

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

Fan Yang,<sup>1\*</sup> Yumin Huang,<sup>2\*</sup> Xianda Chen,<sup>1\*</sup> Lu Liu,<sup>1</sup> Dandan Liao,<sup>1</sup> Huan Zhang,<sup>3,4</sup> Gang Huang,<sup>5</sup> Wenjing Liu,<sup>6</sup> Xianjun Zhu,<sup>6,7</sup> Wengong Wang,<sup>8</sup> Cheryl A. Lobo,<sup>9</sup> Karina Yazdanbakhsh,<sup>10</sup> Xiuli An<sup>2,3,4</sup> and Zhenyu Ju<sup>1,11</sup>

<sup>1</sup>Key Laboratory of Regenerative Medicine of Ministry of Education, Guangzhou Regenerative Medicine and Health Guangdong Laboratory, Institute of Aging and Regenerative Medicine, Jinan University, Guangzhou, China; <sup>2</sup>Department of Hematology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China; <sup>3</sup>Laboratory of Membrane Biology, New York Blood Center, New York, NY, USA; <sup>4</sup>School of Life Science, Zhengzhou University, Zhengzhou, China; <sup>5</sup>Division of Pathology and Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA; <sup>6</sup>The Sichuan Provincial Key Laboratory for Human Disease Gene Study, Institute of Laboratory Medicine, Sichuan Provincial People's Hospital, University of Electronic Science and Technology of China and Chengdu, Sichuan, China; <sup>7</sup>Chengdu Institute of Biology, Sichuan Translational Medicine Research Hospital, Chinese Academy of Sciences, Chengdu, China; <sup>8</sup>Department of Biochemistry and Molecular Biology, Beijing Key Laboratory of Protein Posttranslational Modifications and Cell Function, School of Basic Medical Sciences, Peking University Health Science Center, Beijing, China; <sup>9</sup>Laboratory of Blood-Borne Parasites, New York Blood Center, New York, NY, USA; <sup>10</sup>Laboratory of Complement Biology, New York Blood Center, New York, NY, USA and <sup>11</sup>Institute of Aging Research, School of Medicine, Hangzhou Normal University, Hangzhou, China

\*FY, YH and XC contributed equally to this work.

©2019 Ferrata Storti Foundation. This is an open-access paper. doi:10.3324/haematol.2018.203992

Received: August 7, 2018.

Accepted: February 27, 2019.

Pre-published: February 28, 2019.

Correspondence: ZHENYU JU - zhenyuju@163.com

XIULI AN - xan@nybc.org

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

### **SUPPLEMENTAL METHODS**

#### **DNA extraction and genotyping**

Genomic DNA was extracted using the KAPA Mouse Genotyping Kit. The extracted DNA was diluted 10-fold with water. Identification of the loxP downstream of the target: by *Tmem30a*-Loxp-forward primer: 5'-ATTCCCCTTCAAGATAGCTAC-3'; *Tmem30a*-Loxp-reverse primer: 5'-AATGATCAACTGTAATTCCCC-3'. Length: 214bp (Mutant) and 179bp (WT).

#### **Quantitative real time PCR**

Total RNA was extracted with Trizol (Invitrogen), cDNA was synthesized with the PrimeScript RT reagent Kit (TaKaRa). Parts of cDNA were amplified by qPCR with *SsoFast EvaGreen* Supermix (Bio-Rad) and the following oligo pairs: *Pim1* forward primer-5'-TTCTGGACTGGTTCGAGAGG-3' and *Pim1* reverse primer-5'-GCTCCTCGTTCGGTGATAAA-3'; *Socs3* forward primer-5'-CCGCTTCGACTGTGTACTCAAG-3' and *Socs3* reverse primer-5'-TCTTCTCGCCCCAGAATAGAT-3'; *Bclx* forward primer-5'-ACTGTGCGTGGAAAGCGTAGA-3' and *Bclx* reverse primer-5'-TGCTGCATTGTTCCCGTAGAG-3'; *Actin* forward primer-5'-CGTGCGTGACATTAAGAGAAG-3' and *Actin* reverse primer-5'-TGGATGCCACAGGATTCCATA-3'.

