

# Palmitoyl acyltransferase Aph2 in cardiac function and the development of cardiomyopathy

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**Protein palmitoylation regulates many aspects of cell function and is carried out by acyl transferases that contain zf-DHHC motifs. The in vivo physiological function of protein palmitoylation is largely unknown. Here we generated mice deficient in the acyl transferase Aph2 (Abphilin 2 or zf-DHHC16) and demonstrated an essential role for Aph2 in embryonic/postnatal survival, eye development, and heart development. Aph2<sup>-/-</sup> embryos and pups showed cardiomyopathy and cardiac defects including bradycardia. We identified phospholamban, a protein often associated with human cardiomyopathy, as an interacting partner and a substrate of Aph2. Aph2-mediated palmitoylation of phospholamban on cysteine 36 differentially alters its interaction with PKA and protein phosphatase 1  $\alpha$ , augmenting serine 16 phosphorylation, and regulates phospholamban pentamer formation. Aph2 deficiency results in phospholamban hypophosphorylation, a hyperinhibitory form. Ablation of phospholamban in Aph2<sup>-/-</sup> mice histologically and functionally alleviated the heart defects. These findings establish Aph2 as a critical in vivo regulator of cardiac function and reveal roles for protein palmitoylation in the development of other organs including eyes.**

palmitoyl transferase | phospholamban | cardiac development | eye development | Aph2 gene

**P**rotein S-acylation on cysteine residues by palmitate regulates substrate protein localization, trafficking, and protein–protein interactions (1–3) and could potentially play important roles in vivo (4–9). Palmitoylation is the only reversible lipid modification that can be removed by protein palmitoyl thioesterases. Recently, several palmitoyl acyltransferases (PATs) containing a unique zinc finger domain called zf-DHHC have been identified (10). Comparative genome searches have uncovered 23 PAT-like proteins in mammals (11). However, the physiological function of these PATs remains poorly understood (4).

Heart disease is a leading cause of morbidity and mortality worldwide (12). Recent studies have established a pivotal role for defects of calcium cycling in the onset of heart disease (12–16), with a particularly critical role for the regulation of the sarcoplasmic reticulum (SR) calcium ATPase (SERCA). SERCA2a activity is controlled by phospholamban (PLN), an abundant SR protein. Upon  $\beta$ -adrenergic signaling, PKA can phosphorylate serine 16 of PLN, leading to PLN pentamer formation and loss of ability to inhibit SERCA2a (13, 14, 17). Ser16 phosphorylation is removed by phosphatases such as protein phosphatase 1  $\alpha$  (PP1 $\alpha$ ) (18, 19).

It has been reported that some cardiac proteins are palmitoylated, including Na pump regulatory subunit phospholemman and  $\beta$ 1-adrenergic receptor (20–22). However, there is a lack of genetic evidence that protein palmitoylation plays a role in heart development and function. Here we have taken a reverse genetic approach to study the physiological function of Aph2 (Abphilin 2), first identified as an interacting protein of nonreceptor tyrosine kinase c-Abl (23), and provide direct evidence that Aph2 is a PAT, with PLN as one substrate, and that Aph2 is essential

for embryonic/postnatal survival, eye development, proper embryonic heart development, and in vivo cardiac function. Some of the heart phenotypes can be rescued by PLN deficiency. Thus, Aph2-deficient mice represent an animal model for cardiomyopathy, whose pathogenesis involves defective PLN palmitoylation.

## Results

**Aph2 Is Essential for Mouse Embryonic/Neonatal Survival.** To determine the physiological function of Aph2, we generated an Aph2-deficient mouse line (Fig. 1A, Upper). Southern blot and PCR analysis confirmed the correct recombination driven by the targeting construct (Fig. 1A, Lower panels). Northern blot analysis of liver RNA of newborn pups, using Aph2 cDNA as the probe, demonstrated that Aph2<sup>-/-</sup> mice did not express Aph2 mRNA, whereas the heterozygous mice expressed about half of the level of wild-type animals (Fig. 1B), indicating that the targeted Aph2 gene is a null allele.

Heterozygous mice were phenotypically indistinguishable from wild-type mice up to 14 mo, but homozygotes arising from crosses between heterozygous mice only survived about 1 d after birth. The newborn Aph2<sup>-/-</sup> pups were slightly runted, were generally inactive, and showed difficulties in feeding (Fig. 1C). Genotyping more than 250 newborn pups revealed that the frequency of homozygous mice among the live births was 11% instead of the expected 25%, suggesting that some homozygotes died in utero. Indeed, we detected some underdeveloped homozygous embryos at day 10.5. This is consistent with the observation that Aph2 is expressed at higher levels in day 7 and day 11 embryos than at later stages (Fig. S1). These findings suggest that Aph2 plays an important role in early mouse development, growth, and survival.

