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A sublethal ATP11A mutation associated with neurological deterioration causes aberrant phosphatidylcholine flipping in plasma membranes

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ATP11A translocates phosphatidylserine (PtdSer), but not phosphatidylcholine (PtdCho), from the outer to the inner leaflet of plasma membranes, thereby maintaining the asymmetric distribution of PtdSer. Here, we detected a de novo heterozygous point mutation of ATP11A in a patient with developmental delays and neurological deterioration. Mice carrying the corresponding mutation died perinatally of neurological disorders. This mutation caused an amino acid substitution (Q84E) in the first transmembrane segment of ATP11A, and mutant ATP11A flipped PtdCho. Molecular dynamics simulations revealed that the mutation allowed PtdCho binding at the substrate entry site. Aberrant PtdCho flipping markedly decreased the concentration of PtdCho in the outer leaflet of plasma membranes, whereas sphingomyelin (SM) concentrations in the outer leaflet increased. This change in the distribution of phospholipids altered cell characteristics, including cell growth, cholesterol homeostasis, and sensitivity to sphingomyelinase. Matrix-assisted laser desorption ionization–imaging mass spectrometry (MALDI-IMS) showed a marked increase of SM levels in the brains of Q84E-knockin mouse embryos. These results provide insights into the physiological importance of the substrate specificity of plasma membrane flippases for the proper distribution of PtdCho and SM.

Introduction

Lipids have various roles in cells, including that of energy source, membrane structural component, signaling molecule, and platform for the recruitment and activation of enzymes (1). Their backbone (glycerophospholipids, sphingolipids, and cholesterol), the size of the acyl chains, and the number of double bonds vary widely. Major glycerophospholipids are phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), phosphatidylserine (PtdSer), phosphatidylinositol (PtdIns), and phosphatic acids (PtdOH). Plasma membranes in eukaryotes comprise a lipid bilayer, which is rich in PtdCho (43% of phospholipids), PtdEtn (21%), sphingomyelin (SM) (23%), and PtdSer (4%) (2, 3). They also contain a high level of cholesterol. Plasma membranes of human fibroblasts and rat hepatocytes were reported to contain cholesterol at a level of approximately 45 mol% of all phospholipids (4, 5).

In mammalian cells, the final step in the synthesis of glycerophospholipids (PtdSer, PtdCho, and PtdIns) occurs at the cytoplasmic side of the ER or the mitochondria-associated membrane, whereas SM is synthesized at the luminal side of the Golgi apparatus (2, 3). After their synthesis, glycerophospholipids are transported by vesicular trafficking and lipid transfer proteins to the plasma membrane, where they are asymmetrically distributed between the lipid bilayers. PtdSer, PtdEtn, and PtdIns exclusively localize to the inner leaflet of plasma membranes, whereas most, if not all, of the PtdCho and SM are present in the outer leaflet (6, 7). The translocation of phospholipids and SM from the inner to the outer leaflet has been proposed to be mediated by flippase(s), with some ATP-binding cassette (ABC) transporters being proposed as the candidate transporters in this process (8). However, the contribution of ABC transporters to maintenance of the asymmetrical distribution of PtdCho and SM remains elusive.
trast, type IV–P-type (P4) ATPases are responsible for maintaining PtdSer and PtdEtn at the inner leaflet of plasma membranes (9).

P4-ATPases are membrane proteins that consist of 10 transmembrane segments and a cytoplasmic ATPase domain (10, 11). Loss-of-function mutations in 3 of the 14 P4-ATPases have been implicated in human genetic diseases, such as intrahepatic cholestasis, anemia, and neurological disorders (8, 9, 12). The majority of P4-ATPases require CDC50A for their proper cellular localization and ATPase activity (13–15). We previously demonstrated that 2 members (ATP11A and ATP11C) of the P4-ATPase family were widely expressed in human and mouse tissues, localized in plasma membranes together with CDC50A, and specifically translocated PtdSer and PtdEtn from the outer leaflet to the inner leaflet of plasma membranes (14, 16, 17).

In the present study, we describe a de novo heterozygous point mutation of the ATP11A gene found in a patient with global developmental delays and neurological deterioration. This mutation allowed ATP11A to bind to PtdCho at the phospholipid entry site in order to flip it. The flipping of PtdCho altered various cell characteristics, such as sensitivity to sphingomyelinase (SMase), cholesterol homeostasis, and growth rate, indicating that PtdCho needs to be asymmetrically distributed in the plasma membrane in order to maintain the integrity of mammalian cells.

Results

Clinical features of the patient. The patient was a 26-year-old male born normally at 38 weeks of gestation as a single child of healthy, nonconsanguineous Japanese parents. At birth, his weight, length, and occipitofrontal head circumference were 2186 g (–1.9 SD), 42.0 cm (–3.3 SD), and 31.5 cm (–1.3 SD), respectively. He developed epilepsy at 2 weeks of age, followed by a global developmental delay with mild hypothyroidism and cataracts. At the age of 2 years, he could laugh and gained gazing and eye-tracking ability; however, his overall developmental quotient was 13. He walked with a posture control walker until elementary school. He then gradually lost developmental milestones. Infectious diseases, such as aspiration pneumonia, frequently worsened his neurological deterioration. At the age of 18 years, his height, length, and occipitofrontal head circumference were 140 cm (~4.6 SD), 140 cm (~4.0 SD), and 52.5 cm (~4.0 SD), respectively. He is currently bedridden and on mechanical ventilation. Eye movement is present without gazing or eye tracking. He has stiffness of the posterior neck and limbs and flexion contractures of the limbs without pyramidal signs. The patient also exhibits tongue fasciculation and no deep tendon reflexes. Although his white blood cell count is within the normal range (6.1 × 10^9 cells/L), the percentage of CD19+ B lymphocytes is significantly reduced (1.0% [reference value: 2.9–20.1]). His serum immunoglobulin level is normal. Electroencephalography showed no prominent epileptic activity. Repeated brain MRI revealed nonprogressive, severe cerebral atrophy with ventriculomegaly, an enlarged subarachnoid space, hypomyelinating leukodystrophy, and thinning of the corpus callosum (Figure 1). A motor nerve conduction study indicated mild-to-moderate reductions in velocities of the post-tibial nerve (43.2 m/s, –1.5 SD) and median nerve (50.0 m/s, –3.0 SD) and a severe decrease in compound muscle action potentials in the post-tibial nerve (1.03 mV, –4.0 SD), suggesting axonal neuropathy.

Identification of the de novo heterozygous mutation of ATP11A. We performed whole-exome sequencing of DNA obtained from the patient and his parents. After filtering coding variants against allele frequencies in public and local databases (Table 1), we identified the heterozygous de novo variant c.250C>G in exon 3 of the ATP11A gene in the patient and confirmed this by Sanger sequencing (Figure 2A). The resulting amino acid change (Q84E) affected one of the highly conserved residues in the first transmembrane segment of ATP11A (Figure 2B). Real-time reverse transcription PCR (RT-PCR) of skin fibroblasts from the patient confirmed that the mutant allele was transcribed to mRNA as efficiently as the WT allele (Figure 2C).

