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Materials and Methods

Materials

Reagents were obtained from the following sources: HRP-labeled anti-mouse, anti-rabbit, and anti-goat secondary antibodies and the antibody to LAMP2 from Santa Cruz Biotechnology; antibodies to phospho-T389 S6K1, S6K1, phospho-ULK1, ULK1, phospho-S65 4E-BP1, 4E-BP1, RagA, RagC, p14 (LAMTOR2), p18 (LAMTOR1), mTOR, and the FLAG epitope (rabbit antibody) from Cell Signaling Technology; the antibody to the HA epitope from Bethyl laboratories; the antibody to ATP6V1B2 from Abcam; RPMI, FLAG M2 affinity gel, FLAG-M2 (mouse) and ATP6V0d1 antibodies, and amino acids from Sigma Aldrich; the PNGase F from NEB; Xtremegene 9 and Complete Protease Cocktail from Roche; AlexaFluor-labeled donkey anti-rabbit, anti-mouse, and anti-rat secondary antibodies from Invitrogen and Inactivated Fetal Calf Serum (IFS) from Invitrogen; amino acid-free RPMI and Leucine or Arginine-free RPMI from US Biological; siRNAs targeting indicated genes and siRNA transfection reagent from Dharmacon; Concanamycin A from A.G. Scientific; Torin1 from Nathanael Gray (DFCI); [^{14}C]-labeled amino acids and Opti-Fluor scintillation fluid from PerkinElmer; [^{3}H]-labeled amino acids from American Radiolabeled Chemicals; Egg phosphatidylcholine (840051C) from Avanti lipids; Bio-beads SM-2 from Bio-Rad; and PD-10 columns from GE Healthcare Life Sciences. The antibody to SLC38A9 from Sigma (HPA043785) was used to recognize the deglycosylated protein (according to NEB instructions except without the boiling step) in cell lysates and immunopurifications. A distinct antibody to SLC38A9.1 was generated in collaboration with Cell Signaling Technology and was used to detect the glycosylated protein in Ragulator immunopurifications but is not sensitive enough to detect it in cell lysates.

Cell lines and tissue culture

HEK-293T cells were cultured in DMEM supplemented with 10% inactivated fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 µg/mL) and maintained at 37°C and 5% CO_2. In HEK-293E, but not HEK-293T, cells the mTORC1 pathway is strongly regulated by serum and insulin (*38*).

Mass spectrometric analyses

Immunoprecipitates from 30 million HEK-293T cells stably expressing FLAG-metap2, FLAG-p18, FLAG-p14, FLAG-HBXIP, FLAG-c7orf59, and FLAG-RagB were prepared as described below. Proteins were eluted with the FLAG peptide (sequence DYKDDDDK) from the anti-FLAG affinity beads, resolved on 4-12% NuPage gels (Invitrogen), and stained with SimplyBlue SafeStain (Invitrogen). Each gel lane was sliced into 10-12 pieces and the proteins in each gel slice digested overnight with trypsin. The resulting digests were analyzed by mass spectrometry as described (*6*).

Amino acid or individual amino acid starvation and stimulation of cells

Almost confluent cell cultures in 10 cm plates were rinsed twice with amino acid-free RPMI, incubated in amino acid-free RPMI for 50 min, and stimulated for 10 min with a water-solubilized amino acid mixture added directly to the amino acid-free RPMI. For leucine or arginine starvation, cells in culture were rinsed with and incubated in leucine- or arginine-free RPMI for 50 min, and stimulated for 10 min with leucine or arginine added directly to the starvation media. After stimulation, the final concentration of amino acids in the media was the same as in RPMI. Cells were processed for biochemical or immunofluorescence assays as described below. The 10X amino acid mixture and the 300X individual stocks were prepared from individual amino acid powders. When Concanamycin A (ConA) or Torin1 was used, cells were incubated in 5 μM Concanamycin or 250 nM Torin1 during the 50 min amino acid starvation and 10 min amino acid stimulation periods.

