mice (10 weeks old) to transfuse $2x10^8$ RBC/genotype (full details in supplementary methods).

Osmotic resistance assay

This was performed essentially as described [4] with haematocrit adjusted to 0.8% with 0.9% saline solution.

Statistical analysis

All data was analysed in Prism v6 (Graph Pad) and analysed with an unpaired two-tailed students t test, Mann Whitney test or two way ANOVA as indicated in the figure legend.

Results and discussion

Prkab1$^{tm1b/tm1b}$ mice showed greatly reduced expression of *Prkab1* that was accompanied by a significant (possibly compensatory) increase in *Prkaa1* and *Prkag1* (Supplementary Fig. 1A). This was confirmed by immunoblot analysis which supports observations from *Prkag1* knockout mice [4] and another *Prkab1* knockout mouse line [13] that genetic deletion of one part of the AMPK heterotrimeric complex results in protein dysregulation of other parts of the complex, as there was no detectable alpha protein (pan-AMPK alpha antibody) in *Prkab1*$^{tm1b/tm1b}$ spleen lysates (Supplementary Fig. 1B).

At 16 weeks of age, *Prkab1*$^{tm1b/tm1b}$ mice had significantly reduced haemoglobin (Fig. 1A) and haematocrit (Fig. 1B). A reduction in erythrocyte number (Fig. 1C) and mean corpuscular haemoglobin concentration (Fig. 1D) was only observed in a sex-specific manner, however, erythrocytes in *Prkab1*$^{tm1b/tm1b}$ mice were significantly smaller (Fig. 1E) with an increased red blood cell distribution width (Fig. 1F) in both sexes. These altered erythrocyte indices indicate a microcytic anemia with anisocytosis, similar to that reported in mice deficient in *Prkaa1* or *Prkag1* [4-7]. The leukocyte lineage was unaffected by deletion of *Prkab1* (Supplementary Fig. 1C) and there were no differences in the circulating platelet count (Supplementary Fig. 1D). However, there was an increase in the size of the platelets in both sexes (Supplementary Fig. 1E). At 4 and 6 weeks of age the anemia was normocytic (Supplementary Fig. 2A-G and data not shown).

Scanning electron microscopy confirmed anisocytosis with erythrocytes from *Prkab1*$^{tm1b/tm1b}$ mice showing variable appearances; features of acanthocytes, schistocytes, stomatocytes and echinocytes (Fig. 1G). We then determined the osmotic resistance with *Prkab1*-deficient erythrocytes having a left-shifted curve indicative of increased osmotic resistance (Fig. 1H) in agreement with the previously observed findings in *Prkaa1* and *Prkag1*-deficient mice [4-7].

At necropsy, *Prkab1*$^{tm1b/tm1b}$ mice presented with splenomegaly (Fig. 2A-B), although not to the same degree as *Prkag1*$^{-/-}$ and *Prkaa1*$^{-/-}$ mice [4-7]. We determined the level of total bilirubin in the plasma, an indicator for erythrocyte destruction, and although increased in *Prkab1*$^{tm1b/tm1b}$ mice this did not reach significance in any of the cohorts tested (Fig. 2C). *Prkab1*$^{tm1b/tm1b}$ spleens showed an expansion of the

peripheral red pulp due to increased extramedullary hematopoiesis and increased red cell breakdown with haemosiderin in the red pulp (Fig. 2D). Hemolytic anemia often results in changes in tissue iron deposits and we found a significant increase in splenic iron deposits in *Prkab1*$^{tm1b/tm1b}$ mice (Fig. 2E) with a concomitant increase in circulating levels of ferritin (Supplementary Fig. 3A) and decrease in iron concentration (Supplementary Fig. 3B). Circulating erythropoietin was significantly increased in *Prkab1*$^{tm1b/tm1b}$ mice (Fig. 2F), as was the percentage of reticulocytes (Fig. 2G).

