STEM CELLS, TISSUE ENGINEERING, AND HEMATOPOIETIC ELEMENTS

Tmem30a Plays Critical Roles in Ensuring the Survival of Hematopoietic Cells and Leukemia Cells in Mice

Ning Li,^{*†} Yeming Yang,[‡] Cailing Liang,^{*†} Qiang Qiu,^{*†} Cong Pan,^{*†} Mengyuan Li,^{*†} Shengyong Yang,[†] Lijuan Chen,[†] Xianjun Zhu,^{‡§¶} and Yiguo Hu^{*†}

From the Department of Thyroid Surgery,^{*} State Key Laboratory of Biotherapy & Collaborative Innovation Center for Biotherapy,[†] West China Hospital, Sichuan University, Chengdu, Sichuan; the Sichuan Provincial Key Laboratory for Human Disease Gene Study,[‡] Sichuan Provincial People's Hospital, University of Electronic Science and Technology of China, Chengdu, Sichuan; the Department of Laboratory Medicine,[§] Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, Chengdu, Sichuan; and the Chengdu Institute of Biology,[¶] Chinese Academy of Sciences Sichuan Translational Medicine Research Hospital, Chengdu, Sichuan, China

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Address correspondence to
 Xianjun Zhu, Ph.D., Sichuan
 Provincial People's Hospital,
 University of Electronic Sci-
 ence and Technology of China,
 Chengdu, Sichuan 610072,
 China; or Yiguo Hu, Ph.D., No.
 17, Third part of Ren-Min-Nan Rd.,
 Chengdu, Sichuan 610041, China.
 E-mail: xjzhu@uestc.edu.cn or
huyiguo@scu.edu.cn.

The fundamental structure of eukaryotic cell plasma membrane is the phospholipid bilayer, which contains four major phospholipids. These phospholipids are asymmetrically distributed between the outer and inner leaflets. P4-ATPase flippase complexes play essential roles in ensuring this asymmetry. We found that conditional deletion of *Tmem30a*, the β subunit of P4-ATPase flippase complex, caused pancytopenia in mice. *Tmem30a* deficiency resulted in depletion of lineage-committed blood cells in the peripheral blood, spleen, and bone marrow. Ablation of *Tmem30a* also caused the depletion of hematopoietic stem cells (HSCs). HSC RNA sequencing results revealed that multiple biological processes and signal pathways were involved in the event, including mammalian target of rapamycin signaling, genes for HSC stemness, and genes responding to interferons. Our results also revealed that targeting *Tmem30a* signaling had therapeutic utility in BCR/ABL1-induced chronic myeloid leukemia. (*Am J Pathol* 2018, 188: 1457–1468; <https://doi.org/10.1016/j.ajpath.2018.02.015>)

Plasma membrane is a fundamental structure of eukaryotic cells. The plasma membrane consists of a phospholipid bilayer, which contains four major phospholipids [phosphatidylcholine, phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin], glycolipids, and cholesterol.¹ These phospholipids are asymmetrically distributed between the outer and inner leaflets of plasma membrane. P4-ATPase flippase complexes play essential roles in ensuring the asymmetric distribution of phospholipids.² The P4-ATPase flippase complex is composed of a catalytic α subunit and an accessory β subunit.^{1,2} The α subunit catalyzes the hydrolysis of ATP, and the β subunit assists in binding of the phospholipid substrate.² P4-ATPase flippase complexes transport aminophospholipids from the outer to the inner leaflets by using ATP.¹

P4-ATPases are critical for various biological processes, such as blood coagulation regulation, vesicular protein transport, the recognition of apoptotic cells, and sperm capacitation.^{3–6} P4-ATP flippase complexes also play roles in the

regulation of cell migration through mediating the translocation of PE at plasma membrane.⁷ Phospholipid translocation seems also to be implicated in vesicle formation and in uptake of lipid signaling molecules.² Deficiency of α subunits, such as *ATP8B1*, *ATP8A2*, and *ATP11C*, leads to various human diseases.^{8–11} Deficiency of *Atp11c* causes B-cell lymphopenia, anemia, and intrahepatic cholestasis in mice.^{12–15} However, the functions of β subunits of P4-ATPase flippase complexes remain elusive.