Cell lysis and immunoprecipitations

HEK-293T cells stably expressing FLAG-tagged proteins were rinsed once with ice-cold PBS and lysed in ice-cold lysis buffer (40 mM HEPES pH 7.4, 1% Triton X-100, 10 mM β-glycerol phosphate, 10 mM pyrophosphate, 2.5 mM $MgCl_2$ and 1 tablet of EDTA-free protease inhibitor (Roche) per 25 ml buffer). The soluble fractions from cell lysates were isolated by centrifugation at 13,000 rpm for 10 min in a microcentrifuge. For immunoprecipitates 30 uL of a 50% slurry of anti-FLAG affinity gel (Sigma) were added to each lysate and incubated with rotation for 2-3 hr at 4^{o}C. Immunoprecipitates were washed three times with lysis buffer containing 500 mM NaCl. Immunoprecipitated proteins were denatured by the addition of 50 uL of sample buffer and incubation at RT for 30 min. It is critical that the samples containing SLC38A9 are neither boiled nor frozen prior to resolution by SDS-PAGE and analysis by

immunoblotting. A similar protocol was employed when preparing samples for mass spectrometry.

cDNA manipulations and mutagenesis

The cDNAs for all human SLC38A9 isoforms, both native and codon-optimized, were gene-synthesized by GenScript. The cDNAs were amplified by PCR and the products were subcloned into Sal I and Not I sites of HA-pRK5 and FLAG-pRK5. The cDNAs were mutagenized using the QuikChange II kit (Agilent) with oligonucleotides obtained from Integrated DNA Technologies. All constructs were verified by DNA sequencing.

FLAG-tagged SLC38A9 isoforms and SLC38A9 N-terminal 1-119 were amplified by PCR and cloned into the Sal I and EcoR I sites of pLJM60 or into the Pac I and EcoR I sites of pMXs. After sequence verification, these plasmids were used, as described below, in cDNA transfections or to produce lentiviruses needed to generate cell lines stably expressing the proteins.

cDNA transfection-based experiments

For cotransfection-based experiments to test protein-protein interactions, 2 million HEK-293T cells were plated in 10 cm culture dishes. 24 hours later, cells were transfected with the pRK5-based cDNA expression plasmids indicated in the figures in the following amounts: 500 ng FLAG-metap2; 50 ng FLAG-LAMP1; 100 ng FLAG-RagB and 100 ng HA-RagC; 300 ng FLAG-SLC38A9.1; 600 ng FLAG-SLC38A9.1 Δ110; 200 ng FLAG-SLC38A9.4; 400 ng FLAG-N-terminal 119 fragment of SLC38A9.1; 200 ng FLAG-RagC; 200 ng FLAG-RagC S75N; 200 ng FLAG-RagC Q120L; 400 ng HAGST-RagB; 400 ng HAGST-RagB T54N; 400 ng HAGST-RagB Q99L. Transfection mixes were taken up to a total of 5 µg of DNA using empty pRK5.

For co-transfection experiments to test mTORC1 activity, 1 million HEK-293T cells were plated in 10 cm culture dishes. 24 hours later, cells were transfected with the pRK5-based cDNA expression plasmids indicated in the figures in the following amounts: 500 ng HA-metap2; 50 ng HA-LAMP1; 200 ng HA-SLC38A9.1; 500 ng HA-SLC38A9.1 Δ110; 200 ng HA-SLC38A9.4; 100 ng HA-RagB T54N and 100 ng HA-RagC Q120L; 2 ng FLAG-S6K1. 72 hours post-transfection, cells were washed once prior to 50-min incubation with amino acid-free RPMI. Cells were stimulated with vehicle or amino acids (to a final concentration equivalent to RPMI) prior to harvest.

Lentivirus production and lentiviral transduction

Lentiviruses were produced by co-transfection of the pLJM1/pLJM60 lentiviral transfer vector with the VSV-G envelope and CMV ΔVPR packaging plasmids into viral HEK-293T cells using the XTremeGene 9 transfection reagent (Roche). For infection of HeLa cells, LN229 cells, and MEFs, retroviruses were produced by co-transfection of the pMXs retroviral transfer vector with the VSV-G envelope and Gag/Pol packaging plasmids into viral HEK-293T cells. The media was changed 24 hours post-transfection to DME supplemented with 30% IFS. The virus-containing supernatants were collected 48 hours after transfection and passed through a 0.45 μm filter to eliminate cells. Target cells in 6-well tissue culture plates were infected in media containing 8 μg/mL polybrene and spin infections were performed by centrifugation at 2,200 rpm for 1 hour. 24 hours after infection, the virus was removed and the cells selected with the appropriate antibiotic.

