Inefficient development of syncytiotrophoblasts in the Atp11a-deficient mouse placenta

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The P4-ATPases ATP11A and ATP11C function as flippases at the plasma membrane to translocate phosphatidylserine from the outer to the inner leaflet. We herein demonstrated that Atp11a-deficient mouse embryos died at approximately E14.5 with thin-walled heart ventricles. However, the cardiomyocyte- or epiblast-specific Atp11a deletion did not affect mouse development or mortality. ATP11C may have compensated for the function of ATP11A in most of the cell types in the embryo. On the other hand, Atp11a, but not Atp11c, was expressed in the mouse placenta, and the Atp11a-null mutation caused poor development of the labyrinthine layer with an increased number of TUNEL-positive foci. Immunohistochemistry and electron microscopy revealed a disorganized labyrinthine layer with unfused trophoblasts in the Atp11a-null placenta. Human placenta-derived choriocarcinoma BeWo cells expressed the ATP11A and ATP11C genes. A lack of ATP11A and ATP11C eliminated the ability of BeWo cells to flip phosphatidylserine and fuse when treated with forskolin. These results indicate that flippases at the plasma membrane play an important role in the formation of syncytiotrophoblasts in placental development.

Significance

Plasma membranes are composed of a lipid bilayer in which phosphatidylserine (PtdSer) is confined to the inner leaflet by the action of flippase that translocates PtdSer from the outer to inner leaflets. Two P4-ATPases (ATP11A and ATP11C) work as flippase at plasma membranes. Here, we report that the mouse placenta expresses only ATP11A, and Atp11a-deficient mouse embryos die during embryogenesis due to inefficient formation of syncytiotrophoblasts in the placental labyrinth. The flippase-null mutation inactivates human choriocarcinoma BeWo cells to translocate PtdSer into the inner leaflet and undergo cell fusion. These findings highlight the importance of flippase to regulate the distribution of phospholipids for cell fusion, at least in trophoblast fusion.
Results

Developmental Defects and Embryonic Lethality in Atp11a<sup>fl<sup>ox</sup>fl<sup>ox</sup></sup> Mice. Atp11a<sup>fl<sup>ox</sup>fl<sup>ox</sup></sup> mice harbor the knockout-first allele in the Atp11a gene (SI Appendix, Fig. S1). Atp11a<sup>fl<sup>ox</sup>fl<sup>ox</sup></sup> mice grew normally and had no abnormalities. When they were intercrossed, no Atp11a<sup>fl</sup>fl<sup>/fl</sup> (Atp11a<sup>Δ/Δ</sup>) mice were found among 107 offspring at the age of 4 wk (Fig. 1A). A genotype analysis of embryos indicated that Atp11a<sup>Δ/Δ</sup> mice survived according to Mendelian law until E13.5 but started to die thereafter (Fig. 1A). The surviving E13.5 Atp11a<sup>Δ/Δ</sup> embryos were significantly smaller (by ∼10%) than littermate Atp11a<sup>fl<sup>ox</sup>fl<sup>/ox</sup></sup> or Atp11a<sup>fl<sup>/fl</sup></sup> embryos and showed anemia (Fig. 1B).

The Atp11a<sup>fl<sup>ox</sup>fl<sup>/ox</sup></sup> allele carries the LacZ gene in intron 6 of the Atp11a gene (SI Appendix, Fig. S1). When E13.5 Atp11a<sup>Δ/Δ</sup> embryos were subjected to the in situ 5-bromo-4-chloro-3-indoly-β-D-galactoside (X-gal) staining of whole-mount embryos, intense staining was observed in the choroid plexus, heart, dorsal root ganglion, mesentery, and tongue (Fig. 1C). Strong β-galactosidase (βGal) activity was detected in the ventricles and atrium regions. Cells in the E13.5 heart were dissected and stained for βGal activity using the spirobased immobilisable diethylrhodol-βGal (SPIDER) technique (19). As shown in Fig. 1D, cardiac troponin T (cTnT)—positive cardiac myocytes showed positive signals, indicating that the Atp11a gene was explicitly expressed in cardiac myocytes.