To establish whether the identified heterozygous mutation of ATP11A was responsible for the patient’s phenotypes, we designed a conditional knockin allele (Atp11aQ84E/WT) to express WT Atp11a before Cre-mediated recombination and induced expression of the Q84E mutant (c.250C>G) allele after recombination (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI148005DS1). Crossing of the Atp11aQ84E/WT male mice with homozygous CAG-Cre female mice generated few Atp11aQ84E/WT mice that developed until the weaning stage (3 mutant mice among 15 mice from 3 pregnant mice). Following in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) with sperm from the surviving Atp11aQ84E/WT mice, 80% of mutant mice died within 1 week of birth (number of surviving mice: 38 vs. 6 for Atp11aQ84E/WT and Atp11aQ84E/WT mice, respectively). We observed no apparent sex differences in the mutant mice. Atp11aQ84E/WT mice surviving beyond the perinatal period grew slower than did WT mice (Figure 3A), and 4 of 7 died within 5 weeks of birth (Figure 3B).

Before death, the Atp11aQ84E/WT mice displayed neurological deficit phenotypes, including tremors (Supplemental Videos 1, 2, and 3), an abnormal gait (Figure 3C), and hind limb clasping (Figure 3D). An MRI analysis of mice at 11 weeks showed an apparent reduction in brain size (421.7 vs. 495.4 mm^3) and dilated lateral ventricles in Atp11aQ84E/WT mutant mice (Figure 3E and Supplemental Figure 2). Histological analysis and electron microscopy observations revealed tissue degeneration with pyknotic or TUNEL-positive neurons in various brain regions of Atp11aQ84E/WT embryos and pups (Figure 3, F and G). The average number of TUNEL-positive nuclei in whole-brain sections of mutant mice was approximately 4-fold higher than that in WT mice (Figure 3G). Sequencing analysis of RNA from the brains and spleens of affected mice indicated that half of the mRNA carried the c.250C>G mutation (Supplemental Figure 3). To confirm that the mutant protein was expressed in vivo, we performed SDS-PAGE to separate the membrane fraction of mouse placenta that abundantly expressed ATP11A (18). Proteins of approximately 110–130 kDa were digested with chymotrypsin and subjected to a targeted analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS) (19). Figure 3H and Supplemental Figure 4 show that the Q84-containing WT ATP11A peptide (LVQLIIDTPTSVP'TSLGPL) was present in the chymotryptic digests of the placenta membrane fraction of both Atp11aQ84E/WT and Atp11aQ84E/WT mice. In contrast, we detected the E84-containing mutant peptide (LVQLIIDTPTSVP'TSLGPL) only in digests of the placenta membrane fraction of Atp11aQ84E/WT mice. These results confirmed that the heterozygous point mutation of Atp11a was dominant for causing neuronal defects and lethality.
Flipping of PtdCho by the Q84E-ATP11A mutant. Previous studies of yeast P4-ATPase (Drs2) indicated that Q84, localized to the interfacial region between the extracellular fluid and plasma membrane, functions as a substrate-selecting entry gate (20). To determine whether Q84E has an altered substrate specificity, we stably expressed GFP-tagged WT or Q84E–human ATP11A (hATP11A) in the Atp11a<sup>−/−</sup> Atp11c<sup>−/−</sup> W3 mouse T-lymphoma cell line (double-knockout [DKO]) (17) together with hCDC50A. Both WT and Q84E-hATP11A were present at similar levels in plasma membranes (Figure 4A). We assessed flippase activity in the plasma membrane using a fluorescent nitro-benzoxadiazole-conjugated (NBD-conjugated) phospholipid analog (see Methods). As reported previously (14), WT ATP11A specifically promoted the incorporation of NBD-PtdSer (NBD-PS) and NBD-PtdEtn (NBD-PE), but not NBD-PtdCho (NBD-PC), NBD-SM, or NBD-galactosylceramide (NBD-GalCer) (Figure 4B). Q84E exhibited flippase activity against NBD-PS and NBD-PE, but not against NBD-SM or NBD-GalCer. However, the mutant exhibited flippase activity against NBD-PC.

Some yeast P4-ATPases (Dnf1p and Dnf2p) have been shown to transport lyso-PtdEtn and lyso-PtdCho (21). However, neither Q84E nor WT hATP11A internalized NBD-lyso-PtdCho.

When the Q84E mutant was introduced into WT W3 cells, the transformants showed enhanced flippase activity toward PtdSer and PtdEtn. They also incorporated PtdCho as efficiently as did DKO-Q84E cells (Figure 4C and Supplemental Figure 5A), indicating that the WT alleles (Atp11a and Atp11c) of plasma mem-
branle flippases (14) did not inhibit the PtdCho-flipping activity of Q84E-ATP11A. The PtdSer and PtdCho flippase activity induced by the Q84E mutant of ATP11A were inhibited by orthovanadate, an inhibitor of P4-ATPas (Supplemental Figure 5B), suggesting that PtdCho-flipping activity was due to the mutant ATP11A. An examination of the ATPase activity of purified recombinant WT and Q84E-ATP11A proteins indicated that palmitoyl-oleoyl-glycerophosphoserine (POPS) and palmitoyl-oleoyl-glycerophosphoethanolamine (POPE), but not palmitoyl-oleoyl-glycerophosphocholine (POPC) or SM, activated the ATPase activity of WT ATP11A as previously reported (refs. 13, 14 and Figure 4D). POPS and POPE activated the ATPase activity of Q84E-ATP11A. However, in contrast to WT ATP11A, the ATPase activity of Q84E-ATP11A was activated by POPC with approximately 20% of the efficiency of POPS (Figure 4D).

To clarify whether the replacement of Gln by Glu, but not by other amino acids, is essential for PtdCho flipping, Q84 of ATP11A was replaced by Ala, Asp, or Asn and expressed in DKO cells (Supplemental Figure 5, C and D). As shown in Figure 4E, all mutants exhibited PtdSer-flipping activity to varying degrees. In contrast, PtdCho-flipping activity was only observed with Q84E, indicating that the replacement of Q84 by Glu specifically allowed ATP11A to translocate PtdCho.

**Molecular dynamics simulations for phospholipid flipping.** Among human P4-ATPas, the members of 2 subfamilies (class 1a: ATP8A1 and ATP8A2, class 6: ATP11A and ATP11C) have been shown to have PtdSer-flipping activity at recycling endosomes (22) or plasma membranes (14, 16). The shared identities of the amino acid sequence of hATP11A with those of hATP8A1, hATP8A2, and hATP11C were 37%, 37%, and 66%, respectively. The tertiary structures of hATP8A1 and hATP11C indicated that PtdSer was translocated via a cleft that formed between transmembrane segments (10, 11, 23). The conservation of the amino acid sequence of transmembrane segments was better between hATP11A and hATP11C (shared identity of 79%) than between hATP11A and hATP8A1 or ATP8A2 (shared identity of 42% or 39%, respectively). Since hATP11C and hATP8A2, but not hATP8A1, localized to the plasma membrane (14), we examined the effects of the Q84E mutation on flippase activity with hATP8A2 and hATP11C. We found that Q107 of hATP8A2 or Q79 of hATP11C, corresponding to Q84 of hATP11A (Supplemental Figure 6A), was mutated to Gln or Ala and expressed in DKO cells. WT hATP8A2, hATP11C, and their mutants localized to the plasma membrane (Supplemental Figure 6B). WT hATP8A2 and hATP11C translocated NBD-PS, but not NBD-PC (Figure 5, A-D). On the other hand, PtdSer flippase activity was reduced by approximately 40% and 90% in the Q79E-mutant hATP11C and Q107E-mutant hATP8A2, respectively (Figure 5, A and B). We did not observe this decline in Ala mutants (Q79A-hATP11C and Q107A-hATP8A2), suggesting that Gln at this position interfered with PtdSer flipping. We detected PtdCho flippase activity in cells expressing the Q79E mutant of hATP11C, but not Q107E-hATP8A2 (Figure 5, C and D). As with hATP11A, we did not observe PtdCho-flipping activity with the Q79A mutant of hATP11C. These results suggest that hATP11C, but not hATP8A2, used a mechanism similar to that of hATP11A to transport phospholipids.