Mammalian RNAi

Lentiviruses encoding shRNAs were prepared and transduced into HEK-293T cells as described above. The sequences of control shRNAs and those targeting human SLC38A9, which were obtained from The RNAi Consortium 3 (TRC3), are the following (5' to 3'):
SLC38A9 #1: GCCTTGACAACAGTTCTATAT (TRCN0000151238)
SLC38A9 #2: CCTCTACTGTTTGGGACAGTA (TRCN0000156474)
GFP: TGCCCGACAACCACTACCTGA (TRCN0000072186)

For siRNA-based experiments, 200,000 HEK-293T cells were plated in a 6-well plate. 24 hours later, cells were transfected using Dharmafect 1 (Dharmacon) with 250 nM of a pool of siRNAs (Dharmacon) targeting SLC38A9 or a non-targeting pool. 48 hours post-transfection, cells were transfected again but this time with double the amount of siRNAs. 24 hours following the second transfection, cells were rinsed with ice-cold PBS, lysed, and subjected to immunoblotting as described above. The following siRNAs were used:
Non-targeting: ON-TARGETplus Non-targeting Pool (D-001810-10-05)
SLC38A9: SMARTpool: ON-TARGETplus SLC38A9 (L-007337-02-0005)

Immunofluorescence assays

HEK-293T cells were plated on fibronectin-coated glass coverslips in 6-well tissue culture dishes, at 300,000 cells/well. 12-16 hours later, the slides were rinsed with PBS once

and fixed and permeabilized in one step with ice-cold 100% methanol (for SLC38A9 detection) at -20°C for 15 min. After rinsing twice with PBS, the slides were incubated with primary antibody (FLAG CST 1:300, LAMP2 1:400) in 5% normal donkey serum for 1 hr at room temperature, rinsed four times with PBS, incubated with secondary antibodies produced in donkey (diluted 1:400 in 5% normal donkey serum) for 45 min at room temperature in the dark, and washed four times with PBS. Slides were mounted on glass coverslips using Vectashield with DAPI (Vector Laboratories) and imaged on a spinning disk confocal system (Perkin Elmer).

Whole-cell amino acid transport assay

HEK-293T cells (150,000/well) were plated onto fibronectin-coated 12-well dishes and transfected 12 hours later with the pRK5-based cDNA expression plasmids indicated in the figures in the following amounts using XtremeGene9: 400 ng LAMP1-FLAG, 400 ng FLAG-SLC38A9.1, 400 ng SLC38A2-FLAG, 150 ng PQLC2-FLAG, and 50 ng GFP. Transfection mixes were taken up to a total of 2 µg of DNA using empty pRK5. Cells were assayed 48 hours later by washing twice in transport buffer (140 mM NaCl, 5 mM KCl, 2 mM MgCl2, 2 mM $CaCl_2$, 30 mM Tris-HCl, pH 7.4, 5 mM glucose), incubating in transport buffer for 5 min. at 37^{o}C before replacing the buffer with fresh buffer supplemented with amino acids (unlabeled and 0.1 µCi of [^{14}C]leucine at a total concentration of 380 µM, or unlabeled and 0.1µCi of [^{14}C]amino acid mix at total concentrations found in RPMI, or unlabeled and 0.2 µCi of [^{14}C]arginine at a total concentration of 3 mM) at the indicated pH (pH 5 buffered by MES, pH 8 buffered by Tris) for 10 minutes at 37^{o}C. After uptake, cells were washed twice in ice-cold transport buffer and harvested in 0.5 mL of 1% SDS for scintillation counting. Protocol and amino acid concentrations used were informed by previous whole-cell assays to detect transport by SLC38A2 and PQLC2 (*39, 40*).

Electrophysiology

Whole-cell recordings were made from GFP-positive HEK-293T cells, prepared as described above, 48 to 72 hrs post transfection. Patch pipettes (open-tip resistance 3-4 MΩ) were filled with a solution containing (in mM) K-gluconate 153, $MgCl_2$ 2, $CaCl_2$ 1, EGTA 11, HEPES 10, pH 7.25 adjusted with KOH, and tip resistance was left uncompensated. Cells were continuously superfused (~ 2 ml/min) with extracellular solution containing (in mM) NaCl 150, KCl 3, $CaCl_2$ 2, $MgCl_2$ 1, Glucose 5, HEPES 10, pH adjusted to 7.4 with NaOH. Once whole-cell configuration was established, a homemade perfusion system consisting of several adjacent glass tubes (ID 252 µm) was used to locally perfuse extracellular solution pH 5.5 and to apply

amino acids (in mM) leucine 1.6, arginine 2.4, glutamine 4. Membrane currents were amplified and low-pass filtered at 3 kHz using a Multiclamp 700B amplifier (Molecular Devices), digitized at 10 kHz and acquired using National Instruments acquisition boards and a custom version of ScanImage written in MATLAB (Mathworks) (*41*). Data were analyzed offline using Igor Pro (Wavemetrics), and amino acid-induced currents were quantified as difference in the average membrane currents for the 5 s-windows right before and during application.