A histological analysis showed a defect at the ventricular septum of the heart from the E13.5 Atp11a<sup>Δ/Δ</sup> embryo (Fig. 1E). In addition, the heart myocardium of E13.5 mutant mice was thinner than that of the littermates due to a reduced number of myocardium layers at the ventricular apex (Fig. 1F). These results suggested that developmental disorders in Atp11a<sup>Δ/Δ</sup> mice were caused by the lack of Atp11a in cardiac myocytes. Therefore, we generated the Atp11a allele flanked by loxP (Atp11a<sup>fl<sup>ox</sup>fl<sup>ox</sup></sup> mice) (SI Appendix, Fig. S1) and crossed these mice with mice carrying the Cre gene under the promoter of the α-Mysin heavy-chain gene (αMhc-Cre) that specifically expresses Cre in cardiac myocytes (20). Surprisingly, the Atp11a<sup>fl<sup>ox</sup>fl<sup>/ox</sup></sup> mice carrying αMhc-Cre were born and developed normally (Fig. 1G). Genotyping with DNA from the heart indicated that the Atp11a allele was mutated as expected and designated as Atp11a<sup>Δ<sub>c</sub></sup>. These results showed that abnormal cardiac development in Atp11a<sup>Δ/Δ</sup> mice was not due to the Atp11a deficiency in cardiac myocytes. We previously reported that among 14 members of the P4-ATPase family, ATP11A and ATP11C localized to the plasma membrane were ubiquitously expressed and exhibited redundant activity (8). A real-time RT-PCR analysis indicated that Atp11c messenger ribonucleic acid (mRNA) was present at ∼30% of Atp11a mRNA in the heart (Fig. 1H), suggesting that flipase activity provided by ATP11C was sufficient to maintain homeostasis in the heart.

Lethal Effect of the Abnormal Placenta in Atp11a<sup>Δ/Δ</sup>-Null Mice. Perez-Garcia et al. (21) reported that among embryonic lethal knockout mice, 35% of those dying by E9.5 to E14.5 had placental abnormalities, which were often accompanied by the abnormal development of the heart or brain (21–23). We attributed the lethality of Atp11α-deficient mice to a defect in the placenta. Western blotting with a monoclonal antibody (mAb; clone 4-C11) against mouse ATP11A showed an ∼130-kDa band in the wild-type heart but not in the Atp11a<sup>Δ/Δ</sup> heart (Fig. 2A), which confirmed the specificity of the mAb. The intensity of 130-kDa ATP11A in lysates from the wild-type mouse placenta was approximately 10-fold stronger than in those from the heart. A markedly reduced but still significant level of the Atp11a<sup>Δ/Δ</sup> placenta was observed in the Atp11a<sup>Δ/Δ</sup> placenta, potentially from maternal (Atp11a<sup>Δ<sub>y<sub>ox</sub></sub></sup>fl<sup>ox</sup>)-derived cells. A real-time RT-PCR analysis indicated that the level of Atp11a mRNA normalized to that of glyceraldehyde-3-phosphate dehydrogenase (Gapdh) mRNA was more than 10-fold higher in the placenta (Fig. 2B) than in the heart (Fig. 1H). In contrast, Atp11c mRNA expression was negligible in the E13.5 placenta (Fig. 2B). X-gal staining of the whole placenta revealed that cells in the labyrinthine layer, but not in the spongiotrophoblast layer, expressed the βGal gene under the Atp11a gene promoter (Fig. 2C).

We then deleted the Atp11a gene in an epiblast-specific manner by crossing Atp11a<sup>fl<sup>ox<sub>fl</sub></sub></sup> mice with Sex determining region Y-box 2 (Sox2)-Cre transgenic mice that expressed Cre in epiblasts (24–26). As shown in Fig. 2D, Atp11α-deficient mice with the Sox2-Cre<sup>+</sup>Atp11a<sup>Δ<sub>A</sub></sup> genotype were born at a lower number than expected (Fig. 2D) but grew normally. A Western blot analysis indicated that the Atp11α protein was not present in the heart, kidneys, or lungs of Sox2-Cre<sup>+</sup>Atp11a<sup>Δ<sub>A</sub></sup> mice (Fig. 2E). Histological analysis of the heart from the mice at 16 mo of age showed no apparent abnormality (SI Appendix, Fig. S2). These results showed that Atp11A was dispensable for mouse embryos and adulthood but was indispensable for placental development.

Abnormal Labyrinthine Layer in the Atp11a-Null Placenta. When Atp11a<sup>fl<sup>ox<sub>fl</sub></sub></sup> mice were intercrossed, the E13.5 placentas of Atp11α<sup>Δ/Δ</sup> embryos were smaller and thinner than those of their littermates of the Atp11α<sup>+/+</sup> or Atp11α<sup>fl<sup>ox</sup>fl<sup>/ox</sup></sup> genotype (Fig. 3A). The weight of placentas was ∼70% of those for the control littersmates. The mouse placenta comprises the maternal decidua and fetal parts that consist of the spongiotrophoblast and labyrinthine cell layers and chorion (Fig. 3B). A histological analysis of the placenta in E13.5 Atp11α<sup>Δ/Δ</sup> mice showed that their labyrinthine layer was 30% thinner than that in Atp11α<sup>+/+</sup> mice (Fig. 3D). In contrast, the size of the spongiotrophoblast layer was similar to that of the control littersmates.

A close examination of the wild-type labyrinth showed juxtaposed maternal blood lacunae and fetal blood vessels separated by syncytial and mononuclear trophoblastic cell layers (Fig. 3C). Fetal blood vessels lined by endothelial cells contained primitive nucleated erythrocytes that were easily distinguished from maternal sinusoids carrying nucleated erythrocytes. In comparison with the wild-type placenta labyrinth, the Atp11α<sup>Δ/Δ</sup> placenta labyrinth was less packed and only a few fetal blood vessels containing nucleated erythrocytes were present (Fig. 3C). Maternal sinusoids were also not surrounded well by spongiotrophoblasts. A similar abnormality was observed in the placenta of E11.5 Atp11α<sup>Δ/Δ</sup> placenta (SI Appendix, Fig. S3).