Recently, an inducible or tissue-specific deletion of *Tmem30a* mouse model was generated.¹⁶ *Tmem30a* belongs to

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the β subunit family members of PS flippase, one kind of the P4-ATPase family complexes.¹⁶ PS flippase predominantly transport PS and a lesser PE.² PS is primarily located in the inner cytoplasmic leaflet, and PS flippase transports PS from the exoplasmic to the cytoplasmic leaflet of cell membrane.^{17–19} Deficiency of *Tmem30a* causes an embryonic lethal phenotype.¹⁶ Two studies about the functions of *Tmem30a* have been performed in the retina and liver, respectively, by using *Tmem30a* tissue-specific deletion mouse models.^{16,20} Deletion of *Tmem30a* resulted in a loss of PS flippase activity and exposure of PS on cell outer surface.^{16,20} *Tmem30a* deficiency in mouse cone cells results in loss of cone cells and impaired photopic electroretinogram responses in the retina.¹⁶ Currently, the physiologic roles of *Tmem30a* in hematopoietic cells are unknown. Hematopoietic cells consist of many cell types with specialized functions and are organized as a cellular hierarchy.²¹ In adults, hematopoietic stem cells (HSCs) are evidenced at the apex, which give rise to all the other blood cells through the process of hematopoiesis.²¹ Blood cells play important roles in maintaining the body's functions. In fields of hematopoietic neoplasm, the roles of *Tmem30a* are also not defined, especially its roles in leukemia stem cells (LSCs). It has become apparent that the initiation and propagation of chronic myeloid leukemia (CML) are driven by LSCs.²² Thus, it becomes essential to eliminate LSCs completely to cure CML.²²

In this study, we focused on the roles of *Tmem30a* in the hematopoietic system. Using a *Tmem30a* inducible knockout mouse model, we found that *Tmem30a* is essential for the survival of hematopoietic cells. *Tmem30a* deficiency markedly decreased all lineage-committed blood cells and HSCs and impaired the survival of LSCs of BCR/ABL1-transduced CML.

Materials and Methods

Mice

Conditional gene-targeted *Tmem30a*^{flox/flox} mice were generated as described previously.¹⁶ Briefly, to delete *Tmem30a* in hematopoietic cells *in vivo*, *Tmem30a*^{flox/flox}; CAG-Cre-ER (named *Tmem30a*^{-/-}) mice were generated by breeding *Tmem30a*^{flox/flox} mice with CAG-Cre-ER transgenic mice.²³ Cre expression was induced by i.p. injection of three doses of tamoxifen at a daily dosage of 25 mg/kg body weight at 8 weeks after birth. Littermates of CAG-Cre-ER [wild-type (WT)] mice were used as control and treated at same injection as *Tmem30a*^{-/-} mice. All animal studies were performed in accordance with the guidelines approved by the Institutional Animal Care and Use Committees of Sichuan University and Sichuan Provincial People's Hospital.

Tamoxifen Injection and BM Cell Harvest

Tamoxifen salt (Sigma, St. Louis, MO) was dissolved in ethanol at a concentration of 10 mg/mL, which was kept in -20°C freezer as a stock solution. Before injection, tamoxifen was

further diluted to 1 mg/mL with corn oil (Sigma). *Tmem30a*^{-/-} and WT mice were injected intraperitoneally with a daily dosage of 25 mg/kg body weight for 3 days. At days 7, 15, and 28 after tamoxifen injection, mice were sacrificed and bone marrow (BM) cells harvested from femurs and tibia for analysis.

Flow Cytometry

BM cells from tamoxifen-treated mice were incubated in red blood cell lysis buffer (pH 7.4) to facilitate the removal of erythrocytes. The percentages of the targeting cell population were determined with flow cytometry after the isolation of single cells. Single cell suspensions were incubated for 30 minutes at room temperature with various combinations of the following cell-surface marker antibodies: B220 for B cells, Gr-1 for myeloid cells, Cd3 for T cells, B220⁺IgM⁻ for pro-/pre-B cell, B220^{low}IgM⁺ for immature B cell, B220^{high}IgM⁺ for mature B cell, Lin⁻c-Kit⁺Sca-1⁺ for HSCs, Lin⁻c-Kit⁺Sca-1⁻CD34⁺CD16/32⁻ for common myeloid progenitors (CMPs), Lin⁻c-Kit⁺Sca-1⁻CD34⁺CD16/32⁺ granulocyte-monocyte progenitor (GMP), and Lin⁻c-Kit⁺Sca-1⁻CD34⁻CD16/32⁻ megakaryocyte-erythroid progenitor (MEP). The antibodies were purchased from BD Biosciences (Becton Dickinson, Franklin Lakes, NJ), including PE-CD34. The antibodies were purchased from eBioscience (San Diego, CA), including allophycocyanin (APC)—cyanine 7 (Cy7)—B220, PE-Gr1, PE-Cy7-CD16/CD32, PE-CD34, fluorescein isothiocyanate (FITC)—CD45.2, APC-CD45.1, PE-IgM, FITC-Cd3, Pacific blue—Lin⁻, PE—Sca-1, and APC—c-Kit.

