Deletion of a flippase subunit *Tmem30a* in hematopoietic cells impairs mouse fetal liver erythropoiesis

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Haematologica 2019
Volume 104(10):1984-1994

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Received: August 7, 2018.

Accepted: February 27, 2019.

Pre-published: February 28, 2019.

doi:10.3324/haematol.2018.203992

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/104/10/1984

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ABSTRACT

Transmembrane protein 30A (*Tmem30a*) is the β-subunit of P4-ATPases which function as flippase that transports aminophospholipids such as phosphatidylserine from the outer to the inner leaflets of the plasma membrane to maintain asymmetric distribution of phospholipids. It has been documented that deficiency of *Tmem30a* led to exposure of phosphatidylserine. However, the role of *Tmem30a in vivo* remains largely unknown. Here we found that Vav-Cre-driven conditional deletion of *Tmem30a* in hematopoietic cells led to embryonic lethality due to severe anemia by embryonic day 16.5. The numbers of erythroid colonies and erythroid cells were decreased in the *Tmem30a* deficient fetal liver. This was accompanied by increased apoptosis of erythroid cells. Confocal microscopy analysis revealed an increase of localization of erythropoietin receptor to areas of membrane raft microdomains in response to erythropoietin stimulation in $Ter119^-$ erythroid progenitors, which was impaired in *Tmem30a* deficient cells. Moreover, erythropoietin receptor (EPOR)-mediated activation of the STAT5 pathway was significantly reduced in *Tmem30a* deficient fetal liver cells. Consistently, knockdown of *TMEM30A* in human $CD34^+$ cells also impaired erythropoiesis. Our findings demonstrate that *Tmem30a* plays a critical role in erythropoiesis by regulating the EPOR signaling pathway through the formation of membrane rafts in erythroid cells.

Introduction

Hematopoietic stem cells (HSC) are long lived and able to differentiate into several lineages which are required throughout life.[1] There are two distinct waves of hematopoietic cells during mammalian embryogenesis. The first wave progenitors arise in the circulation of the yolk sac (YS) at embryonic day 7.25 (E7.25), and produce primitive erythrocytes which are essential for the survival of the embryo. The second wave HSC arise at embryonic day 10.5 in the dorsal aorta and differentiate

into mature blood cells in the fetal liver.[2] During embryogenesis, primitive erythroid cells (EryP) first arise from mesodermal progenitors and are detected within 'blood islands' at around E7.5. The maturation of erythroid precursors occurs in the blood circulatory system, where the nucleuses are condensed and embryonic hemoglobin is accumulated.[3] Definitive erythroid cells (EryD) rapidly outnumber the EryP in the growing fetal liver,[4,5] which are identified as β-globin switching and smaller enucleated erythroid cells.[6] The fetal liver is the key organ for definitive erythropoiesis during mid gestation. Definitive erythroid cells can be distinguished into five different sub-populations from R1 to R5 by double staining with the surface markers CD71 and Ter119.[7] Erythropoiesis comprises distinct differentiation stages including burst-forming unit-erythroid (BFU-E), colony-forming unit-erythroid (CFU-E), proerythroblast, basophilic erythroblast, polychromatic erythroblast, orthochromatic erythroblast, reticulocyte and erythrocyte. From the CFU-E stage onwards, the cell starts to express erythropoietin (EPO) receptor (EPOR). CFU-E and proerythroblat require EPO for survival.[8]

Erythroid differentiation occurs at the erythroblastic islands and is regulated by various cytokines and chemokines. EPO and stem cell factor (SCF) play essential roles in erythroid progenitor proliferation and differentiation. EPO is mainly synthesized in liver during embryo genesis and produced in the kidney in adult mammals. EPO/EPOR-mediated signaling transduction is crucial for primitive and definitive erythropoiesis both in the fetal liver (FL) and in the bone marrow.[9] EPO has two receptors: one is a homodimer of two EPO receptors (EPOR), another is a heterodimer consisting of EPOR and CD131.[10] The homodimeric EPO receptor exists in an unliganded state with the pre-bound tyrosine kinase JAK2.[11] Upon binding EPO, EPOR undergoes a conformational change that actives JAK2 which in turn phosphorylates tyrosine residues in the cytoplasmic tail of the EPOR.[12] This binding results in activation of STAT5, which leads to the activation of BCL-XL by direct STAT5 binding to the BCL-X promoter.[13] BCL-XL is a potent inhibitor of programmed cell death and inhibits activation of caspases in cells through direct interaction between caspases and BCL-XL.[14,15] The activation of the JAK2-STAT5 pathway through EPO/EPOR signaling is critical for sustaining the viability of erythroid cells in the fetal liver.[16]

Lipid rafts are small microdomains (10-200 nm) enriched in cholesterol and sphingolipids that can form larger platforms by protein-protein and protein-lipid interactions. The inner leaflet phosphatidylserine is essential for the coupling of actin with lipid-anchored proteins. The actin cytoskeleton clustering determines and immobilizes long saturated acyl chains phospholipids in the inner leaflet.[17] This immobilization engages in glycosylphosphatidylinositol (GPI)-anchored proteins in the outer monolayer interacted by cholesterol, which form the local raft domains. The most important role of lipid rafts is to separate and regulate specific membrane components with other components, thereby increasing the concentration of signaling molecules.

In eukaryotic cells, phospholipids are distributed asymmetrically between the inner and the outer layers of the plasma membrane.[18] Phosphatidylserine (PS) and phosphatidylethanolamine (PE) are mainly located in the inner monolayer while phosphatidylcholine (PC) is essentially present at the outer monolayer.[19,20] Lipids distributions are preserved by many of phospholipid transporters which can be separated into three groups including scramblases, flippases and floppases.[21] One of the most important transporters are the members of the Type-IV P-type ATPases (P4-ATPases) family which possess flippase activity that transports lipids from the outer to the inner leaflet to maintain phospholipid asymmetry. *Tmem30a* (also named CDC50A), the β-subunit of P4-ATPases, is essential for the formation of functional transporter complexes that act as flippase.[22] Maintenance of cell membrane asymmetry by flippase is critical as the loss of this asymmetry usually causes pathological phenotypes.[23]

To investigate the function of *Tmem30a* in embryonic hematopoiesis, we generated hematopoietic-specific *Tmem30a* deficient mice with conditional *Tmem30a* alleles and Cre recombinase expression controlled by the VAV promoter.[24] *Tmem30a* deficient mice (cKO) died *in utero* by E16.5 with severe anemia. Interestingly, *Tmem30a* is not essential for the maintenance of HSC homeostasis, but is essential for the definitive erythropoiesis. Moreover, *Tmem30a* deficiency impaired flippase activity, lipid rafts formation, and activation of EPOR/JAK2/STAT5/BCL-XL pathway. Our findings demonstrate the critical role of *Tmem30a* in erythropoiesis and uncover previously unknown mechanisms by which EPOR signal transduction pathway is initiated.