Abnormal Syncytiotrophoblasts in the Labyrinthine Layer of the Atp11a-Null Placenta. In the mouse placenta, the SynT-I and SynT-II layers surrounded maternal sinusoids in the labyrinthine layer (Fig. 4A). Immunostaining with anti-monomocarboxylate transporter (MCT)1 and anti-MCT4, which are explicitly expressed in SynT-I and SynT-II, respectively (27), showed that SynT-I and SynT-II were closely associated in the wild-type placental labyrinth. On the other hand, the cell layers expressing MCT1 or MCT4 were not well connected.
Fig. 1. Developmental heart defect in Atp11α-null embryos. (A) Atp11α-+/komp mice were intercrossed, and the genotype of littermates at the age of 4 wk or the indicated embryonal day was identified. (B) The appearance of E13.5 Atp11α+/+ and Atp11α+/komp littermate embryos. Atp11α+/+ embryos were smaller and paler than wild-type embryos. Scale bar, 2 mm. (C) E13.5 Atp11α+/komp littermate embryos were stained with X-gal (blue) and counterstained with nuclear fast red (pink). Scale bar, 1 mm. Right: Enlarged area of the dotted box. Scale bar, 500 μm. (D) Cardiomyocytes prepared from E13.5 Atp11α+/komp hearts were stained with SPiDER-β Gal (green), anti-cTnT Ab (red), and DAPI (blue). Scale bar, 50 μm. (E and F) Sections of E13.5 Atp11α+/+ or Atp11α+/komp and Atp11α+/komp littermate embryos were stained with H&E. The ventricular septum (E) and right ventricular apex (F) are shown. Scale bar, 200 μm (E), 100 μm (F). The asterisk in E points to the ventricular septal defect in the Atp11α+/komp embryo. The myocardial layer is thinner in Atp11α+/komp embryos (F). The number of myocardial layers in the boxed area was quantified for four to six sections per mouse (n = 3), and the average values were plotted with SD (bar) on the Right. (G) Atp11αfl/fox and αMhc-Cre-Atp11αfl/fox mice were crossed, and the genotype of obtained pups at the age of 4 wk was identified. The number of mice with the indicated genotype is shown. (H) mRNAs for the Atp11α and Atp11c genes in E13.5 wild-type mouse fetal hearts (n = 3) were quantified by real-time RT-PCR, and their relative levels against Gapdh mRNA were plotted with SD (bar).
in the Atp11a<sup>lox/lox</sup> placenta (Fig. 4B), suggesting that syncytiotrophoblasts were not appropriately developed in the Atp11a<sup>lox/lox</sup> placenta.

To further characterize the defect in the Atp11a<sup>lox/lox</sup> placenta, we observed the E13.5 placenta under a transmission electron microscope. As shown in Fig. 4C, the wild-type labyrinth showed the layer of mononuclear sinusoidal trophoblast giant cells, the SynT-I cell layer, and the vacuole-containing SynT-II cell layer (17, 28). On the other hand, although endothelial cells and vacuole-containing cells were identified in the labyrinth of the Atp11a<sup>lox/lox</sup> placenta, the SynT-I and SynT-II layers were poorly organized (Fig. 4C). Scanning electron microscopy of the wild-type labyrinthine layer from the maternal blood side showed giant cells that were expected to be syncyiotrophoblasts (Fig. 4D). On the other hand, the majority of cells were small in the Atp11a<sup>lox/lox</sup> labyrinth, while few giant cells were present, suggesting the inefficient fusion of trophoblasts. Transmission electron microscopy showed many trophoblasts carrying degenerated nuclei in the Atp11a<sup>lox/lox</sup> labyrinth (Fig. 4C). Accordingly, the number of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells in the Atp11a<sup>lox/lox</sup> placentals labyrinthine layer was about fivefold higher than in the Atp11a<sup>+/+</sup> placental labyrinthine layer (Fig. 4E). In addition, a real-time RT-PCR analysis showed that the mRNA levels of Mct1, transferrin receptor protein (Tfrc), Mct4, and chorion-specific transcription factor (Gcm1) genes, which are expressed in syncyiotrophoblasts (29, 30), were lower in the E13.5 Atp11a<sup>lox/lox</sup> placenta than in the wild-type placenta (Fig. 4F). These results suggested that Atp11a-deficient trophoblasts incapable of forming syncyiotrophoblasts died.

