Mice Deficient in the Putative Phospholipid Flippase ATP11C Exhibit Altered Erythrocyte Shape, Anemia and Reduced Erythrocyte Lifespan

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Background: Asymmetrical distribution of specific phospholipids between the two leaflets of biological membranes is generated and maintained by transporters.

Results: A mutation in murine Atp11c results in altered morphology and shortened lifespan of erythrocytes.

Conclusion: Phospholipid transport by ATP11C maintains phospholipid asymmetry in erythrocytes.

Significance: Defects in phospholipid transport across the cell membrane can lead to anemia.

ABSTRACT

Transmembrane lipid transporters are believed to establish and maintain the phospholipid asymmetry in biological membranes; however, little is known about the in vivo function of the specific transporters involved. Here we report that developing erythrocytes from mice lacking the putative phosphatidylserine (PS) flippase, ATP11C showed a lower rate of PS translocation in vitro compared to erythrocytes from wild-type littersmates. Furthermore, the mutant mice had an elevated percentage of PS-exposing mature erythrocytes in the periphery. While erythrocyte development in ATP11C-deficient mice was normal, the mature erythrocytes had an abnormal shape (stomatocytosis), and the lifespan of mature erythrocytes was shortened relative to that in littermate controls resulting in anemia in the mutant mice. Thus, our findings uncover an essential role for ATP11C in erythrocyte morphology and survival and provide a new candidate for the rare inherited blood disorder stomatocytosis with uncompensated anemia.

Mammalian erythrocytes are unique amongst eukaryotic cells in that they lack nuclei, cytoplasmic structures and organelles. Because of this, the structural and functional properties of erythrocytes are closely associated with their plasma membranes (1). The erythrocyte plasma
membrane consists of a lipid bilayer with an asymmetrical distribution of specific phospholipids between the two leaflets of the bilayer (2). While phosphatidylcholine and sphingomyelin are concentrated predominantly in the outer monolayer of the erythrocyte membrane, phosphatidylserine (PS) and phosphatidylethanolamine (PE) are mainly confined to the cytoplasmic leaflet (2). The generation and maintenance of this asymmetric structure is essential for the function and survival of all cells including erythrocytes (1,2). The concentration of PS in the cytofacial leaflet of the plasma membrane provides important docking sites for a variety of signaling molecules (3) and, in particular in erythrocytes, plays an essential role in the modulation of the mechanical stability of the membrane, through an interaction with the skeletal protein spectrin (4,5). PS exposure on the surface of erythrocytes acts as an “eat me” signal to phagocytes and also can lead to adherence of erythrocytes to the vascular endothelium and activation of surface-dependent plasma blood clotting factors (2), as well as contributing to the anemia observed in a wide variety of blood disorders, including sickle cell anemia and thalassemia (6-10). Three different groups of enzymes are involved in establishing and maintaining phospholipid asymmetry, namely flippases, floppases and scramblases (11,12).

Erythrocytes have served as a model system to characterize phospholipid movement across bilayers. Considerable attempts have been made to isolate and identify the major aminophospholipid flippases from red blood cells and other membranes (13-17). The biochemical characterization suggests that the erythrocyte flippase is a Mg$^{2+}$-dependent, vanadate-sensitive ATPase (13). Studies in yeast suggest that members of the P4-type ATPase family serve as “flippases”, selectively translocating PS, and to a lesser extent PE, into the cytoplasmic leaflet of cell membranes (11,12,18). Although there are 15 members of the P4-type ATPase family in mice (14 members in humans) (12), their biological functions are largely unknown (19), and so far only a few flippases have been linked to biological functions in mammals. Among these, mutations in the gene encoding ATP8A2 in human and mice have been shown to cause neurological disorders (20-22). In addition, ATP8B1 and ATP11C have been implicated bile secretion in mice, which involves translocation of phospholipids (23,24). Furthermore, we and others recently discovered that ATP11C, which is encoded on the X-chromosome in humans and mice, is required for the development of adult B cells (25,26). With regard to erythrocytes, ATP8A1 has been proposed to be the molecular correlate of the erythrocyte flippase activity. The protein was detected in the plasma membrane of mature erythrocytes and flippase activity was demonstrated upon expression in yeast (27). However, the biochemical characteristics of ATP8A1 are consistent with the flippase from chromaffine granules, which is different from the major flippase activity observed in erythrocytes (28). Consistently, erythrocytes from Atp8a1$^{-/-}$ animals exhibit no increased PS exposure on their surface, most likely due to expression of other aminophospholipid flippases (29). In our initial study on the role of ATP11C in B cells we also noted a significant reduction in the number of total erythrocytes in the blood of ATP11C mutant mice (25). Here we investigate this anemia and demonstrate that the mutant erythrocytes have a shortened lifespan and an abnormal shape manifesting itself as a pronounced stomatocytosis. Impaired flippase activity during erythropoiesis was confirmed in the mutant mice and circulating mutant erythrocytes were shown to have elevated surface-exposure of PS that increased with the age of the cells.