BM Cell Competition Assay

BM cells were harvested from 8-week-old B6.SJL-Ptprca-Pepc^{b/BoyJ} (CD45.1) mice and *Tmem30a*^{-/-} (CD45.2) mice. BM cells from CD45.1 and CD45.2 mice were mixed in a ratio of 1:1. CD45.1 mice were subjected to two rounds of 450-cGy X-ray irradiation and injected with 1×10^6 total BM cells (5×10^5 cells of each). After 12 weeks, recipients were randomly divided into two groups. One group was treated with tamoxifen, and another group was treated with placebo (corn oil with ethanol). BM cells were collected and subjected to flow cytometry analysis. The ratio of CD45.1 to CD45.2 cells in recipient BM was determined with their specific antibodies.

Histopathologic Analysis and Immunohistochemistry

Tissues were fixed, processed, sectioned, and stained with hematoxylin and eosin by routine methods. Femurs were additionally treated for 1 hour in decalcifying solution (Fisher Scientific, Shanghai, China). Immunohistochemistry was performed according to standard procedures. The rat anti-mouse Cd19 (EM40308, Huabio, Hangzhou, China) and rat anti-mouse Gr-1 (281-2) monoclonal antibodies were obtained from BD Biosciences; TMEM30A monoclonal antibody (Cdc50-7F4) was a gift from Dr. Robert Molday (University of British Columbia, Vancouver, BC, Canada).

Figure 1 Tmem30a deficiency causes pancytopenia in mice. **A:** Immunohistochemistry photomicrographs of bone marrow (BM) section of wild-type (WT) and *Tmem30a*^{-/-} mice treated with tamoxifen for 15 days were stained with Tmem30a. **B:** Total BM cell counts from femurs and tibia of WT and *Tmem30a*^{-/-} mice treated with tamoxifen for 7, 15, and 28 days. **C:** Photomicrographs of hematoxylin and eosin–stained bone marrow sections of WT and *Tmem30a*^{-/-} mice treated with tamoxifen for 15 days. **D** and **E:** White blood cell counts in peripheral blood (PB; **D**) and spleen (SPL; **E**) at days 15 and 28 after tamoxifen treatment. At least two independent experiments were performed for confirmation. Consistent results were achieved every time. Data are expressed as means ± SD (**B**, **D**, and **E**). *n* = 3 mice. Original magnification, ×200 (**A** and **C**). IB, immunoblot.

RNA Sequencing Analysis

Hematopoietic stem cells [Lin^{low}Sca1⁺c-Kit⁺ (LSK) cells] were sorted from the BM of WT and *Tmem30a*^{-/-} mice at day 7 after tamoxifen injection using a fluorescence activated cell sorter Aria III (Becton Dickinson). Total RNA was extracted from LSK cells using the RNeasy Mini kit (Qiagen, Hilden, Germany). RNA samples were reverse transcribed, amplified using the Ovation Pico WTA System version 2 (NuGEN Technologies, San Carlos, CA), and biotin labeled with the Encore Biotin Module (NuGEN Technologies). RNA sequencing was performed with the Illumina MiSeq system (Illumina, San Diego, CA). Gene set enrichment analysis was performed as previously described.²⁴ RNA sequencing data were provided in [Supplemental Table S1](#). Gene signatures were analyzed with Gene Set Enrichment Analysis (GSEA) software version 3.0 (GSEA, <http://software.broadinstitute.org/gsea/index.jsp>, last accessed September 9, 2017) in the Molecular Signatures Database (Broad Institute, Cambridge, MA).^{25,26}

BCR/ABL1-Transduced CML Mouse Model

The retroviral vector puro murine stem cell virus–BCR/ABL1–internal ribosome entry site–enhanced green fluorescent protein (pMSCV-BCR/ABL1-IRES-eGFP) was used to generate virus stocks as described previously.²⁴ Briefly, donor mice were pretreated with 5-fluorouracil, 200 mg/kg (Sigma), through i.v. tail injection. Four days

later, BM cells were harvested from donor mouse femurs and tibia. BM cells were prestimulated with 10 µg/mL of IL-3 (Novoprotein Scientific Inc., Shanghai, China), 10 µg/mL of IL-6 (Novoprotein), and 50 µg/mL of stem cell factor (Novoprotein). In the next 2 days, BM cells were subjected to two rounds of cosedimentation with retroviral stock at 1000 × *g* for 90 minutes. The transfected marrow cells were transplanted via tail vein injection into same genetic background recipient mice (0.5 × 10⁶ per mouse) as the donor mice. Recipients received two doses of 450 cGy of X-ray irradiation separated by 3 hours. Leukemia cells and LSCs were determined with viable GFP⁺ cells with Gr-1⁺ or Lin⁻Sca1⁺c-Kit⁺, respectively.