**Inefficient Cell Fusion of ATP11A<sup>−/−</sup> ATP11C<sup>−/−</sup> BeWo Cells.** To examine the effects of flipase on the formation of syncyiotrophoblasts, we investigated human BeWo cells, which undergo cell fusion into syncyiotrophoblast-like cells when treated with forskolin (31). Real-time RT-PCR showed that BeWo cells expressed the ATP11A and ATP11C genes (Fig. 5A), and the expression level of ATP11A mRNA increased following the treatment with 20 μM forskolin for 48 h. We established ATP11A<sup>−/−</sup> ATP11C<sup>−/−</sup> (DKO) BeWo cells by the CRISPR-
Cas9 system (32) (SI Appendix, Fig. S4). As shown in Fig. 5B, wild-type BeWo cells incorporated nitrobenzoxadiazole (NBD)-labeled PtdSer (NBD-PS); however, this activity was completely lost in DKO-BeWo cells. The transformation of DKO-BeWo with human ATP11A rescued DKO-BeWo to flip or translocate NBD-PS into cells. To further confirm the contribution of ATP11A to the establishment of an asymmetrical distribution of PtdSer, cells were treated with 5 μM ionomycin, a Ca^{2+}-ionophore, to activate Ca^{2+}-dependent scramblases, such as TMEM16F (33). This treatment resulted in the exposure of PtdSer in most populations of wild-type, DKO, and DKO-ATP11A BeWo cells (Fig. 5C). When the ionomycin was removed, PtdSer on the surface of wild-type BeWo cells gradually disappeared at 25°C, indicating that PtdSer was translocated into the inner leaflet of the plasma membrane. This internalization of PtdSer was severely retarded in DKO-BeWo cells, and more than 80% of the cell population continued to expose PtdSer after a 30-min incubation. On the other hand, DKO-expressing ATP11A regained the ability to internalize PtdSer. These results indicated that ATP11A and ATP11C flippases at the plasma membrane were essential for rapidly establishing the asymmetrical distribution of PtdSer, similar to mouse WR19L lymphoma cells (16).

Fig. 3. The abnormal labyrinthine layer of the Atp11a-deficient placenta. Atp11ak/komp mice were intercrossed, and placentas were removed at E13.5 and genotyped. (A) Left: Representative appearance of the Atp11a^{+/+} (+/+ ) and Atp11a^{k/k} (k/k) placenta. Scale bar, 1 mm. Right: Weights of the placentas from control (Atp11a^{+/+} or Atp11a^{k/komp}) (n = 22) and Atp11a^{k/k} (n = 7) mice were plotted with the average (bars). P values were calculated by Student’s t test. (B and C) Histological analysis of the placenta (B) and labyrinthine layer (C). Paraffin sections from the Atp11a^{+/+} or Atp11a^{k/k} placenta were stained with H&E and observed by microscopy. Bottom: Higher-magnification images of the boxed regions. Scale bar, 500 μm (B), 200 μm (C). (D) The lengths of the Sp and La in the center of the placenta indicated by black arrows in B were measured in three sections per placenta dissected from different pregnant mice (n = 4 for Atp11a^{+/+} and n = 6 for Atp11a^{k/k}). The thickness of each placenta was plotted with the average value (bars). P values were calculated by Student’s t test. ns, not significant.

Wild-type, DKO, and DKO-ATP11A BeWo cells at ~10% confluency were then treated for 48 h with 20 μM forskolin or
its solvent dimethyl sulfoxide (DMSO) (0.05%). Staining with anti-E-cadherin antibody (Ab) indicated that a high level of E-cadherin was present at the site of cell-to-cell contact in aggregated BeWo cells in control DMSO-treated cells (Fig. 5E). As reported by Coutifaris et al. (34), the treatment of wild-type BeWo cells with forskolin strongly reduced the intensity of E-cadherin or even removed it at several locations, suggesting that these cells underwent cell fusion. On the other hand, the expression of E-cadherin in DKO-BeWo cells remained after the forskolin treatment, while it disappeared in forskolin-treated DKO-BeWo-ATP11A cells. Observations by scanning electron micrography indicated that the area size of wild-type BeWo cells increased 4.1-fold from 565 to 2,299 μm² after the treatment with forskolin for 48 h, while the perimeter length increased 2.2-fold from 105 to 228 μm (Fig. 5E). Similar increases in the area size and perimeter length were observed in forskolin-treated DKO-ATP11A cells but not DKO-BeWo cells. Omata et al. (35) previously reported that the forskolin-induced fusion of BeWo cells was accompanied by the expression of a number of genes related to cell differentiation or cell fusion. Real-time RT-PCR indicated that the expression of the SYNCYTIIN2 gene that can be up-regulated by forskolin treatment before cell fusion increased to a similar extent between wild-type and DKO-BeWo cells after the forskolin treatment (Fig. 5F). The up-regulation of the DYSERIN gene that occurs after cell fusion (35) was less pronounced in DKO-BeWo cells, supporting a role of the plasma
membrane flippase, ATP11A or ATP11C, in the fusion of BeWo cells.