## Significance

**The etiology of heart disorders remains poorly understood. In this paper, we show for the first time to our knowledge, that protein palmitoylation plays a critical role in heart structure and function. We demonstrate that Aph2 (Abphilin 2) is a palmitoyl transferase and identify phospholamban, a cardiomyopathy disease gene, as a substrate. Some of the heart defects of an Aph2<sup>-/-</sup> mouse can be rescued by phospholamban ablation. In addition, the present study reveals critical roles for protein palmitoylation in embryonic/postnatal survival and eye development.**

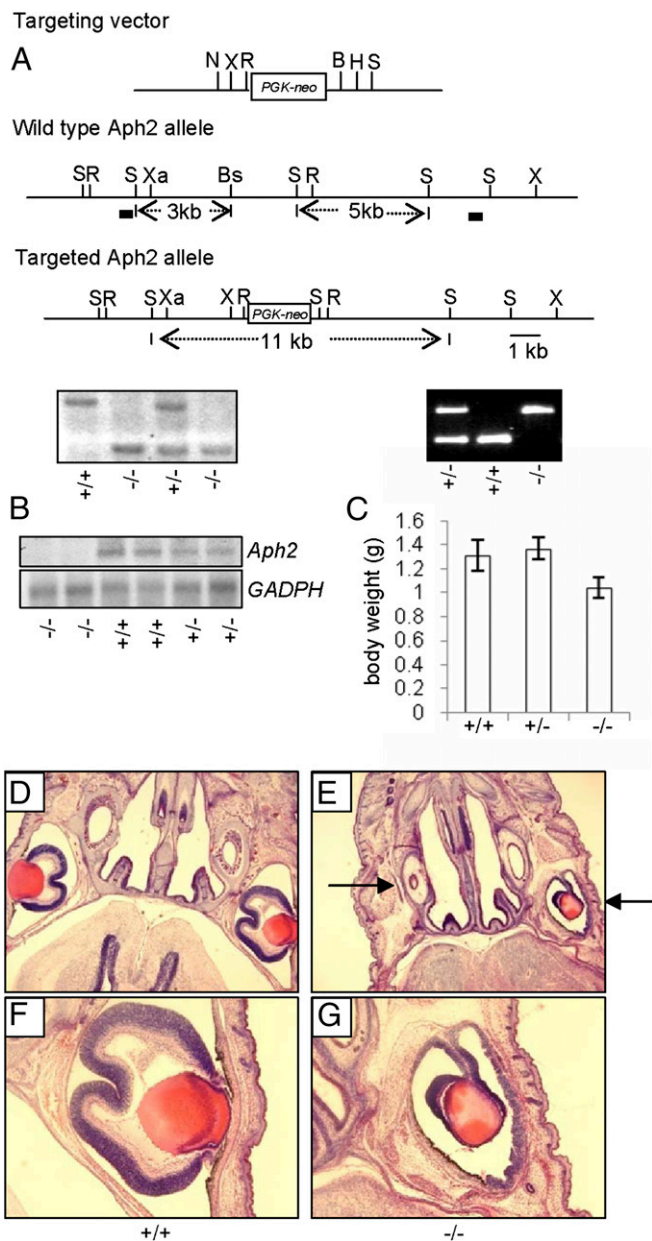
Author contributions: P.Z., L.H., Y.C., Y.-H.C., S.P.G., and B.L. designed research; T.Z., J.L., H.L., D.J., H.J., S.B., H.T., and M.S.-C. performed research; T.Z., J.L., and H.L. analyzed data; and S.P.G. and B.L. wrote the paper.

The authors declare no conflict of interest.

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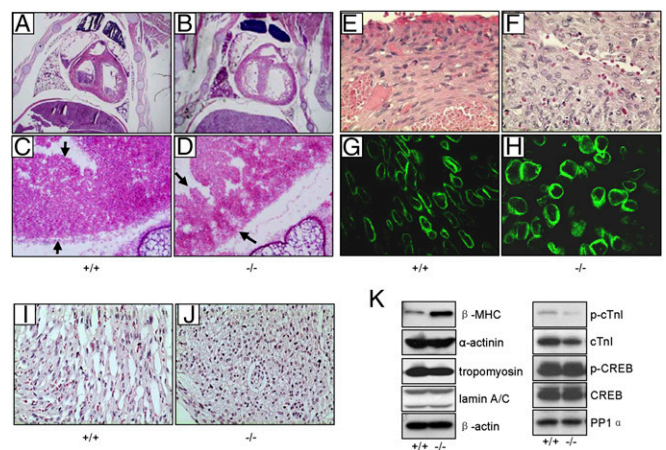
**Fig. 1.** Generation of *Aph2*<sup>-/-</sup> mice and histological analysis of the eye defects. (A) The disruption construct. A 5-kb right arm and a 3-kb left arm were subcloned into vector pGT-N28, flanking the *PGK-Neo* gene, which replaced exons 1 and 2 of *Aph2*. After recombination, the 5' coding sequence including start codon ATG was replaced by the *neo* gene. B, BamH1; Bs, BsaAI; H, HindIII; N, NotI; R, EcoRI; S, SacI; X, XhoI; Xa, XbaI. Southern blot (Lower, Left) and PCR (Lower, Right) results confirmed the disruption of the *Aph2* gene. (B) Northern blot analysis shows that *Aph2* mRNA was not expressed in knockout mice and was reduced to half in heterozygous mice. Total RNA was prepared from the liver tissues of newborn pups and analyzed by Northern blot using radio-labeled *Aph2* cDNA as a probe. (C) *Aph2*<sup>-/-</sup> pups show a slight decrease in body weight. (D and E) H&E staining of the day 1 pups showing the eye defects. (F and G) Higher magnification of the eyes of the *Aph2*<sup>-/-</sup> and control pups.

