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A functional classification of *ABCB4* missense variations causing progressive familial intrahepatic cholestasis type 3

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List of abbreviations: DMEM, Dulbecco's modified Eagle's medium; EndoH, endo-β-N-acetylglucosaminidase H; HEK, human embryonic kidney; ICP, intrahepatic cholestasis of pregnancy; LPAC, low-phospholipid associated cholelithiasis; NBD, nucleotide-binding domain; PFIC3, progressive familial intrahepatic cholestasis type 3; PNGaseF, peptide-N glycosidase F; RT-PCR, reverse transcription-polymerase chain reaction; wt, wild type.

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Abstract **(275 words)**

Progressive familial intrahepatic cholestasis type 3 (PFIC3) is caused by bi-allelic variations of ABCB4, most often (≥70%) missense. In this study, we examined the effects of twelve missense variations identified in PFIC3 patients. We classified these variations on basis of the defects thus identified and explored potential rescue of trafficking-defective mutants by pharmacological means. Variations were reproduced in the *ABCB4* cDNA, and the mutants, thus obtained, expressed in HepG2 and HEK293 cells. Three mutants were either fully (I541F and L556R) or largely (Q855L) retained in the endoplasmic reticulum, in an immature form. Rescue of the defect, *i.e.* increase in the mature form at the bile canaliculi, was obtained by cell treatments with cyclosporins A or C, and to a lesser extent, B, D or H. Five mutations with little or no effect on ABCB4 expression at the bile canaliculi, caused a decrease (F357L, T775M and G954S) or almost absence (S346I and P726L) of phosphatidylcholine secretion. Two mutants (T424A and N510S) were normally processed and expressed at the bile canaliculi but their stability was reduced. We found no defect of the T175A mutant, nor of R652G, previously described as a polymorphism. In patients, the most severe phenotypes appreciated by the duration of transplant-free survival, were caused by ABCB4 variants that were markedly retained in the endoplasmic reticulum, and expressed in a homozygous status. ***Conclusion***: ABCB4 variations can be classified as follows: nonsense variations (I), and on the basis of current findings, missense variations that primarily affect the maturation (II), activity (III) or stability (IV) of the protein, or have no detectable effect (V). This classification provides a first strong basis for the development of genotype-based therapies.

Introduction

Progressive familial intrahepatic cholestasis type 3 (PFIC3) is an autosomal recessive cholestatic liver disease, that occurs early in childhood (1). Evolution towards cirrhosis and liver failure before adulthood, is common. The clinical status of PFIC3 patients can be improved by the therapeutic bile acid, ursodeoxycholic acid, but liver transplantation is often the only effective therapy. PFIC3 is caused by variations in the *ABCB4* gene, which encodes an ABC (ATP-binding cassette) transporter, also called MDR3. ABCB4 is expressed at the bile canalicular membrane of hepatocytes, where it mediates the biliary secretion of phosphatidylcholine (PC) (2). ABCB4 defects result in low levels of biliary phospholipid, coexisting with normal bile salt concentrations in bile. As a consequence, PC biliary micelles are destabilized, allowing non-micellar detergent bile acids to cause epithelial damage and to promote the precipitation of biliary cholesterol. Thereby, ABCB4 defects can cause different types of biliary disorders, including PFIC3 as well as the low-phospholipid associated cholelithiasis (LPAC) syndrome (3), and intrahepatic cholestasis of pregnancy (ICP) (4). LPAC and ICP affect young adults, usually with a monoallelic status whereas PFIC3 patients are homozygous or compound heterozygous.

The ABCB4 predicted structure is typical of ABC transporters, with two cytoplasmic nucleotide-binding domains (NBDs) and two transmembrane domains, each containing six transmembrane segments. The NBDs are large domains, which include conserved sequences for ATP binding such as the Walker A and B domains, the signature sequence and the A-, D-, H-, and Q-loops (5). Since the identification of ABCB4-associated diseases, a growing number of *ABCB4* gene variations, located in various regions of the molecule, have been reported (1, 6-12). Attempts have been made to establish a correlation between the genotype and the phenotype of the patients (1, 11, 15). However, few studies have addressed the effect of mutations at the molecular level. Variations introducing premature stop codons lead to a

defect in protein expression, but more than 70 % of disease-causing *ABCB4* variations are missense variations and how such variations affect ABCB4, is generally unknown. In theory, missense mutations could affect the protein in several ways, either by modulating its expression at the canalicular membrane or by impairing its activity. We have shown that the I541F mutation identified in a PFIC3 patient caused misfolding and retention of the protein in the endoplasmic reticulum (16, 17). Subsequent studies have reported some mutations that reduced PC secretion or affected ABCB4 protein expression (12-14, 18). The aim of these studies was primarily to correlate ABCB4 mutations with the patients' phenotype.

Extending and beyond this goal, the present work aimed i) to determine the effects of a large number of *ABCB4* missense single-nucleotide variations responsible for PFIC3, ii) to classify these variations on the basis of the molecular defects thus identified, and iii) to propose clues for genetic-based therapeutic strategies. Twelve variations, distributed in different regions of the protein, were thus investigated in transfected cells, to determine their impact on the expression and activity of ABCB4 and the potential rescue of trafficking-defective mutants by pharmacological means.

