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Liver Disease without Flipping: New Functions of ATP8B1, the Protein Affected in Familial Intrahepatic Cholestasis Type 1

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Progressive Familial Intrahepatic Cholestasis Type 1 (PFIC1; formerly called Byler's disease and Greenland-Eskimo familial cholestasis) was identified as a chronic intrahepatic cholestasis associated with coarse granular bile in patients with elevated serum bile acids but normal gamma glutamyltranserase (GGT) levels.[1] The gene defect in patients with PFIC1 had been mapped to chromosome 18 in the same region where a similar but milder disease phenotype, Benign recurrent intrahepatic cholestasis (BRIC), was localized. A combined search for the two disease loci identified a P-type ATPase, gene symbol *ATP8B1*, as the defective gene for both PFIC1 and the Type 1 form of BRIC.[2] ATP8B1 is localized on the canalicular membrane of hepatocytes, but is also expressed at the apical membrane of polarized epithelial cells in many other tissues including the pancreas, small intestine, urinary bladder, stomach, and prostate. While cholestasis remains the clinical hallmark of this disorder, ATP8B1 deficiency is also associated with a variety of extrahepatic features including growth retardation, diarrhea, pancreatitis, hearing loss, chronic respiratory problems, and an abnormal sweat composition.[3–5] Notably, many of these extrahepatic symptoms persist after orthotopic liver transplantation.[6]

The positional cloning of ATP8B1 told us "the who" and since that time, investigators have also sought to understand "the what" and "the why": What does ATP8B1 do, and why does ATP8B1 deficiency result in cholestasis and a constellation of extrahepatic symptoms? In comparison to forms of inherited liver disease associated with the canalicular bile salt export pump (BSEP; *ABCB11*) or phosphatidylcholine transporter (MDR3; *ABCB4*), ABC transporters with well-characterized functions, understanding the molecular pathogenesis of ATP8B1 deficiency has proven especially difficult. The study by Verhulst and colleagues in the current issue of HEPATOLOGY brings us closer to answering these questions by identifying possible new roles for ATP8B1 in the regulation of apical membrane protein expression and formation of the microvillus.

P-type ATPases are distinct from ABC transporters and constitute a large family of multispan transmembrane proteins that includes pumps such as the Na/K-ATPase and the copper-transporting Wilson disease gene product (ATP7B). ATP8B1 is a member of the P4-subfamily of P-type ATPases (P4-ATPases), a distinct group that comprises 14 members in humans and requires interaction with an accessory factor, members of the CDC50 protein family, for trafficking from the endoplasmic reticulum and targeting to the proper cellular

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membranes.[7] The P4-ATPases are thought to function as membrane lipid flippases to mediate the inward translocation of aminophospholipids such as phosphatidylserine (PS) and phosphatidylethanolamine (PE) from the outer (exofacial) to inner (cytoplasmic) leaflets of the plasma membrane.[7] Eukaryotic cells synthesize a wide variety of lipid species that are distributed in a non-random fashion between subcellular organelles and even between the two leaflets of individual membranes, an arrangement with important functional consequences. Much of what we understand regarding the role of P4-ATPases in creating and maintaining the aminophospholipid asymmetry in membranes comes from studies in yeast.[8] P4-ATPase-deficient yeast strains exhibit a block in the uptake of fluorescent phospholipid analogues, a loss of plasma membrane lipid asymmetry, and increased exposure of PS and PE on the outer leaflet of the plasma membrane, phenotypes consistent with their proposed flippase function. However as a consequence of, or perhaps in addition to, their aminophospholipid flippase activity, P4-ATPases also function as part of the vesicle, protein, and sterol trafficking machinery operating between the trans-Golgi network and plasma membrane.[9]

The function of P4-ATPases in mammalian cells is also being explored to further our understanding of their role in human disease.[7] In mammalian cells, heterologous expression of human ATP8B1 along with CDC50 proteins also stimulated aminophospholipid flippase activity and reduced the PS content of the outer leaflet of the plasma membrane.[10] These and other findings led to the general hypothesis that ATP8B1 deficiency leads to cholestasis as a result of phospholipid randomization and dysfunction of the canalicular membrane of the hepatocyte. In agreement with this concept, reducing ATP8B1 expression in rat hepatocytes decreased BSEP activity, increased fluorescent PS loss from the canalicular membrane, and was associated with increased canalicular membrane damage in the presence of bile acids.[11] *In vivo* results from Atp8b1-deficient mice demonstrated an increased loss of PS into bile, a reduced cholesterol-to-phospholipid ratio of the canalicular membrane, an increased sensitivity of the canalicular membrane to the detergent action of hydrophobic bile acids, and decreased canalicular transporter activity.[12, 13] Additional support for a specialized membrane defect comes from a recent study that explored the mechanisms responsible for hearing loss in patients with ATP8B1 deficiency. Atp8b1 is abundantly expressed at the stereociliar membrane of the cochlea hair cells in the inner ear. Atp8b1 deficiency in mice was associated with the loss of stereocilia and progressive degeneration of cochlear hair cells, leading to sensorineural hearing loss.[5] However, in light of the complex role of P4-ATPases in vesicle and lipid trafficking and reports of alterations in cell signaling or activation of the farnesoid X receptor (FXR) with ATP8B1 deficiency in hepatocyte or enterocyte models, many questions remain regarding how loss of ATP8B1 leads to the spectrum of hepatic and extrahepatic disease.[14–16]