**Eye Defects of *Aph2*<sup>-/-</sup> Pups.** To explore the *in vivo* phenotype of *Aph2*<sup>-/-</sup> mice, we performed histological examination of the major organs in newborn pups. No gross abnormalities were observed in liver, kidney, spleen, or lungs, even though *Aph2* is expressed in all those organs (Fig. S1). However, some *Aph2*-deficient pups and embryos (~55%) were found to have eye malformation (Fig. 1 D

and E), with one or two eyes missing or underdeveloped, accompanied by structural malformation of cornea and retina (Fig. 1 F and G). *Aph2* is highly expressed in mouse eyes (Fig. S1). These results indicate that *Aph2* plays an important role in eye development, which awaits further investigation.

**Heart Defects of *Aph2*<sup>-/-</sup> Pups.** Our histological study also revealed profound aberrations in *Aph2*<sup>-/-</sup> hearts, where *Aph2* is expressed (Fig. S1). *Aph2*<sup>-/-</sup> mice showed thinner and enlarged ventricular walls (Fig. 2 A–D), cardiomyocyte disarray, and abnormal nucleus morphology (Fig. 2 E and F). It has been established that cell death, especially apoptosis, plays an important role in the etiology of heart diseases (24, 25). However, TUNEL assay revealed very few apoptotic cells on sections of WT or *Aph2*<sup>-/-</sup> hearts, with no obvious difference between the mutant and control mice. Nor did we observe a significant difference in the percentage of proliferating cells between control and *Aph2*<sup>-/-</sup> hearts (Fig. S2). The cellularity on *Aph2*<sup>-/-</sup> heart sections is similar to that of WT hearts (Fig. 1 E and F). It appears that the major defects in *Aph2*<sup>-/-</sup> hearts are disorganization and abnormal nuclear morphology of cardiomyocytes, which were confirmed by immunostaining of nuclear lamina proteins lamin A/C (Fig. 2 G and H). These defects were also observed in E14.5 *Aph2*<sup>-/-</sup> embryos (Fig. 2 I and J). These features are characteristic of cardiomyopathy, a disorder of the cardiac muscle.

Echocardiographic analysis of E18.5 embryos revealed that *Aph2*<sup>-/-</sup> mice had a marked reduction in cardiac output (32% of *Aph2*<sup>+/+</sup> mice), which was due to bradycardia (baseline heart rate down to 54% of *Aph2*<sup>+/+</sup> mice), a modestly reduced left ventricular internal diameter in diastole in mm (LVEDD), and decreased stroke volume (63% of *Aph2*<sup>+/+</sup> mice; Table 1, columns 1–5). The failure of the *Aph2*<sup>-/-</sup> mice to increase their stroke volume to maintain their cardiac output may suggest a decreased cardiac function related to the defects in cardiomyocyte morphology and organization. These findings indicate that *Aph2* may play a role in many aspects of heart structure and function.



**Fig. 2.** *Aph2* deficiency resulted in defects in the heart and cardiomyocyte morphology. (A and B) H&E staining of the day 1 pups showing the hearts. A, WT; B, *Aph2*<sup>-/-</sup> mouse. (C–F) Higher magnification of the left ventricular walls of the *Aph2*<sup>-/-</sup> and control pups, showing the abnormality of the cardiomyocytes. C and E, WT; D and F, *Aph2*<sup>-/-</sup> mouse. C and D, 40× magnification; E and F, 100× magnification. (G and H) Immunostaining for lamin A/C in the heart sections of control (G) and *Aph2*<sup>-/-</sup> (H) pups. (I and J) H&E staining of the heart section of E14.5 control (I) and *Aph2*<sup>-/-</sup> (J) embryos, showing the disorganization of the cardiomyocyte and the cellularity. (K) *Aph2*<sup>-/-</sup> hearts showed elevated β-myosin heavy chain; reduced protein levels of cTnI; normal levels of α-actinin, tropomyosin, lamin A/C, and PP1α; and normal phosphorylation of cTnI and CREB.



**Table 1. Echocardiographic examination of *Aph2*<sup>-/-</sup> and control wild-type embryos (day 18.5)**

Parameters	<i>Aph2</i> <sup>+/+</sup> PLN <sup>+/+</sup>	<i>Aph2</i> <sup>-/-</sup> PLN <sup>+/+</sup>	<i>Aph2</i> <sup>-/-</sup> / <i>Aph2</i> <sup>+/+</sup> , %	t test	<i>Aph2</i> <sup>-/-</sup> PLN <sup>-/-</sup>	<i>Aph2</i> <sup>+/+</sup> PLN <sup>-/-</sup>
HR, bpm	197 ± 66	106 ± 33	54*	0.000471	182.94 ± 66.68	180.95 ± 79.97
LVEDD, mm	0.96 ± 0.15	0.84 ± 0.11	88	0.057672	0.82 ± 0.11	0.86 ± 0.09
LVESD, mm	0.44 ± 0.10	0.42 ± 0.10	96	0.684315	0.45 ± 0.09	0.43 ± 0.08
SV, mL	0.86 ± 0.41	0.54 ± 0.24	63*	0.040768	0.48 ± 0.17	0.56 ± 0.18
FS-L, %	55 ± 8	51 ± 12	92	0.417243	45.59 ± 6.48	49.47 ± 7.86
RVEDD, mm	0.90 ± 0.13	0.87 ± 0.25	97	0.796784	0.73 ± 0.11	0.77 ± 0.09
RVESD, mm	0.51 ± 0.16	0.43 ± 0.11	86	0.254447	0.38 ± 0.10	0.38 ± 0.09
FS-R, %	45 ± 12	50 ± 9	112	0.288736	48.93 ± 8.66	51.08 ± 10.30
CO, mL/min	177 ± 115	57 ± 29	32*	0.001991	88.71 ± 49.45	96.92 ± 43.2

