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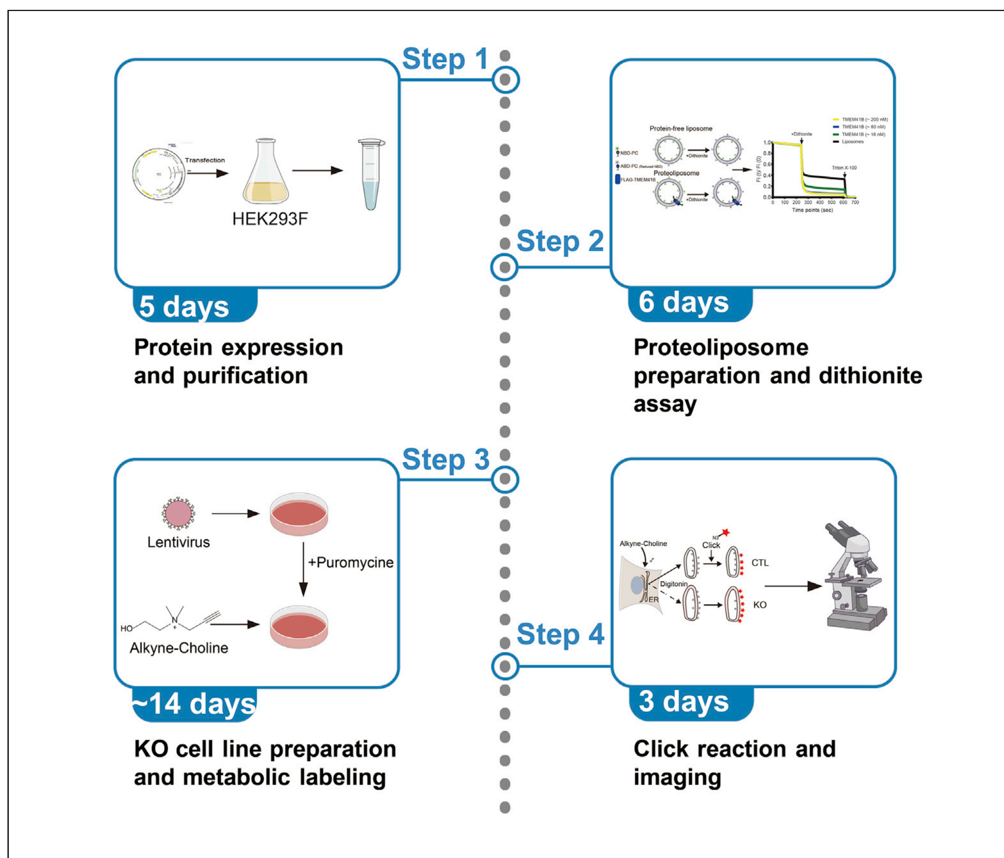
In vitro and *in vivo* assay of the ER lipid scramblase TMEM41B



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Highlights

TMEM41B encodes a phospholipid scramblase at the ER

In vitro scramblase assay of TMEM41B with fluorescent liposomes

In vivo scramblase assay of TMEM41B with metabolic labeling using alkyne-choline

Amphipathic phospholipids translocated by scramblases play a central role in facilitating lipid movement across the membrane bilayer, especially at the endoplasmic reticulum (ER) membranes. Here, we present a protocol for assessing the activity of the ER-localized lipid scramblase TMEM41B. We detail an *in vitro* fluorescent liposome-based phospholipid scrambling assay and *in vivo* metabolic labeling in living cells using alkyne-choline. The scramblase activity of other VTT (VMP1, TMEM41, and Tvp38) domain-containing proteins, such as TMEM41A and VMP1, can be assayed.

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In vitro and *in vivo* assay of the ER lipid scramblase TMEM41B

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SUMMARY

Amphipathic phospholipids translocated by scramblases play a central role in facilitating lipid movement across the membrane bilayer, especially at the endoplasmic reticulum (ER) membranes. Here, we present a protocol for assessing the activity of the ER-localized lipid scramblase TMEM41B. We detail an *in vitro* fluorescent liposome-based phospholipid scrambling assay and *in vivo* metabolic labeling in living cells using alkyne-choline. The scramblase activity of other VTT (VMP1, TMEM41, and Tvp38) domain-containing proteins, such as TMEM41A and VMP1, can be assayed.

For complete details on the use and execution of this protocol, please refer to Huang et al. (2021).

BEFORE YOU BEGIN

Prepare plasmids

Timing: 6–7 days

Plasmids for TMEM41B expression

Bacteria-related operations need to be performed on an ultra-clean bench. Wipe the table with 70% ethanol and then ultraviolet irradiate for 30 min.

1. Download the cDNA sequence of human TMEM41B (GenBank: NM_015012.4) from the NCBI database (https://www.ncbi.nlm.nih.gov/), the web page link of the cDNA sequence of human TMEM41B is: https://www.ncbi.nlm.nih.gov/nuccore/NM_015012.4/.

2. Design the forward and reverse primers and amplify the target gene by PCR. For protein purification, a FLAG-tag (amino acid sequence: DYKDDDK) is added at the N-terminal of the TMEM41B amino acid sequence by two forward primers with 20 bp overlaps.
   a. PCR reaction for amplifying the target gene fragment:

Reagent	Final concentration	Amount
Forward primer1 (10 μM)	0.03 μM	1.5 μL
Forward primer2 (10 μM)	0.01 μM	0.5 μL
Reverse primer (10 μM)	0.03 μM	1.5 μL
2× KOD Fx buffer (TOYOBO™)	1×	25 μL
2 mM dNTPs	200 μM	5 μL

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Continued

Reagent	Final concentration	Amount
KOD Fx	1 U	1 μL
cDNA template (100 ng/μL)	2 ng/μL	1 μL
ddH_2O	N/A	14.5 μL
Total	**N/A**	**50 μL**

b. Thermocycling conditions for the PCR reaction:

Steps	Temperature	Time	Cycles
Initial denaturation	98°C	2 min	1
Denaturation	98°C	10 s	30
Annealing	55°C	30 s	
Extension	68°C	1 min	
Final extension	68°C	10 min	1
Hold	4°C	Forever	

c. Run a 1% agarose gel to verify the fragment (about 900 bp) and purify the PCR product using the OMEGA Gel Extraction Kit (D2500-02), according to the manufacturer's protocol (https://www.omegabiotek.com/wp-content/uploads/2018/07/D2500.D2501-PROTOCOL-E.Z.N.A.-Gel-Extraction-Kit.pdf).

3. Digest the pKH3 (Addgene: 12555, |Mattingly and Macara, 1996|) vectors with restriction enzyme EcoRI and XbaI.
   a. Set up the reactions, as given below:

Reagent	Final concentration	Amount
10× CutSmart® Buffer	1×	5 μL
pKH3 (500 ng/μL)	100 ng/μL	10 μL
EcoRI	0.4 U/μL	1 μL
NotI	0.4 U/μL	1 μL
ddH_2O	N/A	33 μL
Total	**N/A**	**50 μL**

   b. Incubate at 37°C for 1 h.
   c. Run a 0.8% agarose gel to verify the fragment and cut out the band containing the linearized vectors (about 4.5 kb). Then purify the linearized vectors using the OMEGA Gel Extraction Kit (D2500-02), according to the manufacturer's protocol.

4. Assemble the PCR production to the linearized vectors.
   a. Set up the reaction, as given below:

Reagent	Final concentration	Amount
5× TEDA (Xia et al., 2019)	1×	4 μL
Digested pKH3 (50 ng/μL)	1 ng/μL	1 μL
PCR product (20 ng/μL)	0.4 ng/μL	1 μL
ddH_2O	N/A	13 μL
Total	**N/A**	**20 μL**

   b. Incubate at 30°C for 40 min.

⚠ CRITICAL: We recommend the molecule ratio of vectors and inserts should be 1:2. Before transformation, the product should be stored at 4°C, or −20°C for longtime storage.

