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ATP11C is a major flippase in human erythrocytes and its defect causes congenital hemolytic anemia

Nobuto Arashiki,[1] Yuichi Takakuwa,[1] Narla Mohandas,[2] John Hale,[2] Kenichi Yoshida,[3] Hiromi Ogura,[4] Taiju Utsugisawa,[4] Shouichi Ohga,[5] Satoru Miyano,[6] Seishi Ogawa,[3] Seiji Kojima,[7] and Hitoshi Kanno,[4,8]

[1]Department of Biochemistry, School of Medicine, Tokyo Women's Medical University, Japan; [2]Red Cell Physiology Laboratory, New York Blood Center, NY, USA; [3]Department of Pathology and Tumor Biology, Graduate School of Medicine, Kyoto University, Japan; [4]Department of Transfusion Medicine and Cell Processing, School of Medicine, Tokyo Women's Medical University, Japan; [5]Department of Pediatrics, Graduate School of Medicine, Yamaguchi University, Japan; [6]Laboratory of DNA Information Analysis, Human Genome Center, Institute of Medical Science, The University of Tokyo, Japan; [7]Department of Pediatrics, Graduate School of Medicine, Nagoya University, Japan; and [8]Division of Genomic Medicine, Department of Advanced Biomedical Engineering and Science, Graduate School of Medicine, Tokyo Women's Medical University, Japan

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ABSTRACT

Phosphatidylserine is localized exclusively to the inner leaflet of the membrane lipid bilayer of most cells, including erythrocytes. This asymmetric distribution is critical for the survival of erythrocytes in circulation since externalized phosphatidylserine is a phagocytic signal for splenic macrophages. Flippases are P-IV ATPase family proteins that actively transport phosphatidylserine from the outer to inner leaflet. It has not yet been determined which of the 14 members of this family of proteins is the flippase in human erythrocytes. Herein, we report that *ATP11C* encodes a major flippase in human erythrocytes, and a genetic mutation identified in a male patient caused congenital hemolytic anemia inherited as an X-linked recessive trait. Phosphatidylserine internalization in erythrocytes with the mutant *ATP11C* was decreased 10-fold compared to that of the control, functionally establishing that ATP11C is a major flippase in human erythrocytes. Contrary to our expectations phosphatidylserine was retained in the inner leaflet of the majority of mature erythrocytes from both controls and the patient, suggesting that phosphatidylserine cannot be externalized as long as scramblase is inactive. Phosphatidylserine-exposing cells were found only in the densest senescent cells (0.1% of total) in which scramblase was activated by increased Ca^{2+} concentration: the percentage of these phosphatidylserine-exposing cells was increased in the patient's senescent cells accounting for his mild anemia. Furthermore, the finding of similar extents of phosphatidylserine exposure by exogenous Ca^{2+}-activated scrambling in both control erythrocytes and the patient's erythrocytes implies that suppressed scramblase activity rather than flippase activity contributes to the maintenance of phosphatidylserine in the inner leaflet of human erythrocytes.

Introduction

In human erythrocytes, phosphatidylserine (PS) is present exclusively in the inner leaflet of the membrane lipid bilayer as a result of ATP-dependent active transport (flipping) of aminophospholipids (such as PS and phosphatidylethanolamine) from the outer to inner leaflet. PS interacts with spectrin, a cytoskeletal protein underneath erythrocyte membranes, to maintain membrane deformability and mechanical stability of the erythrocytes[1] and protects spectrin from glycation, which

Correspondence:
kanno.hitoshi@twmu.ac.jp

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decreases the membrane deformability necessary for traversing narrow capillaries and the splenic sinuses.[2] More importantly, preventing surface exposure of PS is critical for erythrocyte survival: exposure of PS on the outer surface of the membrane at the end of the 120-day lifespan of erythrocytes is a phagocytic signal for splenic macrophages to remove the senescent cells.[3-5] Indeed in these cells, the Ca^{2+} concentration is elevated to activate lipid scrambling, with consequent surface exposure of PS, which is recognized as an "eat-me signal" by macrophages.[4-7] Besides being exposed on normal senescent cells, PS is exposed prematurely by sickle erythrocytes and thalassemic erythrocytes, resulting in a shortened life span of the red blood cells and consequent hemolytic anemia in these disorders.[7-10] Maintenance, regulation, and disruption of the asymmetric PS distribution are, therefore, important for both erythrocyte survival and death. While it has been well established that PS distribution is determined by flippase and scramblase activities, the molecular identities of these activities in human erythrocytes have not been defined. Furthermore, the relative contributions of these two activities in maintaining the asymmetric distribution of PS under physiological and pathological states are not well understood.