#### **Timed pregnancies and embryo harvest**

*Tmem30a*<sup>fllox/fllox</sup>; *VavCre*<sup>-/-</sup> and *Tmem30a*<sup>WT/fllox</sup>; *VavCre*<sup>Tg/+</sup> breeders were mated at night and vaginal plugs were checked the following morning. The day a vaginal plug

observed was calculated as embryonic day 0.5 (E0.5), the pregnant female mice were then bred separately. Embryo fetal liver was isolated from E14.5 embryos or other time point of gestation under a dissecting microscope. Yolk sac at E9.5 was isolated from embryos and digested in 0.125% collagenase I (Sigma) at 37°C for 30 min. 10% FBS/PBS was added into the mixture to stop the digestion.

### **Colony-forming Unit (CFU) assays**

Cells were suspended in HBSS containing 2% FBS by repeated flushing through needles of 25-gauge. CFU assay was performed following the MethoCult protocols. Briefly,  $3 \times 10^5$  cells were prepared then added the cells into 3 mL of MethoCult M3436 or M3334 medium (Stemcell technologies) for duplicate cultures. A 1.1 mL mixture was dispensed onto 35 mm dish by using blunt end needles. Cells were put into an incubator maintained at 37°C and 5% CO<sub>2</sub>. Colony forming unit erythroid colony cultured in M3334 medium was constituted with more than 8 erythroid cells seen after 2 days of incubation. Burst forming unit erythroid colony cultured in M3436 medium were seen after 10 days of culture.

### **Histology and immunohistochemistry**

For hematoxylin and eosin staining, fetal livers were fixed in 4% paraformaldehyde and embedded in paraffin. Tissue was sectioned and deparaffinised twice in xylene, followed by re-hydration in alcohol and washed in water for 5 min. The sections were then stained in Harris hematoxylin buffer for 10 min and differentiated in 0.3% acid alcohol. The sections were washed with the running water, stained with eosin-phloxine for 1 min, dehydrated and finally mounted. TUNEL assays were carried out with apoptosis detection kit (Roche). Images were scanned and captured by panoramic MIDI (3DHISTECH).

### **Blood collection and cytological analyses**

Embryos were dissected and washed in Hank's balanced salt solution (HBSS) containing 2% fetal bovine serum (FBS). Next, embryos were exsanguinated by decapitating, then blood was dropped into one well of a 24-well plate containing 0.1mL HBSS/2% FBS plus EDTA. Hematocrit was analyzed with an automated hemocytometer (Sysmex).

### **Flow cytometry**

For HSCs, the fetal liver cells were first stained with an antibody cocktail consisting of biotinylated anti mouse -CD4, -CD8, -B220, -Gr-1 and -Ter-119 antibodies (Biolegend). The cells were further stained with APC conjugated anti-mouse/human CD11b (clone: M1170), PE/Cy7 conjugated anti-mouse Sca-1(clone: E13-161.7), FITC conjugated anti-mouse CD48 (clone: HM48-1), PE conjugated anti-mouse CD150 (clone: TC15-12F12.2). Biotinylated antibody was developed with streptavidin APC-Cy7. The above antibodies were all from Biolegend. For HSCs in the yolk sac, the yolk sac was isolated from embryos at E9.5 and digested in 0.125% collagenase I for 30 min at 37 °C. Stop digestion with 10% FBS and stained with APC conjugated anti-mouse c-kit (Clone:2B8) and FITC conjugated anti-mouse CD41(clone: MWReg30, eBioscience). Prepared samples were analyzed on LSRFortessa flow cytometry analyzer (BD Biosciences).

Flow cytometry analyses of human erythroid differentiation *in vitro* were performed by using PE conjugated mouse anti-human CD235a (GPA), APC conjugated mouse anti-human  $\alpha$ 4 integrin and mouse monoclonal antibody against human band3 were generated in our laboratory and labeled with FITC. The preparation and the analysis of the samples were the same for the mice samples.

### **Flippase assay**

Flippase activity was assessed with NBD-PS(1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphoserine (ammonium salt); Avanti Polar Lipids). Fetal liver cells were incubated with the fluorescent PS analog NBD-PS then analyzed at various time points by flow cytometry. Cells ( $2 \times 10^6$ ) were incubated with 1.5  $\mu$ M NBD-PS for the appropriate time at 25°C in 600  $\mu$ L of HBSS. Cells were immediately put on ice and then were incubated for 5 min with cold HBSS including 3 mg/mL BSA to stop the flipping in NBD-PS cells and to remove the unbound NBD-PS from the cell surface. After 3 min, cells were washed twice and analyzed by a BD FACS Fortessa machine.