5. Transformation and colony picking.
   a. Transform 5 μL of the recombination reaction mixture using 50 μL of Stbl3 competent cells.
   b. Keep the transformed competent cells on ice for 30 min, then heat shock cells at 42°C in a water bath for 60 s, put back on ice for 5 min.
   c. Add 500 μL of SOC medium and allow the transformed cells to recover at 37°C for 30 min under 180 rpm shaking.
   d. Plate the transformed cells on agar plates containing 100 μg/mL ampicillin. Incubate the plates at 37°C for 12–14 h.
   e. Pick 2–4 colonies into 3 mL of 2× YT medium containing 100 μg/mL ampicillin and let the culture grow at 37°C for 14–16 h under 200 rpm shaking.
   f. Perform plasmids minipreps using the OMEGA Plasmid Mini Kit (D6943-02) and perform the purification following the manufacturer's instructions (https://www.omegabiotek.com/wp-content/uploads/2013/05/D6942.D6943.D6945-January-2017-Online.pdf).
   g. Perform DNA sequencing to verify the positive expression clones.
   h. Perform plasmids maxipreps using a commercially available kit to obtain enough plasmids (>100 μg) for following use. We use TIANGEN HighPure Maxi Plasmid Kit (DP116) following the manufacturer's instructions (http://www.tiangen.com.cn/asset/imsupload/up0004885001433129475.pdf). The verified plasmids are stored at −20°C for 1 year for subsequent experiments.

Plasmids for CRISPR/Cas9-mediated gene knockout

Bacteria-related operations need to be performed on an ultra-clean bench. Wipe the table with 70% ethanol and ultraviolet irradiate for 30 min.

6. Guide RNAs targeting the human *TMEM41B* gene were designed using the Benchling cloud-based lab notebook, according to the tutorials (https://benchling.com/tutorials/21/designing-and-analyzing-gRNAs).
   a. Select gRNA sequences with on-target scores above 60 and off-target scores below 30 (higher off-target score means lower predicted off-target effects).
   b. Click on the off-target score to examine whether the potential off-target sequence target genes interfere with your studies.

   Note: Three guides for one gene are recommended to make sure the gene would be knocked out.

7. Digest the Lenti-CRISPR-V2 (Addgene: 52961, (Sanjana et al., 2014)) vectors with the restriction enzyme BsmBI.
   a. Set up the reaction, as given below:

Reagent	Final concentration	Amount
10× NEBuffer™ 3.1 buffer	1×	5 μL
Lenti-CRISPR-V2 (500 ng/μL)	100 ng/μL	10 μL
BsmBI	0.2 U/μL	1 μL
ddH$_2$O	N/A	34 μL
Total	N/A	50 μL

   b. Incubate at 55°C for 1 h.
   c. Purify the linearized vectors (about 13 kb), as described in step 3c.

8. Anneal each pair of oligos.
   a. Mix the following reagents in 200 μL PCR tubes:

Reagent	Final concentration	Amount
Forward primer (100 μM)	10 μM	1 μL
Reverse primer (100 μM)	10 μM	1 μL
10× NEB T4 ligase buffer	1×	1 μL
NEB T4 PNK	0.5 U/μL	0.5 μL
ddH_2O	N/A	6.5 μL
Total	**N/A**	**10 μL**

   b. Anneal in a thermocycler using the following program:

Temperature	Time
37°C	30 min
95°C	5 min
Ramp down to 25°C at 5°C/min.	

9. Ligate the annealed oligos with the digested Lenti-CRISPR-V2 vectors.
   a. Dilute the annealed oligos at a ratio of 1:200 in ddH_2O, by adding 2 μL annealed oligos to 398 μL ddH_2O in a 1.5 mL tube.
   b. Set up the ligation reaction, as given below:

Reagent	Final concentration	Amount
10× NEB T4 ligase buffer	1×	1 μL
NEB T4 ligase	20 U/μL	0.5 μL
Digested Lenti-CRISPR-V2 (50 ng/μL)	5 ng/μL	1 μL
Diluted annealed oligo duplex	N/A	1 μL
ddH_2O	N/A	6.5 μL
Total	**N/A**	**10 μL**

10. Transformation and picking colonies, as described in steps 5a–h.

Note: Transform 5 μL of the reaction mixture using 50 μL of Stbl3 competent cells. Stbl3 competent cells are the recommended strain for lentiviral vector system.

Prepare the *TMEM41B* KO cell line

Timing: about 2 weeks

Cell-related operations need to be performed in a Class II biological safety cabinet, wipe the table with 70% ethanol and ultraviolet irradiate for 30 min.

HEK293T cells can passage for 30–40 generations; Huh7 cells and FreeStyle™ 293F cells can passage for 25–30 generations; *TMEM41B* KO Huh7 cells can passage for 10–20 generations.

11. Package of lentivirus: [3–4 days]
   a. Seed the HEK293T cells in 6-well cell culture plates to ~40% confluency at 12 h before transfection.

b. Prepare the transfection mixture: add the plasmid DNA, as given below into 200 μL of serum-free, antibiotic-free DMEM.

Plasmids	Final concentration	Amount
Lenti-CRISPR-V2-*TMEM41B* (1 μg/μL)	10 ng/μL	2 μL
pAX2 (1 μg/μL)	6 ng/μL	1.2 μL
pMD2.G (1 μg/μL)	4 ng/μL	0.8 μL

c. Mix well and add 16 μL of 1 mg/mL PEI (assume the weight ratio of DNA:PEI is 1:4) with the above mixture from step 11b. Incubate at room temperature (RT, 20°C–25°C) for 20 min. And then add into HEK293T cells in one well of 6-well cell culture plates prepared at step 11a.
d. Exchange the medium with DMEM supplemented with 1% Penicillin-Streptomycin (Pen/strep) and 10% FBS 10 h after transfection.
e. Harvest *TMEM41B* CRISPR/Cas9 KO lentivirus in culture medium from step 11d 48 h after transfection and filter-sterilize the medium using a 0.45 μm filter to remove cellular impurities.

⚠ CRITICAL: The lentivirus in the culture medium can be stored at 4°C for one week, or at −80°C for 6 months storage. This work should be done in a Class II biological safety cabinet. When handling lentivirus, wear safety glasses and a face mask if necessary. If the lentivirus has been exposed to eyes or skin, rinse for 15 min at an eyewash station or wash the area with soap and warm water for 15 min.

12. Lentivirus infection of Huh7 cells: [8–12 days]
    a. Seed Huh7 cells in 6-well cell culture plates to ~40% confluency at 12 h before infection.
    b. Discard the culture medium of the cells. Add 600 μL of the medium from step 11e containing lentivirus and 1400 μL DMEM supplemented with 1% Pen/strep and 10% FBS to each well.
    c. Add 1.6 μL polybrene (10 μg/μL) to each well and mix well.
    d. Centrifuge the 6-well cell culture plates at 900 *g* for 30 min at RT (20°C–25°C) using an Eppendorf 5810 R centrifuge with plate holders.
    e. Exchange the medium with DMEM supplemented with 1% Pen/strep and 10% FBS 24 h after infection.
    f. 48 h after infection, exchange the culture medium with DMEM supplemented with 1% Pen/strep, 10% FBS and puromycin (1 μg/mL). Puromycin would kill the uninfected cells and select the infected cells which expressing puromycin resistant gene. Culture the cells for 1 week for selection. The cells can be passaged to avoid over-confluency during the selection process, but the concentration of the puromycin in the culture medium needs to be constant.
    g. Collecting cells for western blotting detection (Figure 3B).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-Alpha Tubulin (1:1000, 0.4 μg/mL)	Proteintech	Cat# 11224-1-AP, RRID: AB_2210206
Rabbit anti-TMEM41B (1:300, 1.3 μg/mL)	Proteintech	Cat# 29270-1-AP
Rabbit anti-FLAG (1:1000, 0.7 μg/mL)	Proteintech	Cat# 80010-1-RR, RRID: AB_2882940
Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP (1:10000, 0.08 μg/mL)	Thermo Fisher Scientific	Cat# 31460, RRID: AB_228341
Bacterial and virus strains		
Stbl3 competent cells	TransGen Biotech	Cat# CD521-01
Chemicals, peptides, and recombinant proteins		
BsmBI	New England Biolabs	Cat# R0739L