Statistical Analysis

All experiments were performed in triplicate and repeated at least twice. Statistical significance was determined by 1-way analysis of variance or *t*-test using GraphPad Prism software version 6 (San Diego, CA). *P* < 0.05 was considered statistically significant.

Results

Tmem30a Deficiency Results in Pancytopenia

To define the physiologic functions of *Tmem30a* in the hematopoietic system, the murine *Tmem30a* gene in adult

Figure 2 *Tmem30a* deficiency causes pancytopenia in mice. **A** and **B**: Immunohistochemistry photomicrographs of bone marrow (BM) section of wild-type (WT) and *Tmem30a*^{-/-} mice treated with tamoxifen for 15 days were stained with Cd19 (**A**) and Gr-1 (**B**), respectively. **C**: The total cell counts of B cells (B220⁺), myeloid cells (Gr-1⁺), and T cells (Cd3⁺) in BM after tamoxifen treatment at indicated days. **D**: Fluorescence-activated cell sorting analysis shows B-cell development stages (pro- and pre-B cell represented with B220⁺IgM⁻; immature B cell represented with B220^{low}IgM⁺; mature B cell represented with B220^{high}IgM⁺) in BM of WT and *Tmem30a*^{-/-} mice at day 15 after tamoxifen treatment. **E**: Percentage of pro- and pre-, immature, and mature B cells in BM of WT and *Tmem30a*^{-/-} mice at days 7, 15, and 28 after tamoxifen treatment (from left to right). Independent experiments were performed two times (**E**) or three times (**C**). Data are expressed as means $\pm$ SD (**C** and **E**). $n = 3$ mice. Original magnification, $\times 200$ (**A** and **B**). IB, immunoblot.

Tmem30a^{-/-} mice was deleted by three i.p. injections of tamoxifen. Immunohistochemistry against *Tmem30a* was used to confirm the deletion in BM of the *Tmem30a*^{-/-} mice, and their littermates were used as WT control (Figure 1A).

Tmem30a^{-/-} mice died between 35 and 50 days after tamoxifen injection (data not shown). *Tmem30a*^{-/-} mice had a marked decrease in leukocyte counts in BM with a time-dependent pattern (Figure 1B). *Tmem30a* deficiency

Figure 3 *Tmem30a* deficiency impairs hematopoietic stem cell (HSC) survival. **A** and **B**: The percentages (**A**) and total numbers (**B**) of HSCs (Lin⁻c-Kit⁺Sca1⁺) are much lower in bone marrow (BM) of *Tmem30a*^{-/-} mice than those in BM of wild-type (WT) mice. **C**: Common myeloid progenitor (CMP; CD34⁺Lin⁻Sca1⁻c-Kit⁺Cd16/32⁺ cell population), granulocyte-monocyte progenitor (GMP; CD34⁺Lin⁻Sca1⁻c-Kit⁺Cd16/32⁺ cell population), and megakaryocyte-erythroid progenitor (MEP; CD34⁺Lin⁻Sca1⁻c-Kit⁺Cd16/32⁺ cell population) cell counts are much lower in BM of *Tmem30a*^{-/-} mice compared with WT mice. **D**: The total CMP, GMP, and MEP cell counts in WT and *Tmem30a*^{-/-} mice after tamoxifen injection for 15 days. Independent experiments were performed two times (**C** and **D**). **E**: Equal BM cell number (5×10^5) from *Tmem30a*^{-/-} (CD45.2) and B6.SJL-Ptprca Pepc^{b/BoyJ} (CD45.1) were mixed and injected into lethally irradiated recipients (CD45.1) via tail vein. Fluorescence-activated cell sorting analysis shows that in the same microenvironment *Tmem30a* deficiency significantly reduces viable cell percentage compared with CD45.1 cells in BM. The percentages of CD45.2 and CD45.1 are shown in the plot images. **F**: BM cells from *Tmem30a*^{-/-} or WT mice were treated with tamoxifen *in vitro* for 48 hours, then were transplanted into lethally irradiated recipients at a cell dose of 1×10^6 . Data are expressed as means $\pm$ SD (**B** and **D**). $n = 3$ mice (**A–E**); $n = 5$ mice (**F**). BMT, bone marrow transplantation.

resulted in an approximate 50% loss of BM cellularity at day 15 after tamoxifen injection. The pancytopenia phenotypes were also obviously observed in bone section from *Tmem30a*^{-/-} mice at day 15. Compared with WT, photomicrographs showed that there were massive cell corpses in the BM section from *Tmem30a*^{-/-} mice, and most cells lost their normal morphologic structure and became debris (Figure 1C). In correlation, *Tmem30a*^{-/-} mice had a marked reduction of white blood cell counts in the peripheral blood

and spleen (Figure 1, D and E). These results indicated that *Tmem30a* was essential for blood cell survival.