### **Immunofluorescence and confocal microscopy**

Ter119<sup>low</sup> fetal liver cells were sorted and cultured on retronectin (Takara)-coated circle glasses in 24-well plate supplemented with IMEM and cytokines for 4 hours, then exposed with 3 U/mL EPO for 30 min. Cells were washed with phosphate buffer saline (PBS) and fixed in 2% paraformaldehyde for 10 min at room temperature (RT). The cells were washed with PBS three times and incubated in normal goat serum for one hour. Cells were permeabilized with saponin and incubated with Anti-EpoR antibody (#SAB4500780, Sigma) and GM1 antibody (Cholera toxin subunit B, Alex Flour 488 conjugate, Invitrogen) which were diluted 1:100 with normal goat serum. Cells were washed with PBS for three times then stained with 4  $\mu$ g/mL Alexa Fluor 546 goat-anti-rabbit secondary antibody (#A11010, Invitrogen) and 1  $\mu$ g/mL 4, 6-diamidino-2-phenylindole (DAPI). Cells were washed with PBS three times and mounted with fluoromount-G. Images were captured by laser scanning confocal microscope (Zeiss LSM710).

### **Cell cycle analysis**

Pregnant mice were injected intraperitoneally with 150  $\mu$ L of 10 mg/mL BrdU in PBS. Mice were euthanized after 1 hour, their uterine horns extracted and placed on ice.

Fetal livers were excised, homogenized via pipetting, and cell numbers were counted. One million cells per sample were then stained with anti-CD71-PE (3  $\mu$ L/100 $\mu$ L) and anti-Ter119-APC (3  $\mu$ L/100  $\mu$ L) for 30 min at RT. BrdU cell-cycle analysis was then performed using the BD-Pharmingen BrdU-FITC flow kit. Cells were acquired on flow cytometry.

### **Lipid raft extraction**

TER119 negative fetal liver cells ( $1 \times 10^8$ ) at E14.5 were enriched and exposed to EPO for 30 min. Cells were collected and resuspended in 2 mL of cold MBS buffer (25mM MES, 150mM NaCl), 1% Triton X-100 supplemented with protease inhibitor. Samples were incubated on ice for 30 min and subjected to sonication for 15 seconds. 2 mL of 80% sucrose (w/v) was mixed gently with the samples then overlaid with 4 mL 35% sucrose and 4 mL of 5% sucrose. The sucrose gradient samples were centrifuged at 41,000 rpm in a SW 41Ti rotor at 4°C for 32 hours. Lipid raft fractions were collected after ultra-centrifugation.

### **SUPPLEMENTAL FIGURE LEGENDS**

#### **Figure S1. *Tmem30a* is dispensable for maintenance of HSCs number.**

(A) Representative PCR results of embryo genotyping (somatic DNA) showing bands for *Tmem30a* (top, WT: 179 bp, flox: 214 bp) and Vav-Cre (bottom, WT: 324 bp, Cre: 236 bp) for all possible genotypes of the offspring of *Tmem30a*<sup>flox/flox</sup>; VavCre<sup>-/-</sup> and *Tmem30a*<sup>WT/flox</sup>; VavCre<sup>Tg/+</sup> timed pregnancy. (B) Western blot analysis showed the protein level of TMEM30A was decreased after *Tmem30a* deletion. (C) Sample plots of FACS analysis for fetal liver HSCs. (D) Comparison of total cell numbers of HSCs from E14.5 FLs from *Tmem30a* control and cKO embryos. Data are shown as mean  $\pm$  SEM. N.S. means no significant difference. (E) HSCs, T cells, B cells and erythroblasts were sorted from E14.5 fetal liver cells by FACS. The expression of

Tmem30a mRNA was measured by qRT-PCR and the relative expression of Tmem30a was normalized to  $\beta$ -actin.