Methods

Mice

All mouse protocols were approved by the Institutional Animal Care and Use Committee of Jinan University, China. Tmem30a$^{WT/flox}$ mice were kindly provided by Prof. Xianjun Zhu and were back-crossed onto a C57/BL6 background. Exon 3 of the *Tmem30a* gene is flanked by loxP sites. The Vav-Cre line we used was B6.Cg-*Commd10*^{Tg}*(Vav*$^{1-icre}$*)*^{A2Kio} to generate hematopoietic deletion, as described previously.[25]

Flow cytometry

Cells were stained with APC-conjugated rat anti–mouse TER-119 (clone: Ter119, Biolegend) and PE conjugated rat anti-mouse CD71 (clone: RI7217, Biolegend) on ice for 30 minutes (min) in the dark. The cells were washed twice, followed by staining with fixable viability DAPI (0.25 μg/10^6 cells) and analyzed within 1 hour (h) of staining. For apoptosis, cells were additionally stained for 15 min in the dark with 10 μL of Annexin V-FITC in 100 μL 1xbinding buffer.[26] Cells were washed and cell pellets were re-suspended in 500 μL 1xbinding buffer containing 5 μL of 7AAD and immediately analyzed by a BD FACS Fortessa machine. Apoptosis was also assessed using TUNEL assay by flow cytometry using APO-BrdU TUNEL assay kit (Invitrogen, A23210).

Western blot analysis

Fetal liver cell samples were separated and then transferred to the polyvinylidene fluoride membrane. Primary antibodies to STAT5 (#9363, CST), Phospho-STAT5 (Tyr694) (#9351, CST), BCL-XL (54H6) (#2764, CST), Flotillin-2 (B-6) (SC-28320, Santa Cruz), TMEM30A (AV47410, Sigma-Aldrich), EPO-R (SAB4500780, Sigma-Aldrich) and β-Actin (A5316, Sigma-Aldrich) were used. The membrane was incubated with horseradish peroxidase enzyme conjugated secondary antibody for 1 h at RT. Clarity western ECL substrate solutions were dropped onto the membrane.

Erythroid differentiation *in vitro*

E14.5 fetal liver cells were labeled with APC-conjugated anti-mouse Ter119 antibody for 30 min followed by staining with anti-APC microbeads. Ter119- cells were purified and cultured in erythroid-differentiation medium containing Iscove modified Dulbecco medium, 15% FBS, 1% detoxified bovine serum albumin, 200 μg/mL recombinant human transferrin, 10 μg/mL recombinant human insulin, 10-4 M β-mercaptoethanol, and 2.5 U/mL recombinant human EPO. After culturing the cells for 24 h, the medium was replaced with IMDM, 20% FBS and 10^{-4} M β-mercaptoethanol for another 24 h. Q-VD-OPh hydrate (#SML0063, Sigma) was added to the erythroid-differentiation medium for every 24 h.

Knockdown of *TMEM30A* by lentivirus in human CD34+ cells

Human CD34-positive (+) cells were purified and harvested from cord blood. The CD34+ cells were differentiated into erythroid cells and the differentiation was assessed by flow cytometry using GPA, Band 3 and α4 integrin as surface markers. pLKO1 vectors which express shRNA against the targeted gene of *TMEM30A* were purchased from Sigma-Aldrich. The sequences were as follows: sh-*TMEM30A*-1: GACAACCTGGAAGAACGATTT. sh-*TMEM30A*-2: GAGATTCTAGTGCTTTGCTTA. Lentivirus was prepared and transfected into CD34+ cells on the culture day 2, as described previously. Knockdown efficiency was checked by real-time polymerase chain reaction (RT-PCR). The primers for TMEM30A were: forward primer-5'-GCGATGAACTATAACGCGAAGG-3'; reverse primer-5'-GCCAATGCCGATGGGAATGA-3'.

Statistical analysis

FACS analysis was performed using FlowJo software (BD, version 10). Statistical analysis was performed using GraphPad prism software (version 7). Band signal intensities were analyzed with ImageJ. The data were shown as the Mean±Standard Error of Mean (SEM). Differences among two groups were calculated by Student unpaired *t*-test. $P<0.05$ was considered statistically significant.

Details of the other methods used in this study are available in the *Online Supplementary Materials.*

Results

Tmem30a deficient mice are embryonic lethal with anemia at mid gestation

To investigate the function of *Tmem30a* in hematopoiesis, we crossed *Tmem30a*$^{flox/flox}$ mice with B6.Cg-Commd10Tg(Vav1-icre)A2Kio mice to generate hematopoietic-specific *Tmem30a*-deficient mice (*Online Supplementary Figure S1A and B*). The *Tmem30a*$^{flox/flox}$, VavCreTg/+ (*Tmem30a* cKO) mice were not viable. The surviving *Tmem30a* cKO embryos at embryonic day 14.5 (E14.5) are pale (Figure 1A), suggesting a defect in fetal hematopoiesis. Genotyping of the progeny embryos showed that the living embryos roughly followed the expected Mendelian ratio with 25% *Tmem30a* cKO embryos up to E12.5, but that ratio dropped to 0% by E16.5 (Figure 1B). The *Tmem30a* cKO fetal liver was noticeably smaller (Figure 1D), and the number of fetal liver cells was significantly reduced in the cKO mice compared to that of control mice at E14.5 (Figure 1C). Hematoxylin & Eosin staining of *Tmem30a* fetal liver sections showed a severe atrophic phenotype (Figure 1E). Red blood cell (RBC) count, hematocrits and hemoglobin levels were significantly decreased in the peripheral blood of the E14.5 *Tmem30a* cKO embryos compared with controls (Figure 1F-H). Wright-Giemsa staining of peripheral blood smears showed a large fraction of nucleated erythrocytes (Figure 1I) in *Tmem30a* cKO blood compared to control. To explore the reasons of the impaired hematopoiesis in *Tmem30a*-deficient embryos, we first analyzed the maintenance of HSC in fetal livers by flow cytometry (*Online Supplementary Figure S1C*).[27] The total number of HSC (Lin⁻Mac⁻1lowSac-1+ CD48⁻CD150+ cells) in cKO embryos was comparable to controls at E14.5 (*Online Supplementary Figure S1D*), indicating the impaired fetal hematopoiesis was not due to a defect in the FL HSC in cKO embryos, although *Tmem30a* is expressed in FL HSC as well as in T cells, B cells and erythroid cells (*Online Supplementary Figure S1E*). Interestingly, colony forming assay showed that the numbers of BFU-E and CFU-E colonies were drastically reduced in cKO embryos (Figure 1J and K). In contrast, no differences were seen in the colonies of CFU-G, CFU-M and CFU-GM between cKO and control (*data not shown*). These findings suggest that loss of *Tmem30a* in mice resulted in a severe mid gestation anemia, likely due to impaired fetal liver erythropoiesis.

Tmem30a is necessary for definitive erythropoiesis in the fetal liver

Next, we investigated fetal liver erythroid progenitor cells in the fetal liver. The number of EryP in cKO fetal livers was comparable with control (*Online Supplementary Figure S2A and B*). We also analyzed erythro-myeloid progenitors at embryo day 9.5 in the yolk sac by flow cytometry (*Online Supplementary Figure S2C*). The absolute number of progenitors in cKO was comparable with the control group (*Online Supplementary Figure S2D*). Finally, we analyzed definitive erythropoiesis using CD71 and Ter119 as surface markers (Figure 2A). The results showed that R3 erythroblast population (CD71^{hi}/Ter119^{hi}) was predominantly affected in the fetal livers of cKO embryos, suggesting a severe blockage in terminal erythroid differentiation from Ter119^{low} to Ter119^{hi} cells (Figure 2B). To further characterize terminal erythroid differentiation, we used FSC to separate Ter119^{hi} cells into three populations: S1 (large), S2 (medium), and S3 (small) (Figure 2C). The number of erythroblasts in S1 and S2 populations (basophilic to orthochromatic stages) and S3 population (reticulocytes) was decreased, suggesting a further defect at very late stage of erythroid maturation (Figure 2D). Next, we analyzed erythroblast enucleation in S1-S3 populations using live-cell nuclear staining with Syto-16 (Figure 2E). Most enucleation events occurred in the S3 population, and *Tmem30a* cKO fetal livers showed a significant reduction of enucleating efficiency in this population (Figure 2F).