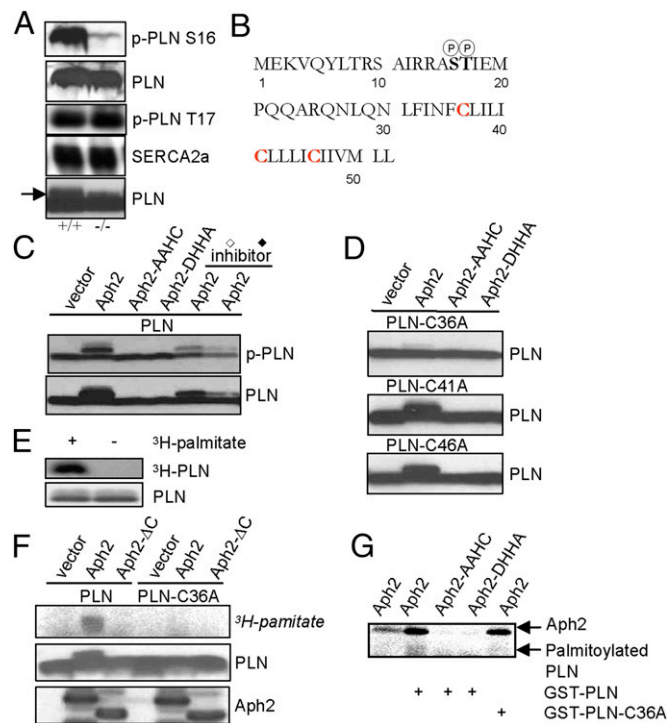
CO, cardiac output in mL/min; FS-L, fractional shortening = (LVEDD - LVESD)/LVEDD; FS-R, fractional shortening = (RVEDD - RVESD)/RVEDD; HR, heart rate in beats per minute; LVEDD, left ventricular internal diameter in diastole in mm; LVESD, left ventricular internal diameter in systole in mm; RVEDD, right ventricular internal diameter in diastole in mm; RVESD, right ventricular internal diameter in systole in mm; SV, stroke volume in mL. \**P* < 0.05.

***Aph2*<sup>-/-</sup> Hearts Show Reduced Ser16 Phosphorylation of PLN.** To gain insights into the mechanisms involved in the abnormal cardiac structure and function, we analyzed the expression of several cardiac muscle-specific structural proteins and Ca<sup>2+</sup> homeostasis regulators in the hearts of newborn *Aph2*<sup>-/-</sup> and WT pups (26). *Aph2* deficiency did not alter the protein levels of  $\alpha$ -actinin, tropomyosin, lamin A/C, or SERCA2a or the levels of active ERK1/2, JNK, p38 MAPK, Akt, mTOR, or Smad1/5/8 (Fig. 2K and Fig. S3A and B) (27). However, *Aph2*<sup>-/-</sup> hearts showed elevated levels of  $\beta$ -MHC and reduced levels of cardiac troponin I (cTnI) (Fig. 2K), a phenomenon observed in some heart disease animal models and human patients (28). More interestingly, phosphorylation of PLN at Ser16 (p-PLN) was markedly reduced in *Aph2*<sup>-/-</sup> hearts, whereas PLN phosphorylation at Thr17, a function of calcium/calmodulin-dependent protein kinases, and PLN total protein levels were not affected (Fig. 3A). PLN Ser16 phosphorylation was also reduced in *Aph2*<sup>-/-</sup> embryonic hearts (Fig. S3C).