Proteoliposome reconstitution

HEK-293T cells stably expressing FLAG-SLC38A9.1 were harvested as described above for immunoprecipitations, except cells were lysed in 40 mM HEPES pH 7.4, 0.5% Triton X-100, 1 mM DTT, and protease inhibitors. Following a 3 hr immunoprecipitation, FLAG-affinity beads were washed twice for 5 min each in lysis buffer supplemented with 500 mM NaCl. Beads were equilibrated with inside buffer (20 mM MES pH 5, 90 mM KCl 10 mM NaCl) supplemented with 0.5% Triton X-100, 1 mM DTT, and 10% glycerol by washing them 5 times. FLAG-affinity purified SLC38A9.1 protein was eluted in glycerol-supplemented inside buffer containing 1 mg/mL FLAG peptide by rotation for 30 min. Protein was concentrated using Amicon centrifuge filters to about 1 mg/mL and snap-frozen in liquid nitrogen and stored at -80^{o}C.

Chloroform-dissolved phosphatidylcholine (PC, 50 mg) was evaporated using dry nitrogen to yield a lipid film in a round bottom flask and desiccated overnight under vacuum. Lipids were hydrated in inside buffer at 50 mg/mL with light sonication in a water bath (Branson M2800H) and split into 100 µL aliquots in eppendorf tubes. Aliquoted lipids were clarified using water bath sonication and recombined and extruded through a 100 nm membrane with 15 passes (Avanti 61000). Reconstitution reaction (15 µg FLAG-SLC38A9.1 protein, 7.5 mg Triton X-100, 10 mg extruded PC, 1 mM DTT in inside buffer up to 700 µL) was initiated by rotating at 4^{o}C for 30 min. Glycerol-supplemented inside buffer was used in lieu of SLC38A9.1 protein in liposome only controls. Bio-beads (200 mg/reaction) were prepared by washing 1 time in methanol, 5 times in water and 2 times in inside buffer. Reconstitution reaction was applied to Bio-beads for 1 hr, transferred to fresh Bio-beads overnight, and transferred again to fresh Bio-beads for 1 hr. Protocol was adapted from a recently reconstituted lysosomal transporter and a recent review (*42, 43*).

Floatation assay

A three-step sucrose gradient was generated by first adding 3.8 mL of the middle buffer (35% glycerol in inside buffer) to the ultracentrifuge tube, then applying 1 mL of the bottom buffer (50% glycerol in inside buffer) with 100 µL of SLC38A9.1 proteoliposomes (or protein only) using a 2 mL pipette to the bottom of the tube, and finally layering 1.2 mL of the top buffer (0% glycerol, inside buffer) on top. For assays containing urea, the proteoliposomes were rotated in bottom buffer containing 6 M urea for 30 min. at 4^{o}C before generating the sucrose gradient with above buffers supplemented with 6 M urea. Gradients were topped with 2 mL paraffin oil and loaded into a SW32.1 rotor and centrifuged at 32,000 g for 24 hours. Fractions (500 µL, excluding the oil) were collected from the top and 20 µL of each subjected to anti-FLAG western analysis. Protocol was adapted from Wuu *et al.* (*44*).

Trypsin protection assay

Trypsin (1 µL of 0.05%, Invitrogen) was added to SLC38A9.1 proteoliposomes (15 µL) and incubated at 37^{o}C for 30 min. As indicated, 1% Triton X-100 was added and rotated for 30 min. at 4^{o}C before addition of trypsin. Reactions were subjected to anti-FLAG western analysis. Protocol was inspired by Brown and Goldstein (*45*).

In vitro amino acid transport assay

All buffers were chilled and assays performed in a 4^{o}C cold room. For time course experiments, SLC38A9.1 proteoliposomes or liposome controls were applied to PD10 columns equilibrated with outside buffer (20 mM Tris pH 7.4, 100 mM NaCl) and eluted according to manufacturer's instructions. Amino acid uptake was initiated by the addition of 0.5 µM [^{3}H]arginine and incubated in a 30^{o}C water bath. Time points were collected by taking a fraction of the assay reaction and applying it to PD10 columns pre-equilibrated with outside buffer. Columns were eluted in fractions or a single elution of 1.75 mL and added to 5 mL of scintillation fluid. Protein used in uptake assays was estimated by assuming 100% incorporation efficiency during reconstitution. To obtain accurate measures of amino acid concentrations, equal volumes of outside buffer was added to scintillation fluid in the standards.