Tmem30a deficiency leads to apoptosis of fetal liver erythroid cells

To explore the underlying mechanisms, we examined proliferation and apoptosis of the fetal liver cells. BrdU incorporation assays showed that the cell cycle profiles were comparable between *Tmem30a* cKO and control mice (*Online Supplementary Figure S3A and B*). We then investigated apoptosis, which is usually assessed by Annexin V binding to exposed PS. *Tmem30a* deficiency lead to increased Annexin V levels (Figure 3A and B). Since

Tmem30a deletion leads to PS exposure because of impaired PS translocation, it is difficult to determine whether the PS exposure is due to apoptotic or impaired PS translocation. Therefore, we employed the TdT-mediated dUTP nick end labeling (TUNEL) assay for determining the intrinsic cellular apoptosis, thereby analyzing the apoptosis-related DNA fragmentation. TUNEL staining showed that the cKO fetal liver cells displayed significantly higher TUNEL positive cells compared with controls (Figure 3C and D). Taken together, these data indicate that the defect of erythropoiesis in the Tmem30a deficient fetal liver is at least partly due to increased apoptosis.

Tmem30a deficiency impairs phosphatidylserine flippase activity in erythroid cells

Tmem30a is the β-subunit of the P4-ATPase, which functions as a flippase to maintain phospholipid asymmetry. Previous study showed that loss of *Tmem30a* resulted in impaired PS translocation.[22] Consistent with the previous study, our data showed that *Tmem30a* deficiency led to increased PS exposure in erythroid cells, as indicated by increased levels of Annexin V positive cells (Figure 4A and B). To examine if the increased PS exposure is at least partly due to impaired flippase activity, we analyzed the aminophospholipid flippase activity. NBD-PS fluorescence

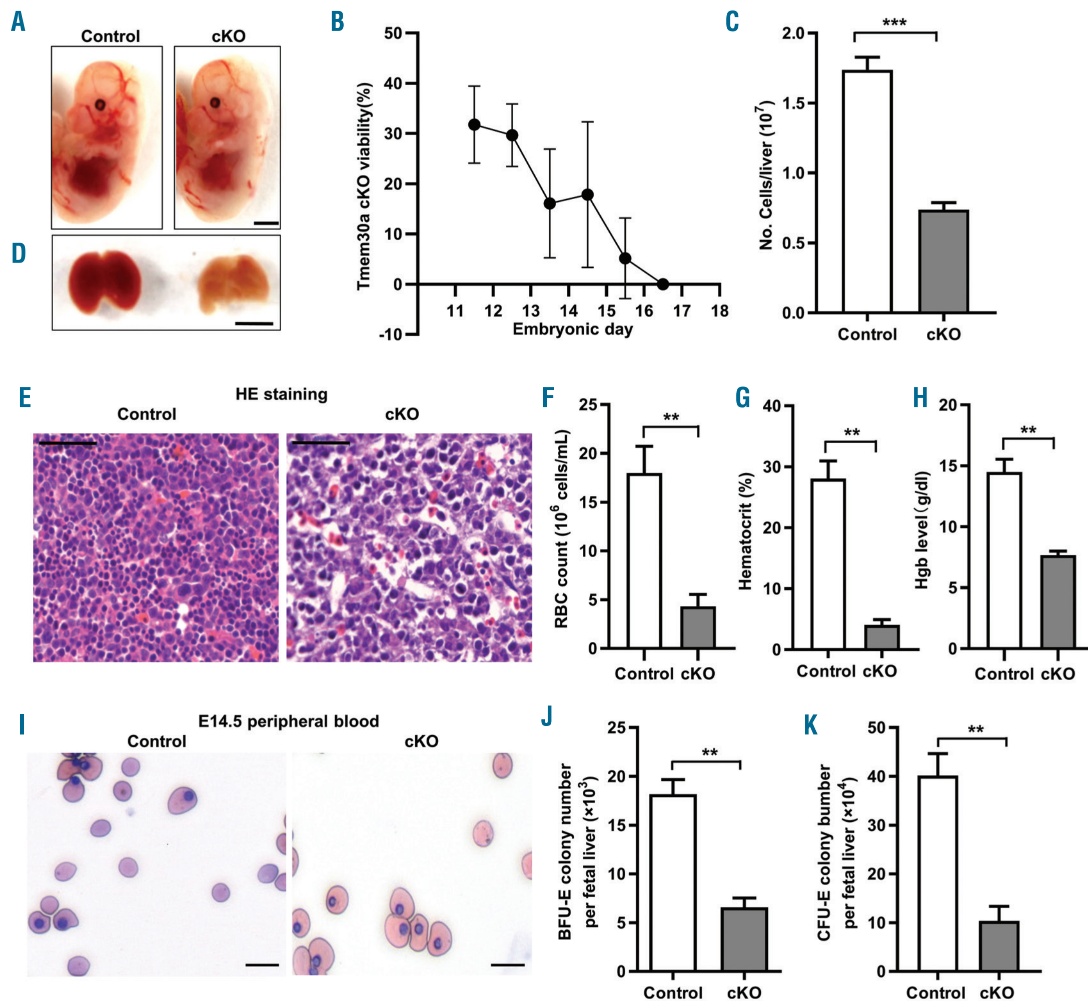

Figure 1. *Tmem30a*-deficient mice (cKO) are embryonic lethal with anemia. (A) Viability of *Tmem30a* embryos was determined. Scale bar represents 2 mm. (B) Timed matings of *Tmem30a*$^{WT/flox}$; VavCre$^{Tg/+}$ and Tmem30a$^{flox/flox}$; VavCre$^{-/-}$ were performed and embryos were harvested at stages E11.5 up to E16.5. The percentages reflect the numbers of living Tmem30a cKO embryos with respect to all embryos harvested in litters at each gestational stage. At least six pregnant mice have been checked at each time point and more than 35 embryos have been examined. (C) Gross appearance of E14.5 fetal livers. The cKO fetal livers were noticeably smaller than fetal livers of controls. Scale bar represents 2 mm. (D) The number of total fetal liver cells was counted in control and cKO embryos at E14.5. Data are presented as Mean ±Standard Error of Mean (SEM) for at least eight embryos per genotype. (E) Hematoxylin & Eosin staining of paraffin sections from *Tmem30a*$^{flox/flox}$; VavCre$^{-/-}$ and *Tmem30a*$^{flox/flox}$; VavCre$^{Tg/+}$ E14.5 fetal livers fixed in 4% formaldehyde solution. Scale bars represent 50 μm. (F) The red blood cell number was decreased in *Tmem30a* cKO peripheral blood. (G) Tmem30a cKO embryos exhibited reduced hematocrit compared with control mice at mid-gestation E14.5. (H) Hemoglobin levels were decreased in the peripheral blood in the cKO embryos. (I) Peripheral blood in Tmem30a cKO embryos reveals a large number of nucleated erythrocytes when sections underwent Wright-Giemsa staining. Scale bars represent 20 μm. (J) The BFU-E colony numbers were decreased in E14.5 cKO embryos compared with cKO. (K) CFU-E colony numbers per fetal liver of cKO were decreased at E14.5 *in vitro*. The graphs are representatives of three biological repeats. Data are indicated as Mean±SEM of three samples per each genotype. **P<0.01; ***P<0.001.