**EXPERIMENTAL PROCEDURES**

**Mice**–The Ambrosius mouse strain with an X-linked N-ethyl-N-nitrosourea-induced point mutation in Atp11c has been described previously (25). All animal procedures were approved by the Australian National University Animal Ethics and Experimentation Committee.

**Analysis of Hematologic Parameters**–An ADVIA 2120 Hematology System (Siemens Healthcare Diagnostics) was used for the analysis of blood parameters.

**Clinical Chemistry**–Serum concentrations of iron parameters were assayed by automated procedures in the Department of Pathology, The Canberra Hospital.

**Flow Cytometry**–Procedures for surface and Annexin-V staining have been described previously (25). For flow cytometric analysis the
following antibodies were used: CD71-Biotin or -PE (Clone C2), Ter119-FITC or -PE-Cy7, CD44-APC (Clone IM7), Streptavidin-APC (all from BD) and Annexin-V-Pacific Blue (Biolegend).

In vivo Erythrocyte Lifespan Analysis—In vivo lifespan measurements using carboxyfluorescein diacetate succinimidyl ester (CFSE), in conjunction with mathematical modeling were performed as described previously (30).

Flippase Assay—Flippase activity was assessed as described previously (25) with the modification that 16:0-06:0-NBD-PS (Avanti Polar Lipids) was used in place of 16:0-12:0-NBD-PS and all incubations were performed at 37 °C instead of 37 °C to avoid non-specific uptake of the PS analogue.

Cation Measurement—Intra- and extracellular Na⁺ and K⁺ levels were determined using Ultra High Pressure Liquid Chromatography (UHPLC; Dionex) linked to a Charged Aerosol Detector (CAD; Dionex) as described elsewhere (31). In brief, 10 µL-heparinized blood was separated by centrifugation into erythrocyte and plasma fractions. The erythrocytes were washed (x1) with a 250 mM MgSO₄ solution to replace extracellular Na⁺ and K⁺. The resulting cell pellet, and 1 uL of the plasma, were added to 200 and 100 µL, respectively, of 40/60 v/v 20 mM ammonium sulphate pH 5/acetonitrile, to precipitate proteins. The samples were centrifuged and 10 µL aliquots of the resulting supernatant solutions were subjected to the HPLC analysis.

Scanning Electron Microscopy (SEM) Analysis—Cells attached to coverslips were fixed with 2% v/v glutaraldehyde in 0.1 M sodium cacodylate overnight at 4 °C and post-fixed with 1% w/v osmium tetroxide for 1.5 hours at room temperature. The samples were dehydrated using a graded ethanol series, then critical point dried, mounted on aluminium stubs, and sputter-coated with gold prior to imaging. Images were taken using a Zeiss Ultraplus FESEM.

Osmotic Fragility Test—Peripheral blood from wild-type and ATP11C-deficient mice was diluted to an approximate hematocrit of 10% with PBS followed by a further 1 in 10 dilution in NaCl solutions of varying osmolarity (0.1%-0.8% w/v NaCl in H₂O). After incubation for 30 minutes at room temperature, samples were centrifuged and the absorbance of the supernatant solutions measured at 540 nm (A₅₄₀) using a microplate reader (ThermoMax; Molecular Devices). Percentage hemolysis of the cells in each solution was calculated based on the A₅₄₀ value relative to that obtained for cells suspended (and thereby lysed) in H₂O.