Discussion

The mouse P4-ATPase family comprises 14 members, with ATP11A, ATP11B, and ATP11C belonging to class 6 of the family (6). We herein demonstrated that a global deficiency of Atp11a caused embryonic lethality and mutant mice died after E14.5, possibly due to heart failure. As previously reported in embryonic lethal mutant mice with heart failure (21–23, 36), the placenta was abnormal or poorly developed in Atp11a-null mice. Organogenesis of the mouse heart, accompanied by the proliferation of cardiac myocytes to thicken the ventricular wall, occurs between E8.5 and E14.5 (37, 38). Since the proliferation of cardiac myocytes is strongly affected by blood flow and hemodynamics (39), a decrease in blood flow due to placental defects may cause abnormal heart development in the embryo.

In contrast to the global Atp11a deficiency, the epiblast-specific Atp11a-null mutation had no apparent effect on mouse development. The mice live healthy at least at the age of 16 mo. ATP11A, ATP11B, and ATP11C have 58 to 64% identities on the amino acid sequence and translocate PtdSer when they are reconstituted in liposomes (40). ATP11A and ATP11B, but not ATP11C, are highly expressed in placental trophoblasts.
(Expression Atlas: https://www.ebi.ac.uk/gxa/home). In contrast, ATP11A and ATP11C are ubiquitously expressed in other tissues (8). ATP11A and ATP11C are present at the plasma membrane, while ATP11B is localized intracellularly or in early or recycling endosomes (8, 41). The severe phenotype of the Atp11a-null placenta indicated that ATP11B, a flipase at endosomes, was unable to compensate for the null mutation of the plasma membrane flipase (ATP11A), whereas ATP11C at the plasma membranes in the embryonal cells can compensate for the Atp11a-null mutation for the development of embryos.

In various biological processes, live cells transiently expose PtdSer to the cell surface (4). Differentiating human trophoblasts expose PtdSer on the cell surface (42), and the Ab against PtdSer to the cell surface (4). Differentiating human trophoblasts can compensate for the distribution of PtdSer during the fusion of trophoblasts. However, the normal development of Tnem16f-null embryos (48) may reject its involvement in this process.

The development of syncytiotrophoblasts was inefficient in the Atp11a-null placenta, which is similar to the phenotype reported in mice deficient in Snytyn-A (28), supporting the role of ATP11A in the efficient fusion of trophoblasts. The function of plasma membrane flipases (ATP11A and ATP11C) is to swiftly internalize PtdSer exposed on the cell surface (16). PtdSer irreversibly exposed on the cell surface due to the lack of flipases functions as an eat-me signal not only for apoptotic cells but also for live cells (16). We previously demonstrated that B-cell lymphopenia in Atp11c-deficient mice was due to the engulfment by macrophages of precursor B cells that failed to internalize the exposed PtdSer due to the lack of plasma membrane flipases (16). On the other hand, the lack of the PtdSer-dependent engulfment system (MerTK and Axl tyrosine kinases) did not rescue the embryonic lethality of Atp11a-null mice (SI Appendix, Table S1). It is likely that the prolonged exposure of PtdSer prevented cell fusion or that PtdSer exposed on the cell surface had to return to the inner leaflet of the plasma membrane to complete cell fusion. PtdSer has been shown to change the surface charge of membranes and regulates protein localization (49). In trophoblasts undergoing fusion, intracellular annexin V localizes via PtdSer and regulates protein localization (49). In trophoblasts under various conditions, was unable to compensate for the null mutation of the Atp11a (28), supporting the involvement in this process.

The involvement of PtdSer in cell fusion has been suggested not only for trophoblasts but also for myoblasts, osteoclasts, and egg/sperm fusion (45). Two groups recently established mouse myoblasts that lack Cdc50a, a shared subunit of multiple P4-ATPases, or ATP11A, and reported contradictory findings (52, 53). One group showed that multiple P4-ATPases were required for the efficient fusion of C2C12 cells (53), while the other claimed that a deficiency in Cdc50a or Atp11a caused the uncontrolled fusion of myoblasts in vitro and in vivo (52). The present results on trophoblasts are consistent with the former findings; however, we cannot rule out the possibility that myoblasts and trophoblasts use different mechanisms for fusion. The exposure of PtdSer has been proposed to serve as a fuse-me signal in the fusion process of various cells (45). The present study showed that the distribution of PtdSer needs to be tightly regulated for efficient fusion, at least for the formation of syncytiotrophoblasts. In order for PtdSer to serve as an eat-me signal, it binds to a specific receptor or receptors on macrophages and activates their engulfment system (54). Further studies are needed to identify which scramblase family members (TMEM16, XKR, or others) are responsible for exposing PtdSer in the fusion process and also to elucidate the molecular mechanisms by which PtdSer is recognized in order to activate the fusion process.