To define whether *Tmem30a* plays important roles for all lineage-committed cells, especially B cells and myeloid cells, immunohistochemistry against Cd19 and Gr-1 was performed. Total B-cell (Cd19⁺) and myeloid cell (Gr-1⁺) counts were drastically decreased in the *Tmem30a*^{-/-} mouse BM section compared with the WT mouse BM section (Figure 2, A and B). Fluorescence-activated cell sorting

Figure 4 Gene expression signatures of *Tmem30a*^{-/-} hematopoietic stem cells (HSCs). **A:** Heat maps showing the expression changes of all 280 genes and hierarchical clustering of the genes in *Tmem30a*^{-/-} Lin^{low}Sca1⁺c-Kit⁺ (LSK) cells from the two biological replicates. Comparison of the global gene transcription profiles of LSK cells of wild-type (WT) and *Tmem30a*^{-/-} mice at day 7 after tamoxifen injection. **B–E:** Gene Set Enrichment Analysis shows enrichment of gene sets up- or down-regulated in *Tmem30a*^{-/-} LSK cells. **F:** Expression level of genes from selected gene sets were confirmed using quantitative real-time PCR. Data are expressed as the means ± SD of triplicate experiments performed at one time. KO, knockout; mTORC1, mammalian target of rapamycin complex 1.

(FACS) analysis further confirmed the results that either the percentages or total cell counts of B, T, and myeloid cells were dramatically decreased at days 15 and 28 after tamoxifen injection in *Tmem30a*^{-/-} mouse BM (Figure 2C). To further determine whether *Tmem30a* deficiency perturbs B-cell development, three well-defined developmental stages of B cells were analyzed in BM with FACS,

and immature cells were found to be the most sensitive and mature B cells less sensitive to *Tmem30a* deficiency (Figure 2, D and E). Taken together, *Tmem30a* was essential for B-cell survival and *Tmem30a* deficiency reduced B-cell progenitors and B-cell maturation at the developmental stages. The results also suggested that efficiency effects of *Tmem30a* deletion were time and cell type dependent.

Table 1 Gene Sets Related to Cell Survival, Proliferation, and Stemness

Gene set	Size	Normalized enrichment score	Nominal <i>P</i> value
HALLMARK_MTORC1_SIGNALING	192	1.51	0.00781
BROWN_MYELOID_CELL_DEVELOPMENT_UP	138	2.38	<0.0001
CHYLA_CBFA2T3_TARGETS_UP	348	1.86	<0.0001
IVANOVA_HEMATOPOIESIS_STEM_CELL	235	-1.74	<0.0001
JAATINEN_HEMATOPOIETIC_STEM_CELL_UP	274	-1.72	<0.0001
GAL_LEUKEMIC_STEM_CELL_DN	207	1.85	<0.0001

Figure 5 Gene expression signatures of hematopoietic stem cells (HSCs) of *Tmem30a*^{-/-} HSCs. **A** and **B**: Genes up-regulated in coagulation and angiogenesis gene sets are down-regulated in *Tmem30a*^{-/-} HSCs. **C**: Interferon response gene sets, such as interferon- α response are up-regulated in *Tmem30a*^{-/-} HSCs. **D**: Genes involved in epithelial mesenchymal transition are down-regulated in *Tmem30a*^{-/-} HSCs. WT, wild type.

Tmem30a Is Essential for HSC and HPC Survival

The widespread effects of *Tmem30a* deficiency on blood lineages prompted us to examine its roles on HSCs and hematopoietic progenitor cells (HPCs). At 15 and 28 days after tamoxifen injection, both the WT and *Tmem30a*^{-/-} mice were sacrificed and BM cells were harvested for LSK cell analysis with FACS (Figure 3A). *Tmem30a* deficiency caused a significant reduction of both the percentage and the absolute number of HSCs (Figure 3, A and B). Both *Tmem30a*^{-/-} and WT BM still contained approximately similar numbers of LSK cells at day 7, but *Tmem30a*^{-/-} BM only contained approximately 50% of WT LSK cells at day 15 and approximately 30% of WT LSK cells at day 28 after tamoxifen injection (Figure 3B). In the HPC compartments, as expected, a decrease of CMPs, GMPs, and MEPs was detected in *Tmem30a*^{-/-} mice compared with WT controls (Figure 3, C and D). Among these HPCs, MEP cells were less sensitive to *Tmem30a* deficiency (Figure 3D).