Flippases are members of the P-IV ATPase family of proteins composed of 10 transmembrane domains (Figure 1A).[11,12] They contribute to localization of PS in the inner leaflet of erythrocyte membranes through ATP-dependent active transport of aminophospholipids (such as PS) from the outer to inner leaflet.[13-15] However, the flippase in human erythrocytes has not yet been definitively identified. Among the 14 family members, ATP8A1, ATP8A2, ATP11A, and ATP11C were previously shown to transport PS in the plasma membrane.[12,16-19] ATP11C has been implicated as one of the candidates in murine erythrocytes based on the finding that its mutation in mice resulted in anemia with stomatocytosis.[20] However, there are differences between the characteristics of human and murine erythrocytes, including their life span, cell volume and cell hemoglobin content. Furthermore, recent studies have documented significant differences in gene expression during human and murine erythropoiesis.[21] For example, GLUT1 which is abundantly expressed in human erythrocytes is not expressed by murine erythrocytes. As such it is important to identify the major flippase in human erythrocytes.

In the present study, we identified a point mutation in *ATP11C* through whole-exome sequencing of DNA from a male patient with mild hemolytic anemia without morphological abnormalities. Detailed analyses established that this mutation is responsible for hemolysis and related clinical features and that ATP11C is a major flippase in

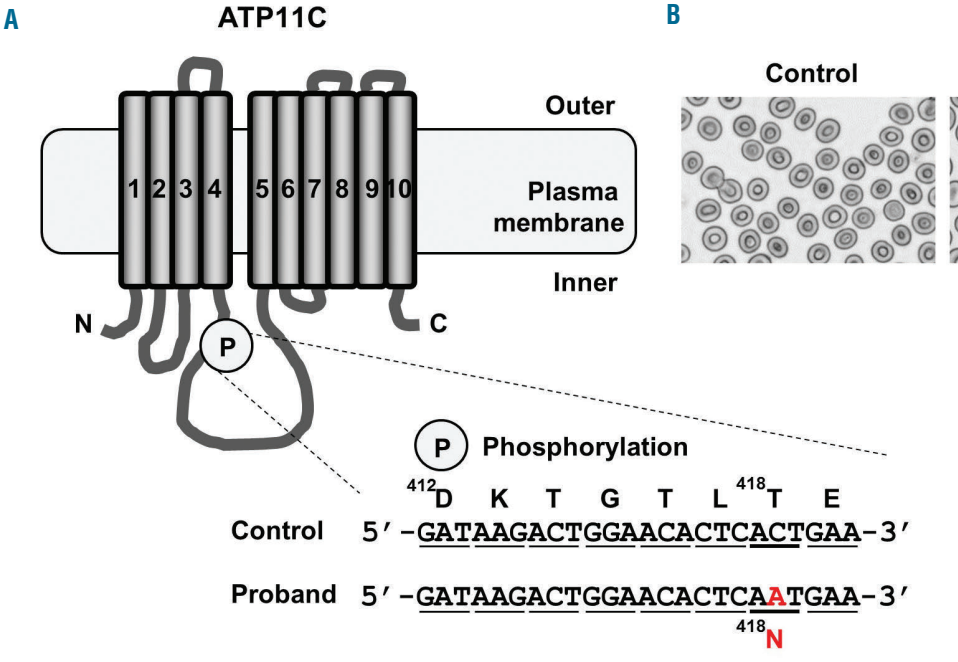

Figure 1. Schematic representation of ATP11C, erythrocyte morphology, and genotyping for the *ATP11C* mutation. (A) Schematic of ATP11C and the site of a c.1253C>A missense mutation coding for Thr418Asn. (B) Phase-contrast microscopy images (×1,000; prepared from original images without any modifications) of Giemsa-stained blood from the healthy control and proband. No morphological abnormality was observed. (C) Wave data from direct sequencing of genomic DNA for exon 13, which includes the coding region of Thr418. The sequence of the antisense strand corresponding to the sense strand represented in (A) is displayed. Arrows indicate position of the mutations.

human erythrocyte membranes. By defining the contribution of ATP11C to PS distribution when scramblase is inactive under physiologically low Ca^{2+} concentrations and when it is activated under elevated high Ca^{2+} concentrations, as in senescent cells, we established that suppressed scramblase activity rather than flippase activity contributes to the maintenance of PS in the inner leaflet of human erythrocytes.