Materials and Methods

Mice. C57BL/6N mice and MRL/lpr/lpr mice were obtained from Japan SLC. Atp11a<sup>fl/fl</sup> mice (Atp11a<sup>fl/fl<i>lox/lox</i></sup>Mmp14<sup>fl/fl</sup>) were from the Mutant Mouse Resource and Research Centers. SI Appendix, Fig. S1 shows the structure of the Atp11a<sup>fl/fl</sup> allele. Atp11<sup>fl/fl</sup> mice in which two loxP elements flanked exons 7 and 8 of the Atp11a gene were prepared by crossing Atp11a<sup>fl/fl</sup> mice with mice carrying the CAG-FLPe gene [C57BL/6-Tg(CAG-FlPe)36TolPorbcRiken]. Atnhc-Cre mice [B6. FVB-Tg[Myh6-cre]2182Msds/J] carrying the Cre gene under the promoter of Atnhc were from the Jackson Laboratory. Sox2-Cre mice [B6.Cg-<i>Ed1</i> <i>StrS</i>Sox2<sup>Crem</sup> <i>Fa<sup>tm1</sup></i>Mc238<i>I</i>] in which Cre is under the control of the SRY box containing gene 2 promoter were from the Jackson Laboratory through R. Nishinakamura (Kumamoto University). All mice were housed in a specific pathogen-free facility at the Research Institute for Microbial Diseases, Osaka University. The Ethics Review Committee at Osaka University approved all mouse studies.

Mouse pregnancy was checked by vaginal plugs and confirmed by weight gain (55). The morning when a vaginal plug was observed was noted as E0.5. In some cases, embryos were generated by in vitro fertilization as previously described (56). Genotyping was performed with DNA prepared from tail snips according to the protocol in the Jackson Laboratory (https://www.jax.org/jax-mice-and-services/customer-support/technical-support/genotyping-resources/dna-isolation-protocols) using the primers described in SI Appendix, Table S2.

Cell Lines, Plasmids, Ab, and Reagents. Human HEK293T (American Type Culture Collection CRL-3216) and BeWo (Riken RB6.768) cells were maintained in Dulbecco's modified eagle medium (DMEM) containing 10% fetal calf serum (FCS) (Gibco) and in Ham's F-12 medium containing 15% FCS, respectively. Mouse cardiac myocytes were prepared as previously described (57). In brief, the hearts of E13.5 embryos were washed with phosphate-buffered saline (PBS), placed in 300 μL of enzyme solution [350 μg/mL pancreatic (Nacalai Tesque) in 136.9 mM NaCl, 11.1 mM D-glucose, 2.6 mM KCl, 41.7 mM NaH2PO4, and 11.9 mM NaHCO3], and incubated at 37 °C for 10 min. The solution excluding the undigested heart was transferred to a new tube, mixed with 300 μL of DMEM containing 10% FCS, and spun at 1,000 rpm for 10 min. Precipitated myocytes were suspended in DMEM-10% FCS. Fresh enzyme solution was concurrently added to the heart and incubated at 37 °C for 10 min. This procedure (the treatment with enzyme solution and collection by centrifugation) was repeated five to eight times, and all myocytes were combined. The plasmids pMAL-p2x and pGEX-5x-1 were purchased from New England Biolabs and GB Healthcare, respectively. pX459V2 was from Addgene. pCMV- VSVG and pCAG-HIVgp were from Riken. The pLVPSN-EF-1x-Pur vector was from Takara Bio.

A horseradish peroxidase (HRP)-labeled mouse anti-FLAG mAb (clone M2) and chicken anti-rat Mct1 Ab were purchased from Sigma-Aldrich. HRP-goat anti-mouse Immunoglobulin (Ig)G<sub>2a</sub>, heavy-chain Ab, rabbit anti-human Cntn mAb (clone EPR39659), Alexa Fluor 568-donkey anti-rat IgG (Heavy and Light chains, H+L) preadsorbed Ab, and Alexa Fluor 568-donkey anti-mouse IgG (H+L) Ab were from Abcam. Rat anti-mouse E-cadherin mAb (clone ECD-2), Alexa Fluor 488-goat anti-rat IgG (H+L) Ab, Alexa Fluor 488-goat anti-chicken

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IgY (H+L) Ab, and Alexa Fluor 568-goat anti-rabbit IgG (H+L) Ab were from Invitrogen. HRP-goat anti-mouse IgG Ab was purchased from Dako Agilent. Mouse anti-human Mt4Ab (clone D-1) was from Santa Cruz.