We conducted molecular dynamics (MD) simulations using the structure of hATP11C, hATP8A1, and their mutants (10, 11, 23). WT hATP11C interacted with PtdSer by forming H-bonds with Q79, T86, T90, S91, and N352 (Figure 5, E and F, and Supplemental Figure 7) at the entry site of the longitudinal groove (11), resulting in high-affinity binding of PtdSer to hATP11C (approximately −25 kcal/mol). Similarly, PtdSer interacted with hATP8A1 via H bonds with Q88, T100, T101, N352, and N353 (binding affinity of −28 kcal/mol). The Q79E mutation of ATP11C and the Q88E mutation of hATP8A1 reduced the number of H bonds (Figure 5, E and F, and Supplemental Figure 7), decreasing the binding affinity of PtdSer by approximately 60%. In contrast, the Ala mutation in these residues did not reduce binding affinity to PtdSer (Q79A-hATP11C and Q88A-hATP8A1; Figure 5, E and F, and Supplemental Figure 7). These results were consistent with the observed flippase activity (Figure 5, A and B). We also investigated the PtdEtn flippase activity of WT ATP11C and its mutants and conducted MD simulations (Supplemental Figure 8 and Supplemental Table 1). The PtdEtn flippase activity of Q79E-hATP11C mutants was 2-fold higher than that of WT hATP11C. The binding affinity of PtdEtn to the Q79E-hATP11C mutant was 5- to 10-fold greater than that of the WT hATP11C or the Q79A-hATP11C mutant, explaining the increase in the PtdEtn flippase activity of the Q79E-hATP11C mutant.

Consistent with the results showing that Q79E-hATP11C, but neither WT ATP11C nor the Q79A-ATP11C mutant, flipped PtdCho, our MD simulations revealed that only the Q79E mutation of hATP11C formed a salt bridge between the choline moiety and E79, resulting in 5-fold stronger binding affinity (from 0.7-3.6 kcal/mol; Figure 5G and Supplemental Figure 9A). In contrast to hATP11C, the carboxyl residue of E88 of hATP8A1 engaged in electrostatic interactions with the nitrogen of peptide bonds between T100 and T101 and between T96 and G97 (Figure 5H).

### Table 1. Variant filtering strategy

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<td>Keep nonsynonymous and splice site variants</td>
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<tr>
<td>(for autosomal-recessive and X-linked inheritance)</td>
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<tr>
<td>Absent in databases (for de novo inheritance)</td>
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</tr>
<tr>
<td>De novo inheritance</td>
<td>1 gene</td>
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<tr>
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<td>X-linked Inheritance</td>
<td>2 genes</td>
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<tr>
<td>Keep variants with CADD Phred score &gt;20</td>
<td>ATP1A</td>
</tr>
<tr>
<td>Sanger sequencing validation</td>
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MAF, minor allele frequency. *1000g2015aug_all, esp6500siv2_all, gnomAD (v2.1.3), HGVD, and ToMMo 8.3. KPN. ATP11A (NM_05205.3) c.250C>G, p.Gln84Glu. SYCE1 (NM_00143764.3) c.585delG; [541G>A], p.[Leu196Serfs*4]; [C(Cy314Glu)]. MAGEB18 (NM_173699.4) c.965C>A, p.[Ala322Asp], XPNPEP2 (NM_003399.4) c.1181A>G, p.[Glu394Gly]. *A CADD Phred-scaled score of 20 represents the top 1% of the most deleterious variants.
A change in the intracellular phospholipid composition often modulates lipogenesis (26). A microarray analysis of gene expression indicated that the sphingomyelin synthase 1 (Sgms1) gene was upregulated by 10- to 25-fold in cells expressing the Q84E mutant (Table 2), which was confirmed by real-time RT-PCR (Figure 6D). We also detected the upregulation of Sgms1 mRNA in Q84E-expressing WT W3 cells (Figure 6D). Western blotting with anti-SGMS1 mAb showed a marked increase in the SGMS1 protein in DKO-W3 and WT W3 cells transformed with Q84E-hATP11A, but not WT hATP11A (Figure 6E). SGMS1 in the Golgi catalyzes the synthesis of SM by transferring the phosphocholine of PtdCho to ceramide (27). LC-MS/MS analysis indicated that the composition of SM species, but not other phospholipids, was approximately 2-fold higher in Q84E cells than in other control cells (parental DKO and DKO cells expressing WT hATP11A; Figure 6F, and Supplemental Figure 10).

Elevated SM at the surface of Q84E-mutant ATP11A-expressing cells. Given the elevated amounts of SM in Q84E cells, we postulated that SM replaced PtdCho to compensate for that removed by Q84E-ATP11A at the outer leaflet of plasma membranes. We observed that staining intensity of nontoxic lysenin (NT-Lys), which specifically binds to SM-rich domains on the cell surface (28), was 6- to 8-fold higher in Q84E-expressing DKO cells than in cells expressing WT ATP11A (Figure 7A). We then metabolically labeled cellular PtdCho and SM by culturing cells for 40 hours in the presence of methyl-14C-choline. Thin-layer chromatography (TLC) analysis of total cellular lipids showed an approximately

and Supplemental Figure 9B). The lack of a salt bridge between E88 and PtdCho reduced the affinity of PtdCho for Q88E-hATP8A1, supporting the lack of PtdCho-flipping activity in the Q107E mutant of hATP8A2 (Figure 5D).

Effects of PtdCho flipping on the plasma membrane. We then assessed subcellular phospholipid dynamics using TopFluor-tagged PtdSer (TopFluor-PS) and PtdCho (TopFluor-PC), which strongly associate with biological membranes (24). DKO and its transformants expressing WT or Q84E-hATP11A were loaded with TopFluor phospholipids and allowed to flip at 20°C. Top-Fluor-PS signals were observed in plasma membranes (Figure 6A). TopFluor-PC also remained in the plasma membranes of DKO cells and DKO cells expressing WT hATP11A. In contrast, in DKO expressing Q84E, a large amount of TopFluor-PC was intracellularly distributed and merged with ER-tracker (Figure 6A). SM-loaded methyl-α-cyclodextrin (MαCD) replaces phospholipids from the outer leaflet of plasma membranes with SM (25). Accordingly, flow cytometry showed that the treatment with SM-loaded MαCD markedly reduced TopFluor-PC fluorescence in DKO and DKO WT ATP11A cells, confirming its localization to the outer leaflet of plasma membranes (Figure 6B). In contrast, the same treatment markedly increased TopFluor-PC fluorescence in Q84E-hATP11A–expressing cells, confirming the lack of SM species, but not other phospholipids, was approximately 2-fold higher in Q84E cells than in other control cells (parental DKO and DKO cells expressing WT hATP11A; Figure 6F, and Supplemental Figure 10).