Methods

This study was approved by the Ethics Committee for Human Genome/Gene Analysis Research of Tokyo Women's Medical University (#223D). The healthy volunteer, the proband, and the proband's mother provided informed consent for blood sample collection and the blood was used in all of the studies outlined. Suppliers of all reagents and laboratory instruments, and the composition of buffer solutions are given in the *Online Supplementary Information*.

Whole-exome sequencing

Whole-exome sequencing was performed as reported previously.[22] Briefly, genomic DNA was extracted from leukocytes, and coding sequences were enriched with a SureSelect Human All Exon V4 kit and used for massively parallel sequencing with the HiSeq 2000 platform with 100-bp paired-end reads. Candidate germline variants were detected through our in-house pipeline for whole exome-sequencing analysis. Single nucleotide variants with an allele frequency >0.25 and insertion-deletions with an allele frequency >0.1 were called. Identified variants were verified by Sanger sequencing of polymerase chain reaction amplicons (details of the methods are given in the *Online Supplementary Information*).

Measurement of phosphatidylserine flipping activity in erythrocytes

To measure PS flipping activity, 1 μL of 1 mg/mL Fluorescent PS (NBD-PS) was added to 1 mL suspension of washed erythrocytes at a hematocrit of 5% in phosphate-buffered saline with glucose (PBS-G) and incubated for 0–20 min at 37 °C. Twenty microliters of the erythrocyte suspension were washed with 1 mL PBS-G with 1% bovine serum albumin [BSA; BSA (+)] to remove NBD-PS remaining in the outer leaflet.[20] To measure loaded NBD-PS after the 20-min incubation, incubated erythrocytes were washed with PBS-G in the absence of BSA [20 min BSA (-)]. NBD-derived fluorescence associated with variously treated erythrocytes was measured by fluorescence activated cell sorting (FACS). For all samples, 100,000 cells were analyzed. To determine the basal level of flippase-independent flipping activity, the cell suspension was treated with 5 mM N-ethylmaleimide (NEM) in PBS-G for 20 min at 37 °C, which irreversibly and non-specifically inactivates flippases.[9]

Analysis of phosphatidylserine-exposing cells

PS exposed on the erythrocyte cell surface was analyzed by Ca^{2+}-dependent binding of fluorescently labeled annexin V.[1] Washed, unfractionated erythrocytes and senescent erythrocytes fractionated by density centrifugation, as we reported previously,[23] were suspended in nine volumes of Tris-buffered saline with glucose (TBS-G). To control the intracellular Ca^{2+} concentration, erythrocytes were treated for 20 min at 37 °C with 2 μM A23187,[5] a Ca^{2+} ionophore, in TBS-G; accurate final concentrations of free Ca^{2+} were determined by adding 1 mM EGTA and calculating the concentration of $CaCl_2$ using Calcon free software (e.g., 0.958 mM for a final 1 μM). After incubation, the erythrocytes were washed three times with 1 mL TBS-G including 1% BSA to remove A23187 from the erythrocyte membranes. Ten microliters of the packed erythrocytes were suspended in 1 mL TBS-G including 5 mM $CaCl_2$. Thereafter, 1 μL 0.25 mg/mL fluorescein isothiocyanate (FITC)-conjugated annexin V was added, and the fluorescence on erythrocytes quantified by FACS as described above. The cut-off for identification of PS-positive cells was set at a fluorescent signal value 20-fold higher than that detected in the absence of FITC-annexin V.

Results

Clinical history and analyses of a male patient with congenital hemolytic anemia

A male proband was born at full-term after a clinically normal pregnancy without any apparent anomalies. At the age of 4 years, his mother noted that the boy had pigmented urine, but medical advice was not sought until he was 13 years old, when he was diagnosed with unknown congenital hemolytic anemia (Table 1). Neither mental nor growth retardation was noted, and he began studying computer sciences at the age of 18. The marriage between his parents was not consanguineous and his parents and siblings (a sister and a brother) are healthy. Laboratory data indicated mild hemolytic anemia without any particular morphological abnormality of the erythrocytes (Table 1, Figure 1B). The leukocyte count was within the normal range, and the platelet count was slightly low. With regards to the lymphocytes, CD2-positive T-lymphocytes accounted for 84% (normal range, 72-90%) and CD20-positive B-lymphocytes for 12% (normal range, 7-30%). Extensive laboratory analyses investigating erythrocyte deformability, membrane proteins and lipids, erythrocyte enzymes, and hemoglobins failed to elucidate a cause, such as hereditary spherocytosis, erythrocyte enzyme

Table 1. Clinical laboratory parameters for the proband at age 13 and 19.