NBD-PS was purchased from Avanti Polar Lipids. X-gal was from Fujifilm Wako Pure Chemical Co. SPIDER-iGal, DAPI, and 0.1% BSA (2-amino-5-ethylethyl)-N,N,N’-tetraacetoxyethyl ester (BAPTA-AM) were from Dojindo Molecular Technologies. Forskolin and ionomycin were from Sigma-Aldrich and Merck, respectively. Cy5-labeled annexin V was purchased from BioVision. Accutase was from Nacalai Tesque.

mAb against Mouse ATP11A. A hybridoma (clone 4-C11) producing mAb against mouse ATP11A was established by immunizing MRL/lpr/lpr mice as previously described (58) with a recombinant mouse ATP11A protein. In brief, two regions of mouse ATP11A (amino acids 427 to 513 and 408 to 892) were fused to glutathione S-transferase (GST) and maltose-binding protein (MBP), respectively, produced in Escherichia coli BL21 and were purified using glutathione Sepharose (GE Healthcare) and amylose resin (New England Biolabs), respectively. Female MRL/lpr/lpr mice at the age of 4 wk were injected subcutaneously twice at a 2-wk interval with 50 μg of GST-ATP11A mixed with TiterMax Gold (TiterMax). Mice were further immunized by intraperitoneal injections of 50 μg of the protein in PBS at a 3-d interval. After six injections, the Ab serum titer was titrated with 0.2 μg/mL HD (Promega). Single clones were isolated by limiting dilutions and genotyped by sequencing the target region of chromosomal DNA. The Ig class of the 4-C11 mAb, identified by the IsoStrip Mouse Monoclonal Antibodyotyping kit (Roche Diagnostics), was IgG2a. In EUSA to assess the Ab titer, 96-well plates were coated with 50 ng of MBP-ATP11A. Samples were serially diluted with PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween 20, added to the wells, and incubated at 4°C for 1 h. Tissues were fixed at room temperature for 48 h in PBS containing 4% PFA, dehydrated, embedded in paraffin, and sliced into 6-μm-thick sections. TUNEL staining was performed using the In Situ Cell Death Detection kit, tetramethylrhodamine (TMR) red (Roche Diagnostics). Sections were counterstained with 1 μg/mL DAPI, mounted on FluoroSave, and observed by fluorescence microscopy (BioRevo BZ-9000, Keyence). TUNEL-positive areas were quantitated using ImageJ software (https://imagej.nih.gov/ij/).

X-Gal and SPIDER-iGal Staining. Tissues were fixed at 4°C for 1 h in PBS containing 4% PFA and washed with X-gal buffer (PBS containing 2 mM MgCl2, 0.02% Nonidet P-40, and 0.01% sodium deoxycholate). After pricking several places with a needle, tissues were immersed in 2 mg/mL X-gal in X-gal buffer containing 5 mM potassium ferricyanide and incubated in the dark at 37°C for 24 to 48 h. Tissues were fixed at room temperature for 48 h in 4% PFA, soaked overnight in 70% ethanol, and dehydrated. Samples were then embedded in paraffin using CT-Pro20 (Japan Genetics) and sliced into 12-μm-thick sections using a microtome (RM2245, Leica). After being deparaffinized and rehydrated, samples were counterstained with 0.1% nuclear fast red (Sigma) in 5% (wt/vol) aluminum sulfate and observed on a fluorescence microscope.

In SPIDER-iGal staining (19), mouse cardiomyocytes cultured on fibrin-coated glass coverslips were fixed at room temperature for 10 min in PBS 0.2% PFA and incubated at 37°C for 1 to 2 h in PBS containing 5 μM SPIDER-iGal and 0.1% Triton X-100. Samples were further incubated at room temperature for 60 min in PBS 2% BSA and stained with 2 μg/mL rabbit anti-human cTnT Ab, followed by incubation with 1 μg/mL Alexa Fluor 568-goat anti-rabbit IgG Ab. Samples were stained with 1 μg/mL DAPI, mounted with FluoroSave, and observed under a confocal fluorescence microscope (IX83, Olympus).

Electron Microscopy. On day 13 postmating, pregnant mice were anesthetized by an intraperitoneal injection of three anesthetics (medetomidine hydrochloride (Zenoaq), midazolam (Astellas Pharma), and butorphanol (Meiji Seika Pharma)) and subjected to cardiac perfusion with 200 to 300 mL of 0.1 M sodium phosphate buffer (pH 7.4) containing 2% glutaraldehyde and 2% PFA. The placenta was removed, fixed at 4°C overnight, and left at room temperature for 3 d in 0.1 M sodium phosphate buffer (pH 7.4). Samples were postfixed with 1% OsO4 at 4°C for 2 h and dehydrated. Samples were incubated twice in propylene oxide (PO) for 20 min, in a 3:1 mixture of PO and epoxy for 1 h, in a 1:1 mixture of PO and epoxy for 1 h, and in epoxy overnight. After incubating in epoxy at 60°C for 3 d, ultrathin sections (thickness of 70 to 80 nm) were prepared with an Ultracut UCT ultramicrotome (Leica), stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope (H-7650, Hitachi High-Technologies) or scanning electron microscope (Helios Nanolab 660, Field Electron and Ion Company).

