PNAS

Supplementary Information for

Mfsd2a utilizes a flippase mechanism to mediate omega-3 fatty acid lysolipid transport

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This PDF file includes:

Other supplementary materials for this manuscript include the following:

Movies S1 to S3

Supplementary methods

Generation of Gallus Mfsd2a Mutants for protein production in insect cells

Gallus Mfsd2a (NCBI number), codon optimized for expression in insect cells, was subcloned into a pFastbac vector containing a TEV cleavage site followed by a His-Tag containing 10 Histidines as previously described (1). Mutants of Gallus Mfsd2a were generated by using the mutagenesis kit, GENEART (ThermoFisher). Insect cell viruses for Gallus Mfsd2a WT and its mutants were generated as described previously (1) to induce protein production in SF9 cells. Virus infected cells pellets were washed by first homogenizing it in low salt buffer,10 mM HEPES, pH7.5 with 10 mM MgCl2 and 10mM KCl, and collecting the pellet at high speed (100,000g, 30 mins at 4 deg). The pellet was further washed in high salt buffer, 1M NaCl, 10 mM HEPES, pH7.5 with 10 mM MgCl2 and 10mM KCl. The washed pellet was then resuspended in a solubilization buffer containing 20 mM HEPES, 200 mM NaCl and 1% Dodecylmaltoside (DDM) for 1.5 hours at 4 deg with gentle agitation. The un-solubilized membrane proteins and cell debris were then cleared away at high speed (100,000g, 30 mins at 4 deg) leaving the clarified supernatant to be used for binding with His-beads (Qiagen) overnight at 4 deg. The bound beads were subsequently washed with buffer, 20 mM HEPES, NaOH, pH7.5, 150 mM NaCl, 60 mM imidazole, 0.1% DDM and eluted with 200 mM imidazole in 20 mM HEPES, pH7.5, 150 mM NaCl, 60 mM imidazole, 0.05% DDM buffer. Proteins were purified in 20 mM HEPES, pH7.5, 150 mM NaCl, 0.05% DDM using size exclusion chromatography on a Superdex200 increase column. Only desired peaks eluted at 10.8 ml were collected and used for subsequent studies. MelB was purified using previously described methods (2, 3).

LPS-oleate transport assay using Mass spectrometry

LPS-oleate, purchased from Avanti, was added at 100 μM into HEK293 cells transfected with the various pcDNA3.1 constructs and incubated for 1 hour at 37°C with 5% CO2. Cells were washed twice with Fatty-acid free BSA (Sigma), dried and the lipids were extracted by adding 3:2 Hexane:Isopropanol. Lipids were dried down and analysed by LC–MS/MS as described previously (4). Transfected cells were lysed and immunoblotted with rabbit anti-Mfsd2a (in-house), anti-His (Bethyl laboratories), β-actin. Blots were analysed using LI-COR Imaging.

Single cell transport assay

Half a million cells were seeded into a well of 12-wells for 24 hours. Cells were transfected with 2 μg of plasmid and Lipofectamine 2000 (Life Technologies) as per manufacturer's protocol for 6 hours and replaced with fresh DMEM (Gibco) High glucose,10% FBS and 1% penicillin-streptomycin. Transfected cells were allowed to grow for another 24 hours prior transport assay. Cells were washed once with charcoal stripped DMEM with no FBS and then increasing concentrations of LPCNBD (16:1 and 12:0) was added to the transfected cells and incubated in a 37°C incubator with 5% CO_2 for 30 minutes. To stop the reaction, the LPCNBD was aspirated and washed once with DMEM containing 0.5% fatty-acid free BSA. Single cells were then obtained by trypsinization with Tryple (GIBCO) and reaction neutralized with PBS containing 0.5% fatty-acid free BSA and 2% FBS and analyzed with BD Fortessa. Single cells were gated and analyzed for Cherry and NBD positive populations. Only 100 cells expressing 10000 to 15000 arbitrary units (A.U.) of cherry fluorescence were analyzed for NBD fluorescence and plotted using Graphpad Prism 9.0. The NBD fluorescence gated for the various concentrations were plotted and fitted using the Hill equation in the program. For mutagenesis scanning of Mfsd2a, mutations were first made onto previously described pcDNA3.1 human Mfsd2a tagged with Cherry using Geneart (Life Technologies). Cells were transfected and assayed for NBD uptake using 2 μM of LPCNBD (16:1), data was plotted using gating strategy established previously (1).