Elevated SM at the surface of Q84E-mutant ATP11A-expressing cells. Given the elevated amounts of SM in Q84E cells, we postulated that SM replaced PtdCho to compensate for that removed by Q84E-ATP11A at the outer leaflet of plasma membranes. We observed that the staining intensity of nontoxic lysenin (NT-Lys), which specifically binds to SM-rich domains on the cell surface (28), was 6- to 8-fold higher in Q84E-expressing DKO cells than in cells expressing WT ATP11A (Figure 7A). We then metabolically labeled cellular PtdCho and SM by culturing cells for 40 hours in the presence of methyl-14C-choline. Thin-layer chromatography (TLC) analysis of total cellular lipids showed an approximately
2-fold higher concentration of SM in Q84E cells than in other control cells (DKO and DKO WT ATP11A) (Figure 7B), confirming the results obtained by LC-MS/MS (Figure 6F). Lipids in the outer leaflet of plasma membranes were then extracted by SM-αCD and analyzed by TLC. We found that the quantity of 14C-PtdCho was 4- to 5-fold lower in Q84E cells than in other cells, whereas the content of SM was approximately 4-fold higher, increasing the ratio of SM to PtdCho by approximately 20-fold (Figure 7B). This
A marked increase in SM on the cell surface substantially altered cellular sensitivity to SMase. Similar to other mammalian cells, such as human proximal tubule cells (29), the treatment of DKO or DKO WT hATP11A cells with 2 units/mL SMase for 5 minutes did not induce plasma membrane rupture leading to lactate dehydrogenase (LDH) release (Figure 7C). However, a 10-fold lower concentration of SMase killed the majority of Q84E cells within 5 minutes, causing the release of LDH. WT W3 cells expressing Q84E-ATP11A showed the same characteristics as the DKO transformants (accumulation of SM on the cell surface and increased sensitivity toward SMase; Supplemental Figure 11). Collectively, these results indicate that the concentration of PtdCho markedly decreased in the outer leaflet of plasma membranes in Q84E-hATP11A–expressing cells, while that of SM increased.

Based on its ability to bind to perfringolysin O (PFO), cholesterol in the plasma membrane is divided into “accessible” and “inaccessible” pools, with that in the accessible pool moving to the ER for esterification and storage (4, 30). Since cholesterol becomes “inaccessible” after binding to SM (4), the large quantity of SM in the outer leaflet of the Q84E cells may alter the distribution of cholesterol. As shown in Figure 7D, mCherry-tagged D4, a cholesterol-binding domain of PFO (31), bound to Q84E-expressing cells with approximately 50% of the efficiency of other control cells (DKO and DKO WT hATP11A). We confirmed the ability of the Q84E mutant to

Figure 4. PtdCho-flipping activity of the Q84E mutant. (A) Membrane localization of Q84E-ATP11A. GFP (green); DRAQ5 (magenta). Scale bars: 10 μm. (B and C) DKO (B) or W3 (C) cells expressing WT or Q84E-ATP11A were incubated with the indicated phospholipids, and incorporated fluorescence was plotted (n = 3), with the SD shown. (D) SDS-PAGE of WT and Q84E-ATP11A (180 ng protein). Proteins were stained with CBB. ATPase activity with the indicated phospholipids is shown as a percentage of POPS-activated ATPase (n = 4) with mean values (bars). P = 0.021, by Student’s t test. (E) DKO and its transformants expressing the indicated mutant ATP11A were incubated with NBD-PS or NBD-PC, and the MFI is plotted (n = 3–6), with the average value shown.
We then assessed SM levels in E16 embryos using matrix-assisted laser desorption ionization–imaging mass spectrometry (MALDI-IMS) (32). As shown in Figure 8A, the levels of N-stearyl-d-erythro-sphingosylphosphorylcholine [SM (d18:1/18:0)], N-oleoyl-d-erythro-sphingosylphosphorylcholine [SM (d18:1/18:1)], and N-palmitoyl-d-erythro-sphingosylphosphorylcholine [SM (d18:1/16:0)] in various regions, particularly the brain and lungs, were higher in Atp11aQ84E/WT than in Atp11a WT/WT embryos. PtdCho and ceramide, substrates for the synthesis of SM, were reduced in the lungs of Atp11aQ84E/WT mice (Figure 8B), in which SM levels increased.

Figure 5. MD simulation. (A–D) Flippase activity. DKO cells expressing the WT or indicated mutant of hATP11C (A and C) or hATP8A2 (B and D) were incubated with NBD-PS (A and B) or NBD-PC (C and D), and the MFI is plotted, with red bars indicating the average value. (E–H) MD analysis of the flipping of PtdSer (E and F) or PtdCho (G and H) by the WT and indicated mutant of ATP11C (E and G) or ATP8A1 (F and H) was simulated as described in Methods. Calculated binding energies (kcal/mol) are shown in the violin plot; black bars indicate the average value (n = 200 structures). Right panels show typical geometries obtained with the indicated molecules for PtdSer and PtdCho. PtdSer and PtdCho are shaded in pale blue and pale green, respectively. Dotted lines indicate electrostatic interactions. Red, oxygen; blue, nitrogen; green, amino acid carbon; gray, hydrogen; orange, phosphorous carbon. Molecules exhibiting flippase activity are underlined in red.

reduce accessible cholesterol on the cell surface in WT W3 cells (Supplemental Figure 11C). Consistent with the reductions in accessible cholesterol, cholesteryl ester levels were more than 70% lower in Q84E-expressing cells (Figure 7E). In contrast, the expression levels of SREBP-regulated genes, such as 3-hydroxy-3-methylglutarylcoenzyme A reductase (Hmgr), low-density lipoprotein receptor (Ldlr), and fatty acid synthase (Fasn), were not affected by the Q84E mutation of ATP11A (Table 2). These results suggest that the expression of Q84E-hATP11A did not change free cholesterol levels in the ER membrane. Q84E cells showed markedly lower proliferation rates than did DKO WT ATP11A (doubling time; 12.6 vs. 9.6 hours) (Figure 7F), and were also smaller than control cells (Figure 7G).
cell biological analyses, we identified a de novo mutation of the ATP11A gene in a patient with severe developmental disorders and progressive neurological decline. The same heterozygous mutation in mice recapitulated the phenotypes (growth retardation, reduction of the brain size, and ataxia) of the patient, confirming it as the dominant mutation responsible for the symptoms. ATP11A is present in the plasma membrane and exclusively translocates PtdSer and PtdEtn, but not PtdCho, from the outer membrane.

Figure 6. Increase in SM in the plasma membrane by Q84E-ATP11A. (A) Cells were loaded with TopFluor-PC or TopFluor-PS (green), incubated at 20°C, and observed by fluorescence microscopy in the presence of ER tracker (red). Scale bars: 10 μm. (B) Cells were loaded with TopFluor-PC (red), treated with SM-MαCD (blue), and analyzed by flow cytometry. Experiments were performed 3 times, and TopFluor-PC signals (MFI) were normalized to signals in cells that were not treated with SM-MαCD and plotted. P = 4.4 × 10⁻⁴ (DKO); P = 0.0025 (DKO WT); P = 0.0017 (DKO-Q84E), by Student’s t test. (C) Cells were loaded with TopFluor-PC (green), treated with SM-MαCD, and observed by fluorescence microscopy. Scale bars: 10 μm. (D and E) SGMS1 mRNA and protein levels in DKO or W3 cells expressing WT hATP11A or Q84E-hATP11A were assessed by (D) real-time RT-PCR (n = 3), relative to the expression of 36B4, or (E) Western blotting (WB) of total cell lysates with anti-SGMS1 mAb. The membrane used for Western blotting was stained with CBB. The asterisks indicate SGMS1. (F) The concentration of SM species in the indicated cells was assessed by LC-MS/MS.

be higher in Atp11aQ84E/WT than in Atp11aWT/WT embryos (Figure 8C). These results suggest that the Q84E mutation of ATP11A caused the flipping of PtdCho at plasma membranes and the upregulation of SM synthesis in these tissues.