**Figure S2. *Tmem30a* deletion is dispensable for maintaining the number of erythro-progenitors .**

(A) Representative flow cytometric profiles of control and Tmem30a cKO fetal liver single cells stained with CD71 and c-kit. (B) The absolute number of erythro-progenitors was calculated in the fetal liver at E14.5. (C) Representative flow cytometric profiles of control and Tmem30a cKO yolk sac single cells stained with CD41 and c-kit. (D) The absolute number of erythro-progenitors was calculated in the yolk sac at embryo day 9.5. Data are represented as mean  $\pm$  SEM of the cell count of 3 embryos for each embryonic data set.

**Figure S3. *Tmem30a* deletion embryos show a normal cell cycle.**

(A-B) Cell-cycle analysis of control and *Tmem30a* cKO fetal liver cells from E14.5 embryos using an *in vivo* BrdU assay indicated a normal cell cycle phenotype. Representative flow cytometry dot plots are shown.

**Figure S4. *Tmem30a* cKO fetal liver cells exhibit low flippase activity.**

(A) NBD-PS fluorescence profiles in each S population of definitive erythropoiesis from control and cKO, assessed after 3 min incubation. (B) NBD-PS in fetal liver erythroid cells from control and cKO, presented as fluorescence intensity. Values are means  $\pm$  SEM (\* $P < 0.05$ , \*\*\*  $P < 0.001$ .) with 3 replicates.

**Figure S5. The response of erythroid cells after EPO exposure.**

(A) Immunofluorescence analysis of isolated Ter119 low erythroid cells were stained

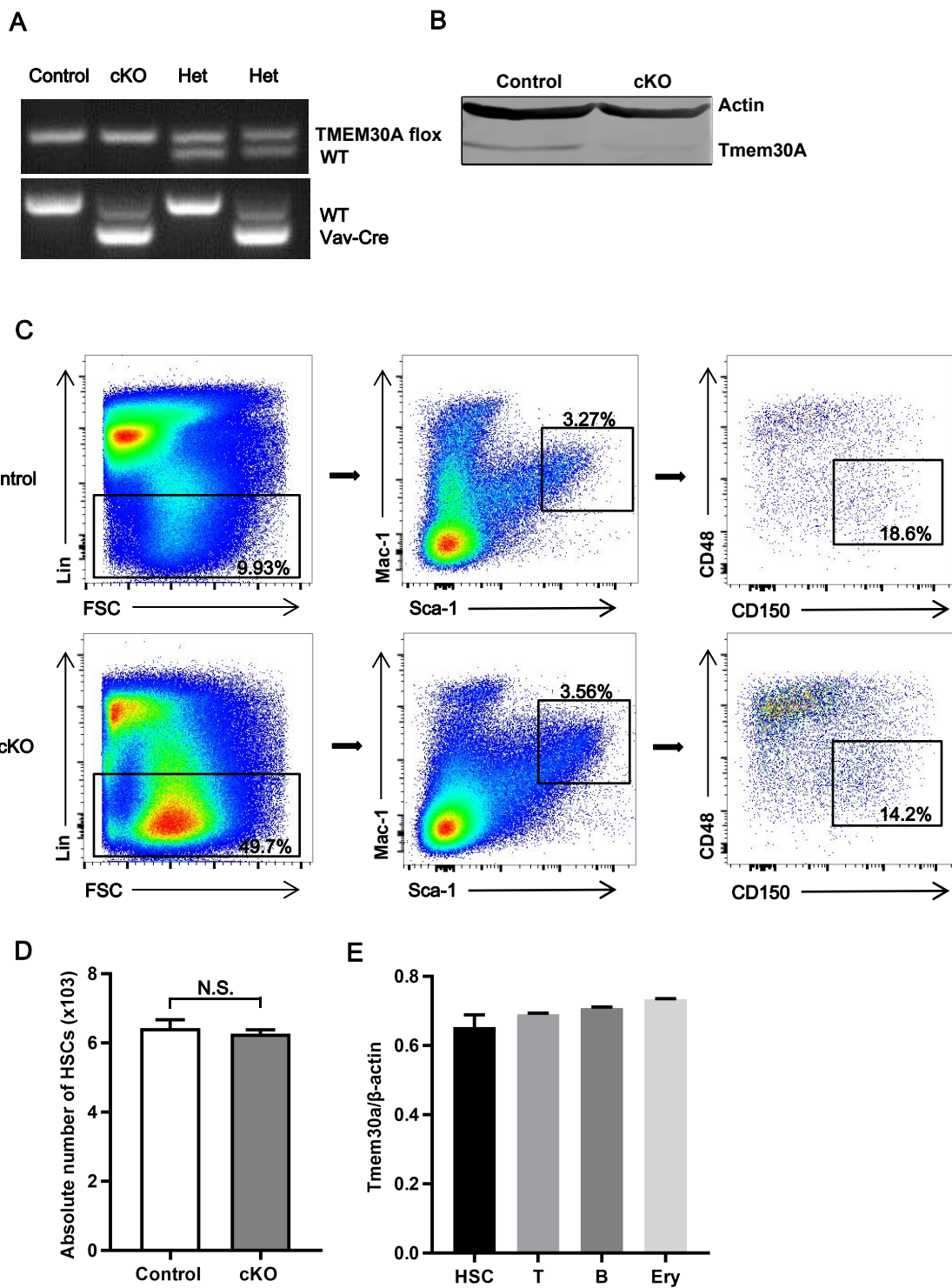
with FITC conjugated GM1 (green) and with an anti-EPOR antibody. A goat anti-rabbit IgG (Alexa Fluor 546) was used as the secondary antibody. PBS instead of the primary antibody EPOR was the negative control. DAPI was used as a nuclear counterstain. **(B)** Lipid raft from TER119<sup>+</sup> cells was purified after EPO treatment by the ultracentrifugation approach. The protein level of EPOR and raft fractions were analyzed for raft-associated molecules, and Flotillin-2 serves as a lipid raft marker. **(C)** Expression of EPOR on erythroid cells of TER119<sup>+</sup> populations incubated with or without EPO was analyzed by FACS.