Statistical Analysis—For comparison of only two groups, the two-tailed Student’s t-test was used. When multiple experimental groups were being compared, One-Way analysis of variance (ANOVA), followed by pair-wise comparison with a Bonferroni post-test were used. All statistical analyses were performed using GraphPad Prism Software.

RESULTS and DISCUSSION

ATP11C Deficiency in Mice Results in Anemia—To extend our previous finding of anemia in ATP11C-deficient mice (25), we analyzed different hematologic parameters in the blood of mutant male mice and their control littermates. Mutant mice showed an approximate 25% reduction in the number of erythrocytes compared to wild-type male mice among all age groups (Fig. 1A). While the relative proportion of immature erythrocytes (reticulocytes) was increased from 2.85 ± 0.2% to 4.24 ± 0.2% in the blood of 6-8 weeks old ATP11C mutant animals, the absolute number of reticulocytes was not significantly increased (Fig. 1A). Interestingly, erythrocytes from mutant mice were larger with a higher mean corpuscular volume (MCV) than controls among all age groups (Fig. 1A). Similar results were found in homozygous mutant female mice (data not shown). In contrast, Atp11camb/+ heterozygous female mice had a normal erythrocyte count in the peripheral blood (data not shown). These results reveal that ATP11C deficiency in mice causes anemia, and suggest that ATP11C plays a significant role in the development and/or survival of erythrocytes.

Normal Erythropoiesis, but Reduced Erythrocyte Lifespan in ATP11C-deficient Mice—To assess if ATP11C is required for erythropoiesis, we examined the early stages of erythroid development in the bone marrow and spleen. Stepwise analysis of different stages of erythroblast differentiation based on expression of the surface marker CD44 and forward scatter profile (32) showed that ATP11C-deficient mice have essentially normal erythropoiesis in the bone marrow and spleen (Fig. 1, B and C). These results
indicate that animals deficient for ATP11C have no block in erythrocyte development.

Thus we next focused on the lifespan of mature erythrocytes to explain the observed anemia in ATP11C-deficient animals. The lifespan of erythrocytes was investigated using dilution of a fluorescent label (CFSE) in vivo and mathematical modeling previously described (30). Erythrocytes from ATP11C-deficient animals exhibited reduced survival compared to cells from their control littermates as determined by the percentage of CFSE-labeled erythrocytes in the blood (Fig. 1D). Erythrocyte survival data showed an average lifespan of 25.2 ± 3.5 days in mutant animals, compared to 38.7 ± 3.6 days in control littermates (presented as means ± S.E.M., p < 0.05) (Fig. 1E). These data indicate that ATP11C plays an important role in the survival of erythrocytes.

ATP11C-deficient Erythrocytes Form Stomatocytes—The composition and asymmetric distribution of phospholipids in the cell membrane is essential for maintaining the normal biconcave shape of erythrocytes (5). Morphological studies of erythrocytes from the blood of ATP11C-deficient and littermate control mice were performed using scanning electron microscopy. As shown in Fig. 2A the majority of mutant erythrocytes showed a distinct change in morphology known as stomatocytosis (Fig. 2A). These results were confirmed with peripheral blood smears (Fig. 2B). Consistent with an increased mean corpuscular volume, flow cytometry analysis revealed that erythrocytes in the blood and spleen from ATP11C-deficient animals were significantly larger than erythrocytes from control animals as determined by an increase in their forward scatter profile (Fig. 2C). This increased size was first apparent in mature erythrocytes in the bone marrow, but also in erythrocyte precursors in the spleen (Fig. 1, B and C).

The effect of the mutation on osmotic fragility was assessed by incubating erythrocytes from ATP11C-deficient mice and wild-type littermates in solutions of varying osmolality, and measuring the extent of hemolysis. Erythrocytes from ATP11C-deficient mice displayed a normal hemolysis profile, suggesting that their surface-to-volume ratio and cell hydration is comparable to that in erythrocytes from wild-type mice (Fig. 2D).