Discussion
By analyzing whole-exome sequencing data from unaffected parents–affected child trios (33) followed by biochemical and...
to the inner leaflet (14, 16). Here, we demonstrated that the flipping of PtdCho by ATP11A causes lethal effects in humans and mice. Approximately 25%–35% of PtdCho in plasma membranes localizes to the inner leaflet (34, 35). However, electron microscopy findings with anti-PtdCho antibody revealed that the majority of PtdCho localizes to the outer leaflet, at least in red blood cells (7, 36). The harmful effects of the translocation of PtdCho to the outer leaflet, or the lack of mechanisms to degrade the excess PtdCho, are also shown. The present results indicate that PtdCho flipped by Q84E-hATP11A traveled intracellularly. Cells carry specific lipid exchange systems. For example, PITPα carries PtdCho from the plasma membrane to the ER, in turn transferring PtdIns from the ER to the plasma membrane (39). PtdSer moves in the other direction. That is, ORP5 and ORP8 transfer PtdSer from the ER to plasma membranes, and PtdIns4P (P14P) from plasma membranes to the ER. In addition, PtdSer can be trapped by the polybasic domains of plasma membrane–localized proteins (40). This may explain why PtdCho, but not PtdSer, at the inner leaflet of plasma membranes traveled intracellularly. The PtdCho that reached the ER directly or indirectly upregulated the Sgms1 gene. This result appears to be consistent with previous findings showing that PtdCho at the ER regulated the lipid metabolic pathway via nuclear receptors (41, 42). It will be interesting to study how PtdCho regulates Sgms1 gene expression.

Despite various attempts (microarray, single-cell RNA-Seq, and slide-Seq), we have so far failed to detect cells with increased Sgms1 gene expression in vivo (K. Segawa and S. Nagata, unpublished observation). This may be because the upregulation of Sgms1 gene expression occurred in a small population of cells, or because the cells with high Sgms1 levels had been killed, for example, by SMase. Using in vivo imaging analysis (MALDI-IMS), we detected several tissues that carried elevated SM levels (the brain, lung, heart, liver, testis, and labyrinth; Figure 8). These results may be attributed to the high expression levels of ATP11A in the cells of these tissues, the weak “floppase” activity translocating PtdCho from the inner to the outer leaflet, or the lack of mechanisms to degrade the excess SM. Regardless of the mechanism, we detected cerebral atrophy in the patient and neuronal degeneration in ATP11A<sup>Q84E/WT</sup> mice, indicating that the loss of PtdCho or enrichment of SM at the outer leaflet of plasma membranes exerted deleterious effects in vivo.

### Table 2. Microarray analysis

<table>
<thead>
<tr>
<th>Gene name (symbol)</th>
<th>W3 signal</th>
<th>DKO signal</th>
<th>DKO WT signal</th>
<th>DKO-Q84E signal</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphingomyelin synthase 1 (Sgms1)</td>
<td>64.5</td>
<td>61.8</td>
<td>86.3</td>
<td>1728.5</td>
<td>24.9</td>
</tr>
<tr>
<td>Hemogen (Hemgon)</td>
<td>126.4</td>
<td>169.9</td>
<td>288.9</td>
<td>1049.6</td>
<td>6.0</td>
</tr>
<tr>
<td>Adhlin (Ahd1)</td>
<td>74.0</td>
<td>194.0</td>
<td>144.2</td>
<td>630.5</td>
<td>5.4</td>
</tr>
<tr>
<td>Glucocorticoid-induced transcript 1 (Circi1)</td>
<td>166.4</td>
<td>109.7</td>
<td>107.1</td>
<td>579.9</td>
<td>4.7</td>
</tr>
<tr>
<td>N-Myc downstream regulated gene 1 (Nmdg1)</td>
<td>137.1</td>
<td>323.6</td>
<td>204.8</td>
<td>809.9</td>
<td>4.1</td>
</tr>
<tr>
<td>Purinergic receptor P2X, ligand-gated ion channel 3 (P2x3)</td>
<td>121.2</td>
<td>84.3</td>
<td>56.9</td>
<td>322.9</td>
<td>4.1</td>
</tr>
</tbody>
</table>

**SREBP-regulated genes**

<table>
<thead>
<tr>
<th>Gene name (symbol)</th>
<th>W3 signal</th>
<th>DKO signal</th>
<th>DKO WT signal</th>
<th>DKO-Q84E signal</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-hydroxy-3-methylglutaryl-coenzyme A reductase (Hmgcr)</td>
<td>2595.1</td>
<td>755.2</td>
<td>932.3</td>
<td>253.7</td>
<td>–5.6</td>
</tr>
<tr>
<td>Cytochrome P450, family 2, subfamily c, 55 (Cyp2c55)</td>
<td>265.0</td>
<td>394.8</td>
<td>376.3</td>
<td>69.8</td>
<td>–5.0</td>
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<tr>
<td>T cell receptor–associated transmembrane adaptor 1 (Trat1)</td>
<td>1450.8</td>
<td>1040.8</td>
<td>575.0</td>
<td>213.3</td>
<td>–4.8</td>
</tr>
<tr>
<td>DIX domain–containing 1 (Dixdc1)</td>
<td>155.7</td>
<td>100.3</td>
<td>101.6</td>
<td>25.8</td>
<td>–4.6</td>
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<tr>
<td>Zinc finger protein 184 (Zfp184)</td>
<td>163.4</td>
<td>146.8</td>
<td>166.0</td>
<td>39.7</td>
<td>–4.0</td>
</tr>
<tr>
<td>Hmgcr</td>
<td>6775.4</td>
<td>7381.5</td>
<td>7109.2</td>
<td>8111.7</td>
<td>1.1</td>
</tr>
<tr>
<td>Slc6a13</td>
<td>2595.1</td>
<td>755.2</td>
<td>932.3</td>
<td>253.7</td>
<td>–5.6</td>
</tr>
<tr>
<td>Fasn</td>
<td>1814.9</td>
<td>1634.8</td>
<td>1837.6</td>
<td>1945.3</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Gene expression in W3, DKO, and DKO cells expressing WT or Q84E-mutant ATP11A was determined by microarray analysis. Genes up- or downregulated by more than 4-fold in Q84E-expressing cells compared with other cells are listed with their signals and fold change. Expression levels of the representative SREBP-regulated genes (Hmgcr, Ldlr, and Fasn) are also shown.
Affected cells may be susceptible to the cell lysis by SMase, since it is highly produced during inflammation (43–45). Or, the growth of these cells is retarded by abnormal cholesterol homeostasis (46). The Atp11a<sup>Q84E</sup>/WT neonate mice developed poorly and died within 20 weeks, but the mutant embryos developed until mid-gestation. In this regard, it may be worth noting that the oxygen stress generated during the fetal-to-neonate circulatory transition can activate SMase (45, 47). In conclusion, the present study showed that the appropriate distribution of PtdCho and SM in plasma membranes is essential for proper development in humans and mice.