**Figure S6. Knockdown of *TMEM30a* impairs human erythroid cell differentiation.**

**(A)** Quantitative RT-PCR analysis showing mRNA expression levels of *TMEM30a* in erythroblasts transduced with lentivirus containing luciferase-shRNA or *TMEM30A*-shRNA, and cultured for 6 days.  $\beta$ -actin was used to normalize the data. Bar plot represents mean  $\pm$  SEM of triplicate analyses. **(B)** Representative flow cytometry plots of GPA expression of cells cultured for 7 days from luciferase control groups and *TMEM30a* knockdown groups. **(C)** Flow cytometry analysis showing the terminal erythroid differentiation as assessed by expression of  $\alpha$ 4 integrin and band 3. **(D)** Growth curves of cells of luciferase control groups and *TMEM30a* knockdown groups. Data was shown as mean  $\pm$  SEM of triplicate analyses. **(E)** Representative flow cytometry profiles of apoptosis as assessed by dual staining of Annexin V and 7AAD of cells cultured for 15 days. **(F)** Quantitative analysis of apoptosis from three independent experiments. **(G)** TUNEL positive cells were measured by flow cytometry. **(H)** *TMEM30a* knockdown cells exhibited higher frequency of apoptosis. **(I)** Levels of STAT5 phosphorylation in CD34<sup>+</sup> cells were assessed by western blotting. **(J)** *TMEM30a* knockdown CD34<sup>+</sup> cells demonstrated decreased STAT5 phosphorylation compared with protein samples of negative control cells upon EPO treatment. Bar plot represents mean  $\pm$  SEM of triplicate analyses. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