Proteoliposome preparation

Unilamellar liposomes were made according to previous studies (5). Briefly, 20 mg of 1-Palmitoyl-2-oleoly-sn-glycero-3phosphocholine (POPC) and 20 mol percent Cholesterol (Avanti) were thoroughly dried down

and reconstituted in 1 ml 100 mM HEPES, KOH ,pH 7.5 buffer at 37 degrees Celsius shaking incubator at 220 rpm for 1.5 hours. 1.5% b-Octylglucoside (Anatrace) was added to the lipid mix and dialyzed with a 100 kDa molecular weight cutoff dialysis tubing (Spectra-Por) overnight at 4 degrees Celsius. The liposomes were collected and diluted to 10 mg/ml, flash frozen and stored at -80 until needed for protein incorporation. 0.25 μM of PSVUE550 (Mtarget) was added to liposomes destabilized with 0.11% Triton-X and nutated at room temperature for 20 mins, followed by dropwise addition of 50 ug of purified proteins that had been buffer exchanged by 7 consecutive additions with 20 mM HEPES, KOH, pH7.5, 140 mM KCl buffer containing 0.05% DodecylMaltoside (DDM) using 50 kDa molecular weight cut-off Amicon (Merck) and incubated for 30 mins at room temperature. Protein incorporation and detergent removal was done by adding 60 mg/ml (weight/reaction volume) of prewashed Bio-beads (Bio-rad) and incubated at room temperature for 1 hour. A further 120 mg/ml of prewashed Bio-beads were added and incubated at room temperature for another hour. Finally, 120 mg/ml of prewashed Bio-beads were added and incubated at 4 degrees Celsius overnight with continuous nutation. Bio-Beads were removed from the mixture using a mini-spin column (Bio-rad). After proteoliposome recovery from Bio-Beads treatment, they are washed in 80 mM HEPES(KOH), pH 7.5 and 25 mM of KCl buffer (reaction buffer). The protein-liposome mix is then centrifuging at 20,000 g for 20 mins at 4 deg after addition of 1.2 ml of 80 mM HEPES (KOH), pH 7.5, 25 mM KCl. The proteoliposome pellet was further washed twice with ice cold 1.2 ml of 80 mM HEPES (KOH), pH 7.5, 25 mM KCl and reconstituted in 1.5 ml of reaction buffer, 80 mM HEPES (KOH), pH 7.5, 25 mM KCl.

Protease protection assay

Gallus Mfsd2a tagged with GFP is purified and incorporated into liposomes as described in previous sections. Liposomes were aliquoted into tubes containing various amounts of trypsin and treated with/without 1% triton-X as described previously (6). The reaction is stopped with soybean trypsin inhibitor and blue loading dye added. Samples are then run onto denaturing SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with anti-His (Bethyl laboratory) (Fig S1).