Methods

*Exome sequencing.* Trio-based exome sequencing was performed for the patient and his parents using the HiSeq 2500 sequencer (Illumina) with 126 bp paired-end reads after enrichment with SureSelectXT Human All Exon V5 (50 Mb) Kits (Agilent Technologies). Reads were mapped against the hg19 human reference genome using Novoalign (version 2.08.05) (http://www.novocraft.com). Variants were called using Genome Analysis Toolkit software (version 1.6–13) (GATK). After quality filtering steps (Table 1), variants were annotated using ANNOVAR (48). Nonsynonymous SNVs, splice site variants, and indels were retained. To prioritize de novo variants, those in the following databases were excluded: 1000 Genomes Project (https://www.internationalgenome.org/), NHLBI GO Exome Sequencing Project (https://evs.gs.washington.edu/EVS/), Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org/), Human Genetic Variation Database (http://www.hgvd.genome.med.kyoto-u.ac.jp/), and ToMMo 8.3KJPNv2 (https://jmorp.megabank.tohoku.ac.jp/).
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RESEARCH ARTICLE

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The mutation of the ATP11A gene (GenBank: NM_015205.3) thus identified in the patient was confirmed by Sanger sequencing.

Animal studies. C57BL/6J and ICR mice were obtained from Japan SLC or the Japan Central Laboratory for Experimental Animals. Atp11afl/WT mice were generated by Unitech as a custom order. We established a conditional knockin allele (Atp11afl) to express WT ATP11A in the absence of Cre recombinase and the Q84E mutant after Cre-mediated recombination. In brief, the mouse Atp11a gene (MGI: 1354735) was retrieved from BAC clones (RP23-81J20 and RP23-312I20) in RIKEN. A DNA carrying a part of intron 2 with a splice acceptor site, WT Atp11a cDNA encoding exons 3–29, triplet SV40 polyadenylation sequences, a neo gene flanked by flippase recognition target (FRT) sequences, and a loxP sequence were inserted into intron 2 of the Atp11a gene (Supplemental Figure 1). This was followed by insertion of exon 3 carrying the c.250C>G mutation, intron 3, exon 4, and a part of intron 4. A DNA for diphtheria toxin A (DT-A) was added at the 5′ terminus and introduced into embryonic stem (ES) cells (Bruce 4). ES cells carrying the targeted allele were selected by PCR and introduced into host embryos to generate chimeric mice. Crossing of chimeric mice with C57BL/6J mice produced Atp11afl mice. Atp11a Q84E/WT mice were produced by crossing male Atp11a fl mice with CAG-Cre mice (RIKEN: B6.Cg-Tg[CAG-Cre]CZ-MO2Osb). Mice were genotyped by PCR with the primers described in Supplemental Table 2.

IVF and embryo transfer. IVF was performed as described previously (49). In brief, sperm from Atp11a Q84E/WT mice were incubated at 37°C for 2 hours in TYH medium (119.37 mM NaCl, 4.78 mM KCl, 1.19 mM MgSO4, 1.19 mM KH2PO4, 25.07 mM NaHCO3, 1.71 mM CaCl2, 5.56 mM d-glucose, 1 mM sodium pyruvate, and 4 mg/mL AlbuMAX [Thermo Fisher Scientific]). C57BL/6J female mice were injected i.p. with 5 units of pregnant mare serum gonadotropin 62 hours before IVF and 5 units of human chorionic gonadotropin 14 hours before IVF. Eggs were treated with 1 mg/mL hyaluronidase (MilliporeSigma) at 37°C for 5 minutes. Sperm were allowed to fertilize eggs at 37°C for 6 hours and were then incubated in TYH for 18 hours. Two-cell stage embryos were transferred to oviducts of pseudo-pregnant ICR females (0.5 days postcoitum [dpc]). For ICSI (50), sperm heads were separated from tails by applying a few piezo pulses and injected into oocytes using a piezo micro manipulator (PrimeTech). Embryos were transferred into the oviducts as described above.

Plasmids. pMXs-puro and pGag-pol-IRES-bsr (51) were obtained from T. Kitamura (University of Tokyo). pCMV-VSV-G, pQE30/His6-mRFP-NT-Lys, and pET28/His6-mCherry-D4 were obtained from RIKEN. pAdVAntage and pX330 were purchased from Thermo Fisher Scientific and Addgene, respectively. cDNAs for hCDC50A (NM_018247.3), hATPI1A (NM_015205.2), hATPI1C (XM_005262405.1), and hATPA82 (NM_016529.4) were prepared by PCR (14), tagged with FLAG or GFP at the C-terminus, and introduced into pMXs-puro. hATPI1A CDNA carrying the c.250C>G mutation was prepared by PCR from the patient’s RNA with the primers 5′-GGCTTAATTAGGAGGAGCCATGGACT-
grown until the OD 600 reached 0.5. After the addition of L-arabinose (MilliporeSigma) and dialyzed against PBS. NT-Lys or mCherry-D4 were concentrated using Amicon Ultra 10K. After removal of cell debris, the supernatant was loaded onto Ni-NTA at 15°C for 24 hours. The cells were disrupted by sonication in 100 mL RPMI 1640 with 10% FCS containing 45 U/mL mouse IL-3. Cells were cultured in RPMI 1640 with 10% FCS. Mouse Ba/F3 cells (54) were cultured in RPMI 1640 with 10% FCS containing 45 U/mL mouse IL-3. Cells were stably transformed as described previously (14). In brief, pMXs-puro carrying the GFP-tagged cDNA was introduced into HEK293T cells by Fugene 6 (Promega) with pGag-pol-IRES-bsr, pCMV-VSV-G, and pAd-Advantage. Two days later, retroviruses in the supernatant were collected with a spatula, and mixed with scintillator cocktail (Clear-sol I; Nacalai Tesque). Radioactivity was measured using the Tri-Carb 4810 scintillation counter (PerkinElmer).

Preparation of NT-Lys and perfringolysin domain 4. To produce red fluorescent protein-tagged (RFP-tagged) NT-Lys and mCherry-domain 4 (D4) of perfringolysin (mCherry-D4), the DNA fragment coding for His6-NT-Lys-RFP or His6-mCherry-D4 (28, 55) was inserted into the pC0d1 vector (Takara) and introduced into chaperone-competent E. coli (pGrot7/BL21; Takara). Transformants were grown until the OD600 reached 0.5. After the addition of L-arabinose and isopropl β-thiogalactopyranoside (IPTG), cells were cultured at 15°C for 24 hours. The cells were disrupted by sonication in 100 mL buffer A (50 mM Tris-HCl buffer [pH 8.0], 150 mM NaCl, and 0.1 mM DTT) containing an EDTA-free protease inhibitor cocktail (Nacalai). After removal of cell debris, the supernatant was loaded onto Ni-NTA Superflow (QIAGEN) equilibrated with buffer A. The column was washed with buffer B (50 mM Tris-HCl buffer [pH 8.0], 500 mM NaCl), and protein bands were eluted with elution buffer (50 mM Tris-HCl buffer [pH 7.4], 150 mM NaCl) containing 10, 100, 300, or 500 mM imidazole in a stepwise manner. The fractions containing NT-Lys or mCherry-D4 were concentrated using Amicon Ultra 10K (MilliporeSigma) and dialyzed against PBS.