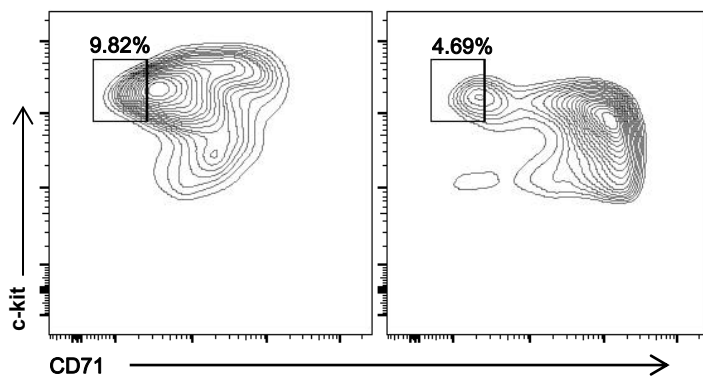


# Supplementary Figure 1

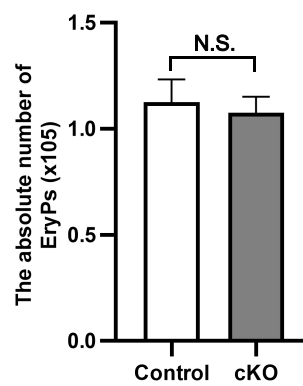


## Supplementary Figure 2

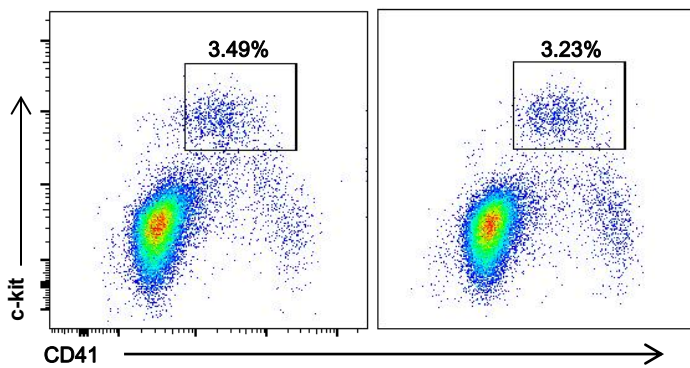
A



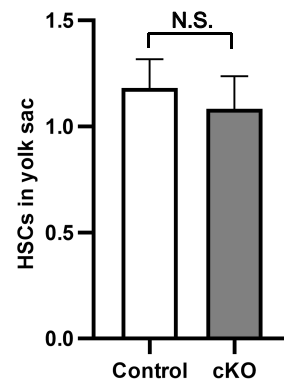
B



C

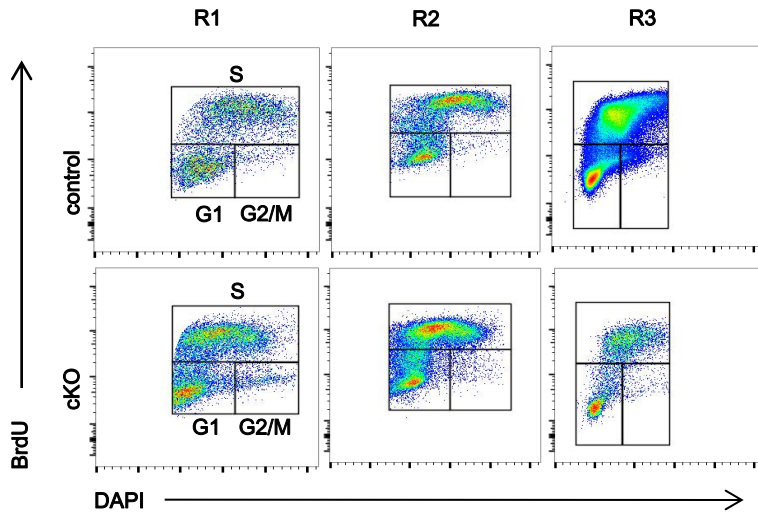


D