increased rapidly in wild-type cells, but this increase was significantly blunted in cKO cells (Figure 4C and D). Interestingly, the flippase activity was mainly compromised in the R1 to R3 cell populations (Figure 4E and F), indicating correlation between flippase activity and cell development. We also analyzed the percentage of NBD-PS fluorescence positive cells in the S1 to S3 cell population and found that the flippase activity was mainly compromised in S1 and S2 cell populations (*Online Supplementary Figure S4A and B*). These data demonstrate that *Tmem30a* is crucial for phospholipid flipping in erythroid cells.

Tmem30a deficiency compromises lipid raft clustering upon erythropoietin treatment

The above data demonstrate that *Tmem30a* is required for phospholipid flipping. PS is essential for the coupling between actin and lipid anchored proteins, and thereby the formation of functional local raft-like domain at the plasma membrane. One of the most important roles of lipid rafts is to separate and regulate specific membrane components with other components and thereby increasing the concentration of signaling molecules. To examine whether depletion of *Tmem30* affects lipid raft formation

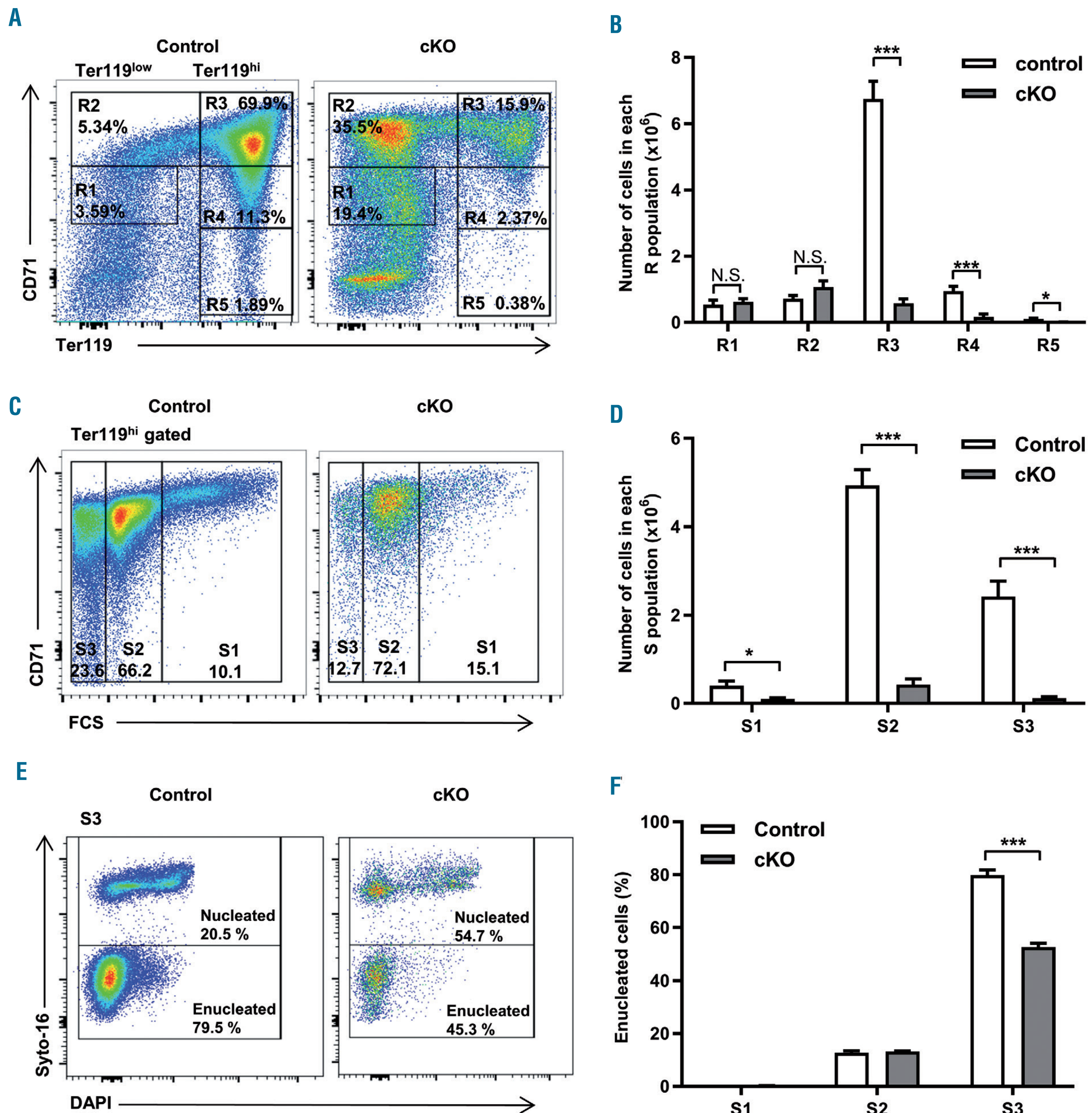

Figure 2. Tmem30a-deficient mice (cKO) embryos are defective in definitive erythropoiesis. (A) Representative flow cytometric profiles of control and *Tmem30a* cKO fetal liver single cells stained with CD71 and Ter119. Gates from R1 to R5 were set as indicated. (B) Absolute number of cells in each R population was calculated in the fetal liver from each embryo. Data are represented as mean±Standard Error of Mean (SEM) of the cell count of six fetal livers for each embryonic data set. (C) Representative CD71/FSC profiles of Ter119hi cells sorted into three populations according to cell size. The percentage of cells in each S population with respect to total Ter119hi cells are indicated for one representative fetal liver. (D) Comparison of the number of S1, S2, and S3 populations. (E) Representative flow cytometry of enucleated cells in the Ter119 hi population of fetal livers, using Syto-16 for nuclei and DAPI for cell viability. (F) Percentages of enucleated cells in the Ter119 hi populations. Data are presented as Mean±SEM of the cell count of six fetal livers for each embryonic data set. **P*<0.05; ***P*<0.01; ****P*<0.001.

and EPOR signaling, we analyzed the lipid raft distribution by using cholera toxin subunit B (CTxB) to label endogenous GM1 ganglioside, a component of lipid rafts. Co-staining of GM1 and EPOR on Ter119^{low} erythroid cells showed that EPO treatment stimulated the EPOR clustering and co-localized with the lipid rafts. Interestingly, *Tmem30a* deletion inhibited lipid raft clustering and EPOR co-localization with the lipid rafts (Figure 5A and B). The specificity of the EPOR antibody was demonstrated by immunofluorescence analysis omitting the primary anti-EPOR antibody as negative control (*Online Supplementary Figure S5A*). To further determine whether TMEM30A deficiency impedes the recruitment of EPOR to lipid raft, lipid rafts were separated from TER119 negative fetal liver cells upon EPO treatment. Western blot showed that the EPOR protein was presented in the extracted lipid rafts, but the level of EPOR was decreased in cKO cells (*Online Supplementary Figure S5B*). Taken together, these data suggest that the impaired lipid raft clustering upon EPO stimulation in *Tmem30a*-deficient fetal liver erythroid cells may compromise the EPO/EPOR signaling, which is essential for fetal liver erythropoiesis.