Flippase assay. Flippase activity was assayed as described previously (13, 14). In brief, 2 × 10^6 cells were incubated at 20°C with 0.25–1.0 μM NBD phospholipids in 600 μL HBSS containing 1 mM MgCl2 and 2 mM CaCl2 (HBSS*). Cells were collected by centrifugation, suspended in HBSS containing 5 mg/mL fatty acid-free BSA to remove nonincorporated NBD lipids, and analyzed using a FACS Canto II (BD Biosciences). Dead cells were omitted from the analysis by standard forward scatter/side scatter (FSC/SSC) gating. The fluorescence that was not removed from cells by the BSA treatment reflected the flipping activity and was shown as NBD phospholipid incorporation.

The flipping activity with TopFluor-conjugated lipids was assayed using the MuCD-mediated lipid exchange system (25). To prepare SM-loaded MuCD, 5.7 mg SM was dried under a nitrogen stream for 1 hour, suspended in 4.5 mL RPMI medium prewarmed to 70°C, and incubated at 70°C for 5 minutes. After adding 500 μL of 40 mM MuCD in PBS, the solution was incubated at 37°C for 1 hour, centrifuged at 10,000g for 5 minutes, and the supernatant was used as SM-loaded MuCD. Cells (2 × 10^5 cells) were incubated on ice for 10 minutes in 1 mL HBSS containing 5 μM TopFluor-PC, resuspended in HBSS* that had been prewarmed to 20°C, and incubated at 20°C for 20 minutes. Cells were collected by centrifugation, suspended in 500 μL SM-loaded MuCD, incubated at 20°C for 1 hour, and analyzed by flow cytometry using FACS Canto II or observed under a confocal microscope (FV1000D; Olympus).

Quantification of cholesterol, staining for SM and cholesterol, and treatment with Smae. Cellular cholesterol and the cholesteryl ester were quantified using the Total Cholesterol and Cholesteryl Ester Colorimetric/Fluorometric Assay kit (BioVision). For staining with NT-Lys or His6-mCherry-D4, 1 × 10^5 cells were incubated on ice for 30 minutes with 10 μg/mL His6-NT-Lys-mRFP or His6-mCherry-D4 in HBSS containing 250 μM SYTOX Blue and analyzed using the FACS Canto II. For treatment with Smae, 3 × 10^4 cells were incubated at 37°C for 5 minutes with neutral SMase or 1 μCi choline chloride [methyl-14C] (55 mCi/mmol, American Radio labeled Chemicals [ARC]). For lipids in the outer leaflet of plasma membranes, 3 × 10^5 to 5 × 10^5 cells were suspended in 1 mL SM-loaded MuCD solution and incubated at 37°C for 1 hour. After removal of MuCD by centrifugation, supernatants were vigorously mixed with 1 mL hexane/isopropanol mixture (3:2, vol/vol), and the organic phase was collected. Lipids in the water phase were re-extracted with the same treatment, dried with the first extract, dried under a nitrogen stream, and dissolved in 25 μL of a 1:1 (v/v) mixture of methanol/chloroform. Extracted lipids (5000 disintegrations per minute [dpm]) were separated on an HP-TLC Silica Gel 60 Plate (Merck) with chloroform/methanol/ammonium hydroxide (65:25:5, vol/vol/vol) as a solvent and visualized with an imaging plate detector (FLA-7000, Fujifilm). Lipids were also visualized by exposure to iodine vapor, collected with a spatula, and mixed with scintillator cocktail (Clear-sol I; Nacalai Tesque). Radioactivity was measured using the Tri-Carb 4810 scintillation counter (PerkinElmer).

LC-MS/MS and MALDI-IMS. For LC-MS/MS analysis, lipids were extracted from the cells by methanol and analyzed by LC-electrospray ionization-MS/MS using a TSQ-Vantage (Thermo Fisher Scientific) at Japan Lipid Technologies as a custom order. For MALDI-IMS, samples...
were embedded in Super Cryoembedding Medium (Section-Lab) and frozen in powdered dry ice. Sections (8 μm thick) were prepared using a Leica CM3050 cryostat, attached to indium tin oxide–coated glass slides (Bruker Daltonics), and coated with 2,5-dihydroxybenzoic acid (50 mg/mL in 80% ethanol) by spraying with an artistic brush (Procon Boy FWA Platinum, Mr. Hobby). The ultrafast extreme MALDI-TOF mass spectrometer (Bruker Daltonics) was equipped with an Nd:YAG laser. Data were acquired in the positive reflection mode with raster scanning by a pitch distance of 50 μm. Signals with an m/z between 400 and 900 were collected in the continuous accumulation of selected ions mode and expressed as the image using FlexImaging 4.1 software (Bruker Daltonics).

**Microscopic analysis and TUNEL staining.** Mouse embryos were fixed at 4°C in 0.1 M phosphate buffer (pH 7.4) containing 2% formaldehyde and 2.5% glutaraldehyde, postfixed for 1 hour with 1% OsO4, dehydrated, and embedded in TAAB Epon 812 (Nissin-EM). Sections (2 μm thick) were stained with toluidine blue and observed under a BX63 Olympus microscope. For electron microscopy studies, fixed brains were cut into small pieces, dehydrated, and embedded in Epon 812. Silver sections (80 nm thick) were prepared with an ultramicrotome (Ultracut UCT, Leica), stained with uranyl acetate and lead citrate, and observed with an electron microscope (HT7700, Hitachi Ltd.). For TUNEL staining, mouse brains were fixed with 4% paraformaldehyde, embedded in paraffin, and sliced into 5 μm thick sections. Sections were subjected to TUNEL staining using the In situ Cell Death Detection Kit, TMR red (Roche), followed by observation with a fluorescence microscope (BIOREVO BZ-9000, Keyence). Six to 7 images that covered the whole-brain tissues were obtained, and the TUNEL-positive foci per brain tissue were counted using Hybrid Cell Count on BZ-H4C (Keyence) image analysis software.

**Mouse MRI analysis.** Mouse MRI was performed using the Avance III BioSpec 117/11 system (Bruker) equipped with a 3T QD coil. During the analysis, mice were anesthetized and their body temperature maintained at 37°C with a warm-water circulating system. The fast spin-echo protocol was applied for T2-weighted imaging. Images were acquired in the positive reflectron mode and expressed as the image using FlexImaging 4.1 software (PerkinElmer).

Microarray analysis was performed using the GeneChip 3′ IVT PLUS Reagent Kit (Thermo Fisher Scientific) as a custom order at Takara Bio. In real-time RT-PCR, RNA was reverse-transcribed with a High Capacity RNA-to-cDNA kit (Thermo Fisher Scientific), and cDNA was amplified in a reaction mixture of LightCycler 480 SYBR Green I Master (Roche Diagnostics). Data are available from the NCBI’s Gene Expression Omnibus database (GEO GSE181171). Primers for real-time RT-PCR are described in Supplemental Table 2.