Experimental procedures

Patients. Nine PFIC3 patients, eight of whom were previously reported (1, 7) were included in the present study (Table 1). *ABCB4* gene analysis was performed as previously described (1, 19).

Mutagenesis. The construction of the human wild type (wt) ABCB4, isoform A (NM_000443.3), in pcDNA3.1 vector was previously reported (16). Site-directed mutagenesis was performed using the Quik-Change II XL mutagenesis kit from Agilent Technologies (Massy, France). Except for I541F mutagenesis that we previously described (16), DNA primers used for ABCB4 mutagenesis were from Invitrogen-Life Technologies (Saint-Aubin, France) and Eurogentec (Angers, France) and are listed in Supplemental Table 1. To reproduce one double mutant (*i.e.* F357L and T775M) identified on the same allele in one patient (No. 7), mutations were done sequentially. All constructs were verified by automated sequencing.

Cell Culture and Transfection. Hepatocellular carcinoma, human (HepG2) cells and human embryonic kidney (HEK293) cells were grown in Dulbecco modified Eagle's medium (DMEM) (GE Healthcare Europe GmbH, Vélizy-Villacoublay, France), as previously described (17). Transfection with plasmids encoding ABCB4-wt or the mutants was performed using Turbofect (Fermentas France, Villebon-sur-Yvette), following the manufacturer's instructions. Stable expression was obtained after selection with 1mg/mL of G418 (GE Healthcare). For the experiments with HEK293 cells, plates were pre-coated with poly-L-lysine (Sigma-Aldrich, Saint-Quentin-Fallavier, France). Cyclosporins used for the

treatments were obtained from Tocris Bio-techne (Lille, France) and cycloheximide from Sigma-Aldrich.

Immunofluorescence and Confocal Microscopy. HepG2 or HEK293 cells were grown on coverslips and fixed with methanol at -20°C. Incubations with primary and secondary antibodies were performed as described (16). The monoclonal P3II-26 anti-ABCB4 antibody was obtained from Enzo Life Sciences (Villeurbanne, France). The polyclonal anti-occludin antibody and Alexa Fluor–labeled secondary antibodies were from Invitrogen-Life Technologies. Nuclei were stained with DRAQ5 (Cell Signaling, Danvers, MA). Confocal imaging was acquired with a Leica TCS SP2 laser scanning spectral system attached to a DMR inverted microscope with a 63/1.4 immersion objective. Digital images were analyzed using the on-line ScanWare software and processed with ImageJ and Photoshop softwares.

Electrophoresis and Immunoblotting. Electrophoresis and immunoblotting were performed as described (16), using the monoclonal P3II-26 anti-ABCB4 antibody and peroxidase-conjugated secondary antibodies from Rockland Immunochemicals (Gilbertsville, PA). Immunoblotting of β-actin with a monoclonal antibody (Sigma-Aldrich) was also performed as a loading control. Deglycosylation treatments with endo-β-N-acetylglucosaminidase H (EndoH; Roche Diagnostics, Basel, Switzerland) or peptide-N glycosidase F (PNGaseF; Roche Diagnostics) were previously described (16). Blot exposure times were within the linear range of detection, and signal intensities were quantified using ImageJ software. Protein concentrations were determined by Uptima bicinchoninic acid protein assay (Interchim, Montluçon, France).

Measurement of Phosphatidylcholine Secretion. PC secretion was measured in the culture medium of HEK293 cells, as described (20). Briefly, cells grown in 6-well plates were

transfected with the plasmids encoding wt or mutant ABCB4. Sixteen hours post-transfection, the medium was replaced by phenol red-free DMEM containing 0.5 mmole/L sodium taurocholate and 0.02% fatty acid–free bovine serum albumin (Sigma-Aldrich). After 24 hours, the medium was collected and lipids were extracted by chloroform/methanol/water partition. The organic phase was evaporated and resuspended in phosphate-buffered saline with 0.1% Triton X-100 (w/v). Measurement of PC content was based on the amount of choline released after phospholipase D treatment, using a fluorimetric assay. Fluorescence was read (λ_{exc}, 320 nm; λ_{em}, 404 nm) with a multiplate cytofluorimeter SpectraFluor from Tecan (MTX Lab Systems, Vienna, VA). Results were normalized to the expression levels of ABCB4-wt or of the mutants, which were quantified from immunoblots of the corresponding cell lysates.

Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-PCR). HEK293 cells were transfected with plasmids encoding ABCB4-wt or ABCB4 mutants. Forty-eight hours later, total RNA was extracted using the RNeasy Mini Kit from Qiagen (Courtaboeuf, France). Complementary DNA was synthesized from 1 µg of total RNA using random hexamer primers and 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies) for 1 hour at 37°C. Quantitative PCR was performed using the Platinum SYBR Green qPCR SuperMix-UDG (Life Technologies) on a Lightcycler 480 (Roche) device, as previously described (21), with actin as a reference gene. Primers are described in Supplemental Table 2.

Statistical Analysis. The Student's *t* test was used for comparisons. A *P* value of less than 0.05 was considered significant.