Tmem30a deficiency compromises STAT5 activation and down-regulates pro-survival protein BCL-X$_L$

It has been well documented that EPO/EPOR signaling activates the JAK2-STAT5 pathway to sustain the viability of erythroid cells in the fetal liver.[13] The above findings strongly suggest that the observed phenotypic changes of Tmem30a knockdown erythroid cells may be due to impaired EPO/EPOR signal transduction. To test this, we investigated the key components of the EPO/EPOR-JAK2-STAT5 signaling pathway. FACS analysis using EPOR antibody staining on living TER119$^-$ erythroid cells showed that the levels of EPOR expression on the cell surface were not reduced in cKO mice compared to control mice (*Online Supplementary Figure S5C*). Intriguingly, after EPO stimulation in culture, the activation of the JAK2-STAT5 signaling pathway was significantly impaired in *Tmem30a* deficient fetal liver cells, as demonstrated by the lack of phosphorylation of STAT5 in cKO cells upon EPO treatment (Figure 6A). In addition, the downstream transcriptional target genes of STAT5 signaling[28] such as Pim1, Socs3 and BCL-XL were significantly decreased after EPO exposure in *Tmem30a* deficient fetal livers compared to the controls (Figure 6B). Among these genes, BCL-X$_L$ is essential for the survival of erythroid cells. Therefore, we further analyzed the protein levels of BCL-X$_L$ by western blotting. The pro-survival protein BCL-X$_L$ was dramatically decreased in cKO fetal liver although exposure to EPO did not increase BCL-X$_L$ expression (Figure 6C). Perhaps 30-min exposure to EPO was not enough to increase the Bcl-X$_L$ protein level. BCL-

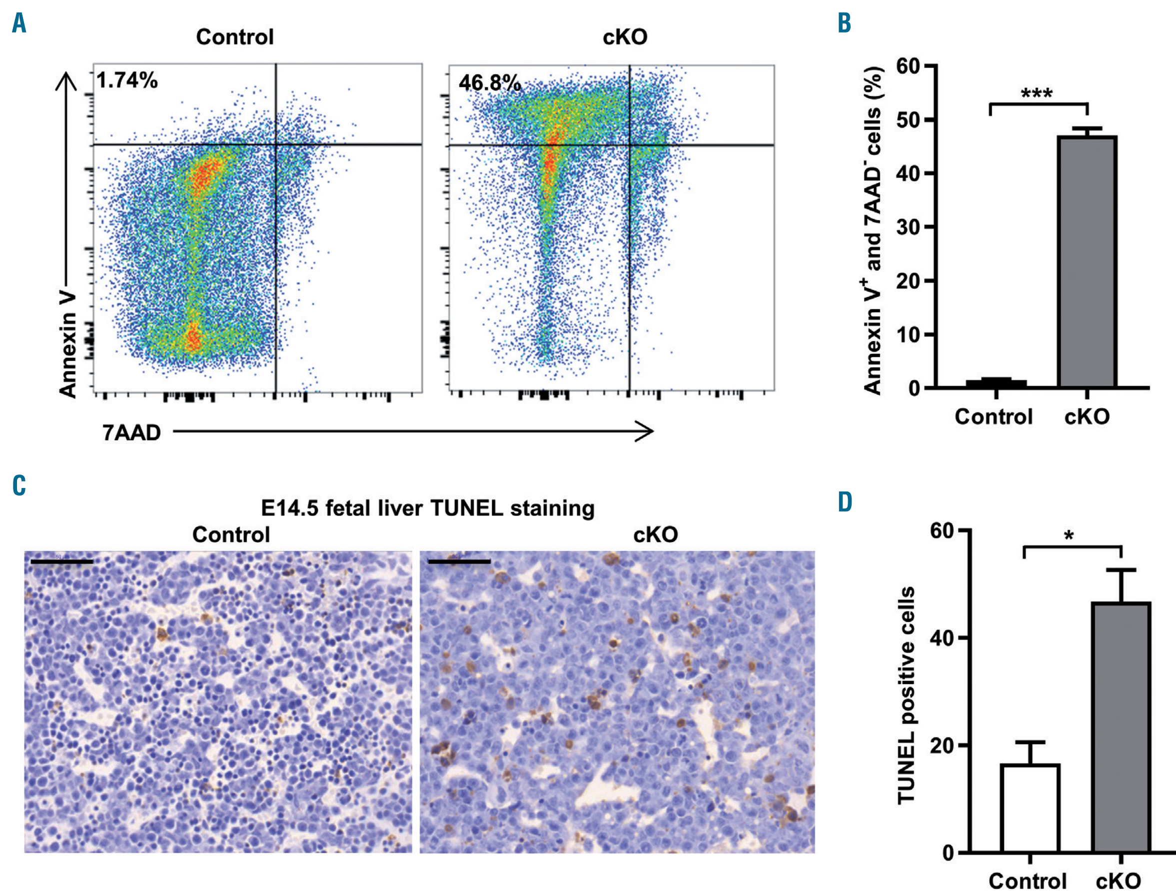

Figure 3. *Tmem30a*-deficient mice (cKO) fetal liver erythroid cells display a higher frequency of apoptosis. (A and B) Flow cytometric analysis of *Tmem30a* cKO fetal livers revealed increased percentages of Annexin V positive cells compared with wild-type littermate controls. (C) TUNEL staining of paraffin sections from fetal livers fixed in 4% formaldehyde solution. Scale bars represent 50 μm. (D) The number of TUNEL positive cells per 1.3*10^5 square micrometers were calculated. All values are presented as Mean±Standard Error of Mean of three fetal livers per each embryonic data. **P*<0.05; *** *P*<0.001.

X_L functions as a substrate to directly inhibit caspases before cleavage;[14] therefore, we measured caspase 3/7 activity using luminescent assays. Caspase 3/7 activity was increased in cKO fetal liver cells (Figure 6D). Next, we used Q-VD-OPh hydrate, a pan-caspase inhibitor to treat the Ter119^{low} erythroid cells in order to rescue the impaired definitive erythropoiesis in *Tmem30a* cKO erythroid cells (Figure 6E).[29] Interestingly, the presence of 50 μM Q-VD-OPh partially rescued erythroid cell maturation of *Tmem30a*-deficient erythroid cells (Figure 6F).