Flippase assay

Each well was preloaded with reaction buffer, with the different assay conditions and mixed well. Proteoliposomes were then added into each well of a 96-well glass-bottomed plate (Perkin-Elmer) and scanned on the Azure Biosystems Imager using 550 nm excitation and 610 nm emission, the reaction was followed by scanning at the start of the reaction (0 time point) followed by every 5 minutes for 15 minutes. The images were quantified using the Azure bio-imager software AzureSpot and plotted with Prism (Graphpad). To calculate the Relative Activity to WT gMFSD2A, obtained intensity (f1) is subtracted from baseline (fo) (gWT proteoliposome treated only with LPS) and normalized to the highest intensity (fmax) obtained by gWT proteoliposome in the experiment, see equation below. For dose experiments the highest intensity is subtracted from baseline and plotted. Curves were fitted to the Michalis-Menten equation to derive Vmax and the transport constant, Km.

$$Relative\ activity\ to\ WT\ gMfsd2a = \frac{f1 - fo}{fmax - fo}$$

Statistical analysis

Statistical analysis comparing between two groups in a single experiment is conducted using GraphPad Prism using unpaired, two-tailed, Student T-test.

Image preparation

All structure images were prepared and analyzed using PyMol. Chemical structures were drawn with ChemSketch. Figures were made with Inkscape. Movies are created by Azure Sapphire Biomolecular Imager (Azure Biosystems).

Figures S1 to S7

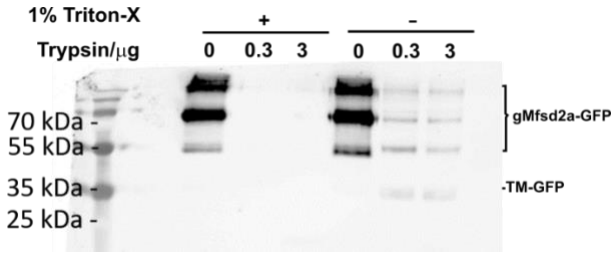

Figure S1. Demonstration that Mfsd2a can be incorporated into sealed proteoliposomes. Proteoliposomes containing Mfsd2a with a green fluorescent protein tag (gMfsd2a-GFP) was treated with increasing amounts of trypsin 0, 0.3 and 3 μg then separated by SDS-PAGE and GFP signal detected by fluorescence scanning using an anti-GFP antibody. Protection of C-terminal tagged GFP (TM-GFP) from trypsin cleavage indicates desired orientation of Mfsd2a with its N- and C- domains facing the liposome lumen. Triton-X treatment to disrupt proteoliposomes resulted in proteolysis of GFP as seen in complete loss of GFP signal. TM-GFP indicates protected Mfsd2a transmembrane 12 attached to the protected GFP, while gMfsd2a-GFP indicates non-digested gMfsd2a-GFP.

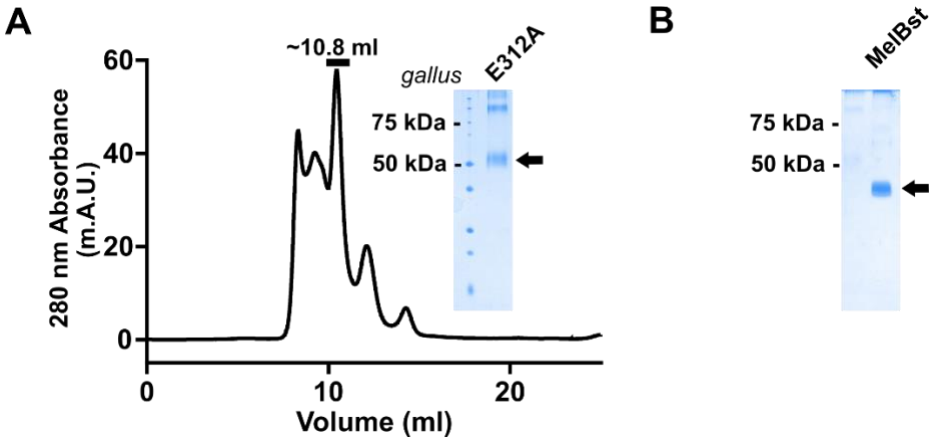

Figure S2. Purity of proteins used in flippase assay. (A) FPLC trace of purified gE312A eluting at the indicated fraction of 10.8 ml. SDS-PAGE showing purity of Mfsd2a mutant gE312A indicated by arrow (B) SDS-PAGE separation of purified MelB indicated by arrow.