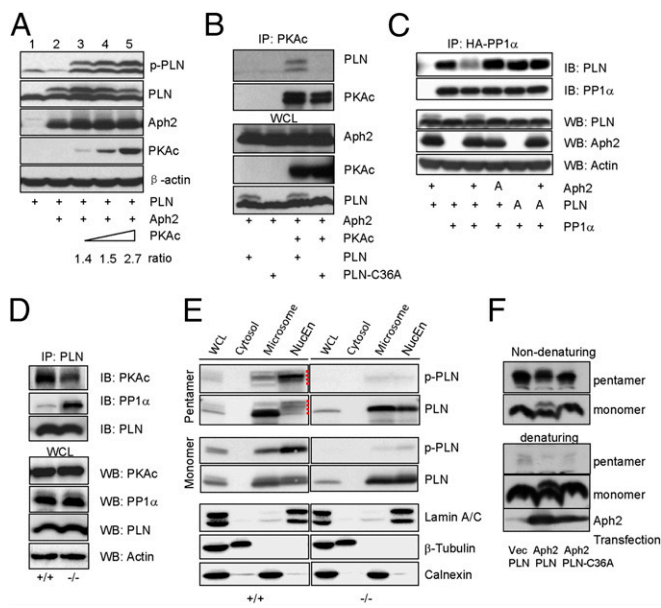
PLN phosphorylation at Ser16 is carried out by PKA and is removed by protein phosphatases, especially PP1 $\alpha$  (18, 19). PLN also forms a complex with PKA and PP1 $\alpha$  (18, 19, 29). Western blot analysis showed that the levels of PKA catalytic (PKAc) and regulatory subunits (PKA R I/II), PP1 $\alpha$ , PP2A (catalytic and structural subunits), and protein phosphatase inhibitor-1 (IPP-1) were not altered in *Aph2*<sup>-/-</sup> hearts (Fig. S4A and B). Assay of PKA activity in vitro revealed no difference between *Aph2*<sup>-/-</sup> and WT heart homogenates (Fig. S4C). Western blot analysis of the heart homogenate using an antibody that specifically recognizes PKA substrates revealed no significant defects in *Aph2*<sup>-/-</sup> mice (Fig. S4D) (30). These results indicate that PLN hypophosphorylation observed in *Aph2*<sup>-/-</sup> mice were not caused by compromised  $\beta$ -adrenergic receptor-PKA signaling. This conclusion is further supported by our observation that the phosphorylation of CREB and cTnI, two other PKA substrates, were not affected by *Aph2* deficiency (Fig. 2K and Fig. S3B). Moreover, the PP1 $\alpha$  activity was not altered in the heart homogenate of *Aph2*<sup>-/-</sup> mice (Fig. S4E). These findings suggest that the decrease in PLN Ser16 phosphorylation observed in *Aph2*<sup>-/-</sup> mice might be due to defects in PLN itself or PKA/PP1 $\alpha$  activation in a microenvironment.

***Aph2* Acts as a PAT and Modifies PLN at Cysteine 36.** In addition to PLN hypophosphorylation, we also noticed that a slow migrating band of PLN was absent in *Aph2*<sup>-/-</sup> hearts (Fig. 3A), suggesting that *Aph2* might be involved in another type of posttranslational modification of PLN, a 52-amino-acid protein (Fig. 3B). Coexpression of *Aph2* resulted in major and minor slow-migrating PLN bands (Fig. 3C), which were not affected by PLN mutations at Ser16 and Thr17 (Fig. S5A). Ectopic expression of *Aph2* in cardiomyocyte cell line HL-1 also resulted in a slow-migrating PLN band (Fig. S5B). In the course of our characterization of the function of *Aph2*, several *zf*-DHHC-containing proteins were shown to be

PATs (10). The following experiments indicate that *Aph2* is a PAT for PLN. Firstly, treatment of cells with 2-bromo-palmitic acid (2BP), a competitive inhibitor for PATs, diminished the slow-migrating PLN band (Fig. 3C). Secondly, the PLN modification could be removed by treatment with hydroxylamine, a chemical



**Fig. 3.** *Aph2* is a PAT for PLN. (A) Phosphorylation of PLN at Ser16 in newborn pups was reduced (see Fig. S3C for quantitation data). Arrow indicates the slow migration band of PLN. (B) The protein sequence of mouse PLN, with potential palmitoylation sites marked in red. (C) Coexpression of *Aph2* and PLN led to posttranslational modification of PLN, an activity depending upon the *zf*-DHHC domain. *Aph2* and PLN were expressed in COS7 cells and analyzed by Western blot. This modification needed the DHHC motif and could be inhibited by 2BP (open diamond, 50  $\mu$ M; filled diamond, 100  $\mu$ M). (D) The modification occurred on Cys36 of PLN. The three cysteine residues were individually mutated to alanine. (E) Endogenous PLN of primary murine cardiomyocytes was modified by palmitoylation. Cardiomyocytes were cultured in the presence of <sup>3</sup>H-palmitic acid for 24 h, and endogenous PLN was immunoprecipitated, fractionated onto SDS/PAGE gels, and exposed to X-ray films. (F) In vivo labeling assay with <sup>3</sup>H-palmitic acid showed that *Aph2* could modify PLN by palmitoylation, in a DHHC domain-dependent manner. (G) In vitro palmitoylation assay showed that *Aph2* could directly palmitoylate PLN at Cys36 in a DHHA motif-dependent manner.