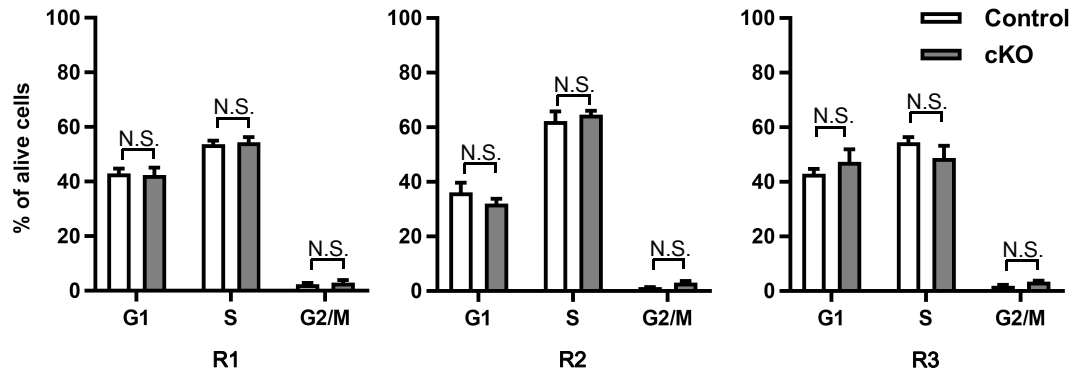


# Supplementary Figure 3

A

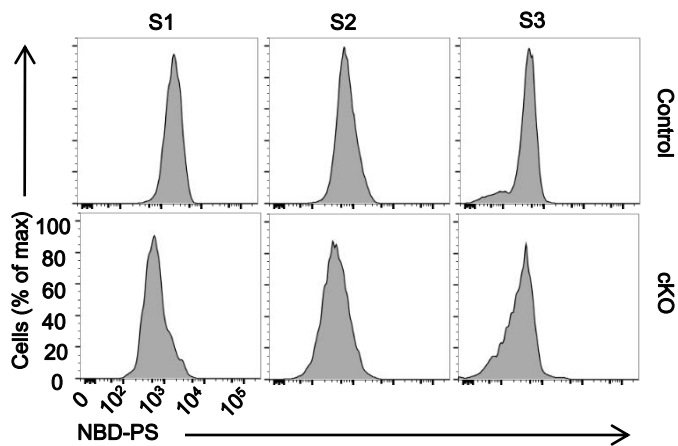


B

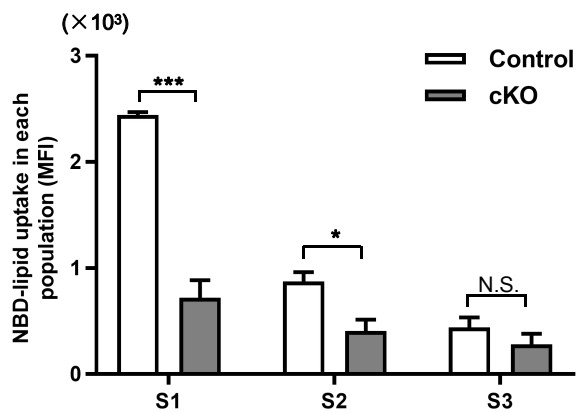


# Supplementary Figure 4

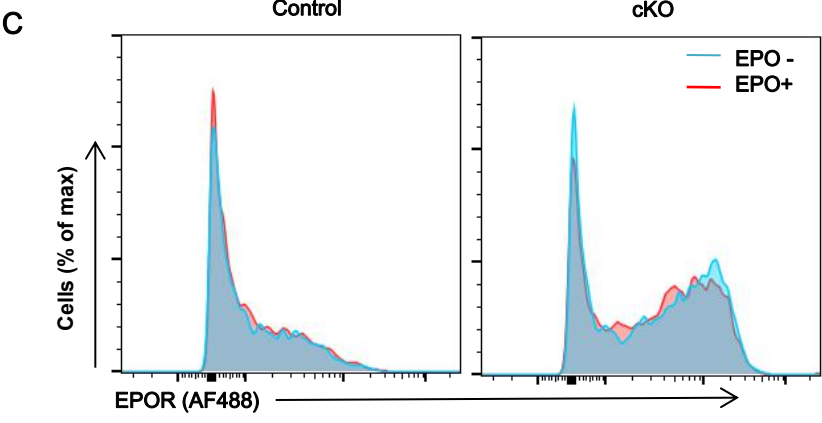
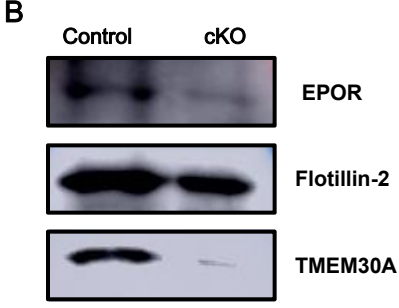
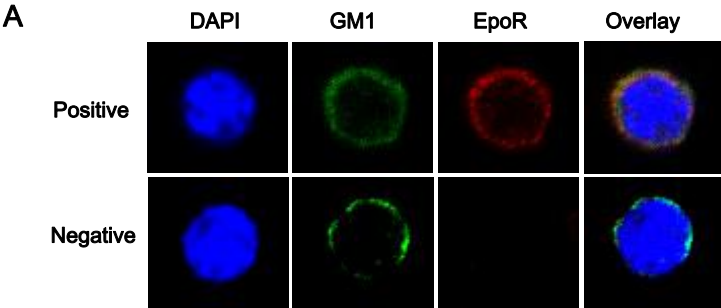
A