No Effect of ATP11C on Na⁺ and K⁺ Homeostasis—Stomatocytosis has been associated with altered cation transport across the erythrocyte membrane, and, consequentially, perturbation of erythrocyte Na⁺ and K⁺ homeostasis (33). In some cases the cation transport abnormality is enhanced upon reduction of the temperature to which the cells are exposed (34). Comparisons of the ratio of Na⁺ to K⁺ in erythrocytes from ATP11C-deficient mice with those from control mice, using ion chromatography, revealed there to be no difference between the two groups (Fig. 2E). Measurement of the plasma K⁺ concentration in blood samples 30 min after blood collection and after incubation for a further 1 and 3 hours at room temperature (in order to enhance any temperature-dependent K⁺ leak that might be present) revealed no significant difference in the plasma K⁺ concentrations in ATP11C-deficient mice and wild-type mice (Fig. 2F). ATP11C deficiency apparently therefore has no significant effect on erythrocyte Na⁺ and K⁺ homeostasis.

Hereditary stomatocytosis in humans can cause iron overload (33). We therefore measured serum iron and transferrin in 8-10 and 18-20 week old ATP11C-deficient mice and their wild-type littermates. Serum iron and transferrin levels were comparable between mutant and wild-type animals and between both age groups (data not shown) thus excluding iron overload as a feature of the stomatocytosis seen in these mice.

Lower PS Flippase Activity in Erythroblasts from ATP11C-deficient Mice—We previously demonstrated that developing B-lymphocytes in ATP11C-deficient mice display impaired PS internalization relative to B-lymphocytes in wild-type mice (25). To test if there is a similar defect in developing or mature erythrocytes we performed an in vitro flippase activity assay using a fluorescent PS analogue (NBD-PS). We first tested flippase activity in the different erythroid stages (Proerythroblasts, Basophilic erythroblasts, Polychromatotic erythroblasts, Orthochromatotic erythroblasts and reticulocytes, and mature red blood cells); gates as regions I, II, III, IV and V respectively in Fig. 1. B and C in the bone marrow and spleen, separated on the basis of their CD44 and forward scatter profiles (32). Erythroid precursors (R I – R III) from wild-type mice took up NBD-PS rapidly whereas orthochromatotic erythroblasts and reticulocytes as well as mature
Annexin-V which binds to PS in the exoplasmic leaflet (37). The percentage of Annexin-V+ from the peripheral blood were stained with increased surface accumulation of PS, erythrocytes flippase activity in mutant animals results in phagocytes (35,36). To test if the defective recognition and clearance of erythrocytes by membranes serves as an “eat-me” signal for the erythrocytes (38). Alternatively, it may also indicate that in ATP11C-deficient erythrocytes the reduced rate of PS flipping during the early developmental stages may have a lasting change on the membrane composition, resulting in reduced erythrocyte deformability, increased intravascular damage and hence PS exposure.

Beside ATP11C, several other P4-type ATPases are expressed during erythropoiesis, with ATP8A1 and ATP11B being particularly prominent in primitive, fetal and adult erythroblasts (38). Interestingly, Atp8a1−/− mice exhibited no erythrocyte phenotype, which can be explained by the compensatory expression of ATP8A2 in Atp8a1−/− erythrocytes (29). The observation of reduced, but not absent PS translocation in ATP11C-deficient erythroblasts suggests that other translocases may still be present in mutant erythroblasts. In addition to their effect on phospholipid translocation P4-type ATPases have also been linked to vesicle trafficking (12,18). This raises the possibility that loss of ATP11C activity may result in defects in membrane or protein trafficking, including trafficking of other translocases like ATP8A1 that are expressed in erythrocytes. Furthermore, lack of ATP11C may lead to a compensatory overexpression of ATP8A1 and other P4-type ATPases that partially compensates for the lack of ATP11C. Differentiation between a direct or more indirect effect of ATP11C defects on PS translocation will require further studies, including the development of better antibodies against all P4-type ATPases that go beyond the scope of this study.