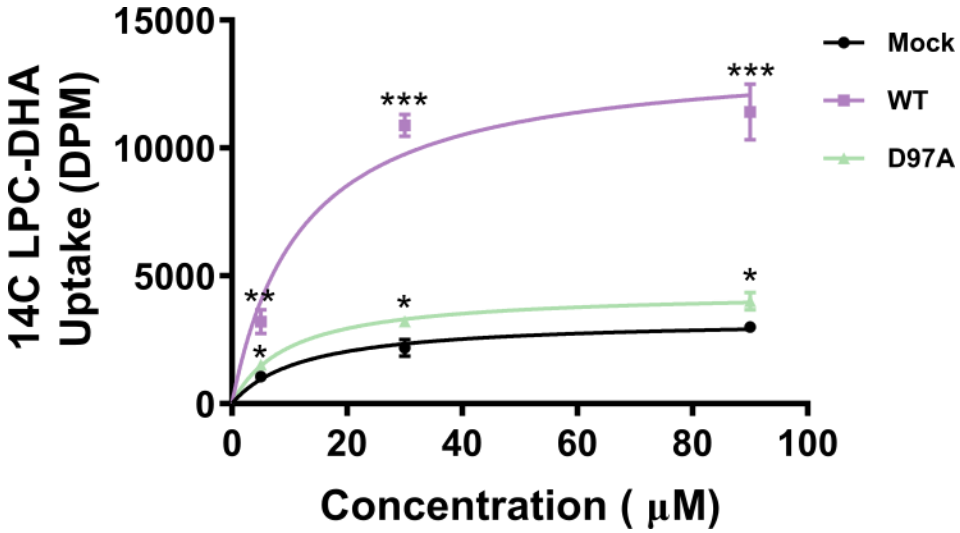

Figure S3. D97A shows residual LPC transport in cells. Transport curve of increasing ^{14}C-labeled LPC-DHA on HEK293 cells transfected with human Mfsd2a and the sodium binding site mutant D97A. Data are represented as mean +/- SD; *p <0.05 , **p < 0.005, ***p < 0.002 relative to Mock.