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
EcoRI	New England Biolabs	Cat# R0101L
XbaI	New England Biolabs	Cat# R0145L
CutSmart® Buffer	New England Biolabs	Cat# B7204S
10× NEBuffer™ 3.1 buffer	New England Biolabs	Cat# B7203S
T4 Polynucleotide Kinase	New England Biolabs	Cat# M0201L
T4 DNA ligase	New England Biolabs	Cat# M0202L
10× NEB T4 ligase buffer	New England Biolabs	Cat# B0202S
T5 exonuclease	New England Biolabs	Cat# M0363S
KOD Fx	Toyobo	Cat# KFX-101
PEG 8000	Pharmabiology	Cat# P32973
Polybrene	MilliporeSigma	Cat# TR-1003-G
Puromycin	Thermo Fisher Scientific	Cat# A1113803
Ampicillin	Inalco	Cat# 1758-9314
Tryptone	Oxoid	Cat# Lp0042
Yeast extract	Oxoid	Cat# Lp0021
NaCl	Beihua	Cat# S0219
KCl	Xilong	Cat# * 21-1
NaOH	Xilong	Cat# S0205
$MgCl_2$	Amresco	Cat# 0288
$CuSO_4$	Jena Bioscience	Cat# CLK-MI004-50
Sodium ascorbate	Aladdin	Cat# S105024
Dithionite	MilliporeSigma	Cat# 71699
Agarose	Vetec	Cat# V900500
EDTA	Yuanye	Cat# S30020-250g
DTT	Thermo Fisher Scientific	Cat# R0861
Tris-base	Vetec	Cat# WXBD1194V
HEPES	MilliporeSigma	Cat# V900477-500G
PEI	Polysciences	Cat# 23966
PBS	Hyclone	Cat# SH30256.01
DMEM	Hyclone	Cat# SH30022.01B
SMM 293-T1 medium	Sino Biological	Cat# M293T1
Penicillin-Streptomycin solution (Pen/strep)	CAISSON	Cat# PSL01
Fetal Bovine Serum (FBS)	VISTECH	Cat# SE100-011
EDTA-free Roche complete protease inhibitor cocktail	Roche	Cat# 4693132001
FLAG peptide	MilliporeSigma	Cat# F3290
POPC	MilliporeSigma	Cat# 42773
POPG	Avanti Polar Lipids	Cat# 840457P
NBD-PC	Avanti Polar Lipids	Cat# 810133P-1MG
ATP	MilliporeSigma	Cat# A1852-1VL
DDM	Qisong Biological	Cat# QS81007015
Triton X-100	MilliporeSigma	Cat# X100
Digitonin	MilliporeSigma	Cat# D141
Bio-beads	Bio-Rad	Cat#1528920
Alkyne choline	CONFLUORE	Cat# BCP-44
5-TAMRA azide	CONFLUORE	Cat# BCP-44
BTTAA	CONFLUORE	Cat# BDJ-4
4% (w/v) formaldehyde	Leagene	Cat# DF0135
Chloroform	Beijing Tong Guang Fine Chemicals Company	Cat# 112050
Methanol	Beijing Tong Guang Fine Chemicals Company	Cat# 104028
Ethanol	Beijing Tong Guang Fine Chemicals Company	Cat# 104021
Critical commercial assays		
OMEGA Gel Extraction Kit	OMEGA	Cat# D2500-02
OMEGA Plasmid Mini Kit	OMEGA	Cat# D6943-02

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
TIANGEN HighPure Maxi Plasmid Kit	TIANGEN	Cat# DP116
ANTI-FLAG M2 Affinity Gel	MilliporeSigma	Cat# A2220
Pierce™ BCA Protein Detection Kit	Thermo Fisher Scientific	Cat# 23227
Experimental models: Cell lines		
HEK293T	ATCC	Cat# CRL-3216
Huh7	JCRB Cell Ban	Cat# JCRB0403
Huh7 *TMEM41B* KO	This paper	N/A
FreeStyle™ 293F	Thermo Fisher Scientific	Cat# R79007
Oligonucleotides		
EcoRI-FLAG F1: ctgcacctcggttctaagcttGCGG CCGCcaccATGGACTACAAAGACGATGAC	This paper	N/A
FLAG-TM41B F2: TACAAAGACGATGACG ACAAGTCTAGAgcgaaaggcagagtcgcc	This paper	N/A
TM41B-XbaI R: ATTCGGGCCCCTCG AGGGATCCttactcaaatttctgctttag	This paper	N/A
TMEM41B gRNA F: caccgG TCGCCGAACGATCGCAGTTT	This paper	N/A
TMEM41B gRNA R: aaacAA CTGCGATCGTTCGGCGACc	This paper	N/A
Recombinant DNA		
Plasmid: pKH3	Mattingly and Macara (1996)	Addgene Cat# 12555
Plasmid: FLAG-hTMEM41B	This paper	N/A
Plasmid: GFP- SEC61β	Ma and Mayr (2018)	Addgene Cat# 121159
Plasmid: Lenti-CRISPR-V2	Sanjana et al. (2014)	Addgene Cat# 52961
Plasmid: Lenti-CRISPR-V2-TMEM41B	This paper	N/A
Plasmid: psPAX2	a gift from Didier Trono Lab, École Polytechnique Fédérale de Lausanne (unpublished)	Addgene Cat# 12260
Plasmid: pMD2.G	a gift from Didier Trono Lab, École Polytechnique Fédérale de Lausanne (unpublished)	Addgene Cat# 12259
Software and algorithms		
ImageJ (Fiji)	Schindelin et al. (2012) National Institutes of Health (NIH)	RRID: SCR_0030. 70 https://imagej.net/software/fiji/
GraphPad Prism8	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
Gen5™	BioTek	https://www.biotek.com/products/software-robotics-software/gen5-microplate-reader-and-imager-software/
Other		
Dounce homogenizer	Active motif	Cat# 40401
Beckman TLA 100.3 rotor	BECKMAN COULTER	Cat# 349490
Open-Top Thinwall Polypropylene Tube	BECKMAN COULTER	Cat# 326819
Fisherbrand™ Borosilicate Glass Square Coverslip	Thermo Fisher Scientific	Cat# 3406
Glass slide	NorthGlass	Cat# B2237
Flasks (250 mL)	NorthGlass	Cat# B0051
Single neck round bottom ball bottle (100 mL)	Beijing Synthware Glass	Cat# F309100
Axygen® 1.5 mL Snaplock Microcentrifuge Tube	Axygen® Brand Products	Cat# MCT-150-C-S
Axygen® 2 mL MaxyClear Snaplock Microcentrifuge Tube	Axygen® Brand Products	Cat# MCT-200-C
15 mL Centrifuge Tubes	NEST	Cat# 601001
50 mL Centrifuge Tubes	NEST	Cat# 602051
6-Well Cell Culture Plates	NEST	Cat# 703001
60 mm Cell Culture Dishes	NEST	Cat# 705001
100 mm Cell Culture Dishes	NEST	Cat# 704001
Amicon 0.5 mL concentrators (10 KDa cutoffs)	SEP	Cat# UFC501008

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Dialysis bag MD77, retained molecular weight: 30,000 Da, RC membrane	D&B	Cat# L106100-1/pk
MX-RD-Pro LCD digital Rotator	DLAB	Cat# MX-RD-Pro
Centrifuge 5424 R	Eppendorf	Cat# 5404000090
Centrifuge 5810 R	Eppendorf	Cat# 5811000690
Extruder Set with Holder/Heating Block	Avanti polar lipids	Cat# 610000
Filter Support	Avanti Polar Lipids	Cat# 610014
Polycarbonate Membranes 0.4 μm	Avanti Polar Lipids	Cat# 610007
Polycarbonate Membranes 0.2 μm	Avanti Polar Lipids	Cat# 610006
Synergy H1 Hybrid Multi-Mode Reader	BioTek	N/A
NanoDrop 2000 Spectrophotometer	Thermo Fisher Scientific	N/A
Rotary evaporation	Ruideyiqi	Cat# N-1100-D
Gel Loading Tips, standard, round	Thermo Fisher Scientific	Cat# LC1001
CO2 oscillation incubator	Shanghai Zhichu Instrument Co., LTD	Cat# ZCZY-AN