Results

ABCB4 variations and PFIC3 disease severity. Table 1 shows the *ABCB4* variations identified in PFIC3 patients of the present study and the durations of transplant-free survival, recorded to estimate disease severity in these patients (Table 1). The distribution of *ABCB4* variations, in the different protein domains, is shown in Figure 1. They were located in (or at the border of) transmembrane domains (S346I, Q855L and G954S), in the first intracellular loop (T175A), in the linker region (R652G) or in the first nucleotide-binding domain (I541F, L556R) of ABCB4 (Fig.1). The variation T424A was close to the Walker A motif, N510S was located between Walker A and the signature, and L556R laid within the Walker B motif. *In silico* analyses generally predicted that these variations would be deleterious, with the exception of the R652G variation, considered as a polymorphism (Supplemental Table 3). It was previously reported that the R652G variation was detected with the same frequency in 5 affected patients and in 50 unaffected unrelated healthy individuals (1).

Effect of ABCB4 mutations on cellular localization and expression of the protein at the plasma membrane. The mutated cDNAs reproducing the ABCB4 variations identified in PFIC3 patients, were transfected in polarized HepG2 cells and in non polarized epithelial HEK293 cells. The former derived from a human hepatocarcinoma form neo-bile canaliculi in culture and allow localization studies, whereas the latter are suitable for studies of transport activity. Forty-eight hours after transfection of HepG2 cells, immunofluorescence showed that ABCB4-wt was exclusively localized at the canalicular membrane (Fig. 2A), as previously shown (16). Most mutants (T175A, S346I, T424A, F357L, N510S, R652G, P726L, T775M and G954S), and the double mutant F357L/T775M also displayed apical predominant localization (Fig.2A). Two mutants (I541F and L556R) were fully intracellular, while the Q855L mutant was detected both at the bile canalicular membrane and intracellularly. In

HEK293 cells, the expression of the mutants was studied after selection of stable cell populations. As in HepG2 cells, most mutants were localized predominantly at the plasma membrane, except the I541F and L556R mutants, which were intracellular and the Q855L mutant, which was detected both at the plasma membrane and in the cytoplasm. Western blot analysis showed that the wt protein was expressed as a single band migrating with an apparent molecular weight of 160 kDa (Fig.3A). All mutants localized at the plasma membrane, displayed the same pattern of migration, except the S346I, which often showed a minor (<10%) form of lower M_r (140 kDa). The I541F and L556R mutants were found essentially under the fast-migrating form. A balance between the two forms was observed for the Q855L mutant. Further analysis by endoglycosidase treatment showed that the lower band was endoH sensitive and therefore, corresponded to the immature high mannose form found in the endoplasmic reticulum (Fig.3B), whereas the higher band was sensitive to N-glycosidase F only and consisted in the mature complex-glycosylated protein. These results indicated that nine of the mutations did not impair maturation or trafficking of the protein to the cell surface. Three mutations caused a maturation defect and retention in the endoplasmic reticulum. Two of them in particular, led to almost complete absence of the fully glycosylated form and of its expression at the plasma membrane.

Effect of Cyclosporins on the Rescue of Processing Defective ABCB4 Mutants. We previously showed that treatment with cyclosporin A partially rescued the I541F mutant, allowing its maturation and traffic to the plasma membrane (17). We therefore tested the effect of cyclosporin A and several analogs (cyclosporins B, C, D and H) on the rescue of the trafficking-defective mutants in stably transfected HEK293 cells (Fig. 4). Confluent cells were treated with each drug (5 µmole/L) for 24 hours. All cyclosporins, particularly cyclosporins A and C, markedly increased the amount of the mature form of the I541F,

L556R and Q855L mutants. Cyclosporins A and C also slightly increased the amount of ABCB4-wt. The rescue by cyclosporin A was further examined by immunofluorescence in HepG2 cells. This showed the appearance of the I541F and L556R mutants at the membrane of the bile canaliculi, and an increase of the Q855L mutant at the bile canaliculi (Fig.4C, to compare with Fig. 2A).

Effect of ABCB4 Mutations on Phosphatidylcholine Secretion. The activity of ABCB4 mutants was measured in HEK293 cells. The amount of PC released over 24 hours was corrected for the level of the mature protein expressed in the corresponding cell culture well. Cells transfected with the I541F or R556L mutants, which were intracellular, released almost no PC, as compared to the wt protein (Fig.5). Two other mutants (S346I and P726L) were almost inactive. The F357L, T775M, Q855L and G954S were only partially active. The double mutant F375L/T775M released very little PC, suggesting that the effects of the two mutations were additive. These results show that amongst the mutations expressed at the plasma membrane, six caused a defect in PC secretion. However, the T175A, T424A, N510S and R652G mutants apparently, were fully active.