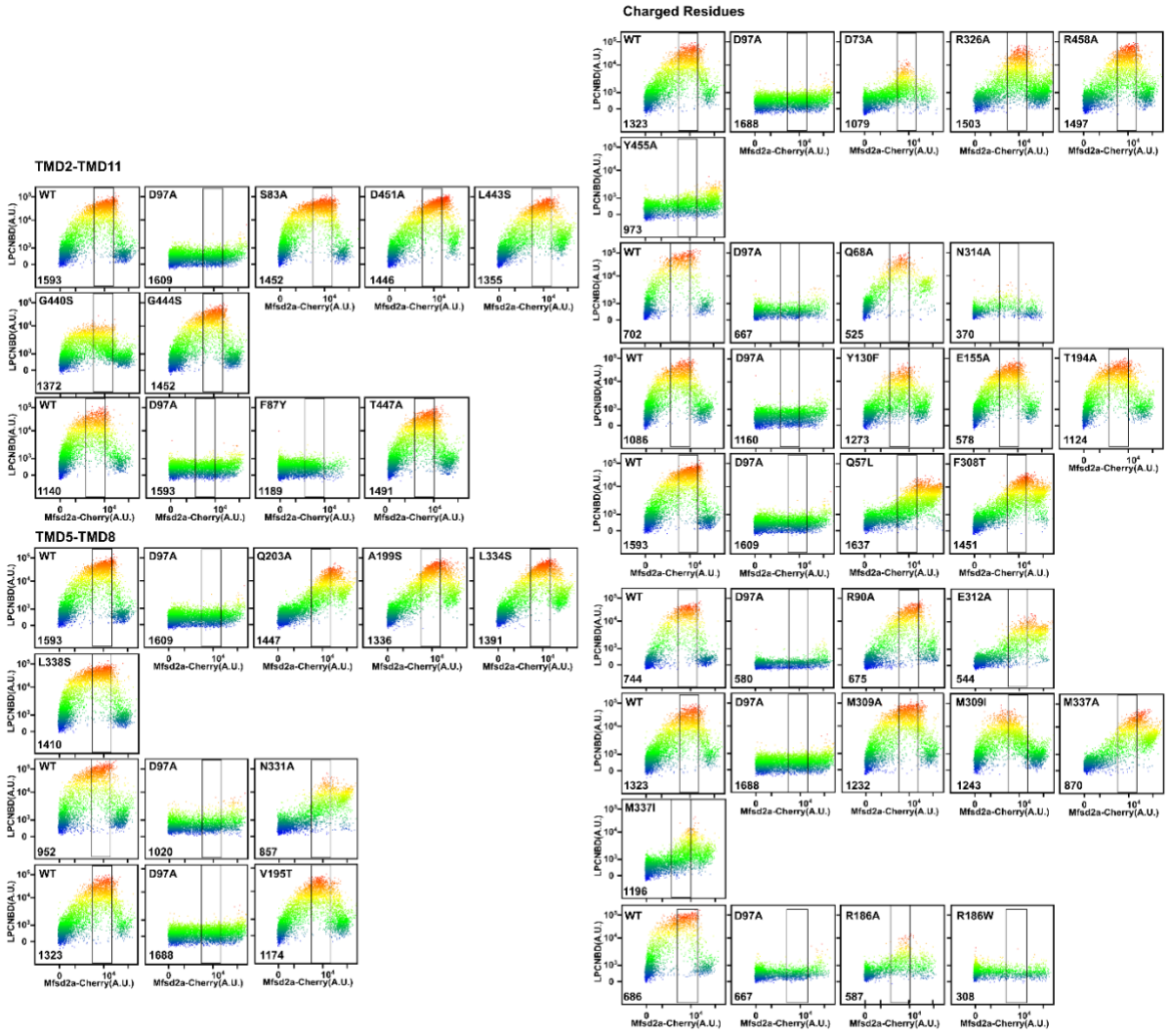

Figure S4. Analysis of Mfsd2a point mutants using a single cell Flow cytometry based transport assay. The experimental set up and gating strategy were previously validated (6). Flow cytometric analysis of LPC-NBD uptake relative to mCherry fluorescence for wild-type and indicated mutant hMfsd2A-mCherry constructs expressed in HEK 293 cells. LPC-NBD fluorescence intensity is represented as a gradient from blue (low) to red (high). Gates and denoted by a black rectangle and the number of cells within each gate indicated at the bottom left corner of each plot. Both WT and D97A were included in all experiments and served as positive and negative controls, respectively.