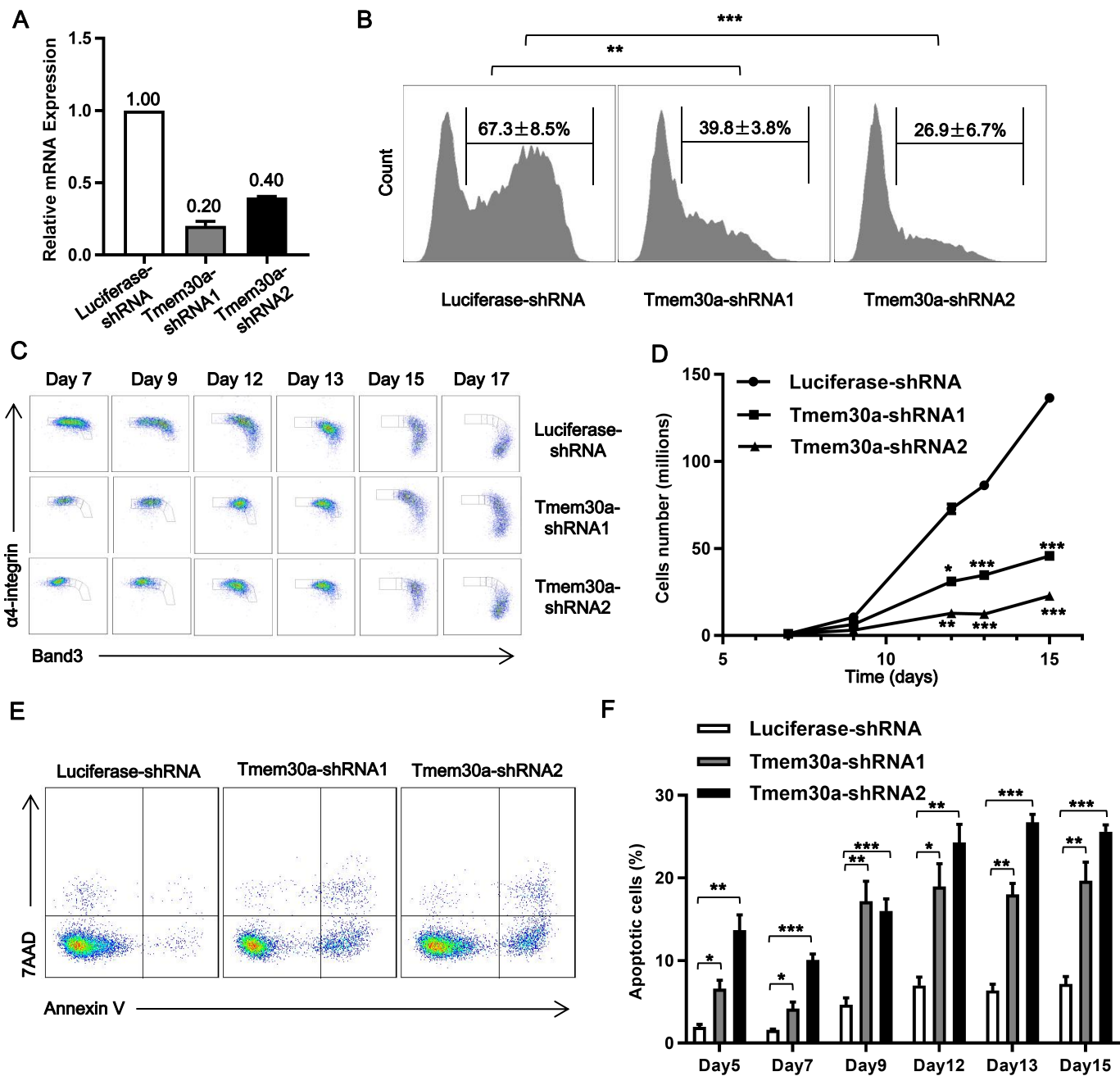
B



# Supplementary Figure 5



# Supplementary Figure 6



# Supplementary Figure 6