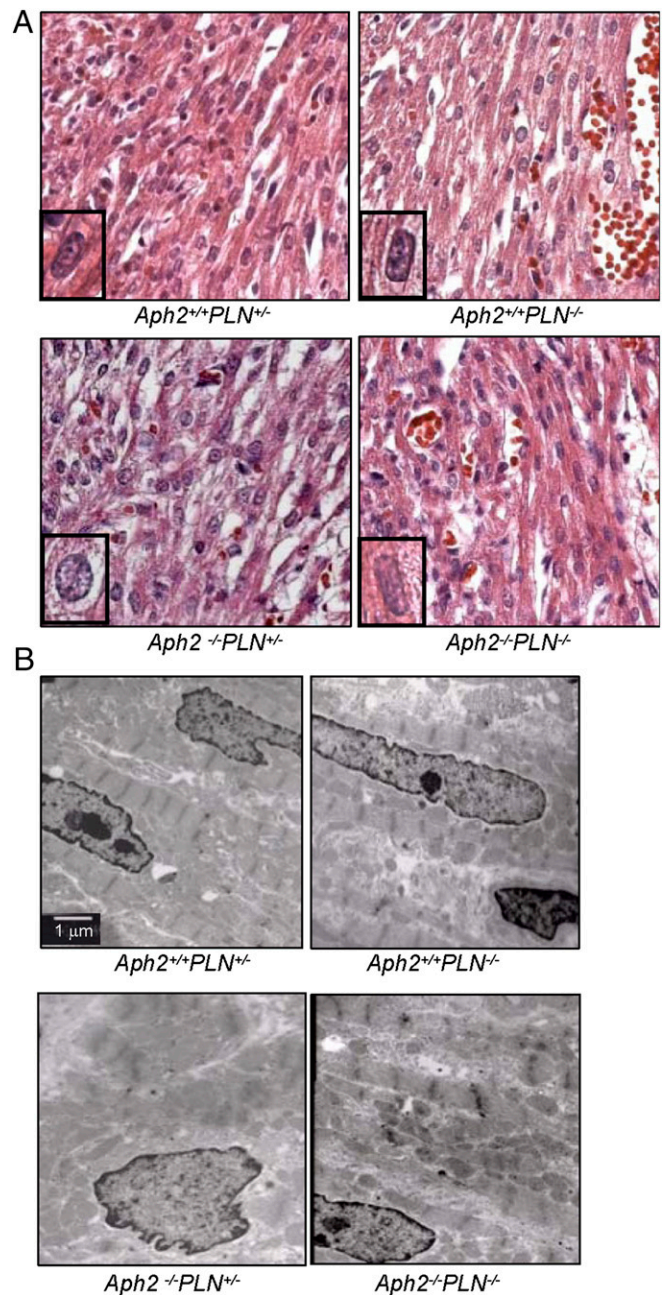


**Fig. 4.** Palmitoylation altered the interaction between PLN and its partners and pentamer formation. (A) Ectopically expressed PKAc preferred palmitoylated PLN as a substrate and showed an inhibitory effect on PLN palmitoylation. PKAc, Aph2, and PLN constructs were transfected into COS7 cells, and the whole-cell lysate (WCL) was fractionated onto SDS/PAGE gel. Then they were analyzed by Western blot. The ratios of p-PLNpal/PLNpal to p-PLNnon-pal/PLNnon-pal were shown at the bottom of the panel. (B) Palmitoylated PLN showed higher affinity for PKAc in coimmunoprecipitation experiments. The ectopic expression levels of Aph2, PKAc, and PLN in WCL were checked by Western blot. Immunoprecipitation of PKAc was able to bring down more palmitoylated PLN than nonpalmitoylated PLN. (C) Palmitoylated PLN showed a decrease in interaction with PP1 $\alpha$ . (D) Aph2 deficiency led to decreased PLN–PKAc interaction and increased PLN–PP1 $\alpha$  interaction in vivo. (E) Aph2 deficiency led to PLN hypophosphorylation and loss of three slower migrating forms of pentamers. Four hearts from newborn pups were pooled, and the homogenates were fractionated into cytosolic ( $\beta$ -tubulin as a marker), microsomal (Calnexin as a marker), and NucEn (Lamin A/C as markers) portions. PLN and p-PLN were determined by Western blot under nondenaturing conditions. The different migrating forms of PLN pentamers were designated as bands 1–4. (F) Palmitoylated PLN seemed to be present mainly in pentamers (Left panel). PLN molecules modified by Aph2 were analyzed by Western blot analysis under denaturing and nondenaturing conditions.

that can remove protein palmitoylation (Fig. S5C). Thirdly, PLN modification by Aph2 requires the *zf*-DHHC domain, as deletion of the DHHC domain ( $\Delta$ C) or mutation of DHHC to DHHA or AAHC abolished PLN modification (Fig. 3C and Fig. S5A). The closest paralog of Aph2, DHHC6, did not modify PLN (Fig. S5D), indicating a specific role of Aph2. Fourthly, PLN modification occurred mainly on cysteine 36 (Cys36). PLN has three cysteine residues at 36, 41, and 46 (Fig. 3B), but only the C36A mutation affected the modification (Fig. 3D). Finally, endogenous PLN from mouse cardiomyocyte cultures in the presence of <sup>3</sup>H palmitic acid was radio-labeled, suggesting that PLN is palmitoylated in vivo (Fig. 3E). To validate that Aph2 is a PAT, cells expressing Aph2 and PLN were labeled with <sup>3</sup>H-palmitate, and PLN was immunoprecipitated and fractionated on a SDS/PAGE gel, which was dried and exposed to X-ray film. Coexpression of Aph2, but not Aph2- $\Delta$ C, led to palmitoylation of PLN on Cys36 (Fig. 3F). In an in vitro assay, purified Aph2 was able to palmitoylate recombinant GST-PLN on Cys36 in the presence of <sup>14</sup>C-palmitoyl-CoA (Fig. 3G). Moreover, Aph2 could be self-palmitoylated as well, whereas DHHA and AAHC mutants could not (Fig. 3G). Taken together, these results indicate that Aph2 is a bona fide PAT for PLN. This palmitoylation could be enhanced by higher concentrations of palmitic acid but not

by adrenergic stimulation (Fig. S5E and F), suggesting that PLN palmitoylation is not dynamically regulated by adrenergic signaling.