The abnormal stomatocyte shape of erythrocytes in ATP11C mutant mice has been found to be independent of cation leaks or hydration status, and suggests that an alternative mechanism is responsible for the stomatocytosis in these mice. One possibility is a mechanism similar to that responsible for Mediterranean stomatocytosis (39), in which a lack of control over sterol absorption and excretion leads to...
changes in the plasma lipid composition and presumably the membrane lipid composition of circulating cells. Alterations in the relative lipid composition of the erythrocyte membrane can lead to an expansion or contraction of either the inner or outer leaflet, resulting in altered erythrocyte shape (40-42). In addition, reduced accumulation of PS at the cytofacial leaflet is predicted to interfere with the interaction between skeletal proteins and the membrane (4,5). It is plausible, therefore, that a disturbance in these interactions underlies the observed formation of stomatocytes in ATP11C mutant mice.

In conclusion, the results presented in this study reveal an important functional role for ATP11C in erythrocyte biology, and raise the question of whether mutations in ATP11C serve as a previously unclassified cause of stomatocytosis in humans. Whether the observed blood phenotypes in ATP11C-deficient mice occur as a result of changes to the erythrocyte membrane composition and/or through changes to the interaction between erythrocytes and other cells, for example splenic macrophages, will be an important area for future studies.

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FOOTNOTES
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6The abbreviations used are: PS, phosphatidylserine; PE, phosphatidylethanolamine; P4-type ATPase, type IV P-type ATPase; CFSE, carboxyfluorescein diacetate succinimidyl ester; NBD-PS, 1-palmitoyl-2-\{6-[7-nitro-2–1,3-benzoxadiazol-4-yl]amino\}hexanoyl-\{sn\}-glycero-3-phosphoserine; SEM, Scanning Electron Microscopy.

FIGURE LEGENDS

FIGURE 1. Anemia in mice with a point mutation in Atp11c due to a reduced lifespan despite normal erythropoiesis. A, graphs show the number of erythrocytes, hemoglobin, hematocrit, the number of reticulocytes, mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) in the blood of Atp11c<sup>+/0</sup> (WT, open circle) and Atp11c<sup>amb/0</sup> (Amb, black filled circle) animals of the indicated age groups. The symbols represent mean ± S.E.M. with four to eight mice per genotype in each age group. B and C, representative flow cytometric contour plots of CD44 and forward scatter (FSC) profiles in the bone marrow (B) and spleen (C) of Atp11c<sup>+/0</sup> (WT) and Atp11c<sup>amb/0</sup> (Amb) animals. The plots are pre-gated on CD71<sup>+</sup> and Ter119<sup>+</sup> erythrocytes. Bar graphs represent mean ± S.E.M. of the percentage of cells in the outlined areas in the flow cytometric plots. Data are representative of at least five independent experiments with one to four mice per genotype in each. D, graph shows mean ± S.E.M. of the percentage of CFSE-labeled erythrocytes in the blood of Atp11c<sup>+/0</sup> (WT, open circle) and Atp11c<sup>amb/0</sup> (Amb, black filled circle) mice after in vivo labeling. E, average erythrocyte lifespan calculated by lognormal modeling from the data presented in (D). Bar graph represents mean ± S.E.M. of the average survival of erythrocytes. Data are representative of two independent experiments with four to five mice per genotype in each. Statistical significance was calculated using One-Way ANOVA analysis, followed by the
Bonferroni post-test, with the \( p \) values comparing each age pair of groups shown on the plots (A) or the two-tailed Student's \( t \)-test (B-E). ns, not significant; \( { }^\text{\#} \), \( p < 0.0001; \ { }^{**} \), \( p < 0.001; \ { }^{*} \), \( p < 0.01; \ { }^* \), \( p < 0.05. \\