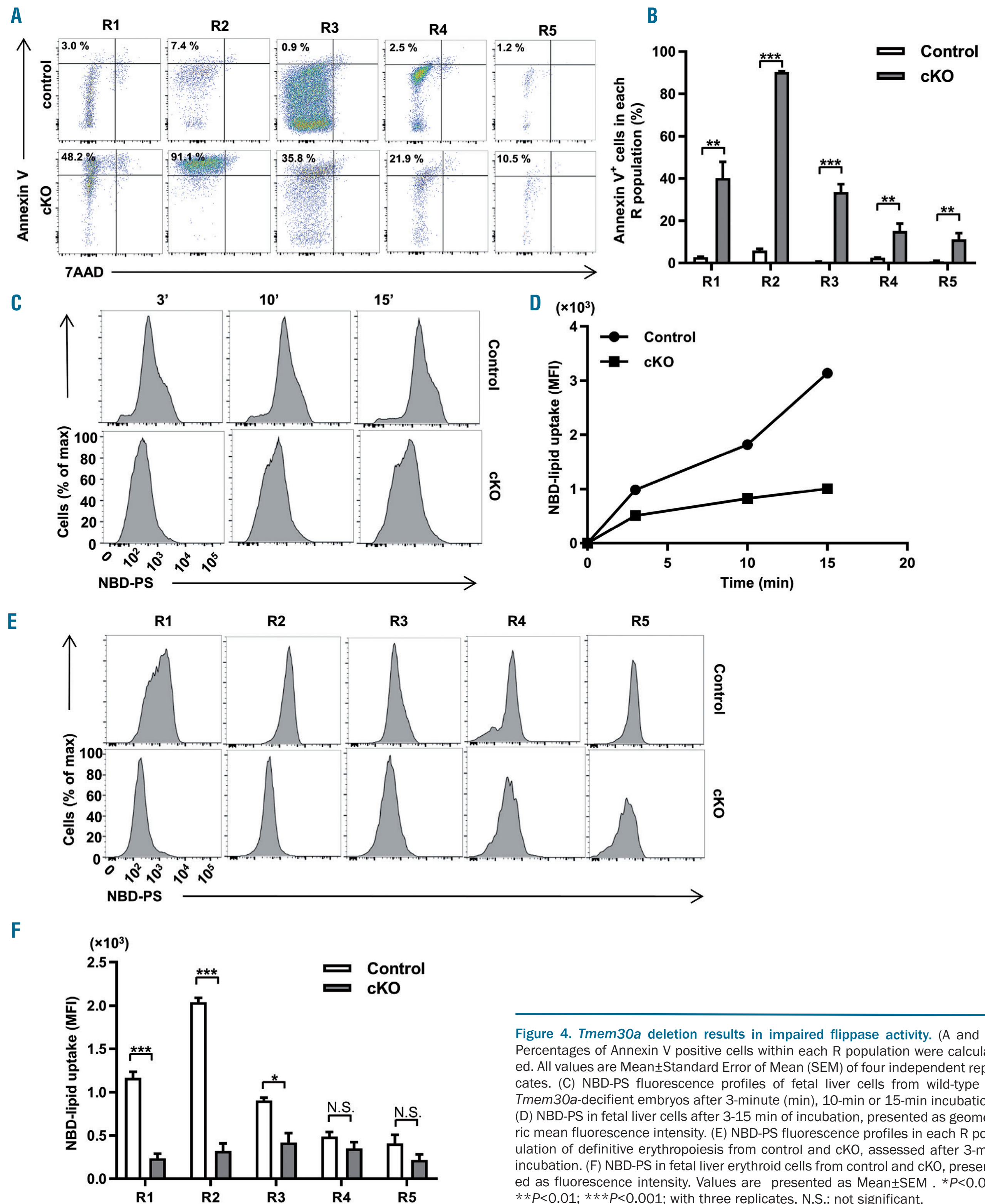

Figure 4. *Tmem30a* deletion results in impaired flippase activity. (A and B) Percentages of Annexin V positive cells within each R population were calculated. All values are Mean±Standard Error of Mean (SEM) of four independent replicates. (C) NBD-PS fluorescence profiles of fetal liver cells from wild-type or *Tmem30a*-decifient embryos after 3-minute (min), 10-min or 15-min incubation. (D) NBD-PS in fetal liver cells after 3-15 min of incubation, presented as geometric mean fluorescence intensity. (E) NBD-PS fluorescence profiles in each R population of definitive erythropoiesis from control and cKO, assessed after 3-min incubation. (F) NBD-PS in fetal liver erythroid cells from control and cKO, presented as fluorescence intensity. Values are presented as Mean±SEM . **P*<0.05; ***P*<0.01; ****P*<0.001; with three replicates. N.S.: not significant.

TMEM30A is required for human erythroid differentiation

To examine whether *TMEM30A* also plays a role in human erythropoiesis, we used shRNA-mediated knockdown approach in human cord blood CD34$^+$ cells.[30-32] *Online Supplementary Figure S6A* shows efficient knockdown of *TMEM30A*. As demonstrated by the decreased expression of GPA (*Online Supplementary Figure S6B*), delayed upregulation expression of band 3/downregulation of α4 integrin (*Online Supplementary Figure S6C*), *TMEM30A* knockdown impaired erythroid differentiation. Knockdown of *TMEM30A* also led to reduced cell growth (*Online Supplementary Figure S6D*). Moreover, similar to the murine data, *TMEM30A* knockdown also induced a significantly increase in the frequency of Annexin V positive cells (*Online Supplementary Figure S6E and F*). We further detected apoptosis of control and *TMEM30A* knockdown human erythroid cells by TUNEL assay. *TMEM30A* knockdown led to increased apoptosis (*Online Supplementary Figure S6G and H*). Finally, we examined the effect of *TMEM30A* on EPOR-mediated signal transduction in human erythroid cells. *TMEM30A* knockdown resulted in attenuated phosphorylation of EPOR downstream target STAT5 (*Online Supplementary Figure S6I and J*). Thus, *TMEM30A* plays a conserved function in both human and murine erythropoiesis.

Discussion

Studies over the past decade have clearly documented that *Tmem30a* is required for the flippase activity of P4-ATPases.[33] However, the function of *Tmem30a in vivo* remains largely unexplored. In the present study, we found that, unexpectedly, selective deletion of *Tmem30a* in hematopoietic cells severely impaired fetal liver erythropoiesis which contributes to the embryonic lethality of the mice. Our findings have, therefore, uncovered a novel role for *Tmem30a* in erythropoiesis.

In exploring the underlying mechanisms for the impaired erythropoiesis, we found that, while *Tmem30a* deletion did not affect cell cycle, it led to increased apoptosis of erythroid cells. Interestingly, the increased apoptosis is due to significantly impaired activation of JAK2-STAT5 signal transduction pathway, the essential pathway for survival of erythroid cells. Further examination revealed that *Tmem30a* deletion impaired lipid rafts formation accompanied with impaired EPOR clustering. Our findings provide new insights into the mechanisms by which EPO/EPOR signal transduction pathway is regulated.

The type 4 subfamily of P-type adenosine triphosphatases (P4-ATPases) actively transports phospholipids across the membrane bilayer. There are 14 P4-ATPases (ATP1-14) in eukaryotes whereas only three *Tmem30* (termed *Tmem30a*, *Tmem30b*, *Tmem30c*) homologs are identified, each *Tmem30* protein interacting with multiple P4-ATPases.[21,34] It is very interesting to note that, of the three Tmems, only *Tmem30a* is expressed in both murine and human erythroid cells. Since members of the *Tmem* family can compensate each other, the lack of expression of other Tmem family members in erythroid cells may explain the severe phenotypic changes of erythroid cells when *Tmem30a* is depleted. These findings imply the important role of flippase activity in erythropoiesis. Together with previous findings that ATP11C mutated mice showed a lower rate of PS translocation in pre-B cells and defective differentiation of B lymphocytes,[35] we suggest that flippase activity may play important roles in hematopoiesis in general, and this warrants future studies. It is likely that members of P4-ATPases family and Tmem family may contribute to hematopoiesis in a lineage-specific manner.