For competition experiments with unlabeled amino acids, high concentrations of amino acids were required due to the high K_m (~39mM) of SLC38A9.1 import activity. SLC38A9.1 proteoliposomes or liposome controls were centrifuged at 100,000 g for 30 min. in a TLA-100.3 rotor and resuspended in a smaller volume of outside buffer such that they could be added to a

larger volume of 100 mM unlabeled amino acid (final concentration) supplemented with outside buffer components. We had to resort to this procedure due to the solubility limit of leucine at ~130 mM. At such high concentrations, it is important to adjust all amino acid solutions to pH 7.4. Assays were initiated by addition of 0.5 µM [^{3}H]arginine to the amino acid buffer solution followed by the addition of SLC38A9.1 proteoliposomes or liposome controls. For steady-state kinetics experiments, time points were collected as described above and to assess substrate specificity, competition experiments were collected at 75 min.

For efflux experiments, SLC38A9.1 proteoliposomes or liposome controls were loaded with [^{3}H]arginine as described above for an import assay for 1.5 hrs. To remove external amino acids, the reactions were applied to PD10 columns pre-equilibrated with outside buffer, and time points were collected as described above. Scintillation counts from liposome controls were subtracted from that of SLC38A9.1 proteoliposomes.

Generation of knockout clones using CRISPR/Cas9

The CRISPR guide sequences designed to the N-terminus (1-119 a.a.) of SLC38A9 or the AAVS1 locus using http://crispr.mit.edu were cloned into pX459 (*46*).

AAVS1: GGGGCCACTAGGGACAGGAT
SLC38A9_1: GGCTCAAACTGGATATTCATAGG
SLC38A9_2: GGAGCTGGAACTACATGGTCTGG

HEK-293T cells (750,000/well) were plated into 6 well dishes and transfected 16 hours later with 1 µg of pX459 expressing above guides using XtremeGene9. Cells were trypsinized 48 hours later, 2 mg/mL puromycin was applied for 72 hours, and allowed to recover for a few days. When cells were approaching confluency, they were single-cell sorted into 96-well dishes containing 30% serum and conditioned media. Clones were expanded and evaluated for knockout status by western analysis for SLC38A9. These clones were evaluated for amino acid response as described above.

Figure S1: Membrane topology of SLC38A9.1**.** (**A**) Representation of the TMHMM topology prediction for SLC38A9.1. (**B**) Visualization of SLC38A9.1 topology as generated by Protter.

Figure S2: Ragulator and the Rag GTPases do not interact with all lysosomal amino acid transporter-like proteins. (**A**) SLC38A9.1, but not SLC38A7 or SLC36A1, interacts with the Ragulator complex and the Rag GTPases. HEK-293T cells were transfected with the indicated cDNAs in expression vectors and lysates were prepared and subjected to FLAG immunoprecipitation followed by immunoblotting for the indicated proteins. (**B** and **C**) The interaction with Ragulator requires the presence of the intact N-terminal domain of SLC38A9.1, which is lacking in SLC38A9.2 (B), SLC38A9.1 Δ110 (C), and SLC38A9.4 (C). HEK-293T cells were transfected with the indicated cDNAs in expression vectors and processed as in (A).

Figure S3: Localization of SLC38A9 isoforms 2 and 4 and signaling effects of siRNA-mediated SLC38A9 knockdown. SLC38A9 isoforms lacking part (**A**) or all (**B**) of the N-terminal region of SLC38A9.1 still localize to the lysosomal membrane. HEK-293T cells stably expressing the indicated FLAG-tagged SLC38A9 isoforms were immunostained for FLAG and LAMP2. (**C**) The interaction between SLC38A9.1 and Ragulator occurs only when Ragulator is anchored at the lysosomal membrane through lipidation of the N-terminus of p18. Ragulator containing the lipidation-deficient $p18^{G2A}$ mutant fails to interact with SLC38A9.1. HEK-293T cells were transfected with the indicated cDNAs in expression vectors and lysates prepared and subjected to FLAG immunoprecipitation followed by immunoblotting for the indicated proteins. (**D**) Knockdown of SLC38A9 in HEK293T cells with a pool of short interfering RNAs suppresses the phosphorylation of S6K1.