MATERIALS AND EQUIPMENT

SOC medium		
Reagent	Final concentration	Amount
Tryptone	20 g/L	20 g
Yeast extract	5 g/L	5 g
NaCl	0.5 g/L	0.5 g
250 mM KCl	2.5 mM	10 mL
5 M NaOH	N/A	Adjust pH to 7.0
ddH_2O	N/A	Bring up to 1 L
Total	N/A	1 L
Combine above and sterilize by autoclaving, then add the reagents below		
*2 M $MgCl_2$	10 mM	5 mL
*1 M glucose	20 mM	20 mL

The SOC medium should be autoclaving and can be stored at 4°C for a couple of months. *Should be filtered by a 0.22 μm filter to remove microbial contamination.

2× YT medium		
Reagent	Final concentration	Amount
Tryptone	16 g/L	16 g
Yeast extract	10 g/L	10 g
NaCl	5 g/L	5 g
5 M NaOH	N/A	Adjust pH to 7.0
ddH_2O	N/A	Bring up to 1 L
Total	N/A	1 L

The 2× YT medium should be autoclaving and can be stored at 4°C for a couple of months.

TBS buffer		
Reagent	Final concentration	Amount
5 M NaCl	150 mM	15 mL
1 M Tris-HCl, pH 7.5	20 mM	10 mL
ddH_2O	N/A	Bring up to 500 mL
Total	N/A	500 mL

The TBS buffer can be stored at RT (20°C–25°C) for a couple of months.

5× TEDA		
Reagent	Final concentration	Amount
1 M Tris-HCl, pH 7.5	500 mM	0.5 mL
1 M DTT	50 mM	50 μL
1 M $MgCl_2$	50 mM	50 μL
PEG 8000	0.25 g/mL	0.25 g
T5 exonuclease	0.01 U/μL	1 μL
ddH_2O	N/A	Bring up to 1 mL
Total	**N/A**	**1 mL**

Mix well before use. Aliquots of 50 μL can be stored at −80°C for 1 year.

Buffer A		
Reagent	Final concentration	Amount
1 M Tris-HCl, pH 7.5	20 mM	0.2 mL
5 M NaCl	500 mM	1 mL
1 M DTT	1 mM	10 μL
EDTA-free Roche complete protease inhibitor cocktail (50×)	1×	200 μL
ddH_2O	N/A	Bring up to 10 mL
Total	**N/A**	**10 mL**

Mix well before use and store 4°C. Dissolve one tablet of EDTA-free Roche complete protease inhibitor cocktail (Cat# 4693132001) in 1 mL ddH_2O to prepare stock solution (50×), the solution can be stored at −20°C for 12 weeks.

Buffer B		
Reagent	Final concentration	Amount
1 M Tris-HCl, pH 7.5	20 mM	2 mL
5 M NaCl	500 mM	10 mL
10% (w/v) DDM	0.02% (w/v)	0.2 mL
ddH_2O	N/A	Bring up to 100 mL
Total	**N/A**	**100 mL**

Buffer B can be stored at 4°C for a couple of months.

Buffer C		
Reagent	Final concentration	Amount
1 M HEPES-NaOH, pH 7.5	50 mM	50 mL
5 M NaCl	200 mM	40 mL
ddH_2O	N/A	Bring up to 100 mL
Total	**N/A**	**1 L**

Buffer C can be stored at 4°C for a couple of months.

100 mM alkyne-choline		
Reagent	Final concentration	Amount
Alkyne-choline	100 mM	12.82 mg
ddH_2O	N/A	1 mL
Total	**N/A**	**1 mL**

Aliquots of 100 μL can be stored at −80°C for 1 year. Avoid multigelation.

10 mM 5-TAMRA azide		
Reagent	Final concentration	Amount
5-TAMRA azide	10 mM	1 mg
DMSO	N/A	180 μL
Total	**N/A**	**180 μL**
Keep out of light and store at −20°C for a couple of months.		

50 mM sodium ascorbate		
Reagent	Final concentration	Amount
Sodium ascorbate	50 mM	9.906 mg
ddH_2O	N/A	Bring up to 1 mL
Total	**N/A**	**1 mL**
Should be prepared just before use. Sodium ascorbate is purchased from Aladdin, Cat# S105024.		

BTTAA/CuSO4 complex		
Reagent	Final concentration	Amount
BTTAA	8.57 mM	3.69 mg
$CuSO_4$-$5H_2O$	1.43 mM	0.36 mg
ddH_2O	N/A	Bring up to 1 mL
Total	**N/A**	**1 mL**
Can be stored at −20°C for a couple of months.		

STEP-BY-STEP METHOD DETAILS

Assay of TMEM41B scramblase activity *in vitro*

Here we describe the reconstitution of TMEM41B into liposomes to characterize its scramblase activity using a fluorescence liposome-based *in vitro* scramblase assay (Brunner et al., 2014; Ghanbarpour et al., 2021; Hrafnsdóttir and Menon, 2000; Marek and Günther-Pomorski, 2016; Mathiassen et al., 2021; Matoba et al., 2020; Menon et al., 2011; Ploier and Menon, 2016; Vehring et al., 2007). Trace amounts of 7-nitro-2-1,3-benzoxadiazol-4-yl acyl chain-labeled phosphatidylcholine (NBD-PC) adapts a ~50% distribution between the inner and the outer leaflet of a symmetric liposome bilayer. The addition of membrane-impermeant dithionite quenches outer leaflet NBD fluorescence of the liposomes, resulting in ~50% fluorescence bleaching of NBD. In the presence of a scramblase (TMEM41B), which catalyzes the exchange of NBD-lipids between the leaflets, greater fluorescent quenching will be observed due to exposure of inner leaflet NBD to dithionite on the outer leaflet. Similar studies have been carried out previously on related scramblases such as Triton-extracted ER scramblases, opsin, TMEM16, ATG9 and VMP1 (Brunner et al., 2014; Ghanbarpour et al., 2021; Hrafnsdóttir and Menon, 2000; Mathiassen et al., 2021; Matoba et al., 2020; Menon et al., 2011; Suzuki et al., 2013; Vehring et al., 2007).

Expression and purification of recombinant TMEM41B

Timing: 7 days

Cell-related operations need to be performed in a Class II biological safety cabinet. Wipe the table with 70% ethanol and the ultraviolet irradiate for 30 min.

1. FreeStyle™ 293F cells are expanded in 250 mL glass flasks in SMM 293-T1 medium (Sino Biological Inc.) supplemented with 0.5% Pen/strep and 1% FBS. The cells are incubated in a 37°C

oscillation incubator with 5% CO_2, with optimal shaker speed at approximately 100–130 rpm. The cells are seeded at 0.5 × 10^6 viable cells/mL when cell density reaches 2.0 × 10^6 viable cells/mL (troubleshooting 1).