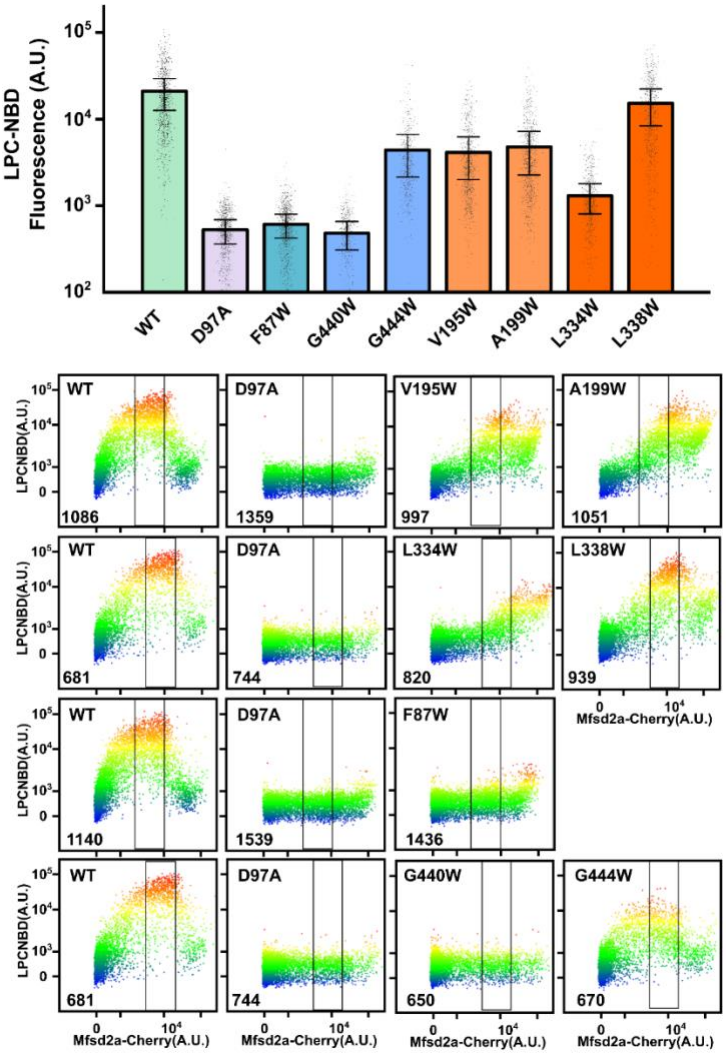

Figure S5. Analysis of Tryptophan substitution at TM2-TM11 and TM5-8. Single cell transport assay using Flow cytometry (similarly to Figure S1) was used to analyze hMfsd2a mutants. Top panel shows LPC-NBD uptake for indicated mutants with WT-mCherry and D97A-mCherry serving as positive and negative controls, respectively. Data are represented as mean +/- SD with each dot representing an individual cell. Bottom panel is the primary Flow cytometry data with gates denoted by a black rectangle and the number of cells within each gate indicated at the bottom left corner of each plot.