Figure S4: (A) Transient overexpression of SLC38A9.1, but not truncation mutants lacking the N-terminal Ragulator-binding domain, makes the mTORC1 pathway insensitive to amino acid starvation. Cell lysates were prepared from HEK-293T cells deprived for 50 min for amino acids and, then, where indicated, stimulated with amino acids for 10 min. Cell lysates and FLAG immunoprecipitates were analyzed for the levels of the specified proteins and for the phosphorylation state of S6K1. **(B)** Stable overexpression of SLC38A9.1 in HeLa cells, LN229 cells, and MEFs makes the mTORC1 pathway partially resistant to amino acid deprivation. Cells transduced with retroviruses encoding the specified proteins were deprived for 50 min of all amino acids and, where indicated, stimulated for 10 min with amino acids. Cell lysates were analyzed for the levels of the specified proteins and the phosphorylation state of S6K1. **(C)** Stable overexpression of SLC38A9.1 suppresses autophagy induction upon arginine starvation

as indicated by detected by p62 accumulation and suppressed LC3 degradation. HEK-293T cells stably overexpressing FLAG-SLC38A9.1 were simultaneously deprived of arginine and, where indicated, treated with 30 uM chloroquine for the indicated time. Cell lysates were analyzed for the levels of the specified proteins and the phosphorylation state of S6K1. **(D)** Stable overexpression of SLC38A9.1 in HEK-293E cells does not perturb the response of mTORC1 signaling to serum starvation and insulin stimulation. **(E)** Stable overexpression of SLC38A9.1 does not protect mTORC1 signaling from the inhibitory effects of MK2206, which blocks growth factor signaling by allosterically inhibiting Akt.

Figure S5: Endogenous immunoprecipitation of Rag and Ragulator components recovers SLC38A9 in an amino acid-sensitive fashion. Cell lysates were prepared from HEK-293T cells deprived for 50 min for amino acids and, then, where indicated, stimulated with amino acids for 10 min. Cell lysates as well as control, p18, RagA, and RagC immunoprecipitates were analyzed for the levels of the indicated endogenous proteins.

Figure S6: SLC38A9.1 is a low-affinity amino acid transporter. (**A**) Immunostaining of HEK-293T cells transiently overexpressing SLC38A9.1 at levels that cause spillover to the plasma membrane. These cells were used for whole-cell amino acid transport assays and amino acid-induced current recordings. HEK-293T cells transiently expressing indicated cDNAs were incubated with [^{14}C]arginine (**B**), [^{14}C]amino acid mix (**C**), or [^{14}C]leucine (**D**) containing buffer at the indicated pH and washed before harvested for scintillation counting. (**E**) (Left) Whole-cell recordings from HEK-293T cells expressing indicated cDNAs at -80 mV. Quantified is the change in steady-state current following local application of 2.4 mM arginine, 1.6 mM leucine, and 4 mM glutamine (4x DMEM concentrations). All recordings were performed at pH 5.5. Statistical comparison was performed by Kruskall-Wallis test, followed by Dunn's test. (Right) Representative examples of individual recordings. Grey bars indicate application of amino acids. (**F**) Coomassie stain of FLAG-affinity purified LAMP1 or SLC38A9.1 from HEK-293T cells stably expressing respective protein. (**G**) Floatation assay shows successful insertion of SLC38A9.1 into proteoliposomes. Where indicated, 6 M urea was added following the reconstitution reaction. (**H**) SLC38A9.1 is unidirectionally inserted into proteoliposomes, with the N-terminus facing the outside of liposomes. Proteoliposomes containing N-terminally FLAG-tagged SLC38A9.1 were exposed to trypsin and immunoblotted for FLAG. The addition of 1% Triton X-100 did not reveal any protected FLAG-tagged fragments. (**I**) SLC38A9.1 proteoliposomes uptake [^{3}H]arginine. 0.5 μM [^{3}H]arginine was incubated with the indicated components for 60 min. and the reaction was applied to a column that traps free amino acids. Proteoliposomes

pass through the column and fractions were subjected to scintillation counting and FLAG immunoblotting. To recapitulate the pH gradient across the lysosomal membrane, the lumen of the proteoliposomes is buffered at pH 5.0, while the external buffer is pH 7.4.

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