Effect of Mutations on ABCB4 Protein Expression. The results above indicated that several mutations (T175A, T424A, N510S and R652G) apparently had no effect on the processing, membrane localization or activity of ABCB4. Moreover, two other mutations (T775M and G954S) had only moderate effect on ABCB4 function. We therefore studied whether these mutations induced defective protein expression, by comparing the levels of mRNA and protein expression in HEK293 cells. Forty-eight hours after transfection, RNA was extracted and ABCB4 mRNA was quantified by RT-PCR. ABCB4 protein was quantified on immunoblots after gel electrophoresis. Both the mRNA and protein levels of the T175A, R652G, T775M and G954S mutants were not different from those of wt (Fig.6A). By

contrast, the protein levels of the T424A and N510S mutants were significantly reduced as compared to those of wt, despite similar mRNA levels (Fig. 6A). These results suggested that the T424A and N510S mutations affected the stability of the protein. The stability of these mutants was thus compared to that of the wt protein by analyzing the decay after inhibition of protein synthesis by cycloheximide. Twenty-four hours after transfection, 25 µmole/L cycloheximide was added to the culture medium and the cells were harvested at time points 0, 4 and 18 hours. At time point 0, a large amount of the immature form both of ABCB4-wt and the mutants was detected (Fig.6B). This immature form disappeared at later time points, consistent with inhibition of protein synthesis. Between the 4 hour- and 18 hour-time points, the mature form of ABCB4-wt decreased by 30%, while those of T424A and N510S mutants decreased significantly more, *i.e.* by 55% and 62%, respectively (Fig.6C). These findings confirmed that the stability of the T424A and N510S mutants was reduced as compared to the wt protein.

Discussion

In this study, we report the effect of twelve PFIC3-causing missense variations reproduced into human ABCB4 cDNA. Based upon complete functional characterization, we propose to classify ABCB4 variations, depending on whether they affect the traffic, activity or stability of the protein (Table 2). We show a relation between this classification and the severity of the disease, estimated by the duration of transplant-free survival, and we bring clues for future targeted therapies in PFIC3.

Classifications already exist for variations in other genes. Such classifications display some similarities, but also differ from each other, depending on which gene is involved. To fit in with these classifications, we assigned class I to variations that cause defective synthesis, mainly nonsense and frameshift mutations, which were not investigated in the present study.

Class II includes variations that induce a maturation defect with retention in the endoplasmic reticulum. This is the case of the I541F and L556R mutants, which were almost fully expressed as immature forms, and of the Q855L mutant, which was only partially retained. Accumulation in the endoplasmic reticulum was already known for the I541F variation, previously shown to cause a folding defect (16, 17). The L556R variant seems to behave similarly. Both variations are located in the first NBD. Folding of this domain may be crucial to pass the quality control system of the endoplasmic reticulum. These variations, in the homozygous status appear to be particularly deleterious. Indeed, patients No. 1 and No. 2 who were homozygous for I541F and L556R respectively, underwent liver transplantation early in childhood (Table 1). The Q855L mutant, which was incompletely retained, was less deleterious as shown in patient No. 9, homozygous for this mutation, who did not require liver transplantation up to at least 25 years.

Class III includes variations, with little or no effect on maturation, that cause defective activity. A large number of variations studied here fell into this category. The S346I and P726L mutations caused a profound decrease in the activity, while three others (F357L, T775M and G954S) had less dramatic effects. Noteworthy is that variations, which affected ABCB4 function laid within or at the boundaries of transmembrane segments. The most deleterious (S346I and P726L), were located in the middle of the sixth (TM6) and seventh (TM7) transmembrane segments, respectively. It was shown in the closely related protein ABCB1, which transports amphiphilic drugs, that the substrate-binding pocket site of the transporter laid within the TMDs, including TM6 and TM7 (22- 23). Replacement of crucial residues in the transmembrane segments may thus prevent entry or translocation of the substrate.

Class IV includes variations that affect the stability of ABCB4. The T424A and N510S variants fit into this category. Both were fully processed and active. However, their stability

was decreased. The phenomenon was assessed by the difference between the level of specific mRNA and protein, and by the accelerated degradation after cycloheximide treatment. These two variations are located in the first NBD. They are not part of crucial motifs implicated in the binding or hydrolysis of ATP, but may alter the overall structure of the molecule and make it more susceptible to degradation. The severity of instability may vary between such variations. Patient No. 8 was heterozygous for the N510S mutation and did not require liver transplantation until at least the age of 19 years, although his second allele bore the P726L mutation, which strongly impairs ABCB4 activity (Table 1). Patient No. 4, who was heterozygous for the T424 mutation, required liver transplantation at the age of 13 years, suggesting that the intronic insertion he carried in a homozygous status was deleterious (Table 1).

Class V includes variants without detectable effect in our model system. The T175A and R652G variations affected neither the localization, nor the activity or stability of ABCB4. The R652G variation is reported in the nucleotide polymorphism database as a polymorphism (rs2230028), and the absence of effect in our cell models reinforces this conclusion. More surprising is the lack of effect of the T175A mutation. This variant has been reported in association with the LPAC syndrome (6, 9, 11), ICP (4, 25), and unexplained anicteric cholestasis (26). However, this mutation can also be found in healthy Caucasians (27, 28). Therefore its pathogenicity remains uncertain. It cannot be excluded that the effect of this mutation is minor and that it could be revealed when associated with another gene defect.