	Age 13	Age 19
White blood cell count (10^9/L)	2.3	4.0
Red blood cell count (10^{12}/L)	3.80	3.76
Hemoglobin (g/dL)	11.8	12.3
Hematocrit (%)	36.4	39.3
MCV (fL)	95.8	105
MCH (pg)	31.1	32.7
MCHC (%)	32.4	31.3
RDW-CV (%)	13.2	12.4
Platelet count (10^9/L)	125	149
Reticulocytes (%)	1.4	1.7
Haptoglobin (mg/dL)	10	17
Total bilirubin (mg/dL)	1.8	1.8
Direct bilirubin (mg/dL)	0.8	0.6
Serum total protein (g/dL)	7.7	7.2
Aspartate aminotransferase (IU/L)	23	16
Alanine aminotransferase (IU/L)	13	11
Lactate dehydrogenase (IU/L)	183	138
γ-Glutamyl transpeptidase (IU/L)	10	9

MCV: mean corpuscular volume; MCH; mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width.

deficiency, or unstable hemoglobinopathy, of the hemolysis (*Online Supplementary Figure S1, Online Supplementary Table S1*).

To identify the molecular etiology of the hemolytic anemia in the proband, we performed whole-exome sequencing as described previously[22] and identified 349 candidate variants: 46 indels and 303 non-synonymous single nucleotide variants: none of which was previously known to be causative genes for hemolytic anemia (*Online Supplementary Table S2*). We focused on ATP11C since its defect has been associated with anemia in mice.[20] We identified a missense mutation in *ATP11C* (GenBank Accession Number NM_001010986) on the X chromosome, c.1253C>A, corresponding to p.Thr418Asn. The proband is hemizygous and the mother is heterozygous for this mutation, determined by direct sequencing (Figure 1C).

Flippase activity in the patient's erythrocytes

Flipping activity was measured by monitoring PS internalization using flow cytometry. Fluorescent PS (NBD-PS) was loaded exogenously onto membranes of control, the patient's, and maternal erythrocytes in similar amounts and incubated for up to 20 min at 37 °C [Figure 2A; BSA (-)]. After the indicated times, NBD-PS remaining in the outer leaflet was extracted with 1% BSA such that the remaining cell-associated fluorescence represented PS that flipped to the inner leaflet (Figure 2). In control erythrocytes, all cells were clearly NBD-positive after 5 min, indicating that NBD-PS translocated from the outer to inner leaflet by flippase activity (Figure 2A). In contrast, the patient's erythrocytes showed very little NBD fluorescence even after 20 min, indicating dramatically decreased flippase activity. Quantitative analyses confirmed the importance of ATP11C for PS internalization: 35% of loaded PS was transported to the inner leaflet at 20 min in normal erythrocytes, but only ~3% (10-fold decrease) was transported in the patient's erythrocytes (Figure 2B). To confirm the contribution of ATP11C in normal cells, erythrocytes were treated with NEM, a non-specific flippase inhibitor,[9] before loading NBD-PS. NEM-treated control erythrocytes showed very little PS internalization, confirming the importance of ATP11C for flipping activity (Figure 2B, *Online Supplementary Figure S2*). The residual flippase activity in the patient's erythrocytes was also diminished by NEM treatment (Figure 2B).

The maternal erythrocytes comprised two populations: 55-60% showed exactly the same peak positions as the control for all incubation periods, and the other 40–45% were identical to those of the patient (Figure 2A). The former possessed normal PS transport activity, and the latter lacked flippase activity. The total internalized PS was 55-60% of the control amount, reflecting the proportion of erythrocytes with normal activity (Figure 2B). The presence of two populations suggests random inactivation of the X chromosome in the erythroblast populations.

Phosphatidylserine-exposing erythrocytes in the circulation

To understand the mechanisms underlying the proband's "mild" hemolytic anemia, the percentages of PS-exposing erythrocytes in whole blood samples and cell fractions enriched for senescent cells were measured by analyzing the binding of FITC-conjugated annexin V to PS on the cell surface using flow cytometry (Figure 3). Senescent cells were collected as the densest fraction (0.1% of total cells) by density gradient centrifugation.[23] There was no apparent difference in the proportion of the dense cell populations among the different blood samples studied (*data not shown*). The percentage of PS-positive cells among total erythrocytes was slightly higher for the patient (8.86%) than in the control (6.32%) and increased further in the patient's senescent erythrocytes: 15.86% *versus* 9.17% in the control. Maternal erythrocytes had a profile similar to that of the control cells. PS-positive erythrocytes were greatly increased only in the densest senescent cells, suggesting that PS exposure did not occur until very late stages of the erythrocytes' lifespan.

