The flip side of cardiolipin import

To the Editor:

We read with interest the paper by Ray and colleagues[1] reporting that elevated cardiolipin levels during pulmonary infection impair lung and lung surfactant function. They suggest that Atp8B1 is a cardiolipin importer that helps clear this lipid from lung surfactant and that it contains a 40-amino-acid cardiolipin-binding domain that reduces lung injury when introduced into infected lungs. We do appreciate the inference that elevated cardiolipin contributes to the etiology of pneumonia and the observation that Atp8B1 overexpression improves lung function after experimental pneumonia. However, the mechanism of pulmonary cardiolipin accumulation and the role of Atp8B1 therein is not illuminated by the experiments of Ray and colleagues[1].

Atp8B1 deficiency not only causes progressive familial intrahepatic cholestasis type 1 but also extrahepatic symptoms arising from effects on the apical membranes of epithelial cells[2]. For instance, Atp8B1-deficient hepatocytes are prone to apical membrane damage resulting from extraction of membrane constituents by hydrophobic bile salts[3–5], and stereocilia and cochlear hair cells suffer from progressive degeneration probably as a result of impaired Ca^{2+}-ATPase (PMCA2) activity[6]. *In vitro*, Atp8B1-deficient Caco-2 cells show loss of microvilli and reduced membrane protein expression[7]. Collectively, these data support a role for Atp8B1 in preserving a stable apical membrane environment and suggest that enhanced pulmonary cardiolipin in Atp8B1 deficiency may result from damaged apical membranes in lung epithelia.

Ray and colleagues suggest that Atp8B1 is a cardiolipin transporter, on the basis of measurements of cardiolipin internalization[1]. To be transported, cardiolipin must traverse the aqueous space to reach the membrane. The authors used fluorescently labeled nitrobenzoxadiazole (NBD)-cardiolipins with four long fatty acid side chains. Such molecules are essentially insoluble in water, and thus are unable to partition into membranes. If the solubility of NBD-cardiolipin were unexpectedly high enough to permit exchange between membranes, the probe transported to the cytosolic leaflet would exchange into interior membranes. In Figure 4a of Ray *et al.*[1], there is no sign of such redistribution, suggesting that the cardiolipin remains trapped at the external surface. Because cardiolipin is a nonbilayer lipid, which prefers the so-called inverted-hexagonal phase, it has fusogenic properties[8], so that cardiolipin at the cell surface may result from fusion and not transport. Together, the experiments provide no clear evidence for cardiolipin transport into cells, and it therefore remains unclear that Atp8B1 can be described as a cardiolipin importer.

The isolation of the cardiolipin-binding domain peptide (CBD) from Atp8B1 might seem to confirm the idea that Atp8B1 is a cardiolipin importer. Atp8B1 is a P-type ATPase, and structural studies have shown that these transporters combine transmembrane domains, which bind the transported substrate, with three cytoplasmic domains that hydrolyze ATP to drive transport[9,10]. The CBD is a hydrophilic amino acid sequence in the ATP-hydrolyzing domain in the aqueous cytoplasm, and it cannot be reached by cardiolipin molecules that must be imported from the external membrane leaflet, where they are anchored by their four fatty acid side chains.

The data presented by Ray and colleagues[1] do not justify the conclusion that Atp8B1 is a cardiolipin transporter. However, the discovery of excess cardiolipin in lung surfactant is noteworthy as cardiolipin is normally confined to the mitochondrial inner membrane, and it thus will be of interest to discover how the cardiolipin reaches the surface. The mechanisms behind the beneficial effects of CBD administration in pulmonary infection will await further investigation.

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COMPETING FINANCIAL INTERESTS
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Ray *et al.* reply:

In their correspondence, Paulusma and colleagues[1] question whether Atp8B1 functions in cardiolipin import in lung epithelia. Of note, the natural substrate for Atp8b1 has not been conclusively identified. Unlike many studies using functional assays showing phosphatidylserine flippase (out-to-in) activity of Atp8b1 and its yeast homologs (for example, Dnf2p; ref. 2), we have used both functional and biochemical approaches to map a cardiolipin binding domain (CBD). With regard to nitrobenzoxadiazole (NBD)-cardiolipin used in our studies[3], we optimized solubility of this probe in medium by including apoprotein-containing Infasurf (5%). We show that NBD-cardiolipin is internalized in mouse embryonic cells[4] and in lung epithelia where it is detected within lysosomes but not mitochondria (**Fig. 1**). We also observe that ectopically expressed Atp8b1 increases NBD-cardiolipin uptake in lung epithelia (**Fig. 2**).