In the study published in this issue of HEPATOLOGY, Verhulst and colleagues investigated the impact of blocking ATP8B1 expression on the aminophospholipid flippase activity of the apical brush border membrane as well as on the functional organization of polarized Caco-2 cells. Using biochemical and microscopic approaches, differentiated Caco-2 cells were shown to express abundant amounts of ATP8B1 protein, which was localized to the apical brush border membrane. The cells also expressed the mRNA for CDC50A, the interacting protein required for plasma membrane localization of ATP8B1. Short hairpin RNA constructs were introduced into the cells to block ATP8B1 expression. Despite significant reductions in ATP8B1 protein expression, there was no measurable effect on aminophospholipid transport or the asymmetric distribution of aminophospholipids across the apical brush border membrane. Surprisingly, knockdown of ATP8B1 was associated with dramatic morphological changes in the apical brush border membrane. Microvilli were sparse or aberrant and F-actin bundles that lie at the core of the microvilli were largely absent. In contrast to the microvilli, the tight junctions and desmosomes appeared well

developed and unaffected. Biochemically, knockdown of ATP8B1 expression was associated with decrease in the expression of apical membrane proteins such as alkaline phosphatase, aminopeptidase N, and sucrase-isomaltase. This was not due to a generalized cell stress such as activation of the unfolded protein response, secondary to depletion of ATP8B1. Although changes in the assembly of the apical brush border might be expected to decrease apical membrane protein abundance by hastening degradation, there was no observed change in protein half-life but instead a selective decrease in the level of apical protein synthesis. Based on these findings, the authors propose that, in addition to its lipid flippase activity, ATP8B1 may function as a molecular scaffold in the apical membrane to recruit components of the actin cytoskeleton and other factors involved in microvilli formation.

A complex brush border apical membrane and the associated microvilli are an important component of many cell types such as the enterocyte, renal proximal tubule cell, placental syncytiotrophoblast, pancreatic acinar cell, and hepatocyte.[17] These specialized regions of membrane are enriched in proteins critical for the high volume secretory and absorptive functions of these cells. Morphologically, the enterocyte apical brush border is composed of numerous microvilli (up to ~3000 per cell), which are cylindrical membrane protrusions (~1 μm long by 0.1 μm in diameter) that are supported by polarized bundles of actin filaments. These projections are separated by invaginations of cell membrane situated between adjacent villi. With regard to apical membrane and protein trafficking, these smaller invaginations represent the dynamic region of the brush border membrane where transport vesicles are able to bud or fuse.[17] Where does ATP8B1 come into play in this process? The aminophospholipid flippase activity of ATP8B1 could certainly be playing a role in maintaining the unusual membrane composition and structure of the apical membrane.[12, 13] Alternatively, ATP8B1 may function through protein-protein interactions to recruit membrane or cytoskeletal elements involved in microvilli formation. Indeed, a recent proteomics-based strategy showed that the yeast P4-ATPase, Drs2p, interacted with a variety of proteins involved in membrane trafficking as well as phosphoinositide metabolism.[18] Notably, this loss of microvilli is similar to the disruption of the enterocyte apical brush border membrane organization associated with microvillus inclusion disease and gluten-sensitive enteropathies, and may account in part for the malabsorption and diarrhea observed in PFIC1.[19]

In summary, the study by Verhulst and colleagues has expanded our understanding of the repertoire of ATP8B1 functions in mammalian cells. In addition to affecting the phospholipid asymmetry of the apical membrane, ATP8B1 may also either directly or indirectly affect the formation and/or stabilization of the microvillar structures essential for the normal function of numerous polarized cells types.

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Abbreviations

ABC	ATP binding cassette
BRIC	Benign Recurrent Intrahepatic Cholestasis
GGT	gamma glutamyltransferase
PE	phosphatidylethanolamine
PFIC	Progressive Familial Intrahepatic Cholestasis

PS phosphatidylserine

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