Phosphatidylserine exposure promoted by Ca^{2+}-activated scrambling in the patient's erythrocytes

In senescent erythrocytes, PS exposure on the cell surface is promoted by the Ca^{2+}-activated scramblase, which translocates phospholipids, including PS, between the inner and outer leaflets.[4-7] To examine whether ATP11C prevents Ca^{2+}-activated PS externalization, the proportion of PS-exposing erythrocytes was measured under different Ca^{2+} concentrations controlled by treatment with A23187, a Ca^{2+} ionophore (Figure 4). With increasing Ca^{2+} concentrations up to 50 μM, the proportion of PS-positive cells increased. The control and patient's erythrocytes exhibited similar annexin V binding profiles with no apparent differences in the percentages of PS-positive cells at all Ca^{2+} concentrations tested.

Discussion

In the present study ATP11C was identified as a major flippase molecule of human erythrocyte membranes through whole-exome sequencing of a male patient with an *ATP11C* missense mutation on X chromosome, c.1253C>A, corresponding to p.Thr418Asn. This mutation is not recorded in SNP databases (dbSNP132 and 135) or our in-house database for Japanese patients with congenital anemia due to bone marrow failure, red cell aplasia, or hemolytic anemia. The patient's erythrocytes showed 10-fold less flipping activity compared with control cells, clearly demonstrating that ATP11C is a major flippase in human erythrocytes. Thr418 is near Asp412, the phosphorylation site for forming the 4-aspartyl phosphate intermediate essential for active transport of PS.[24] The amino acid sequence between Asp412 and Thr418 is conserved among all P-type ATPases. We, therefore, hypothesized that this mutation could disturb the functional activity of ATP11C. It should be noted that the residual flipping activity (~3%) in the patient's red cells may arise from other flippases such as ATP8A1, ATP8A2, and ATP11A. RNAseq analyses of normal human erythroblasts generated from CD34-positive cells in an *in vitro* culture system[21] demonstrated that mRNA of the three candidate flippase genes, *ATP8A1*, *ATP11A*, and *ATP11C*, were indeed expressed at all stages of human terminal erythroid differentiation (*Online Supplementary Figure S3A*). Alternatively, the missense mutation may not completely abolish the enzymatic activity of ATP11C. This residual activity may contribute to PS internalization during erythropoiesis, especially in the patient's erythrocytes. Investigation of the molecular basis of the congenital hemolytic anemia in the proband (Table 1) using several diagnostic tests for already-known hemolytic anemias