**PLN Palmitoylation Differentially Alters Its Interaction with Partners.** We also found that Aph2 and PLN could form a complex. When the two were coexpressed, PLN was found to be associated with myc-tagged Aph2 immunoprecipitated by anti-myc antibodies. Conversely, Aph2 was present in the complex with PLN immunoprecipitated by anti-PLN antibodies (Fig. S6A–C). This



**Fig. 5.** PLN ablation rescued the structural phenotypes of *Aph2*<sup>-/-</sup> heart. (A) The histomorphological defects of *Aph2*<sup>-/-</sup> heart phenotypes were restored by PLN ablation. Shown is the H&E staining of the heart sections of newborn pups of *Aph2*<sup>+/+</sup>PLN<sup>+/+</sup>, *Aph2*<sup>+/+</sup>PLN<sup>-/-</sup>, *Aph2*<sup>-/-</sup>PLN<sup>+/+</sup>, and *Aph2*<sup>-/-</sup>PLN<sup>-/-</sup>. Insets in the four panels are representative images of the nucleus of cardiomyocytes. (B) Electronic microscope images of the heart sections of *Aph2*<sup>+/+</sup>PLN<sup>+/+</sup>, *Aph2*<sup>+/+</sup>PLN<sup>-/-</sup>, *Aph2*<sup>-/-</sup>PLN<sup>+/+</sup>, and *Aph2*<sup>-/-</sup>PLN<sup>-/-</sup> mice.



interaction does not require the *zf*-DHHC motif (Fig. S6 A–C). Moreover, Aph2 and PLN were also colocalized on the ER and nucleus envelope (NucEn) when expressed in COS7 cells (Fig. S6 D–F). We tried several times to raise anti-Aph2 antibodies but failed, probably due to the unique topology of this protein (four transmembrane domains). We were thus not able to perform a co-IP experiment with endogenous Aph2 and PLN. The complex formation between Aph2 and PLN might facilitate PLN palmitoylation or affect the complex formation between PLN and PKA, PP1 $\alpha$ , or SERCA2a.

As expected, coexpression of the PKAc subunit enhanced PLN phosphorylation at Ser16 (Fig. 4A), and more palmitoylated PLN molecules were phosphorylated than nonpalmitoylated PLN when normalized to PLN protein levels, suggesting that palmitoylated PLN is a better substrate. Ectopic expression of PKA also slightly inhibited PLN palmitoylation probably due to its competition with Aph2 for PLN interaction (Fig. 4A). Coimmunoprecipitation assays indicated that palmitoylated PLN had a higher affinity for PKA and a reduced affinity for PP1 $\alpha$  (Fig. 4 B and C), which were confirmed by coimmunoprecipitation assays of endogenous proteins from mouse hearts (Fig. 4D). These results suggest that one function of the palmitoylation is to differentially modulate the interaction between PLN and its kinase and phosphatase partners and thus promote PLN Ser16 phosphorylation. A similar case has been reported in which palmitoylation of adrenergic receptor 2 beta enhanced its phosphorylation by PKA (31).

**Palmitoylation Promotes PLN Pentamer Formation.** Fractionation experiments, following a widely used protocol (32), confirmed that PLN is always in the membrane fraction, independent of palmitoylation status, and is present in both microsome and NucEn fractions, but showed that the palmitoylated PLN molecules were mainly localized at the NucEn (Fig. S7). The NucEn-localized PLN molecules (monomers and pentamers) were found to be more heavily phosphorylated on Ser16 than microsome-localized PLN (Fig. 4E). This may contribute to PLN hypophosphorylation in *Aph2*<sup>-/-</sup> heart as well.

PLN pentamers showed several forms (bands 1–4, from bottom to top) when analyzed under nondenaturing conditions, with microsome fraction showing a dominant band 1 and a minor band 4 and the NucEn showing all four bands. Surprisingly, these forms of PLN were not evenly phosphorylated on Ser16, with band 3 (NucEn) heavily phosphorylated and band 1 (microsome) the least phosphorylated (Fig. 4E). In *Aph2*<sup>-/-</sup> mice, heart samples showed mainly a band 1 pentamer that was hypophosphorylated, suggesting that palmitoylation might regulate assembly/disassembly of different forms of pentamers, as bands 2 and 4, which are not usually phosphorylated, were absent in *Aph2*<sup>-/-</sup> heart. This notion was substantiated by the observation that palmitoylated PLN tends to be present in pentamers, as 18% of the monomeric PLN was palmitoylated under nondenaturing conditions, whereas about 36% of the monomeric PLN was palmitoylated under denaturing conditions (Fig. 4F).

**Rescue of Heart Phenotypes in *Aph2*<sup>-/-</sup> Mice by PLN Ablation.** The above results predict that *Aph2* deficiency would lead to PLN hypophosphorylation. This was validated with inhibition of PATs with 2BP in mouse (Fig. S8A). We wanted to test whether Aph2 deficiency affects intracellular calcium transient using fluorescence imaging. Unfortunately, primary *Aph2*<sup>-/-</sup> cardiomyocytes isolated from neonatal pups were highly susceptible to the toxicity of calcium chelators such as Fura-2/AM. We then used 2BP to inhibit PATs in cardiomyocytes and found that this severely diminished Ca<sup>2+</sup> transients in primary murine cardiomyocytes (Fig. S8B).