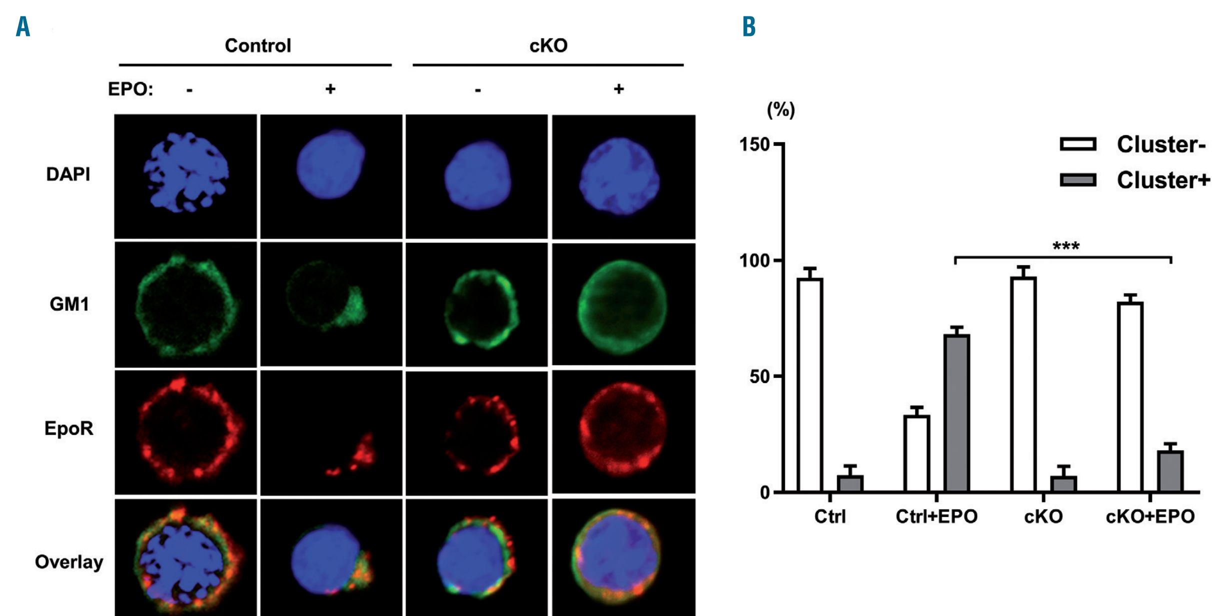

Figure 5. *Tmem30a* deletion impairs erythropoietin receptor (EPOR) clustering. (A) Isolated Ter119 low erythroid cells were stained with DAPI (blue) and with an anti-EPOR antibody (red). Lipid rafts were detected using GM1 ganglioside with FITC conjugated CTB (green). (B) Proportions of cells that showed lipid raft clustering are indicated as cluster+. Values are presented as Mean±Standard Error of Mean. ***P<0.001. N=3 slides per group.

The fact that EPO/EPOR mediated signal transduction pathway in erythroid cells is essential for erythropoiesis has been well documented.[36] However, how the pathway is initiated remains unclear. One striking finding of the current study is that EPO induces the clustering of EPOR at the areas of lipid raft domains on the plasma membrane. Importantly, disruption of lipid rafts formation due to *Tmem30a* deletion led to failure of EPOR clustering as well as impaired activation of JAK2-STAT5. These findings imply that initiation of EPOR signal pathway requires lipid rafts-mediated EPOR clustering. It has been shown that SCF receptor is essential for erythropoiesis because mutation of c-kit caused severe anemia.[37] Therefore, in addition to EPO receptor, other receptors may also be affected after *Tmem30a* deletion. Moreover, a recent study showed that *Tmem30a* plays an essential role in ensuring the survival of hematopoietic cells in adult mice,[38] suggesting that *Tmem30a* play different functions between embryo and adult hematopoiesis.

In summary, our study has uncovered a critical role of *Tmem30a* in erythropoiesis and identified the underlying mechanisms. As *Tmem30a* is required for the flippase activity, our findings suggest the role of flippase in erythropoiesis. Together with other findings, our study has