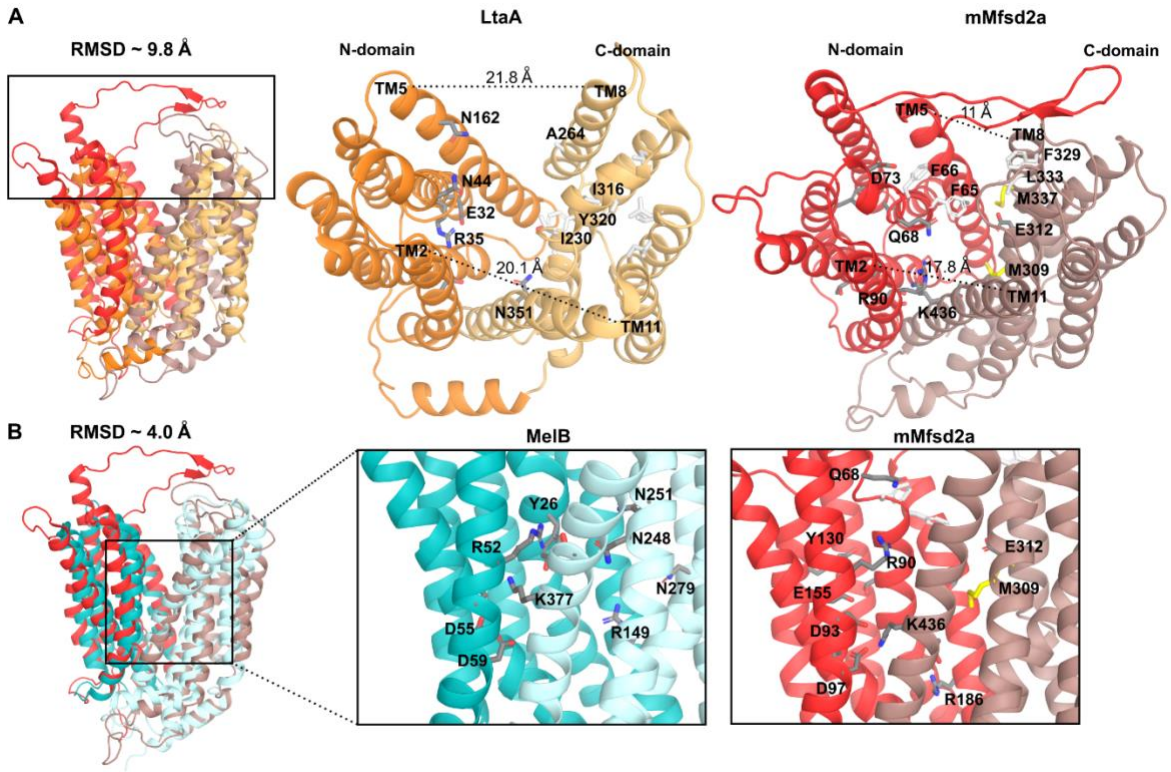

Figure S6. Comparison of outward-open mMfsd2a, LtaA, and MelB structure. (A) Left most panel showing Pymol alignment of mMfsd2a (7N98) (7) and LtaA (6S7V) (8) with black box indicating the regions that are shown in detail on the right. Middle panel showing LtaA structure N-domain in deep orange and C-domain in light orange. Right panel showing Mfsd2a structure N-domain in red and C-domain in dark pink. Residues lining the central translocation cavity are shown in sticks. The dotted lines in the structures denote the distances between indicated TMs and shows that such distances are smaller for Mfsd2a. (B) Left most panel showing Pymol alignment of mMfsd2a (7N98) and MelB (7L16) (9). Indicated black box showing regions magnified for MelB and mMfsd2a, residues denoting the sodium binding site for MelB (D55, D59, and K377) and mMfsd2a (D93, D97 and K436), and other residues lining the transport cleft of MelB and mMfsd2a are depicted in sticks.