2. Protein expression and purification (a culture of 50 mL).
   a. Transfect 1 μg plasmids (the weight ratio of DNA:PEI is 3:1) per mL cells when cell density reaches 1.5–2.0 × 10^6 viable cells/mL. Prepare DNA-PEI complexes, as follows:
      i. Dilute 50 μg plasmid DNA into 1.5 mL SMM 293-T1 medium, mix gently and incubate at RT (20°C–25°C) for 5 min.
      ii. Dilute 150 μg PEI into 1.5 mL SMM 293-T1 medium, mix gently and incubate at RT (20°C–25°C) for 5 min.
      iii. Mix the plasmid DNA with the PEI. Incubate at RT (20°C–25°C) for 10–20 min.
      iv. Add the mixture from step 2a-*iii* to 50 mL of 1.5–2.0 × 10^6 cells/mL in a 250 mL glass shaker flask and place it back into the 37°C oscillation incubator with 5% CO_2.
   b. Collect the cells in 50 mL centrifuge tubes 48 h after transfection, and centrifuge at 1,000 *g* for 10 min at 4°C using an Eppendorf 5810 R centrifuge with a 50 mL tube holder.
   c. Resuspend the cells in 10 mL cold (4°C) TBS buffer. Centrifuge at 1,000 *g* for another 10 min at 4°C. Snap freeze the cell pellet in liquid nitrogen, and store at −80°C until further use.
3. Thaw the cell pellet at 4°C, resuspend in 5 mL buffer A, and lyse with a Dounce homogenizer (10 passes). Transfer the lysate to a 15 mL centrifuge tube.

   Note: DTT and protease inhibitor cocktail should be added to prepare buffer A just before use. Avoid the formation of bubbles when using the Dounce homogenizer to minimize protein degradation.

4. Add 0.5 mL DDM (10% (w/v) stock concentration) to the cell lysate (1% (w/v) final concentration), and gently rotate (10 rpm) the lysate using a MX-RD-Pro LCD digital Rotator at 4°C for 90 min.
5. Transfer the lysate to an Open-Top Thinwall Polypropylene Tube. Centrifuge the cell lysate at 100,000 *g* for 30 min at 4°C (using a TLA 100.3 rotor) to remove cell debris and undissolved membranes.
6. Transfer the supernatant to a new 15 mL centrifuge tube. Incubate the supernatant with 50 μL ANTI-FLAG M2 beads (MilliporeSigma, Cat# A2220) and rotate (10 rpm) the mixture at 4°C for 4 h.

   Note: The ANTI-FLAG M2 beads should be pre-equilibrated with 1 mL buffer B in a 1.5 mL microcentrifuge tube, by centrifuging the ANTI-FLAG M2 beads at 4,000 *g* for 1 min at 4°C to remove the supernatant in Centrifuge 5424 R, repeat the wash step for 3 times.

7. After incubation, pellet the ANTI-FLAG M2 beads by centrifuge at 1,000 *g* for 10 min at 4°C using an Eppendorf 5810 R centrifuge with a 15 mL tube holder. Transfer the ANTI-FLAG M2 beads to a new 1.5 mL tube, wash the beads with 1 mL buffer B about 4 times.
8. To remove chaperones, incubate the washed ANTI-FLAG M2 beads with 1 mL buffer B supplemented with 1 mM $MgCl_2$, 2.5 mM ATP and 1× EDTA-free Roche complete protease inhibitor cocktail, rotate (10 rpm) at 4°C overnight (12–14 h).
9. Wash the ANTI-FLAG M2 beads about 6 times with buffer B until the OD280 of the supernatant is close to 0 detecting by a NanoDrop 2000 Spectrophotometer.
10. Elute the recombinational FLAG-TMEM41B protein from the ANTI-FLAG M2 beads using 0.1 mg/mL FLAG peptide in buffer B supplemented with 1× EDTA-free Roche complete protease inhibitor cocktail, the elution volume is 500 μL. Rotate (10 rpm) at 4°C for 2 h.
11. Concentrate the recombinant human FLAG-TMEM41B protein in Amicon 0.5 mL concentrators (10 kDa cutoffs) by centrifuging the concentrators at 10,000 *g* for 20 min at 4°C in centrifuge 5424 R. When the liquid is reduced to a fifth of its volume, add 450 μL buffer B.
12. Repeat step 11 for 3 times to change the buffer of the recombinant human FLAG-TMEM41B protein with buffer B. Concentrate the volume to 50–100 μL.

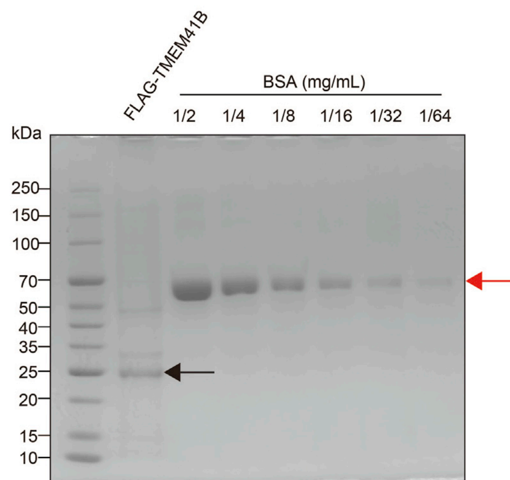

Figure 1. SDS-PAGE analysis of the recombinant human FLAG-TMEM41B

The black arrow: recombinant human FLAG-TMEM41B protein; the red arrow: serial diluted BSA standards.

13. Concentrated human FLAG-TMEM41B protein can be analyzed following SDS-PAGE. BSA protein from Pierce™ BCA Protein Detection Kit (Thermo Fisher) are diluted in a series of concentrations (1/2, 1/4, 1/8, 1/16, 1/32 and 1/64 mg/mL) as the standards. As shown in Figure 1.

Note: We recommend to load the same volume of purified human FLAG-TMEM41B and BSA protein, which could make it easier to determine the protein concentration of recombinant human FLAG-TMEM41B by Coomassie blue staining.

Liposome preparation

⏲ Timing: 2 days

14. Dissolve POPC (final concentration 25 mg/mL), POPG (final concentration 25 mg/mL) and 18:1–12:0 NBD-PC (final concentration 1 mg/mL) in chloroform (HPLC grade) on ice. Lipids powder could be dissolved in chloroform within one minute.

Note: The lipids stock solutions can be stored at −20°C for several months in glass jars. Powdered lipids can be stored at −20°C for 1 year, according to the reagent specification of Avanti Polar Lipids.

15. Add 143.5 μL POPC, 16 μL POPG and 9.5 μL 18:1–12:0 NBD-PC (about 0.2 mol% of the total phospholipids) to a 100 mL single neck round bottom ball bottle. The total lipids used here is about 3.98 mg, and the molar ratio of POPC versus POPG is 9:1 (mol/mol).
16. Dry the lipids by rotary evaporation (Ruideyiqi, Cat# N-1100-D) under an argon or nitrogen stream at a rotation speed of 120 rpm, for 5–15 min until the chloroform has evaporated. Then transfer the flask to a vacuum desiccator at RT (20°C–25°C) for at least 3 h or overnight (12–14 h), the pressure of vacuum desiccator is about -12 – -15 psi.

Note: as the lipids dry, the inside of the glass bottle will be covered with a green-colored lipid film.

⚠ CRITICAL: Dry the lipids at a slow speed to make sure the lipids form a thin lipid film. If it dries too quickly, a green film with uneven thickness can be observed on the inside of the glass bottle.

17. Resuspend the lipid film in 0.5 mL buffer C to generate a 10.5 mM lipid solution, by gently swirling the flask and incubate at 37°C for 10 min, until forming a homogenous turbid solution. The

lipids would form multilamellar vesicles (MLVs). Transfer the solution to a 2 mL microcentrifuge tube.

Pause Point: The MLVs solution can be stored at −80°C for 1–3 months. We avoid using MLVs stored longer than 3 months due to concerns of lipids oxidation.

18. Freeze-thaw the MLVs for 10 cycles by alternately placing it in liquid nitrogen and at a 37°C water bath, 3–5 min is needed for each operation.
19. Assemble the mini-extruder (Avanti, Cat# 610020), as instructed by Avanti (https://avantilipids.com/divisions/equipment-products/mini-extruder-assembly-instructions). Extrude the lipids suspension 20 times through a membrane with a 400 nm pore size, followed by 10 times extrusion through a membrane with 200 nm pore size at RT (20°C–25°C). Transfer the samples to a new 2 mL microcentrifuge tube.
20. Store the liposomes at 4°C and use within the day of preparation.