As Aph2 deficiency leads to an increase in inhibitory PLN molecules, we wanted to determine whether PLN mediated the heart defects in *Aph2*<sup>-/-</sup> mice. We crossed *Aph2*<sup>+/-</sup> and *PLN*<sup>-/-</sup> mice to generate *Aph2*<sup>-/-</sup>*PLN*<sup>-/-</sup> mice (33) and found that PLN

ablation rescued the histopathologic defects, such as cardiomyocyte disarray and nuclear malformation (Fig. 5A), and alleviated the heart dysfunction of *Aph2*<sup>-/-</sup> mice, especially bradycardia (Table 1). Electronic microscopy analysis revealed that *Aph2*<sup>-/-</sup> hearts showed sarcomere organization defects, which were rescued by PLN ablation (Fig. 5B). The sarcomere numbers in *Aph2*<sup>-/-</sup> hearts were decreased to 42% of the control, whereas the numbers in *PLN*<sup>-/-</sup> and *Aph2*<sup>-/-</sup>*PLN*<sup>-/-</sup> hearts were 92% and 87% of the control, respectively. The genetic evidence suggests that the roles of Aph2 in cardiogenesis are at least partially mediated by PLN. Although PLN ablation has been reported to improve cardiac functions in a few cardiomyopathy models (34–37), rescue of Aph2-null mice is unique in two aspects: *Aph2*<sup>-/-</sup> mice display an increase in inhibitory PLN molecules in vivo, and PLN ablation rescued not only some aspects of heart dysfunction but also the histopathologic defects. Although the double knockout mice failed to survive to day 2 and still showed eye defects, almost all double knockout embryos survived to birth (with frequency increased from 11% to the expected 25%), suggesting that altered PLN underlies the heart defects of *Aph2*<sup>-/-</sup> embryos and that heart dysfunction is likely the cause of embryonic lethality.

## Discussion

Our studies of the *zf*-DHHC16 knockout mouse line demonstrate a crucial role for protein palmitoyl transferase Aph2 in the regulation of embryonic/postnatal survival, eye development, cardiac function, and heart development, suggesting that protein palmitoylation plays important roles in these organs/tissues. Aph2-null mice also displayed overt phenotypes characteristic of cardiomyopathy including depressed cardiac function, histological abnormalities, and altered expression of  $\beta$ -MHC and cTnI, and bradycardia. This is the first study, to our knowledge, to show that protein palmitoylation plays important roles in in vivo heart function.

It is noteworthy that c-Abl, the bait used to identify Aph2 in a yeast two-hybrid system, is also involved in heart development. Embryonic and newborn pups deficient for c-Abl showed enlarged hearts due to hyperproliferation of cardiomyocytes (38). Moreover, the c-Abl inhibitor imatinib mesylate, a drug used to treat chronic myelogenous leukemia, has been reported to cause heart contractile dysfunction in human and mice (39). The function of the c-Abl–Aph2 interaction in heart development and function warrants further investigation.

Our in vitro and in vivo studies suggest that Aph2 is a PAT, with PLN as a substrate. Mutations in PLN, Lys39stop, Arg9Cys, and Arg14del are associated with the development of cardiomyopathy (30, 40–42). This study identifies Aph2 as a PLN interacting partner and a PLN modifying enzyme. PLN palmitoylation at Cys36 differentially regulates its interaction with PKA and PP1 $\alpha$  and alters Ser16 phosphorylation, suggesting that PLN palmitoylation regulates its phosphorylation. Our genetic evidence shows that Aph2's roles in heart function are at least partially mediated by PLN. Interestingly, PLN deficiency also rescued the bradycardia phenotype of the *Aph2*<sup>-/-</sup> mouse, suggesting that PLN may play a role in cardiac pacemaker under certain pathological conditions. The fact that PLN deficiency rescued the heart phenotypes and embryonic lethality but not eye defects or postnatal survival of *Aph2*<sup>-/-</sup> pups suggests that Aph2 has other important substrates.

## Materials and Methods

**Generation of the Mice.** The first and second exons of the *Aph2* gene were replaced by PGK-neo cassette in the targeting vector (SI Materials and Methods), which was introduced into mES w9.5 to generate the mice. Mice were handled following the recommendations in the National Research Council Guide for the Care and Use of Laboratory Animals (43), with the protocols approved by the Institutional Animal Care and Use Committee of Shanghai, China [SYXK(SH)2011-0112].

**PAT Activity Assays.** For in vivo assay, Aph2 and PLN were expressed in COS7 cells in the presence of 0.1 mCi/ml  $^3\text{H}$ -palmitic acid (NEN). Then PLN was immunoprecipitated, separated on SDS/PAGE gels, and radiographed. For in vitro assay, Aph2 was expressed in COS7 cells and purified by immunoprecipitation, which was mixed with GST-PLN in the presence of  $^{14}\text{C}$ -palmitoyl CoA for 1 h at 30 °C. PLN was analyzed as described above and in *SI Materials and Methods*.

Immunoprecipitation and Western blot were carried out as previously described (23).

Histological analysis and electronic microscopy are carried out as described previously and in *SI Materials and Methods* (38).

Echocardiographic examination was performed as described in *SI Materials and Methods*.

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