Because CAG-CreER expression is not restricted in hematopoietic cells, the observed HSC phenotypes in *Tmem30a*^{-/-} mice may be due to a perturbed BM microenvironment. To investigate the possibility, *Tmem30a*^{-/-} (CD45.2) and B6.SJL-*Ptpca Pepc*^{b/BoyJ} (CD45.1) BM cells,

which were mixed at ratio of 1:1, were transplanted into lethally irradiated CD45.1 mice. Recipients received tamoxifen or vehicle at 12 weeks after BM transplantation (BMT). At day 15 after tamoxifen injection, recipients were sacrificed to harvest BM cells and the percentages of CD45.2 and CD45.1 were analyzed with FACS (Figure 3E). The mean ratio of CD45.2 versus CD45.1 was 29.2% to 47.5% in recipients received vehicle, but it was 12.8% to 66.5% in the tamoxifen injection group mice (Figure 3E). These results indicated that *Tmem30a* deficiency may directly cause leukocyte death. To avoid the BM microenvironment effects, BM cells from *Tmem30a*^{-/-} or WT mice were treated with tamoxifen *in vitro* for 48 hours and then transplanted into lethally irradiated recipients at a cell dose of 1×10^6 . Recipients that received *Tmem30a*^{-/-} BM cells or no cell died within 10 days after irradiation, and only one mouse died in the group that received BM cells from WT mice (Figure 3F). This result indicates that deletion of *Tmem30a* in HSCs was sufficient to impair HSC survival.

Tmem30a Deletion Alters Multiple Signal Pathways in HSCs

To gain insights into the mechanism of HSC deficiency in *Tmem30a*^{-/-} mice, two independent sets of RNA

Table 2 Gene Sets Related to Important Biological Functions or Biological Processes

Gene set	Size	Normalized enrichment score	Nominal <i>P</i> value
HALLMARK_COAGULATION	131	1.58	0.00518
HALLMARK_ANGIOGENESIS	138	1.58	0.01329
HALLMARK_COMPLEMENT	180	1.35	0.01235
HALLMARK_INTERFERON_ALPHA_RESPONSE	87	-1.85	<0.0001
HALLMARK_INTERFERON_GAMMA_RESPONSE	182	-1.47	<0.0001
BROWNE_INTERFERON_RESPONSIVE_GENES	56	-1.7	<0.0001
HALLMARK_INFLAMMATORY_RESPONSE	193	1.21	0.03488
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	192	1.3	0.01923
HALLMARK_CHOLESTEROL_HOMEOSTASIS	69	1.48	0.03578
HALLMARK_GLYCOLYSIS	193	1.27	0.01493

Figure 6 *Tmem30a* is also essential for BCR/ABL1-transduced chronic myeloid leukemia in mice. **A:** Kaplan-Meier survival curves for recipients of puro murine stem cell virus—BCR/ABL1—internal ribosome entry site—enhanced green fluorescent protein (pMSCV-BCR/ABL1-IRES-eGFP)—transduced bone marrow (BM) cells from wild-type (WT) or *Tmem30a*^{-/-} donor mice. The recipient numbers of each group and survival days are indicated. Independent experiments were performed twice. **B:** Fluorescence-activated cell sorting analysis shows appearance of GFP⁺Gr-1⁺ cells in peripheral blood (PB), BM, and spleen (SPL) of recipients of pMSCV-BCR/ABL1-IRES-eGFP—transduced BM cells from WT or *Tmem30a*^{-/-} donor mice at day 13 after bone marrow transplantation (BMT; day 5 after tamoxifen injection). **C:** Photomicrographs of hematoxylin and eosin—stained lung and spleen sections from recipients of pMSCV-BCR/ABL1-IRES-eGFP—transduced BM cells from WT or *Tmem30a*^{-/-} donor mice. **D and E:** FACS analysis (**D**) and total numbers (**E**) of leukemia stem cells (GFP⁺Lin⁻c-Kit⁺Sca-1⁺) in BM of recipients of BM cells of WT or *Tmem30a*^{-/-} donor mice transduced with pMSCV-BCR/ABL1-IRES-eGFP, 8 days after BMT and before tamoxifen injection and 13 days after BMT (5 days after tamoxifen injection). Data are expressed as means ± SD (**E**).

sequencing were performed for gene profiling analysis. BM cells from *Tmem30a*^{-/-} mice and their littermates, which were treated with tamoxifen for 7 days, were harvested and LSK cells were subsequently sorted with FACS. Total RNA was isolated, reverse transcribed to cDNA, and amplified. RNA sequencing was performed with the Illumina MiSeq system. Genes were considered significantly altered based on a twofold or greater change in mean expression ($P < 0.05$). With these criteria, 280 genes were determined to be significantly altered. Among these genes, 89 genes were up-regulated and 191 genes were down-regulated in *Tmem30a*^{-/-} LSK cells (Figure 4A).