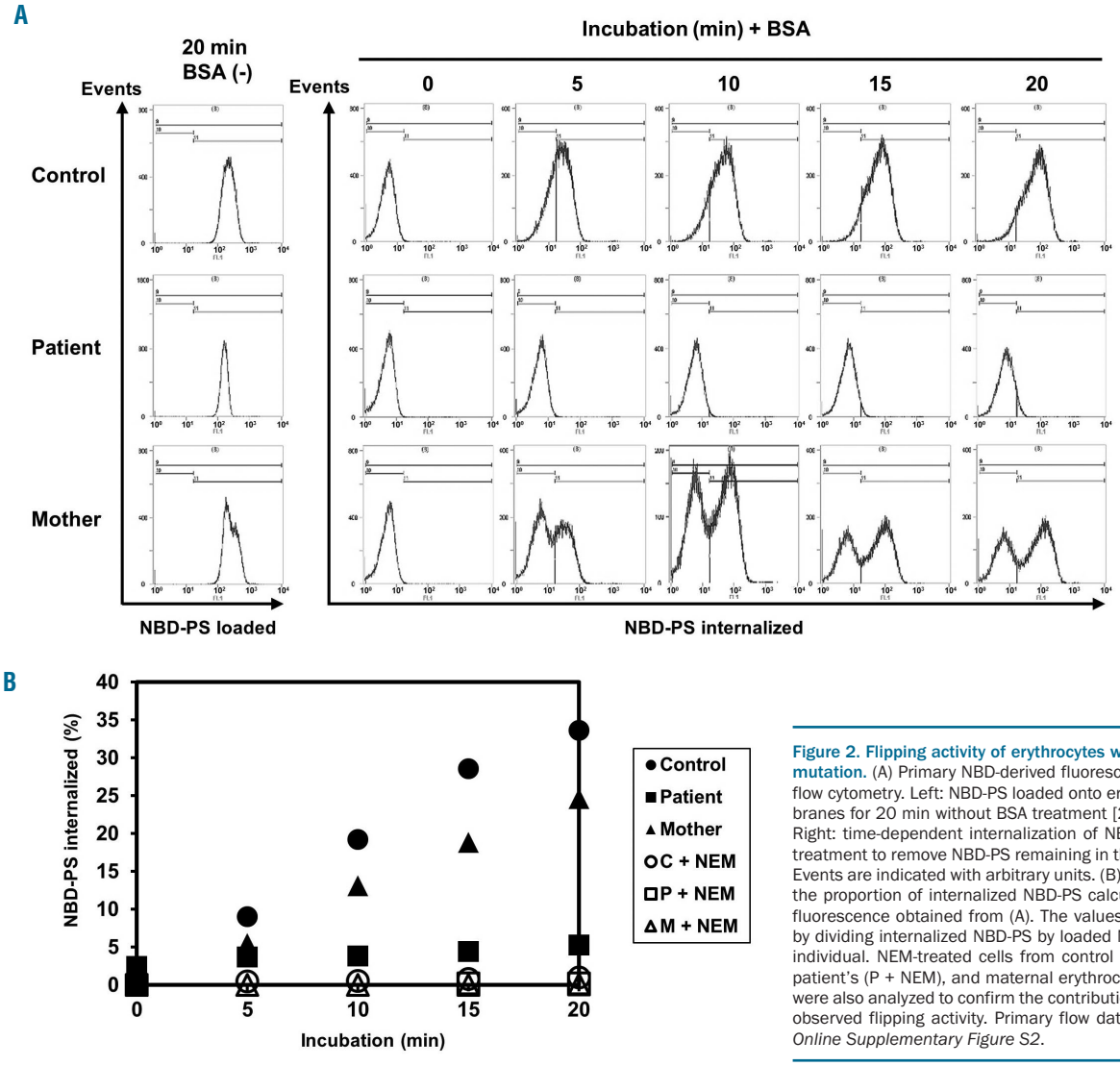

Figure 2. Flipping activity of erythrocytes with the *ATP11C* mutation. (A) Primary NBD-derived fluorescence data from flow cytometry. Left: NBD-PS loaded onto erythrocyte membranes for 20 min without BSA treatment [20 min BSA (-)]. Right: time-dependent internalization of NBD-PS with BSA treatment to remove NBD-PS remaining in the outer leaflet. Events are indicated with arbitrary units. (B) Quantitation of the proportion of internalized NBD-PS calculated by mean fluorescence obtained from (A). The values were obtained by dividing internalized NBD-PS by loaded NBD-PS in each individual. NEM-treated cells from control (C + NEM), the patient's (P + NEM), and maternal erythrocytes (M + NEM) were also analyzed to confirm the contribution of ATP11C to observed flipping activity. Primary flow data are shown in *Online Supplementary Figure S2*.

failed to elucidate the cause of hemolysis as hereditary spherocytosis, a red cell enzyme deficiency, or unstable hemoglobinopathy (*Online Supplementary Figure S1, Online Supplementary Table S1*). The identification of a mutation in the gene encoding the flippase, *ATP11C*, enabled us to discover a new candidate gene responsible for human congenital hemolytic anemia.

Although we predicted that loss of flipping activity could lead to a definitive increase of PS-exposing (positive) erythrocytes in the majority of the patient's circulating cells, this was not found to be the case. PS was retained in the inner leaflet of the vast majority of both control erythrocytes and those from the patient, suggesting that PS is not exposed on the erythrocyte cell surface as long as scramblase is inactive, regardless of flippase activity. The proportion of PS-exposing cells increased only in the densest senescent cells (0.1% of total) in which scramblase was activated to transport PS from the inner to outer leaflet by increased Ca^{2+} concentration. The proportion increased further in the patient's senescent cells with deficiency of flippase activity, indicating that ATP11C does play a role in active transport of externalized PS back to the inner leaflet to some extent in senescent erythrocytes. The distinct increase in PS-exposing cells in a small population of senescent cells from the patient indicated that PS exposure occurred at a very late stage of the patient's erythrocyte lifespan. It should be emphasized that PS-positive cells are continuously removed from the circulation by phagocytosis and those remaining in the circulation reflect the population that has not yet been cleared. The increased percentage of PS-positive senescent cells in the patient's circulation does, therefore, indicate persistent and mild hemolysis, corresponding to the clinical symptoms such as mild jaundice.