The classification of ABCB4 variations as proposed in the present work, provides the first strong basis for the development of genotype-based therapies for PFIC3, as each class of defect will justify a specific strategy. Class II misfolded mutants retained in the endoplasmic reticulum may be rescued by pharmacological chaperones. We have previously shown that the intracellular retention of the I541F mutant could be rescued by cyclosporin A (17). Here,

we show that cyclosporin A also allowed the L556R mutant to exit the ER and reach the bile canaliculi. Several other cyclosporins also rescued these mutants, the most effective being cyclosporins A and C. Cyclosporins likely have several effects. First they act as chaperones and facilitate folding. This effect was most visible on the I541F and R556L mutants, which underwent maturation under cyclosporin treatment. Second, they appear to stabilize the protein. Indeed, an increase in the mature protein was an effect clearly visible towards the wt protein, and to even greater extend towards the Q855L mutant. These results make cyclosporins or related molecules potential therapeutic alternatives. In patients who harbor variants with defective activity (class III) or defective stability (class IV) it may be worthwhile to increase ABCB4 expression, provided that the mutant is at least partially active. ABCB4 expression is controlled by the nuclear receptors Farnesoid Receptor (FXR) (29) and Peroxisome Proliferator-Activator Receptor α (PPARα) (30). Several drugs that target these receptors are known to up-regulate ABCB4 in the animal (31-34). It has also been shown that statins enhance ABCB4 expression in the rat (35, 36). Recently, clinical trials have shown that targeting nuclear receptors with the FXR agonist obeticholic acid (37), or with the PPARα agonist bezafibrate (38), was promising in the treatment of chronic liver diseases with poor response to UDCA. All these drugs are clinically approved and could be proposed as therapeutic alternatives for PFIC3, alone or in combination with UDCA therapy.

References

1. Jacquemin E, De Vree JM, Cresteil D, Sokal EM, Sturm E, Dumont M, et al. The wide spectrum of multidrug resistance 3 deficiency: from neonatal cholestasis to cirrhosis of adulthood. Gastroenterology 2001;120:1448-1458.

2. Falguières T, Aït-Slimane T, Housset C, Maurice M. ABCB4: Insights from pathobiology into therapy. Clin Res Hepatol Gastroenterol. 2014;38:557-63.

3. Rosmorduc O, Hermelin B, Poupon R. MDR3 gene defect in adults with symptomatic intrahepatic and gallbladder cholesterol cholelithiasis. Gastroenterology 2001;120:1459-1467.

4. Pauli-Magnus C, Lang T, Meier Y, Zodan-Marin T, Jung D, Breymann C, et al. Sequence analysis of bile salt export pump (ABCB11) and multidrug resistance p-glycoprotein 3 (ABCB4, MDR3) in patients with intrahepatic cholestasis of pregnancy. Pharmacogenetics 2004;14:91-102.

5. Zolnerciks JK, Andress EJ, Nicolaou M, Linton KJ. Structure of ABC transporters. Essays Biochem 2011;50:43-61.

6. Rosmorduc O, Hermelin B, Boelle PY, Parc R, Taboury J, Poupon R. ABCB4 gene mutation-associated cholelithiasis in adults. Gastroenterology 2003;125:452-459.

7. Degiorgio D, Colombo C, Seia M, Porcaro L, Costantino L, Zazzeron L, et al. Molecular characterization and structural implications of 25 new ABCB4 mutations in progressive familial intrahepatic cholestasis type 3 (PFIC3). Eur J Hum Genet 2007;15:1230-1238.

8. Poupon R, Barbu V, Chamouard P, Wendum D, Rosmorduc O, Housset C. Combined features of low phospholipid-associated cholelithiasis and progressive familial intrahepatic cholestasis 3. Liver Int 2010;30: 327-331.

9. Denk GU, Bikker H, LekanneDitDeprez RH, Terpstra V, van der Loos C, Beuers U, et al. ABCB4 deficiency: A family saga of early onset cholelithiasis, sclerosing cholangitis and cirrhosis and a novel mutation in the ABCB4 gene. Hepatol Res 2010;40:937-941.

10. Giovannoni I, Santorelli FM, Candusso M, Di Rocco M, Bellacchio E, Callea F, Francalanci P. Two novel mutations in African and Asian children with progressive familial intrahepatic cholestasis type 3. Dig Liver Dis 2011;43:567-570.

11. Poupon R, Rosmorduc O, Boëlle PY, Chrétien Y, Corpechot C, Chazouillères O, et al. Genotype-phenotype relationships in the low-phospholipid-associated cholelithiasis syndrome: a study of 156 consecutive patients. HEPATOLOGY 2013;58:1105-1110.

12. Andress EJ, Nicolaou M, Romero MR, Naik S, Dixon PH, Williamson C, Linton KJ. Molecular mechanistic explanation for the spectrum of cholestatic disease caused by the S320F variant of ABCB4. HEPATOLOGY 2014;59:1921-1931.

13. Kluth M, Stindt J, Dröge C, Linnemann D, Kubitz R, Schmitt L. A Mutation within the extended X loop abolished substrate-induced ATPase activity of the human liver ATP-binding cassette (ABC) transporter MDR3. J Biol Chem 2015;290:4896-4907.

14. Gordo-Gilart R, Andueza S, Hierro L, Martínez-Fernández P, D'Agostino D, Jara P, Alvarez L. Functional analysis of ABCB4 mutations relates clinical outcomes of progressive familial intrahepatic cholestasis type 3 to the degree of MDR3 floppase activity. Gut 2015;64:147-155.