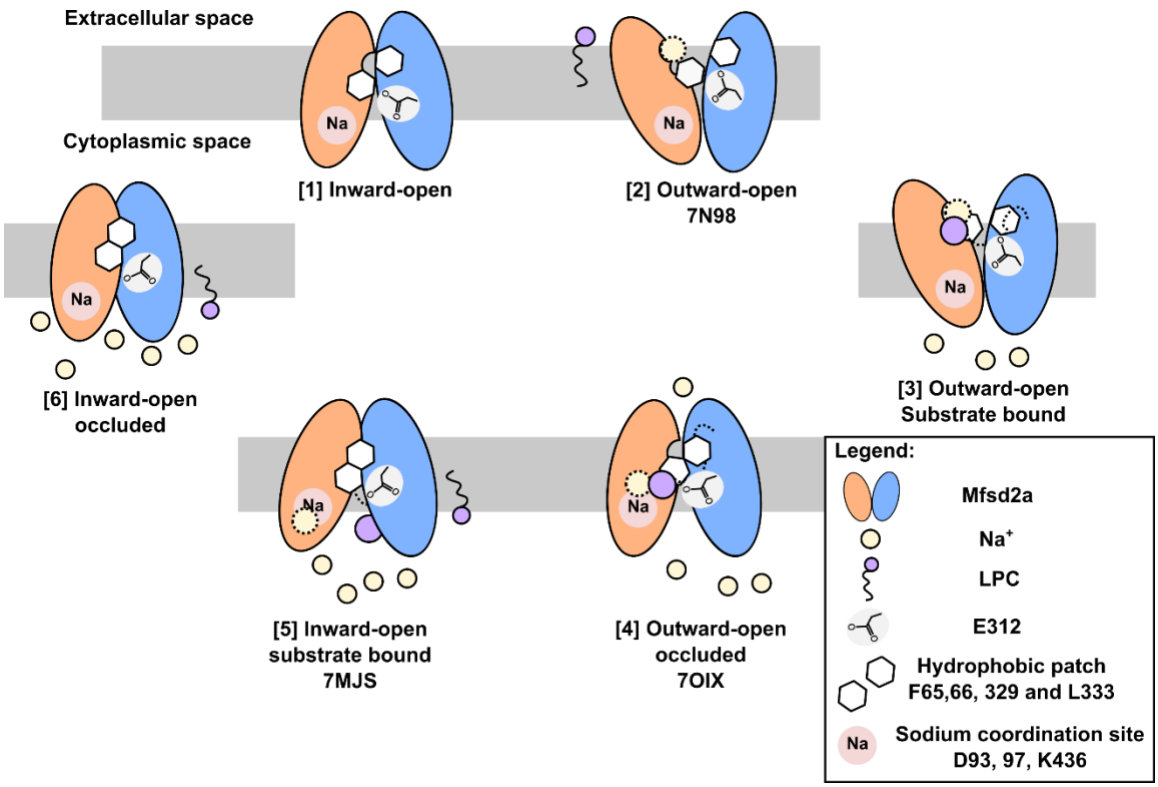

Figure S7. Postulated transport cycle showing Mfsd2a-mediated LPC flipping with conformational changes in key residues involved in alternating access. Mfsd2a undergoes several conformational changes from [1] inward-open state, that is destabilized by sodium and LPS headgroup binding to form the [2] outward-open structure that exposes the hydrophobic patch of residues F65, F66, F329 and L333 that possibly interacts with the acyl chain to form the [3] outward-open substrate bound structure. Movement of the Na^+ and LPS headgroup and subsequent interaction with E312 likely results in the [4] outward-open occluded state, that places the Na^+ and LPS closer to the sodium coordination site resulting in the [5] inward-open substrate bound state, and [6] inward-open occluded (Apo) to expose binding sites to the intracellular milieu to facilitate transport across the membrane bilayer. The Mfsd2a N-domain is represented in orange and the C-domain in blue.

Legends for Datasets S1 to S3

Dataset S1_LPS-oleate Mass Transport Assay: Lipidomic data file and calculation derived from HEK293 cells transfected with HMfsd2a and gMfsd2a and their respective sodium binding site mutant. This data was used to derive Figure 1A.

Dataset S2_Mfsd2a Proteoliposome Flippase Assay: Azurespot quantification of each well from 96-well plates from testing flippase activity and assay conditions. Values are used in Figure 3.

Dataset S3_NBD values of gated cells: NBD values of gated cells from Flow cytometry analysis of single cell transport assay (primary Flow plots shown in Figure S1 and Figure S2) used to plot Figures 4, 5 and supplemental Figure S2.

Legends for Movie files

Movie S1_WT Mfsd2a flippase activity: 5-minute scans at 550 nm and 610 nm emission for a total of 15 minutes for gWT proteoliposomes under the following three conditions: leftmost three vertical lanes containing 140 mM NaCl, middle three vertical lanes containing 140 mM NaCl and 25 µM LPS, rightmost containing only 25 µM LPS. Images are in three 700 ms loops encompassing four time points (0, 5,10,15 min).

Movie S2_Specificity of Mfsd2a flippase activity: 5-minute scans at 550 nm and 610 nm emission for a total of 15 minutes for empty liposomes, gWT and gD92A proteoliposomes in wells containing 50 µM LPS-oleate and 140 mM of NaCl. Images are in three 700 ms loops at four time points (0, 5,10,15 min).

Movie S3_Sodium-dependent Mfsd2a flippase activity: 5-minute scans at 550 nm excitation and 610 nm emission for a total of 15 minutes for gWT proteolipsomes in 50 mM NaCl (wells 1, 2, 3), 25 mM NaCl (wells 4, 5, 6), 1 mM NaCl (wells 7, 8, 9), 0 mM NaCl (for wells 10,11,12). Images are in three 700 ms loops of 4 time points (0, 5,10,15 min).

SI references

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