There was an increase in the ratio of Ter119^{+} to CD45^{+} cells in the spleen (Fig. 2H) as well as an increase in percentage of erythroblasts (Fig. 2I) and reticulocytes, with a concomitant decrease in mature erythrocytes (Fig. 2J). The bone marrow showed a reduction in adipocytes of the marrow stroma and a mild hematopoietic hyperplasia with a mild increase in the erythroid subsets (Supplementary Fig. 3C-E). These observations would suggest a reactive increase in erythroid hematopoiesis in both bone marrow and spleen in response to the observed hemolytic anemia. A similar hemolytic anemia with compensatory extramedullary hematopoiesis has been found in *Prkaa1* and *Prkag1*-deficient mice [4-7].

Previous studies on *Prkag1*$^{-/-}$ and *Prkaa1*$^{-/-}$ mice have demonstrated that deficiency in either gene results in a decreased half-life *in vivo* [4, 5, 7]. Via adoptive transfer of fluorescently labelled erythrocytes we observed no difference in the half-life of *Prkab1*$^{tm1b/tm1b}$ erythrocytes when transferred into wild type mice compared to the co-transferred wild type erythrocytes (Supplementary Fig. 3F) or when transferred into *Prkab1*$^{tm1b/tm1b}$ mice (Supplementary Fig. 3G). However, we cannot rule out the possibility that the method employed skews the analysis if the *ex vivo* fluorescent labelling preferentially occurs in "normal" erythrocytes given the heterogeneous morphological alterations to the erythrocytes in *Prkab1*$^{tm1b/tm1b}$ mice.

In summary we report a key role for the AMPK beta 1 subunit in erythrocyte development similar to that observed for alpha 1 and gamma 1 subunits. Deletion of *Prkab1* resulted in regenerative hemolytic anemia, splenomegaly and splenic iron deposition with enhanced erythropoiesis in the spleen and to a lesser extent bone marrow. Erythrocytes from deficient mice presented with multiple morphological alterations and an increased osmotic resistance.

Acknowledgements

This work was funded by the Wellcome Trust (grant number: WT098051). The authors wish to thank Yvette Hooks, Wellcome Trust Sanger Institute, for histology technical support and Professor Elspeth Milne and Dr Paola Cazzini, Royal (Dick) School for Veterinary Studies, University of Edinburgh, for expert opinions on murine red blood cell morphology

Author contributions

ELC, ZM, SC, MJA, DAG, CI, SC, CBR, AS, LK, KH, The Sanger Mouse Genetics Project and AOS generated the data. ELC, ZM, MJA, DAG, CI and AOS analysed the data. The Sanger Mouse Genetics Project generated, genotyped and phenotyped the mice, DJA, JKW and AOS led the project. AOS wrote the manuscript with contributions from all authors.

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Figure legends

Figure 1 *Prkab1* deficient mice present with anemia, erythrocyte morphological abnormalities and increased erythrocyte osmotic resistance

A) hemoglobin, B) hematocrit, C) red blood cell count, D) mean corpuscular hemoglobin concentration, E) mean corpuscular volume and F) red blood cell distribution width of 16 week old $Prkab1^{+/+}$ and $Prkab1^{tm1b/tm1b}$ mice * P<0.05, ** P<0.01, *** P<0.001 and **** P<0.0001 unpaired two-tailed students t test; G) representative SEM images of erythrocytes from $Prkab1^{+/+}$ and $Prkab1^{tm1b/tm1b}$ mice; H) osmotic resistance of $Prkab1^{+/+}$ and $Prkab1^{tm1b/tm1b}$ erythrocytes (combined males and females), ****P<0.0001 as determined by a repeated measures 2-way ANOVA with Sidak's multiple comparisons test adjusting for multiple testing, insert derived % of NaCl for 50% hemolysis of erythrocytes **** P<0.0001 unpaired two-tailed students t test. All data representative of three independent experiments or two mice for SEM analysis, each symbol represents an individual mouse with the line at the mean except for H) where n = 10 for $Prkab1^{+/+}$ and n = 9 for $Prkab1^{tm1b/tm1b}$ with mean ± standard error of the mean.