15. Colombo C, Vajro P, Degiorgio D, Coviello DA, Costantino L, Tornillo L, et al. Clinical features and genotype-phenotype correlations in children with progressive familial intrahepatic cholestasis type 3 related to ABCB4 mutations. J Pediatr Gastroenterol Nutr 2011;52:73-83.

16. Delaunay JL, Durand-Schneider AM, Delautier D, Rada A, Gautherot J, Jacquemin E, et al. A missense mutation in ABCB4 gene involved in progressive familial intrahepatic cholestasis type 3 leads to a folding defect that can be rescued by low temperature. HEPATOLOGY. 2009;49:1218-1227.

17. Gautherot J, Durand-Schneider AM, Delautier D, Delaunay JL, Rada A, Gabillet J, et al. Effects of cellular, chemical, and pharmacological chaperones on the rescue of a trafficking-defective mutant of the ATP-binding cassette transporter proteins ABCB1/ABCB4. J Biol Chem 2012;287:5070-5078.

18. Degiorgio D, Corsetto PA, Rizzo AM, Colombo C, Seia M, Costantino L, et al. Two ABCB4 point mutations of strategic NBD-motifs do not prevent protein targeting to the plasma membrane but promote MDR3 dysfunction. Eur J Hum Genet 2014;22:633-639.

19. Gonzales E, Spraul A, Jacquemin E. Clinical utility gene card for: progressive familial intrahepatic cholestasis type 3. Eur J Hum Genet 2014;22.

20. Gautherot J, Delautier D, Maubert MA, Aït-Slimane T, Bolbach G, Delaunay JL, et al. Phosphorylation of ABCB4 impacts its function: insights from disease-causing mutations. HEPATOLOGY 2014;60:610-621.

21. Firrincieli D, Zúñiga S, Rey C, Wendum D, Lasnier E, Rainteau D, et al. Vitamin D nuclear receptor deficiency promotes cholestatic liver injury by disruption of biliary epithelial cell junctions in mice. HEPATOLOGY 2013;58:1401-1412.

22. Loo TW, Bartlett MC, Clarke DM. Transmembrane segment 7 of human P-glycoprotein forms part of the drug-binding pocket. Biochem J 2006;399:351-359.

23. Loo TW, Clarke DM. Mutational analysis of ABC proteins. Arch Biochem Biophys 2008;476:51-64.

24. Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R, et al. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. Science 2009;323:1718-1722.

25. Floreani A, Carderi I, Paternoster D, Soardo G, Azzaroli F, Esposito W, et al. Hepatobiliary phospholipid transporter ABCB4, MDR3 gene variants in a large cohort of Italian women with intrahepatic cholestasis of pregnancy. Dig Liver Dis 2008;40:366-370.

26. Ziol M, Barbu V, Rosmorduc O, Frassati-Biaggi A, Barget N, Hermelin B, et al. ABCB4 heterozygous gene mutations associated with fibrosing cholestatic liver disease in adults. Gastroenterology 2008;135:131-141.

27. Pauli-Magnus C, Kerb R, Fattinger K, Lang T, Anwald B, Kullak-Ublick GA, et al. BSEP and MDR3 haplotype structure in healthy Caucasians, primary biliary cirrhosis and primary sclerosing cholangitis. HEPATOLOGY 2004;39:779-791.

28. Lang T, Haberl M, Jung D, Drescher A, Schlagenhaufer R, Keil A, et al. Genetic variability, haplotype structures, and ethnic diversity of hepatic transporters MDR3 (ABCB4) and bile salt export pump (ABCB11). Drug Metab Dispos 2006;34:1582-1599.

29. Huang L, Zhao A, Lew JL, Zhang T, Hrywna Y, Thompson JR, et al. Farnesoid X receptor activates transcription of the phospholipid pump MDR3. J Biol Chem. 2003;278:51085-51090.

30. Ghonem NS, Ananthanarayanan M, Soroka CJ, Boyer JL. Peroxisome proliferator-activated receptor α activates human multidrug resistance transporter 3/ATP-binding cassette protein subfamily B4 transcription and increases rat biliary phosphatidylcholine secretion. HEPATOLOGY 2014;59:1030-1042.

31. Chianale J, Vollrath V, Wielandt AM, Amigo L, Rigotti A, Nervi Fet al. Fibrates induce mdr2 gene expression and biliary phospholipid secretion in the mouse. Biochem J 1996;314:781-786.

32. Kok T, Bloks VW, Wolters H, Havinga R, Jansen PL, Staels B, Kuipers F. Peroxisome proliferator-activated receptor alpha (PPARalpha)-mediated regulation of multidrug resistance 2 (Mdr2) expression and function in mice. Biochem J 2003;369:539-547.

33. Liu Y, Binz J, Numerick MJ, Dennis S, Luo G, Desai B, et al. Hepatoprotection by the farnesoid X receptor agonist GW4064 in rat models of intra- and extrahepatic cholestasis. J Clin Invest 2003;112:1678-1687.

34. Shoda J, Okada K, Inada Y, Kusama H, Utsunomiya H, Oda K, et al. Bezafibrate induces multidrug-resistance P-Glycoprotein 3 expression in cultured human hepatocytes and humanized livers of chimeric mice. Hepatol Res 2007;37:548-556.