CRITICAL: The liposomes preparation should be stored above 0°C. Because the gel-liquid crystal transition temperature of POPC is −2°C, low temperature might damage the liposomes. The samples should be kept out of the light to avoid photobleaching of NBD.

Proteoliposome preparation

Timing: 3 days

21. Dilute the liposomes in buffer C to a final lipids concentration of 5.25 mM (~3.98 mg/mL).
22. Add Triton X-100 to the liposomes to a final concentration of 7 mM. Rotate (10 rpm) at RT (20°C–25°C) for 2–3 h to destabilize the liposomes.

Note: After incubating with 7 mM Triton X-100, the solution would become clear because the destabilizing of liposomes particles.

23. After 2–3 h of Triton X-100 destabilization, add 0.2% (w/v) DDM-solubilized recombinant human FLAG-TMEM41B protein preps (Figure 1, ~0.1 mg/mL) to the samples and gently rotate (10 rpm) at RT (20°C–25°C) for 1 h. The volume of destabilized liposomes per sample is 250 μL in a 1.5 mL microcentrifuge tube.

Note: Here the recombinant human FLAG-TMEM41B protein concentrations 200 nM, 80 nM and 16 nM have been used to assay its scramblase activity, at a protein-to-lipid molar ratio ~1:26000, 1:65000 and 1:320000, respectively. Add additional buffer B to ensure consistent phospholipids concentration across groups, as well as protein-free liposomes.

24. Meanwhile, prepare the bio-beads. Wash the bio-beads 2 times with methanol, 3 times with ddH_2O and 1 time with buffer C. For each washing step stir slowly for 10 min.

Note: It is recommended to prepare the 100 mg bio-beads for each sample, you can prepare the bio-beads for several samples at once, the specific weight of bio-beads depends on the sample you prepared.

25. Add 20 mg of the prepared bio-beads to each sample and rotate (10 rpm) the mixture at RT (20°C–25°C) for 1 h.
26. Add additional 20 mg of bio-beads and rotate (10 rpm) at RT (20°C–25°C) for another 2 h.
27. Transfer the liquid by gel loading tips (Cat# LC1001) to a new 1.5 mL microcentrifuge tube containing 40 mg of fresh bio-beads and rotate (10 rpm) at 4°C overnight (12–14 h).

Note: It is recommended to use gel loading tips (Cat# LC1001) to transfer the liquid because of the slenderness and easy access of the tips, which could avoid pipetting the particles of bio-beads.

28. Transfer the sample to a 1.5 mL microcentrifuge tube with a hole in the lid. Cover the hole with a pre-washed (with buffer C) dialysis membrane (30 kDa). Close the lid and make sure there is no leakage. Put the lid under the liquid in 500 mL buffer C in a 1 L beaker, magnetic rotor stirring (150 rpm) and dialyze against buffer C at 4°C for 2 days to remove the remaining detergent.

Note: Make sure the dialysis membrane and 1.5 mL microcentrifuge tube cover lid are tightly closed to prevent fluid from flowing out of the side. After incubation with bio-beads and dialysis, the solution would be turbid when the samples are observed against light. The samples should be kept from light to avoid photobleaching of NBD.

Scramblase assay

Timing: 1 day

29. Dilute the liposomes/proteoliposomes (~5 mM lipids concentration) to ~200 μM final lipids concentration with buffer C. The scramblase assay is performed at 30°C in 96-well plates, and the reaction volume per sample is 100 μL.
30. To assess scrambling activity of recombinant human FLAG-TMEM41B protein, the NBD fluorescence (excitation at 460 nm, emission at 538 nm) per sample is monitored using Gen5™ software in the Synergy H1 Hybrid Multi-Mode Reader (BioTek), with an inspection interval of 10 s.
31. After ~200 s recording, the addition of dithionite (to final concentration 10 mM) is added to monitor the reduce of the NBD fluorescence (troubleshooting 2, troubleshooting 3).

Note: 1 M dithionite is dissolved in 0.5 M unbuffered Tris (e.g., 10 mg of dithionite dissolve in 57 μL ice-cold 0.5 M Tris immediately before use) and keep on ice. The dissolved dithionite should be used within 10 min after preparation.

32. After ~600 s recording, add 2.5 μL 20% (w/v) Triton X-100 to each sample. The Triton X-100 will destabilize the liposomes/proteoliposomes, resulting in complete bleaching of the NBD fluorescence (troubleshooting 2, troubleshooting 3).
33. Data analysis: Calculate the relative fluorescent of NBD according to the following formula:

$$FI(t)/FI(0) = [F_t - F_{Triton}]/[F_0 - F_{Triton}]$$

F_0 represent the initial fluorescent intensity before dithionite, F_t represent the fluorescent intensity at "t" sec and F_{Triton} is the final background fluorescence after adding Triton X-100. The curves of scramblase assay data are generated using GraphPad Prism8.

Assay of TMEM41B scramblase activity *in vivo*

Phosphatidylcholine (PC), the most abundant phospholipid, is synthesized at the cytosolic leaflet of the ER (Vance, 2015), and subsequently translocated to the luminal leaflet by scramblases in wild-type (WT) cells (Pomorski and Menon, 2016). Alkyne-choline could be incorporated into PC in the living cells (Jao et al., 2009; Sun et al., 2021), and visualized by azide-alkyne following so-called click reaction. In mutant cells devoid of scramblases at the ER, the newly synthesized alkyne-PC is expected to accumulate on the cytosolic leaflets. The PC on the cytosolic leaflet of the ER can be selectively visualized with digitonin permeabilization, which leaves the organelle biolayer intact. Here we describe the click-chemistry and imaging approach to visualize the cytosolic leaflet of the *TMEM41B* WT/KO cells to examine the TMEM41B scramblase activity *in vivo*.

In vivo metabolic labeling to visualize PC

Timing: 4 days

Cell-related operations need to be performed in a Class II biological safety cabinet. Wipe the table with 70% ethanol and ultraviolet irradiate for 30 min.

34. WT and CRISPR/Cas9-mediated *TMEM41B* knockout Huh7 cells are seeded on Fisherbrand™ Borosilicate Glass Square Coverslip (Cat# 3406) in 6-well Cell Culture plates at ~25% confluence (troubleshooting 4).
35. After 12 h, transfect 1 μg GFP-SEC61β plasmids to each well, which labels the ER membrane.
    a. Add 1 μg GFP-SEC61β plasmids to 200 μL serum-free DMEM, mix gently and incubate at RT (20°C–25°C) for 5 min.
    b. Add 4 μg PEI to the mixture, mix gently and incubate at RT (20°C–25°C) for 15 min.
    c. After incubation, add the medium from step 35b to the cells.
36. 8 h after transfection, exchange the media with DMEM supplemented with 1% Pen/strep, 10% FBS and 100 μM alkyne-choline. Culture the cells for another 12–24 h (troubleshooting 5).

Note: Alkyne-choline should be thaw at RT (20°C–25°C) and added to the medium before use. Vortex vigorously before added to the cells.

37. Wash the cells with 1× PBS 3 times. Fix cells with 4% (w/v) formaldehyde at RT (20°C–25°C) for 15 min. Wash the cells with 1× PBS 3 times.
38. Permeabilize the cells with 25 μg/mL digitonin in 1× PBS for 10 min and wash the cells with PBS 3 times.
39. The cells on coverslips are incubated with 10 μM 5-TAMRA azide, BTTAA-$CuSO_4$ complex (50 μM $CuSO_4$, BTTAA/$CuSO_4$ (6:1, mol/mol)) and 2.5 mM sodium ascorbate in 1× PBS at RT (20°C–25°C) for 1 h.
40. Prepare 200 μL click reaction mixture, as given below:

Reagent	Final concentration	Amount
10× PBS	1×	20 μL
10 mM 5-TAMRA azide (1,000×)	10 μM	0.2 μL
10 mM BTTAA/$CuSO_4$ complex (6:1, mol/mol)	50 μM	1 μL
50 mM sodium ascorbate (freshly prepared)	2.5 mM	10 μL
ddH_2O	N/A	Bring up to 200 μL
Total	**N/A**	**200 μL**

Note: Sodium ascorbate should be prepared just before use. It is important to make sure the liquid phase cover the coverslips throughout the experiment.