Results of whole transcriptome comparisons using GSEA showed that mammalian target of rapamycin (mTOR) complex 1 pathway gene set was down-regulated in *Tmem30a*^{-/-} LSK cells (Figure 4B). Previous studies already demonstrated that the mTOR signal pathway was essential for HSC self-renewal and multilineage hematopoiesis and mTOR deficiency was sufficient to cause pancytopenia in mice.²⁷ This result indicates that the pancytopenia in *Tmem30a* deficiency mice is partially caused by the impairment of mTOR signal pathway.

Interestingly, a myeloid cell development gene set was enriched and down-regulated in *Tmem30a*^{-/-} LSK cells (Figure 4C), indicating that the differentiation capability of HSCs without *Tmem30a* was limited. In correlation, two previously published HSC gene sets of HSC stemness were significantly enriched in *Tmem30a*^{-/-} LSK cells but not WT LSK cells (Figure 4, D and E).^{28,29} In addition, another two gene sets, CBFA2T3 and LSC down, were also significantly enriched and down-regulated in *Tmem30a*^{-/-} LSK cells (Table 1).

To confirm the RNA sequencing results, nine genes (*Anxa3*, *Cbx2*, *Cttnl1*, *Dnmt3a*, *F13a1*, *Laptn4b*, *Prc1*, *Slpi*, and *Tgfb1*), which were listed in the above-mentioned gene sets and had well-established functions related to HSCs, were selected.^{28–30} Consistent with RNA sequencing data, all nine genes were either up-regulated or down-regulated in RT-PCR analysis (Figure 4F).

Previous studies have demonstrated that P4-ATPases were critical for blood coagulation.³ Significant enrichment of coagulation sets was also detected by GSEA (Figure 5A). More gene sets related to important biological processes, such as angiogenesis and epithelial mesenchymal transition, were also significantly enriched (Figure 5, B and D, Table 2). The fundamental biological function of *Tmem30a* is to ensure plasma membrane asymmetry and its deficiency causes cell death.^{16,20} This phenotype is further supported by some significant enrichment of gene sets, such as interferon response and DNA repair (Figure 5C, Table 2).

Tmem30a Is Also Essential for the Induction of CML by BCR/ABL1

Currently, CML is still an incurable disease. The main reason is that LSCs in CML are not sensitive to chemotherapy.²² *Tmem30a* is essential for blood cell survival,

including HSCs in mice. To further investigate whether *Tmem30a* is also critical for LSC survival, a BCR/ABL1-transduced CML mouse model was used. WT or *Tmem30a*^{-/-} donor BM cells were used for CML induction. Two recipient groups were further divided into four groups and treated with tamoxifen or vehicle at day 8 after BMT. In the group that received *Tmem30a*^{-/-} BM cells and tamoxifen treatment, only four recipients died of CML, and seven mice finally were leukemia cell free; all recipients in the other three groups died of CML within 4 weeks (Figure 6A). This defective disease phenotype was correlated with many fewer leukemia cells in peripheral blood, BM, and spleen at day 13 after BMT (day 5 after tamoxifen injection) (Figure 6B). In addition, the lungs and spleens of the mice in the *Tmem30a*^{-/-} donor and tamoxifen treatment group were less severely infiltrated with leukemia cells compared with the lungs and spleens of the other mice (Figure 6C).

It is essential to completely remove LSCs to cure CML. To test whether loss of *Tmem30a* impairs LSCs, LSCs in BM of CML mice were quantified at day 8 (before tamoxifen injection) and day 13 (day 5 after tamoxifen injection) after BMT. At day 8, there was no significant difference of LSC count in BM between WT and *Tmem30a*^{-/-} mice (Figure 6, D and E). This result indicated that there was no deficiency of *Tmem30a*^{-/-} BM cells in BM cell engraftment and leukemia transformation. However, at day 5 after tamoxifen injection, the percentage and number of LSCs transformed from *Tmem30a*^{-/-} BM cells were significantly reduced compared with that in other group recipients (Figure 6, D and E). This result indicated that *Tmem30a* deficiency not only strongly impaired normal HSCs but also was required for LSC survival.