35. Carrella M, Feldman D, Cogoi S, Csillaghy A, Weinhold PA. Enhancement of mdr2 gene transcription mediates the biliary transfer of phosphatidylcholine supplied by an increased biosynthesis in the pravastatin-treated rat. HEPATOLOGY 1999;29:1825-1832.

36. Hooiveld GJ, Vos TA, Scheffer GL, Van Goor H, Koning H, Bloks V, et al. 3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) induce hepatic expression of the phospholipid translocase mdr2 in rats. Gastroenterology 1999;117:678-687.

37. Hirschfield GM, Mason A, Luketic V, Lindor K, Gordon SC, Mayo M, et al. Efficacy of obeticholic acid in patients with primary biliary cirrhosis and inadequate response to ursodeoxycholic acid. Gastroenterology 2015;148:751-761.

38. Honda A, Ikegami T, Nakamuta M, Miyazaki T, Iwamoto J, Hirayama T et al. Anticholestatic effects of bezafibrate in patients with primary biliary cirrhosis treated with ursodeoxycholic acid. HEPATOLOGY 2013;57:1931-1941.

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Legend to figures

Fig.1. Schematic representation of ABCB4. The positions of twelve PFIC3 variations are indicated by stars. The Walker A (A) and Walker B (B) motifs and the signature (S) in the nucleotide binding domains are indicated.

Fig.2. Localization of ABCB4-wt and mutants in HepG2 and HEK293 cells by immunofluorescence. HepG2 cells transiently expressing ABCB4-wt or the mutants (A), and HEK293 cells stably expressing ABCB4-wt or the mutants (B) were fixed with methanol and processed for indirect immunofluorescence using the anti-ABCB4 P3II-26 monoclonal antibody and Alexa 488–conjugated anti-mouse immunoglobulin G. In HepG2 cells transfected with the I541F and L556R mutants, the presence of bile canaliculi was confirmed by immunolabeling of occludin (in red). Nuclei were stained with Draq5 (in blue). Bile canaliculi are indicated by asterisks. Bars, 10 µm.

Fig.3. Immunoblot of ABCB4-wt and mutants. (A) HEK293 cells stably expressing ABCB4-wt or the mutants were lysed and processed for electrophoresis and immunoblotting using the anti-ABCB4 P3II-26 antibody. (B) Same as in A except that the lysates were treated or not with endoglycosidase H (endo H) or peptide-N glycosidase F (PNGase F). Arrows point to the mature (1) immature (2) and deglycosylated (3) forms of ABCB4.

Fig.4. Effect of cyclosporins on ABCB4-wt and mutants. (A) HEK293 cells stably expressing ABCB4-wt or the indicated mutants were treated with 5 µmol/L cyclosporin A, B, C, D or H for 24 hours. Cells were fixed and processed for electrophoresis and immunoblotting with the anti-ABCB4 P3II-26 antibody. (B) Quantification of the immunoblots, as shown in A. The amount of the mature form (band 1) in each condition was quantified by densitometry and expressed relative to controls which were considered as 100%. Results are means (± SD) of three to five experiments. *P<0.05, **P<0.01, ***P< 0;005. (C) HepG2 cells were

transfected with plasmids encoding ABCB4-wt or the indicated mutants. After 24 hours, cells were treated with 5 µmol/L cyclosporin A and processed for immunofluorescence of ABCB4 as in Fig.2. Bile canaliculi are indicated by asterisks. Bar, 10 µm.

Fig.5. Phosphatidylcholine secretion by ABCB4-wt and by the mutants. PC secretion was measured in HEK293 cells 24 hours after transfection with plasmids encoding ABCB4-wt or the mutants. Activity was normalized to the levels of the respective proteins (wt or variant) and expressed as percent of activity of wild type. Results are means (± SEM) of at least three independent experiments. *$P<0.05$, **$P<0.001$.

Fig.6. Effect of mutations on the expression and stability of ABCB4. (A) HEK293 cells were transfected with plasmids encoding ABCB4-wt or the indicated mutants. After 48 hours, mRNA expression of ABCB4 was quantified by RT-PCR and ABCB4 protein expression was analyzed by immunoblotting and quantified by densitometry. Data were normalized to β-actin. Results are expressed as a percentage of the expression level of ABCB4-wt and represent means (± SEM) of at least four independent experiments. (B) HEK293 cells were transfected with plasmids encoding ABCB4-wt or the T424A or N510S mutants. After 24 hours, cycloheximide (25 µg/mL) was added to the culture medium to inhibit protein synthesis. Expression of ABCB4 and mutants was analyzed by immunoblotting at the indicated time points, using equal amounts per lane. Beta-actin served as a loading control. (C) Amounts of ABCB4 were quantified from (B) at 4 and 18 hours after cycloheximide addition. The amount of ABCB4 at time 4 hours was considered as 100%. Remaining ABCB4 at 18 hours was expressed as a percentage of time 4 hours. Means (± SEM) of three independent experiments are shown. * $P<0.05$.