FIGURE 2. Abnormally shaped erythrocytes in the periphery of ATP11C-deficient mice. A, scanning electron microscopic analysis of erythrocytes from the blood of \( \text{Atp11c}^{+/0} \) (WT) and \( \text{Atp11c}^{amb/0} \) (Amb) animals. B, peripheral blood smears from \( \text{Atp11c}^{+/0} \) (WT) and \( \text{Atp11c}^{amb/0} \) (Amb) animals. C, representative overlay histograms of forward scatter (FSC) profile in Ter119 \(^+\) erythrocytes from the bone marrow, spleen and blood of \( \text{Atp11c}^{+/0} \) (WT, shaded grey) and \( \text{Atp11c}^{amb/0} \) (Amb, black line) animals. Data are representative of three independent experiments with four to six mice per genotype in each. D, osmotic fragility of erythrocytes from \( \text{Atp11c}^{+/0} \) (WT, open circle) and \( \text{Atp11c}^{amb/0} \) (Amb, black filled circle) mice. The symbols represent mean \( \pm \) S.E.M. of the percentage of hemolysis. Data are representative of three independent experiments with four to five mice per genotype in each. E, ratio of intra-erythrocytic Na\(^+\) and K\(^+\) of \( \text{Atp11c}^{+/0} \) (WT) and \( \text{Atp11c}^{amb/0} \) (Amb) animals analyzed by high-performance liquid chromatography within 30 min of blood collection (0.5 h) and after resting at room temperature for 1.5 and 3.5 hours. Bar graph represents mean \( \pm \) S.E.M. of the ratio of intra-erythrocytic Na\(^+\) and K\(^+\) content. Data are representative of two independent experiments with four to five mice per genotype in each.

FIGURE 3. Reduced flippase activity in mutant erythroblasts and increased PS exposure in mature erythrocytes from ATP11C-deficient animals. A and B, graphs show \( C_6\)-NBD-PS uptake after 0, 1, 3, 5, 10, 20 and 30 min incubation in Proerythroblasts, Basophilic erythroblasts, Polychromatic erythroblasts, Orthochromatic erythroblasts and reticulocytes and mature red blood cells (regions I, II, III, IV and V respectively, gated as in Fig. 1, B and C) in the bone marrow (A) and spleen (B) from \( \text{Atp11c}^{+/0} \) (WT, open circle) and \( \text{Atp11c}^{amb/0} \) (Amb, black filled circle) mice. Graphs represent mean \( \pm \) S.E.M. of the percentage of \( C_6\)-NBD-PS uptake relative to wild-type region II at 30 min at which the highest rate of PS internalization is observed. Data are pooled from five independent experiments with one mouse per genotype in each. C, representative overlay histogram of Annexin-V staining in Ter119\(^+\) erythrocytes in the blood of \( \text{Atp11c}^{+/0} \) (WT, shaded grey) and \( \text{Atp11c}^{amb/0} \) (Amb, black line) animals. Bar graphs represent mean \( \pm \) S.E.M. of the percentage of Annexin-V\(^+\) cells in \( \text{Atp11c}^{+/0} \) and \( \text{Atp11c}^{amb/0} \) erythrocytes. Data are representative of at least three independent experiments with four to six mice per genotype in each. D, graph shows mean \( \pm \) S.E.M. of the percentage of Annexin-V\(^+\) cells among circulating CFSE\(^-\) or CFSE\(^+\) erythrocytes in the blood of \( \text{Atp11c}^{+/0} \) (WT) and \( \text{Atp11c}^{amb/0} \) (Amb) mice after \textit{in vivo} labeling. E, representative overlay histogram of Annexin-V staining in Ter119\(^+\) erythrocytes in the spleen of \( \text{Atp11c}^{+/0} \) (WT, shaded grey) and \( \text{Atp11c}^{amb/0} \) (Amb, black line) animals. Bar graphs represent mean \( \pm \) S.E.M. of the percentage of Annexin-V\(^+\) cells in Ter119\(^+\) erythrocytes. Data are representative of at least three independent experiments with four to six mice per genotype in each. Statistical significance was calculated using the two-tailed Student's \( t \)-test. \( { }^\# \), \( p < 0.0001 \).
FIGURE 2

A

WT
Amb

Gated on Ter119
(Bone Marrow)
Gated on Ter119
(Spleen)
Gated on Ter119
(Blood)

C

WT
Amb

D

Hemolysis (%) vs NaCl (%)

E

Intra-erythrocytic Na/K ratio vs Time samples rested at RT (h)

F

K+ (mmol/L) vs Time samples rested at RT (h)
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