Gene Editing and Transformation of Cell Lines. The ATP11A and ATP11C genes in BeWo cells were knocked out using the CRISPR-Cas9 system (9, 32). Complementary oligonucleotides carrying the single-guide (sg)RNA target sequence for human ATP11A and ATP11C genes (SI Appendix, Table S2) were annealed and ligated into px4S592. Plasmid DNA was introduced into BeWo cells using FuGENE® HD (Promega). Single clones were isolated by limiting dilutions and genotyped by sequencing the target region of chromosomal DNA. Human ATP11A cDNA (NM_032189.4) was previously described (8). The DNA fragment for Flag-tagged ATP11A was inserted into pLVSVN EF-1α Neo and introduced into HEK293T cells with pCAG-HIVgp and pCMV-VSV-G-RSV-rev using Fugenex (Promega). After culturing for 2 d, the virus in the supernatant was collected with the Lentivirus Concentrator (Takara Bio) and used to infect DKO-BeWo cells. Transfectants were selected with 250 μg/mL G418.

Flippase Assay. Flippase activity for endogenous PtdSer or exogenous PtdSer analog was assessed as previously described (16). In brief, 5.0 × 10^6 BeWo cells in a 12-well plate were cultured overnight, washed with Hanks’ balanced salt solution (HBSS) (–) without phenol red (buffer B), and incubated at 15°C with for 20 min, cells were permeabilized by treatment with 0.1% Triton X-100 at room temperature for 5 min. Sections were incubated for 60 min with 2 μg/mL chicken anti-rat Mct1 Ab and 5 μg/mL mouse anti-human Mt4Ab, followed by incubation with Alexa Fluor 488-goat anti-chicken IgY Ab and Alexa Fluor 568-donkey anti-mouse IgG (H+L) Ab. Samples were mounted on FluoroSave and observed by confocal fluorescence microscopy (IX81, Olympus).

Histological Analyses and TUNEL Staining. In the histological analysis, tissues were fixed at room temperature for 48 h in PBS 4% paraformaldehyde (PFA), soaked overnight in 70% ethanol, dehydrated, embedded in paraffin, sliced into 4- to 6-μm-thick sections, and mounted on glass slides. Samples were then stained with hematoxylin and eosin (H&E) and observed under a fluorescence microscope. Regarding immunohistological staining, placenta was mounted in optimal cutting temperature (OCT) compound (Sakura Finetek), frozen at −80°C, and sectioned at a thickness of 5 μm. After fixing with 4.0% PFA
1 μM NBD-PS in 200 μL of buffer B. Cells were incubated at 4 °C for 1 min in buffer B containing 5 mg/mL fatty acid-free BSA, treated at room temperature for 3.5 min with Accutase, mixed with 200 μL of buffer B containing 5 mg/mL fatty acid-free BSA and 500 nM Sytox blue (Thermo Fischer Scientific), and analyzed by fluorescence-activated cell sorting (FACS) Canto II (BD Biosciences). Data were assessed by FlowJo software (BD Biosciences). To evaluate cell membranes, cells from a six-well plate were treated for 48 h with 20 μM M forskolin with a medium change at 24 h. Cell fusion was evaluated by immunohistochemical staining followed by morphological observations and by scanning electron microscopy. Cells were fixed with 4% PFA, permeabilized with 0.1% Triton X-100, and stained at room temperature for 30 min with Alexa Fluor 488-goat anti-rabbit IgG (H+L) Ab. Samples were counterstained with 5 μg/mL DAPI, mounted with FluorSave, and observed under a confocal fluorescence microscope.

Cell Fusion with BeWo Cells. The fusion of BeWo cells was performed according to Omata et al. (35). In brief, 1 × 10⁵ BeWo cells in a six-well plate were treated for 48 h with 20 μM forskolin with a medium change at 24 h. Cell fusion was evaluated by immunohistochemical staining followed by morphological observations and by scanning electron microscopy. Cells were fixed with 4% PFA, permeabilized with 0.1% Triton X-100, and stained at room temperature for 30 min with Alexa Fluor 488-goat anti-rabbit IgG (H+L) Ab. Samples were counterstained with 5 μg/mL DAPI, mounted with FluorSave, and observed under a confocal fluorescence microscope.

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Regarding scanning electron microscopy, cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), postfixed with 2% OsO₄, and dehydrated with a graded series of ethanol. After treatment with ruthenyl alcohol, samples were freeze-dried with a freeze dryer (VFD-30, Vacuum Device) and mounted on aluminum stubs with carbon paste. Dried specimens were coated with osmium with a Neox-Pro osmium coat (Meiwafosis) and observed under the field-emission scanning electron microscope (JSM-IB8000, JEOL). To estimate cellular areas and perimeters, cell boundaries were traced and analyzed using ImageJ software. Data were expressed as the area (in square micrometers) and perimeter (in micrometers) of the sycnitzym surrounded by the cell boundary.

Statistical Analysis. Data obtained from each experiment were expressed as the mean ± SEM or the mean ± SD. Statistical analyses were performed using Student’s t-test.

Data Availability. All study data are included in the article and/or SI Appendix.

ACKNOWLEDGMENTS. We thank M. Kamada for secretarial assistance. This work was supported in part by grants-in-aid from the Japan Society for the Promotion of Science (21H04770) to S.N.

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