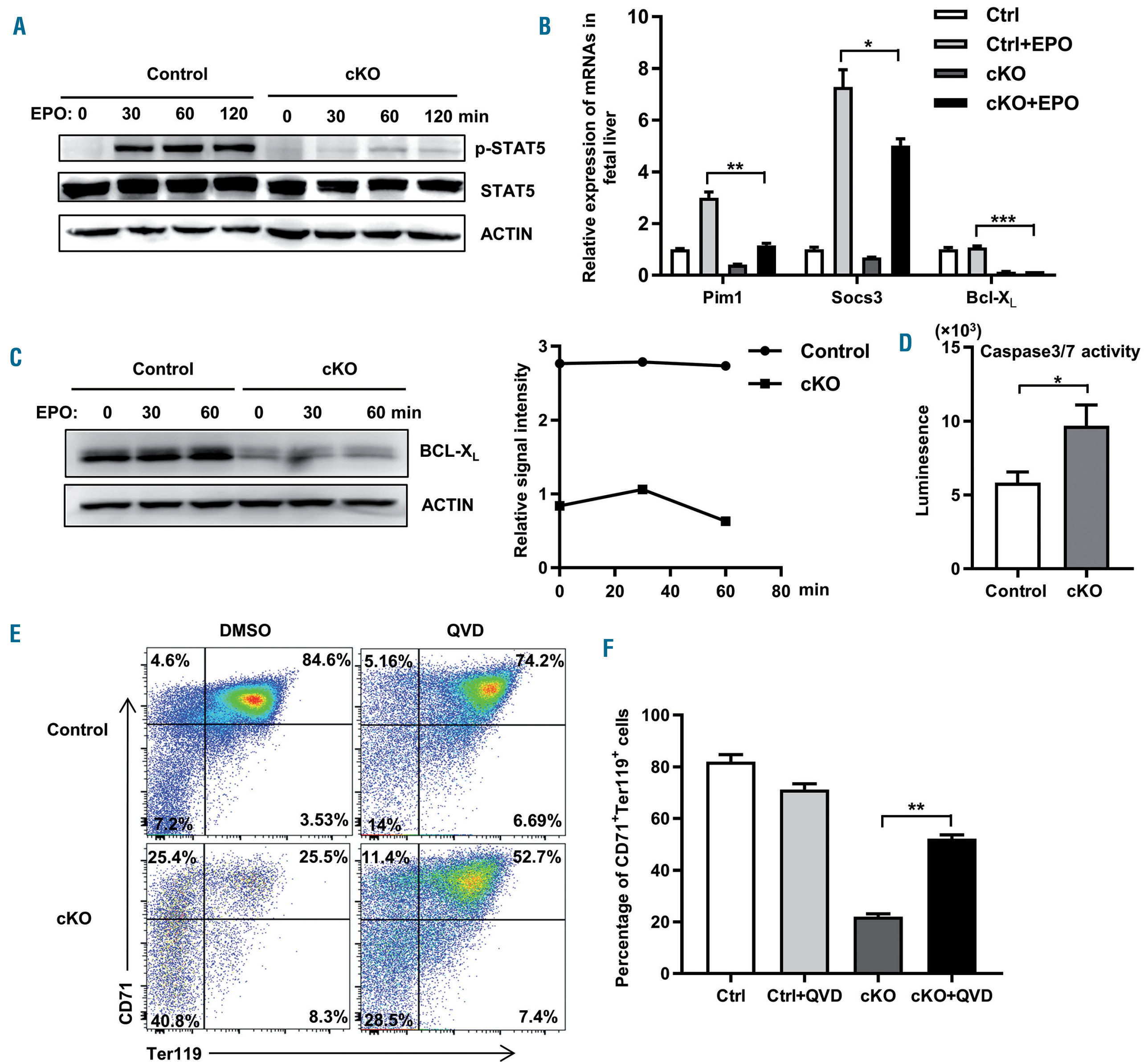

Figure 6. ***Tmem30a*** **deficiency leads to decreased STAT5 phosphorylation.** (A) STAT5 activation with or without erythropoietin (EPO) exposure, decreased STAT5 phosphorylation at Tyr694 which was demonstrated by western blotting of protein of fetal liver cells isolated from E14.5 cKO fetal livers compared with protein samples of fetal liver control cells. (B) Expression of STAT5 responsive genes in E14.5 control and cKO fetal liver cells measured by qualitative polymerase chain reaction (n=3 for each genotype). Data are presented as Mean±Standard Error of Mean (SEM) of fold-expression relative to control; expression normalized *versus* actin. *$P<0.05$; **$P<0.01$; ***$P<0.001$. (C) Decreased expression level of BCL-X_L after EPO exposure in cKO cells compared to control. (D) Levels of caspase-3/7 activity in total fetal liver cells were assessed. N=5 embryos for each group. (E) Enriched Ter119 negative fetal liver cells were cultured for 48 hours *in vitro* and stained with anti-CD71 and anti-Ter119 markers. (F) The pan-caspase inhibitor Q-VD-OPh partially reversed *Tmem30a* erythroid cell differentiation defects as analyzed by FACS.

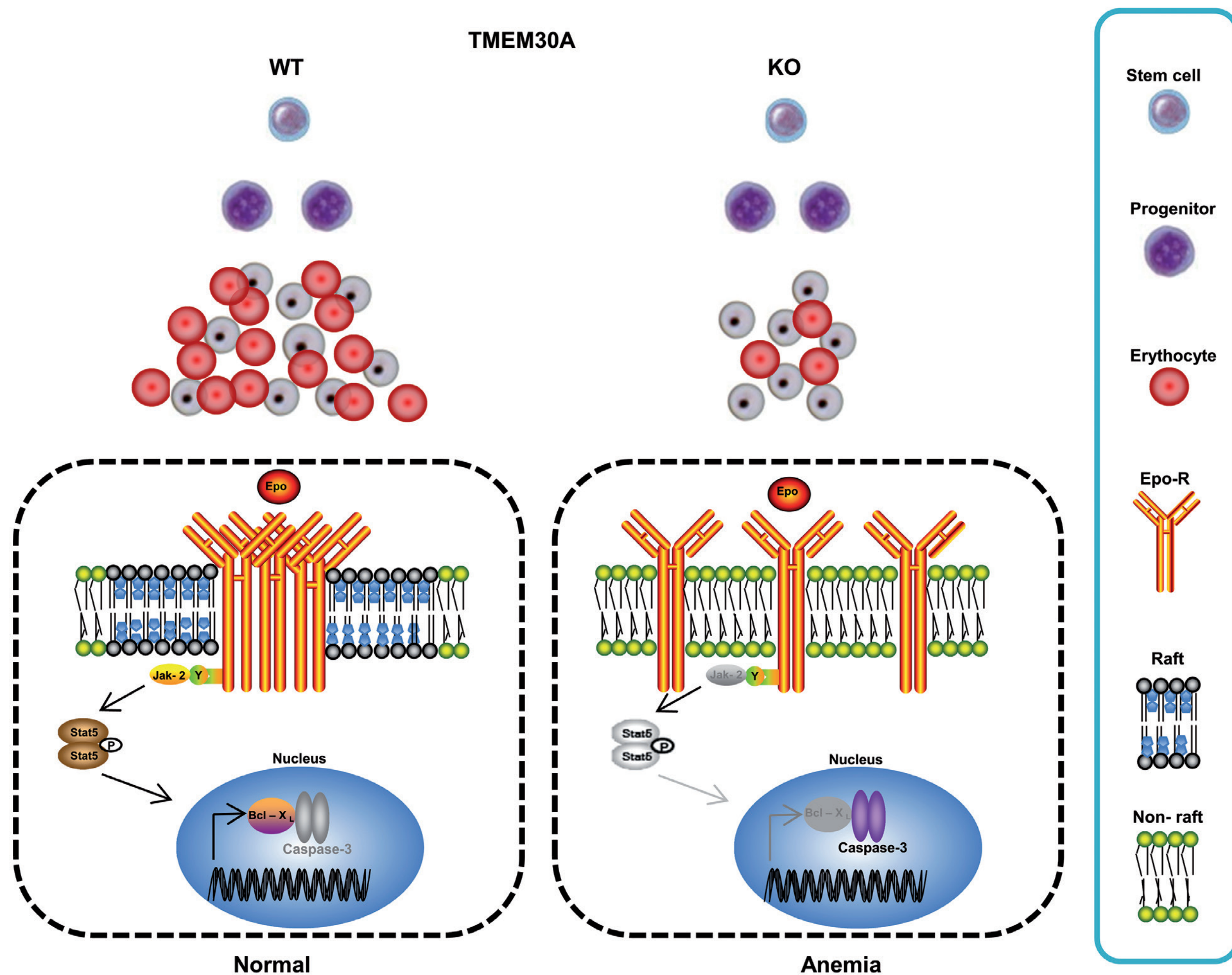

Figure 7. Model of *Tmem30a* function in erythropoiesis. Under normal conditions, upon erythropoietin (EPO) exposure, EPO-receptor (EPOR) localizes to lipid rafts and enhance EPOR-JAK2-STAT5-BCL-X_L-Caspase3 signal transduction which is essential for the survival of the embryos during erythropoiesis. In the absence of *Tmem30a*, lipid raft formation is disturbed, resulting in impaired downstream signal transduction.

elucidated a previously, unknown connection among membrane phospholipid partitioning, lipid raft clustering, receptor signal transduction, and erythroid differentiation (Figure 7).

Acknowledgments
The authors would like to thank the National Key Research and Development Program of China (grants 2016YFA0100602, 2017YFA0103302), National Natural Science Foundation of China (grants 91749203, 81525010, 81420108017 and 81530005), Program for Guangdong Introducing Innovative and Enterpreneurial Teams (2017ZT07S347), and by the Innovative Team Program of Guangzhou Regenerative Medicine and Health Guangdong Laboratory (grants 2018GZR110103002).

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