**Data-dependent LC-MS/MS analysis of immunoprecipitated GFP-tagged hATP11A.** DKO cells stably expressing GFP-tagged WT or Q84E-hATP11A were lysed in RIPA buffer (20 mM HEPES-NaOH [pH7.5], 150 mM NaCl, 1 mM EGTA, 1 mM MgCl2, 0.25% sodium deoxycholate, 0.2% SDS, and 1% NP-40) supplemented with a protease inhibitor cocktail and a PhosSTOP phosphatase inhibitor cocktail (Roche). After centrifugation at 20,000g at 4°C for 15 minutes, supernatants were incubated with a 4 μL slurry of GFP-Trap_MA (ChromoTek) at 4°C for 2 hours. The beads were washed 4 times with RIPA buffer and twice with 50 mM ammonium bicarbonate. Proteins on the beads were digested with 200 ng chymotrypsin (Roche) at 25°C for 16 hours. The digests were reduced, alkylated, acidified, and desalted using GL-Tip SDB (GL Sciences). The eluates were evaporated in a SpeedVac concentrator and dissolved in 0.1% trifluoroacetic acid and 3% acetonitrile (ACN). LC-MS/MS analysis was performed on an EASY-nLC 1200 UHPLC connected to an Orbitrap Fusion mass spectrometer through a nanoelectrospray ion source (Thermo Fisher Scientific). Peptides were separated on a C18 reversed-phase column (75 μm × 150 mm) (Nikkoyo Technos) with a linear 4%–36% ACN gradient for 0–100 minutes, followed by an increase to 80% ACN for 10 minutes. The mass spectrometer was operated in the data-dependent acquisition mode with a maximum duty cycle of 3 seconds. MSI spectra were measured with a resolution of 60,000, an automatic gain control (AGC) target of 4 × 106, and a mass range from 350 to 1500 m/z. Collision-induced dissociation...
an AGC target of 1.5 \times 10^5, an isolation window of 1.6 m/z, a maximum injection time of 100 ms, and a normalized collision energy of 35. Dynamic exclusion was set to 5 seconds. Raw data were analyzed against the SwissProt database restricted to *Mus musculus* supplemented with human ATP11A (WT and Q84E) and EGFP protein sequences using Proteome Discoverer version 2.4 (Thermo Fisher Scientific) with the SEQUEST HT search engine. The search parameters were as follows: (a) chymotrypsin as an enzyme with up to 3 missed cleavages; (b) precursor mass tolerance of 10 ppm; (c) fragment mass tolerance of 0.02 Da; (d) the carbamidomethylation of cysteine as a fixed modification; and (e) acetylation of the protein N-terminus and oxidation of methionine as variable modifications. Peptides were filtered at an FDR of 1% using the percolator node.

Parallel reaction monitoring analysis of ATP11A peptides from mouse placenta. The frozen placenta was disrupted using an ultrasonic homogenizer (Q55, QSonica) in 6.5 mL of 10 mM Tris-HCl buffer (pH 7.5) supplemented with 0.5 mM DTT and a protease inhibitor cocktail (buffer C). After addition of an equal volume of buffer C containing 0.5 M sucrose, 0.1 M KCl, 10 mM MgCl$_2$, and 2 mM CaCl$_2$, the mixture was incubated on ice for 15 minutes with 10 units/mL benzonase (Merck) and serially centrifuged at 800 g and 8000 g each at 4°C for 10 minutes. The supernatant was then centrifugated at 100,000 g for 1 hour, and the precipitates were recovered as a crude membrane fraction.

The placenta membrane fractions were lysed in 1× SDS sample buffer, incubated at 25°C for 20 minutes, separated by 5%–20% SDS-PAGE, and stained with Coomassie brilliant blue (CBB). Bands with a MW between 100 and 130 kDa were cut out and digested in gel with chymotrypsin at 25°C for 16 hours as described previously (56). Several selected peptides of ATP11A (WT and Q84E) were measured by parallel reaction monitoring (PRM) (19). Targeted CID MS/MS scans were acquired by a time-scheduled inclusion list at a resolution of 60,000, an AGC target of 1.5 \times 10^5, an isolation window of 1.6 m/z, a maximum injection time of 1 second, and a normalized collision energy of 35. The time alignment of the transitions was performed using Skyline software.

Molecular model and MD simulations. We used the cryo-EM structures of hATP11C (PDB: 7BSU; ref. 23) and hATP8A1 (PDB: 6K7M; ref. 10) in the PtdSer-occluded E2-P state. Hydrogen atoms were generated and energetically optimized with the CHARMM program (version 40b) (57). The positions of all nonhydrogen atoms were fixed, and all titratable groups were maintained in their standard protonation state at pH 7. The hATP11C–CDC50A or hATP8A1–CDC50A flippase complex in the PtdSer-occluded E2-P state (WT PtdSer) was embedded in a lipid bilayer consisting of approximately 1000 POPC molecules and soaked in approximately 180,000 water molecules. Fifteen chloride ions were added to the model using the CHARMM–GUI program (58). After structural optimization with positional restraints on heavy atoms of the WT PtdSer assembly, (a) the system was heated from 0.1 to 300 K over 5.5 ps with intervals of 0.01 fs, equilibrated at 300 K for 1 ns with 0.5 fs intervals, and annealed from 300 to 0 K over 5.5 ps with intervals of 0.01 fs. (b) The same procedure was repeated with positional restraints on the heavy atom of the protein backbone. (c) The same procedure, except for the annealing process, was repeated without positional restraints on any atoms. (d) The WT PtdSer structure was obtained after the system was equilibrated at 300 K for 67.5 ns with intervals of 1.5 fs. For WT PtdCho and WT PtdEtN, initial WT lipid structures were modeled on the basis of the WT PtdSer structure, and steps (c) and (d) were repeated. Mutant lipid structures were modeled on the basis of the WT lipid structures, and steps (c) and (d) were repeated. All equilibrating simulations were conducted using the NAMD program (version 2.12; ref. 59). The SHAKE algorithm (60) was used for hydrogen constraints with a time step of 1.5 fs where applicable. The Langevin thermostat and piston were used for temperature and pressure control, respectively (61). The binding energy between the lipid and flippase complex (excluding cavity-filling water molecules) was calculated on the basis of 200 conformations in the last 22.5 ns MD simulation using the Molecular Operating Environment (MOE) program (Chemical Computing Group). The population of the H-bond pattern at the binding moiety was analyzed from 3000 conformations in the last 22.5 ns MD simulation (refer to Supplemental Table 1 for the number of water molecules that formed H-bonds with lipids).

Statistics. The significance of differences was calculated with a 2-tailed Student’s t test. Differences were considered significant when the P value was less than 0.05.

Study approval. The use of patient samples was approved by the ethics committees of the Research Institute for Microbial Diseases at Osaka University (protocol no. 30-2) and the Tohoku University Graduate School of Medicine (protocol no. 2015-1-694). Written informed consent was obtained from the parents of the patient. Mice were maintained under specific pathogen-free conditions at Osaka University. Experiments were conducted according to protocols approved by the IACUC of the Research Institute of Microbial Diseases at Osaka University.

Author contributions

KS, AK, SK, and SN conceived and designed the project. AK, SI, and SK identified the ATP11A mutation in the patient. KS, AK, KY, YO, T Nishimura, MM, SI, and WS performed the experiments. YS and MS performed MALDI-IMS analysis. T Noji, K Hiragi, and HI conducted MD simulations. CS and YU performed the electron microscopic analyses. K Haginoya and YK collected and analyzed clinical data and provided patient samples. FS performed the MRI analysis. HK and KN performed LC-MS/MS-based targeted proteomics analysis. MI conducted IVF and ICSI experiments. KS, AK, SK, YS, NH, HI, and SN wrote the manuscript. All authors read and approved the manuscript.

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