Interestingly, the maternal erythrocytes comprised two populations; 55-60% possessed normal PS transport activity, and 40-45% lacked flippase activity, like those of the patient, suggesting random inactivation of the X chromosome in the erythroblast populations. The proportion of PS-positive circulating erythrocytes in the mother was similar to that in controls and the woman is not anemic. These findings imply that hemolytic anemia in the proband with *ATP11C* mutation is inherited as an X-linked recessive trait.

A balance between flipping and scrambling activities maintains the asymmetric distribution of PS. Our finding that the proportion of PS-positive cells in which lipid scrambling was promoted by exogenous Ca^{2+} incorporation up to 50 μM was very similar between control erythrocytes and the patient's erythrocytes implies that ATP11C cannot com-

pete sufficiently with Ca^{2+}-activated PS scrambling to maintain PS asymmetry. In normal erythrocytes, the Ca^{2+} concentration increases transiently under shear stress-induced deformation during passage through narrow vessels and gradually increases during red cell senescence.[25,26] Under these conditions, scramblase is activated to scramble PS from the inner to outer leaflet, and the concerted effort of ATP11C and other flippases may not be sufficient to prevent Ca^{2+}-activated PS externalization in these cells. Together, our findings imply that suppression of scramblase activity rather than flippase activity is the major contributor to maintenance of PS in the inner leaflet of normal erythrocytes and that PS externalization as an "eat-me signal" depends primarily on scramblase activity at the end of the erythrocytes' lifespan.

Asymmetric PS distribution is important in other human cells. For instance, in platelet membranes PS is distributed in the inner leaflet probably by flippase activity under static conditions and exposed to the cell surface by Ca^{2+}-activated scrambling *via* TMEM16F when blood coagulation is initially activated.[27,28] Based on our findings concerning erythrocyte senescence, flippase activity cannot fully compensate for significantly increased scrambling activity.

No morphological change was observed in human erythrocytes with *ATP11C* mutation, while *Atp11c* mutant mice have anemia with stomatocytosis.[20] Other differences between the human and murine systems is that while there is 10-fold less flippase activity in mature human erythrocytes with mutant *ATP11C*, flippase activity is nearly normal in mature erythrocytes from *Atp11c* mutant mouse. In addition, while mRNA levels of both *ATP11A* and *ATP11C* were very similar in human erythroblasts, only *Atp11c* mRNA was highly expressed in mice, with no expression of *Atp11a* (*Online Supplementary Figure S3*). These findings suggest that total flippase activity might be significantly decreased or absent in the ery-

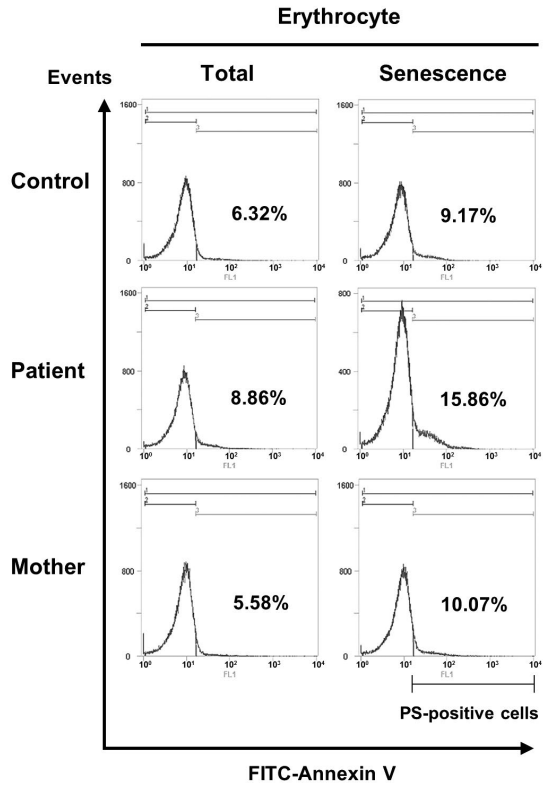

Figure 3. PS cell surface exposure in circulating erythrocytes with the *ATP11C* mutation. Exposed PS was detected by Ca^{2+}-dependent specific binding of FITC-annexin V. Unfractionated erythrocytes (total) and fractionated erythrocytes obtained from density gradient centrifugation (senescence) for the control, patient, and mother were suspended in isotonic buffer including 5 mM $CaCl_2$ before adding FITC-annexin V. Primary data obtained from flow cytometry analyses of FITC-derived fluorescence on erythrocytes are displayed. Events are shown with arbitrary units. The values in each panel indicate the proportion of PS-positive cells.