41. Wash the cells with 1× PBS 3 times, 10 min per time, and block the cells in 1× PBS with 1% BSA at RT (20°C–25°C) for 15 min.
42. The coverslips are mounted to a glass slide (NorthGlass, Cas# B2237), wait until the mounting medium dries. It's optional to seal the slides with colorless nail polish. Then subject to confocal microscopy (troubleshooting 6).
43. Imaging is performed on a Zeiss LSM 880 with an Airyscan confocal microscope. GFP and TAMRA channels visualize ER (SEC61β) and PC (alkyne-choline), respectively. The parameters used here are as follows: excitation/emission wavelength = 493/517 nm, 577/603 nm, exposure time = 500 ms, gain = 700, pinhole = 1 AU, at RT (20°C–25°C). The images are saved as LSM files, and the image resolution is 1024 × 1024.

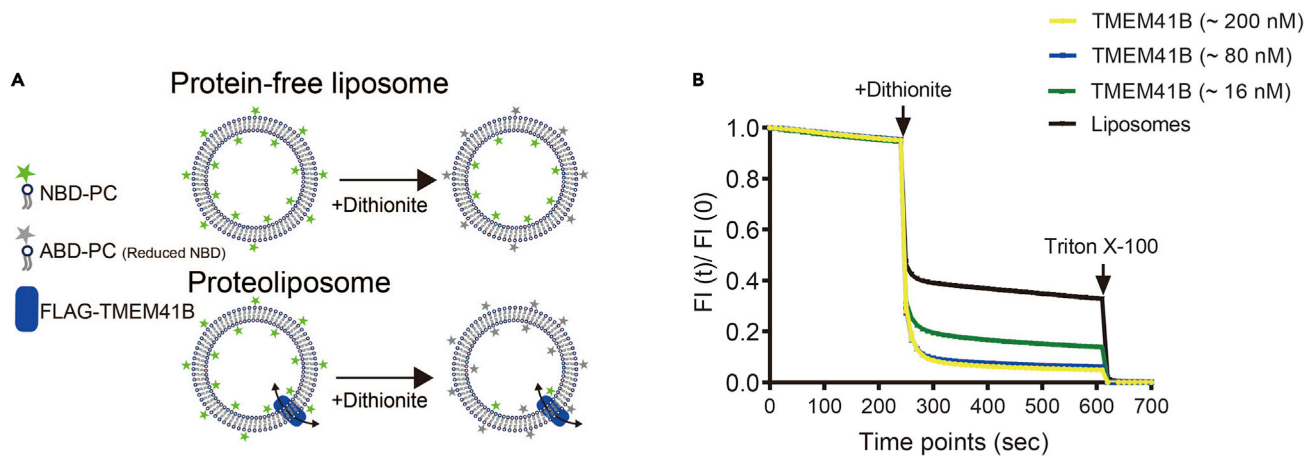

Figure 2. The fluorescence liposome-based *in vitro* scramblase assay of TMEM41B

(A) Schematic of the fluorescence liposome-based *in vitro* scramblase assay. Trace amounts of 7-nitro-2-1,3-benzoxadiazol-4-yl (NBD) acyl chain-labeled fluorescent phosphatidylcholine (PC) is symmetrically distributed over the liposome bilayer. Addition of dithionite bleaches outer leaflet NBD-PC to 7-amino-2,1,3-benzoxadiazol-4-yl (ABD)-PC, but cannot access inner leaflet NBD-PC. In the presence of a scramblase, NBD-PC is exchanged between the two leaflets, therefore causing greater reduction in fluorescence over time upon dithionite addition. Triton X-100 is added to destabilize the liposomes/proteoliposomes, resulting in complete bleaching of the NBD fluorescence.

(B) Representative NBD fluorescence traces over time (in sec) after dithionite treatment showing the scrambling activity of TMEM41B. The fluorescence reduction of proteoliposomes is ~92%, ~90% and ~80% (for 200 nM, 80 nM and 16 nM of FLAG-TMEM41B concentration, respectively) comparing to the liposomes (~60%) at ~300 s.

44. Co-localization analysis and fluorescence intensity are quantified using ImageJ Fiji, as described in the following:
    a. Open the LSM files by ImageJ Fiji.
    b. Crop images to select the region on the periphery ER using the selection tools.
    c. From the Analyze menu select "Set Measurements". Make sure area integrated intensity and mean gray value are selected.
    d. Select "Measure" from the Analyze menu. Then there will be a pop-up box with a stack of values.
    e. Select a region next to your cell that has no fluorescence, take this as the background signal. The value of the target area will be subtracted from the value of the background area as its signal value.

    The following are the operation for colocalization analysis:

    f. Open the LSM files by ImageJ Fiji.
    g. Crop images to select a region of interest using the selection tool.
    h. From the Image menu select "Color-Split Channels" to divide pictures into different channels.
    i. Select "Colocalization-Coloc 2" from the analyze menu for the colocalization analysis.
45. Analyzed by GraphPad Prism8 to compare the data difference from *TMEM41B* WT and KO Huh7 cells, and the two-tailed Student's t-test is used.

⚠ CRITICAL: Because the perinuclear regions contain stacks of ER membrane, it is difficult to avoid out-of-focus signals or over saturation of fluorescence. For more accurate quantification, it is recommended to quantify the fluorescent intensity of periphery ER (GFP-SEC61β) of the TAMRA channel for both *TMEM41B* WT and KO cells.

EXPECTED OUTCOMES

As shown in Figure 2B, the fluorescence reduction ($1-FI_{(300)}/FI_{(0)}$, at 300 s, after adding dithionite for a period of time, the fluorescence signal tends to be stable) of proteoliposomes is ~92%, ~90% and ~80% for 200, 80 and 16 nM recombinant human FLAG-TMEM41B protein concentration, respectively. The fluorescence reduction of liposomes is ~60%. Recombinant human FLAG-TMEM41B protein reconstituted into liposomes, which facilitated translocation of NBD-PC bidirectionally over the