Discussion

In this study, we found that *Tmem30a* deficiency caused severe pancytopenia by impairing the survival of lineage-committed blood cells and HSCs in mice, and its deficiency also impaired the survival of BCR/ABL1-transduced CML LSCs in mice. *Tmem30a* deficiency caused a rapid BM failure in mice (Figure 1B). Apoptosis status of BM cells was examined at several time points after tamoxifen injection. Although an obvious BM cell count decrease was observed (Figure 1, B and C), no difference was found in apoptosis of available cells from WT and *Tmem30a*^{-/-} mice (data not shown). *Tmem30a* deficiency causes a rapid cell death.^{16,20} Therefore, most biological events, such as apoptosis and cell cycle, are difficult to determine in *Tmem30a*^{-/-} mice.

The leukocyte counts in WT mice were also reduced after tamoxifen injection (Figure 1B) because tamoxifen, which usually is used to treat and prevent some types of breast cancers by blocking the actions of estrogen,³¹ also has cytotoxic effects on leukocytes. Compared with lineage-committed blood cells, HSCs were less sensitive to the cytotoxicity caused by tamoxifen (Figure 3B). Therefore, the total HSC count increased at day 15 compared with that

at day 7 after tamoxifen injection in the *WT* group mice, whereas the HSC count in the *Tmem30a*^{-/-} group mice decreased at all examined time points (Figure 3B). Interestingly, not all lineage-committed cells were equally sensitive to *Tmem30a* deletion. Among B-cell development stages, pre- and pro- as well as immature B cells were more sensitive to *Tmem30a* deficiency compared with mature B cells (Figure 2D). Among HPCs, MEP cells were less sensitive to *Tmem30a* deficiency compared with CMP and GMP (Figure 3D).

To understand the molecular mechanisms of the pancytopenia, gene profiling for *Tmem30a*^{-/-} LSK cells was performed. One critical prosurvival signaling gene set of mTOR was significantly down-regulated in *Tmem30a*^{-/-} cells (Figure 4B). mTOR deficiency alone is sufficient to cause pancytopenia in mouse.²⁷ Unexpectedly, two gene sets related to HSC stemness were highly up-regulated in *Tmem30a*^{-/-} LSK cells (Figure 4, D and E). The increased expression of these genes might be a compensatory effect of the increased death of LSK cells in *Tmem30a*^{-/-} mice. In correlation, *Tmem30a*^{-/-} LSK cells displayed a diminished expression of myeloid differentiation relation genes (Figure 4, C and F), such as genes in myeloid cell development up³⁰ and CBFA2T3 target up³² gene sets. CBFA2T3 is a member of the myeloid translocation family genes.³³ CBFA2T3 interacts with DNA-bound transcription factors, recruits a range of corepressors to facilitate transcriptional repression, and is involved in HSC differentiation.³² In addition, *Tmem30a*^{-/-} LSK cells displayed an increased level of interferon response genes (Figure 5, C and E).^{34,35} Interferon response genes are usually linked to growth inhibition, senescence, and apoptosis.^{36–40} Together, these results indicated that *Tmem30a* was involved in multiple signal pathways. A systematic exploration of the human interactome data also demonstrated that *Tmem30a* interacted with many other proteins, not only P4-ATPase family proteins.^{41,42}

Unexpectedly, in BCR/ABL1-induced CML model, some recipients still died of CML (Figure 6A) after tamoxifen injection likely because the inducible deletion of *Tmem30a* by tamoxifen takes some time. In addition, tamoxifen-induced deletion of *Tmem30a* was not completed in all leukemia cells, including LSCs. This explanation was further confirmed with PCR from leukemia cell genomic DNA. PCR results showed that the *Tmem30a* gene was not deleted in the leukemia cells from mice that died of CML. If the recipient could overcome this critical period, it became leukemia cell free (Figure 6A). Of interest, LSCs were more sensitive to the loss of *Tmem30a* compared with normal HSCs. LSC count rapidly reduced at day 5 (Figure 6, D and E), but there was no significant decrease of HSC count at day 7 after tamoxifen injection. This may provide a time window for potential CML therapy by targeting *Tmem30a*. Although it is hard to directly target *Tmem30a* with compounds now, in the P4-ATPase complex, the α subset, such as ATP8A1, ATP8B1, ATP11A, ATP11B, and ATP11C, is potentially targetable.^{43–45}

Collectively, our results demonstrate that *Tmem30a* is essential for the survival of hematopoietic system cells and BCR/ABL1-transduced leukemia cells. *Tmem30a* deficiency results in rapid pancytopenia in mice and impairment of leukemia cell survival. These results highlight the roles of *Tmem30a* in the hematopoietic system.

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Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.02.015>.

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