Figure 2 *Prkab1* deficient mice have splenomegaly, extramedullary hematopoiesis and splenic iron deposits

A) spleen weight B) spleen/body weight ratio (mg/g); C) plasma bilirubin concentration; D) H and E stained sections of spleen (100x magnification); E) Perls' stained sections of spleen (100x magnification); F) plasma erythropoietin; G) circulating reticulocyte %; H) splenic erythroid (Ter119)/Leukocyte (CD45) ratio; I) splenic erythroblast %; J) splenic reticulocyte and erythrocyte %. For all * P<0.05, ** P<0.01, *** P<0.001 and **** P<0.0001 unpaired two-tailed students t test except for spleen/body weight ratio and Ter119/CD45 ratio which were analysed with a Mann Whitney test. All data is representative of three independent experiments or four mice for histology analysis, each symbol represents an individual mouse with the line at the mean.

Supplementary Figure 1 molecular and phenotypic characterisation of *Prkab1* deficient mice

A) mean gene expression of AMPK subunits in *Prkab1*$^{+/+}$ and *Prkab1*$^{tm1b/tm1b}$ spleen RNA n = 5 per genotype with error bars illustrating standard error of the mean; B) Immunoblot analysis of AMPK subunits in *Prkab1*$^{+/+}$ and *Prkab1*$^{tm1b/tm1b}$ spleen protein lysates * IgG heavy chain; C) platelet count, D) mean platelet volume, E) white blood cell count of 16 week old *Prkab1*$^{+/+}$ and *Prkab1*$^{tm1b/tm1b}$ mice * P<0.05, ** P<0.01, *** P<0.001 and **** P<0.0001 students t test, haematology data is representative data of 3 independent experiments each symbol represents an individual mouse with the line at the mean.

Supplementary Figure 2 *Prkab1*$^{tm1b/tm1b}$ mice have altered hematological parameters at 4 weeks of age

A) hemoglobin, B) hematocrit, C) red blood cell count, D) mean corpuscular hemoglobin concentration, E) red blood cell distribution width, F) mean corpuscular volume and G) mean platelet volume of 4 week old *Prkab1*$^{+/+}$ and *Prkab1*$^{tm1b/tm1b}$ mice * P<0.05, ** P<0.01, *** P<0.001 and **** P<0.0001 unpaired two-tailed students t test representative data of 2 independent experiments each symbol represents an individual mouse with the line at the mean.

Supplementary Figure 3 characterisation of circulating iron, bone marrow erythropoiesis and erythrocyte half-life of *Prkab1* deficient mice

A) plasma ferritin concentration; B) plasma iron concentration; C) representative H&E stained bone marrow sections from *Prkab1*$^{+/+}$ and *Prkab1*$^{tm1b/tm1b}$ mice (400x magnification); D) erythroid (Ter119)/Leukocyte (CD45) ratio on bone marrow from *Prkab1*$^{+/+}$ and *Prkab1*$^{tm1b/tm1b}$ mice; E) characterisation of erythropoiesis in the bone marrow of *Prkab1*$^{+/+}$ and *Prkab1*$^{tm1b/tm1b}$ mice; *in vivo* half-life of erythrocytes transferred into *Prkab1*$^{+/+}$ F) or *Prkab1*$^{tm1b/tm1b}$ G) mice. For all * P<0.05, ** P<0.01, *** P<0.001 and **** P<0.0001 unpaired two-tailed students t test except for Ter119/CD45 ratio which was analysed with a Mann Whitney test, representative data of 2 independent experiments or four mice for histology analysis, each symbol represents an individual mouse with the line at the mean except for F and G) where n = 5 with mean ± standard error of the mean.