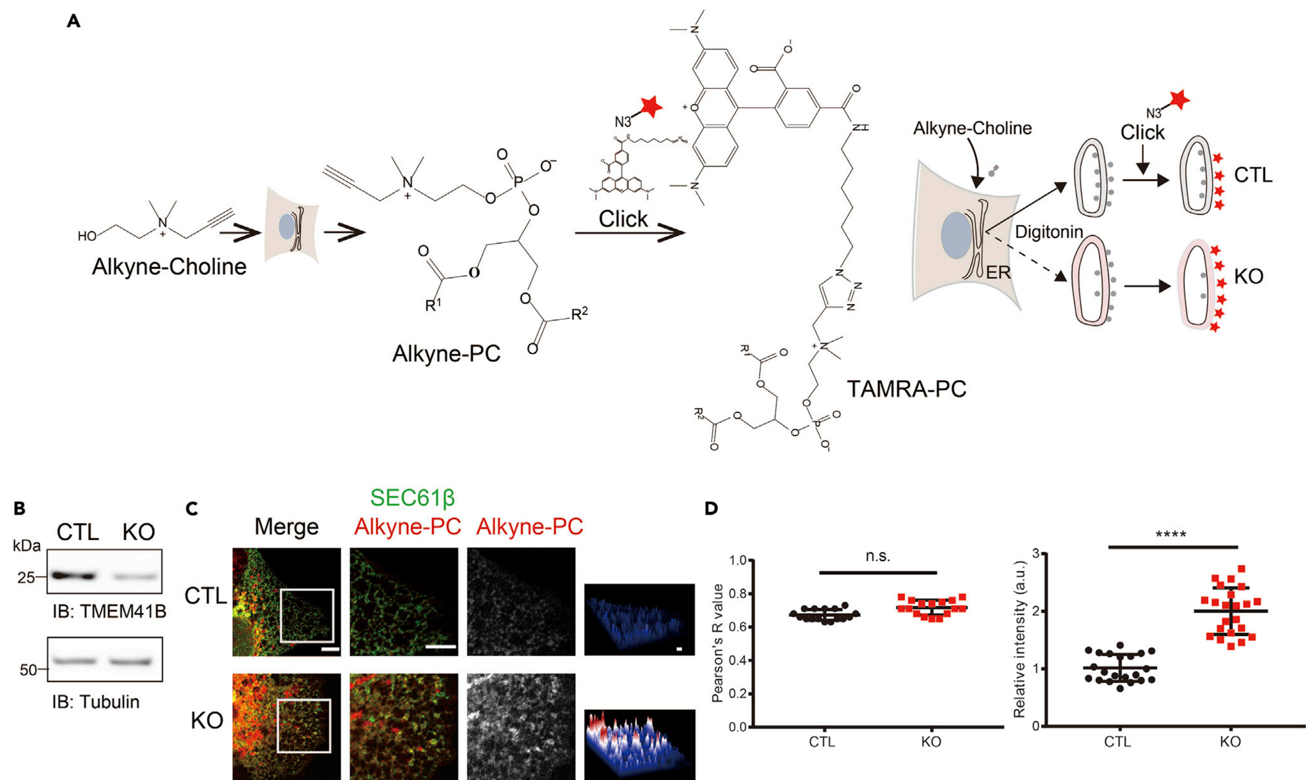

Figure 3. *In vivo* scrambling activity assay showing TMEM41B shuttles PC between the ER membrane leaflets

(A) Schematic of metabolic labeling and click-chemistry to detect PC in Huh7 *TMEM41B* WT/KO cells. Cells are incubated with alkyne-choline for synthesis into alkyne-PC at the ER outer leaflet. In wild-type cells, alkyne-PC will shuttle across the bilayer into the inner leaflet of the ER. Digitonin can permeabilizing the cell surface membrane, but the endo-membrane would be intact. Therefore, the click-chemistry reagents could only label alkyne-PC on the outer leaflet of the ER but could not access the inner leaflet of ER membrane. The absence of scramblases in the ER of mutant cells would trap more alkyne-PC on the outer leaflet and increase the fluorescent signal.

(B) Representative western blots for validation of TMEM41B deficiency in Huh7 cells.

(C) Representative confocal images showing accumulation of alkyne-PC signal on the outer leaflet of the ER in the absence of TMEM41B (n=3 independent experiments). CRISPR/Cas9-mediated control or *TMEM41B* KO Huh7 cells incubating with alkyne-choline are clicked with 5-TAMRA azide and visualized by confocal microscopy. The ER is marked by GFP-SEC61β. The middle image is a zoomed in image from the white box in the left image. Right: surface plots depicting alkyne-PC signals. Scale bars, 5 μm.

(D) Quantification of colocalization of signals of alkyne-PC and GFP-SEC61β (left) and total fluorescence signals (a.u.) of alkyne-PC (right) from (B). Data are presented as mean ± SEM. n.s., no significance, ****$p < 0.0001$ (two-tailed Student's t test).

bilayer, greater NBD fluorescent quenching is expected to result in due to increased exposure of NBD to dithionite (Figure 2B). And there is a positive correlation between the fluorescence reduction and the protein concentration of recombinant human FLAG-TMEM41B (Figure 2B).

In the metabolic labeling and imaging assay in cells, the alkyne-PC signal of *TMEM41B* KO cells is expected to significantly increase on the ER surface compared to control cells permeabilized with digitonin (Figure 3), due to failure in the shuttling of newly synthesized PC in the cytosolic leaflets to the luminal leaflets.

The scramblase activity assays reveal that TMEM41B has phospholipid scramblase activity *in vitro* and *in vivo* (Huang et al., 2021).

LIMITATIONS

In this protocol, we demonstrated phospholipids scramblase activity of the recombinant human FLAG-TMEM41B by a fluorescence liposome-based *in vitro* scramblase assay (Brunner et al., 2014; Ghanbarpour et al., 2021; Hrafnsdóttir and Menon, 2000; Marek and Günther-Pomorski,

2016; Mathiassen et al., 2021; Matoba et al., 2020; Menon et al., 2011; Ploier and Menon, 2016; Vehring et al., 2007). We showed that the phospholipids scramblase activity of TMEM41B is concentration dependent. However, the scramblase activity of TMEM41B inactivation mutation is absent in this protocol, which should be investigated in future work.

TROUBLESHOOTING

Problem 1

During FreeStyle™ 293F cell culture, cell clumping is detected which decreases cell vitality and influences the protein expression (step 1).

Potential solution

Thawing new FreeStyle™ 293F cells should always be the first choice. The cell density should maximal be 3.0×10^6 viable cells/mL during cell culture.

Problem 2

The fluorescence intensity of NBD is not reduced to ~50%, but by ~20%–~40% after dithionite treatment and does not drop to the background fluorescent intensity after adding 0.5% (w/v) Triton X-100. Buffer C was used to monitor the background intensity (steps 31 and 32).

Potential solution

Dithionite oxidizes fast. It is important to make sure dithionite is used shortly after being dissolved and kept on ice. It is recommended to increase the concentration of dithionite at the first step (~200 s). If dithionite is oxidized over time, prepare a new dithionite solution.

Problem 3

After adding dithionite, the NBD fluorescence intensity of protein-free liposomes is decreased by more than 50% (~60%) and/or the intensity keep reducing (~70%, at ~600 s) over time (steps 31 and 32).

Potential solution

The fluorescence intensity of NBD is quenched by the excitation laser during the monitoring. We suggest to set a suitable period of time to monitoring the sample and reduce the number of samples to reduce inspection interval (because the more samples you have, the longer inspection interval time you need). It is also recommended to determine the presence of Triton X-100 (absorbance at 275 nm) after reconstitution (Hrafnsdóttir and Menon, 2000) to exclude dithionite leakage over the liposome bilayer over time (Mathiassen et al., 2021). Decreasing fluorescence traces may also be due to the use of a plate reader, as the vesicles would sediment over time in the absence of stirring. A suggestion could be to use a fluorometer cuvette with stirring instead of the 96-well plate, as described in (Marek and Günther-Pomorski, 2016; Mathiassen et al., 2021; Ploier and Menon, 2016).

Problem 4

TMEM41B is not completely knocked out for the *in vivo* metabolic labeling assay to visualize PC in cells (step 34).

Potential solution

TMEM41B KO cells showed slower growth rate compared to the WT cells. Therefore, KO cells may be outcompeted by residual WT or heterozygous cells. We recommend not to culture the *TMEM41B* KO cells for over 20 passages.

Problem 5

After metabolic labeling and click reaction, the signal of alkyne-PC is weak in cells (step 36).

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Potential solution

Make sure cells are in good condition in this part. Adding alkyne choline to the culture medium is the most important step. We recommend vortex the culture containing alkyne choline vigorously before being added to cells. Labeling efficiency is different between different cell line due to the different metabolic rates. We recommend to test a series of alkyne-choline concentrations and labeling times when setup this experiment.

Problem 6

The signal of alkyne-PC in cells is quenched over time (step 42).

Potential solution

The unsaturated phospholipids may be oxidized, which might damage the membrane integrity and the signal of clicked TAMRA-PC might be quenched over time. We recommend that proceeding the labeled cells immediately to the next step to avoid potential signal loss or distortion.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for reagents and resources will be fulfilled by the lead contact, Xiao-Wei Chen (xiaowei_chen@pku.edu.cn).

Materials availability

Plasmids generated in this study are available from the lead contact upon request.

Data and code availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

L.Z.W., L.L., and X.W.C. conceptualized the study, designed the experiments, and analyzed the data. H.D. and B.L.X. helped with the design and experiments. L.Z.W. and L.L. performed the experiments. L.Z.W. and X.W.C. wrote the paper. All authors approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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