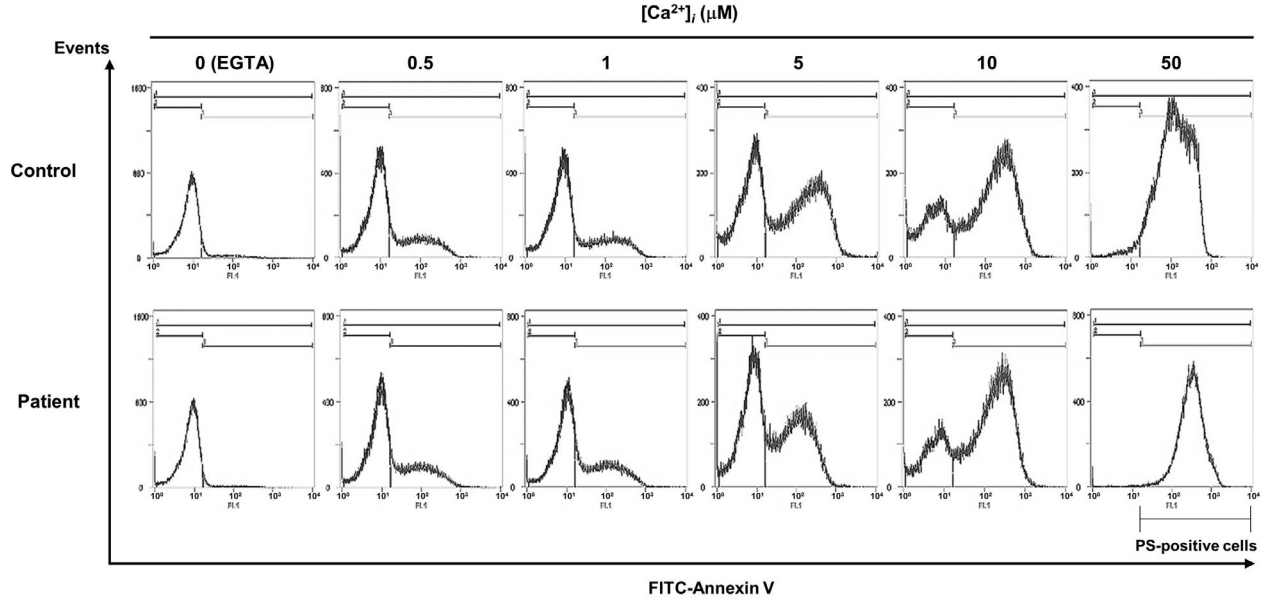

Figure 4. PS cell surface exposure in Ca^{2+}-loaded erythrocytes with the *ATP11C* mutation. Effect of Ca^{2+}-stimulated scrambling on PS exposure in control and patient's erythrocytes. Ca^{2+} was introduced by a Ca^{2+} ionophore, A23187, for 20 min at 37 °C. After removal of the Ca^{2+} ionophore, PS-exposed cells were analyzed by monitoring FITC-annexin V binding to erythrocytes.

throblasts of *Atp11c* mutant mice with resultant PS exposure on the outer membrane with subsequent gradual flipping back of the PS to the inner leaflet due to other flippases in mature erythrocytes, inducing the stomatocytic shape change.[29,30] On the other hand, the flippase activity is presumably maintained in the erythroblasts of human subjects with ATP11C deficiency, due to compensation by other flippases including ATP11A. As a result, mature erythrocytes with ATP11C deficiency may maintain the biconcave disc shape because most PS is located in the inner leaflet from the erythroblast stage to the mature erythrocyte stage. Anemia in the mutant mouse may result from PS-positive erythroblasts being possibly eliminated (ineffective erythropoiesis) as previously documented in the case of pyruvate kinase deficient mice.[31] Based on the expression of ATP8A1 in mature murine erythrocytes,[32] it is likely that flippase activity in murine erythrocytes is primarily driven by ATP8A1 while ATP11C is the primary flippase in human erythrocytes.

In summary, our analyses of a patient with mild hemolytic anemia identified ATP11C as a major flippase in human erythrocytes and showed that genetic mutation of *ATP11C* causes congenital mild hemolytic anemia inherited as an X-linked recessive trait. We suggest that the contribution of ATP11C to the maintenance of PS in the inner leaflet is important in senescent cells when scramblase is active but very subtle under physiological, low Ca^{2+} concentrations when scramblase is inactive.

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