Table 1: Classification of *ABCB4* variations and disease course in PFIC3 patients

Patients No.	Allele 1 and protein defect	Allele 2 and protein defect	Age at onset, first symptoms and evolution	Transpla survi
1	c.1621A>T **I541F**	c.1621A>T **I541F**	1 year icterus, pruritus then hepatomegaly, PH	LT at 5
2	c.1667T>G **L556R**	c.1667 T>G **L556R**	5 months hepatomegaly, discoloured stools then pruritus, icterus, hepatomegaly, PH, LF	LT at 8.5
3	c.1037G>T **S346I**	c.1037G>T **S346I**	3 months icterus then icterus, pruritus, hepatomegaly, PH	LT at 10
4	c.1270A>G **T424A** **+** c.2925-10_2925-9insC	c.2925-10_2925-9insC	1 year hepatomegaly then pruritus, hepatomegaly, PH, LF	LT at 13
5 and 6	c.2860G>A **G954S** + c.1954A>G **R652G**	c.2860G>A **G954S** **+** c.1954A>G **R652G**	4 years pruritus then pruritus, icterus, hepatomegaly, PH, LF 6 months pruritus then pruritus, hepatomegaly	LT at 21 No LT at with UD
7	c.523A>G **T175A**	c.1069T>C **F357L** **+** c.2324C>T **T775M**	6 months pruritus, hepatomegaly then pruritus, hepatomegaly	No LT at with UD
8	c.1529A>G **N510S**	c.2177C>T **P726L**	15 years abnormal liver tests then hepatomegaly	No LT at with UD
9	c.2564A>T **Q855L**	c.2564A>T **Q855L**	1.5 years pruritus, hepatomegaly then pruritus, hepatomegaly	No LT a with UD

LT, liver transplantation; PH, portal hypertension; LF, liver failure; UDCA, ursodeoxycholic acid[(a)] Status at last clinical record[(b)] The defect and classification were determined in the present study (see table 2).

Table 2. **Classification of ABCB4 missense variations associated with PFIC3.**

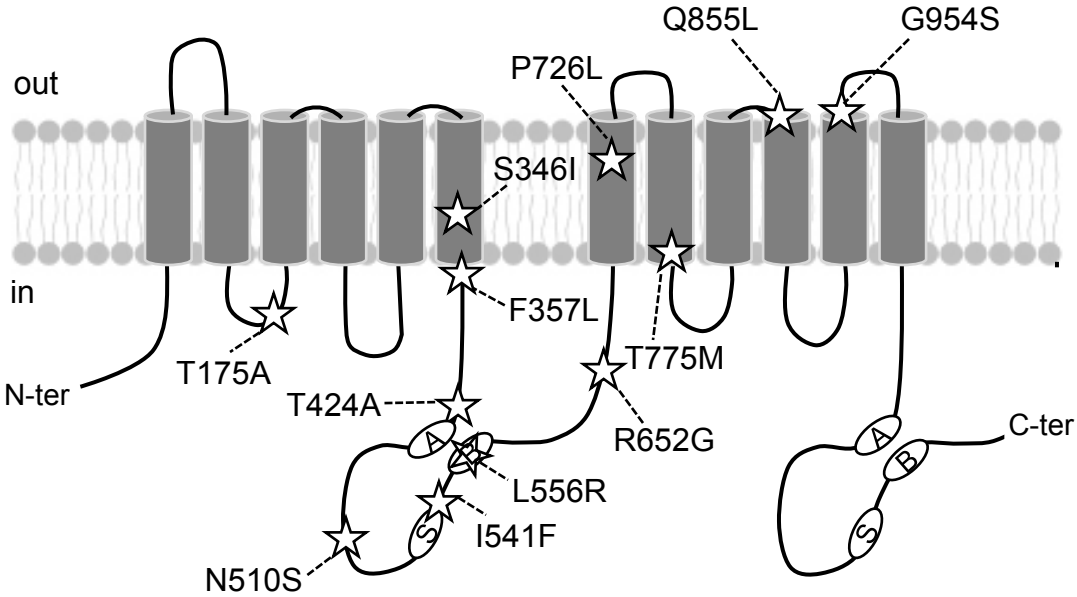
	Class I [(a)]	**Class II**	**Class III**	**Class IV**	**Cl**
Defect		**Maturation**	**Activity**	**Stability**	**No de de**
Variants	Non sense Frame shift Deletions	I541F L556R Q855L	S346I F357L P726L T775M G954S	T424A N510S	R6 T1
Potential therapy[(b)]		Pharmacological chaperones (e.g. cyclosporines)	Nuclear receptor agonists (e.g. PPARα), statins	Nuclear receptor agonists (e.g. PPARα), statins	

[(a)]Class I variations were not investigated in the present study.[(b)]likely to be tested in association with UDCA.

Supplemental Table 3. **Scoring systems for nonsynonymous *ABCB4* variants**

Nucleotide change	Aminoacid change	Grantham	SIFT	Polyphen 2	EC/EU	Mutation Taster
c.523A>G	**T175A**	58	0.00	0.841	EC	DC (p-value 1.0)
c.1037G>T	**S346I**	142	0.01	0.149	EC	DC (p-value 0.854)
c.1069T>C	**F357L**	22	0.00	0.075	EC	DC (p-value 1.0)
c.1270A>G	**T424A**	58	0.00	0.999	EC	DC (p-value 1.0)
c.1529A>G	**N510S**	46	0.00	0.862	EC	DC (p-value 1.0)
c.1621A>T	**I541F**	21	0.00	0.998	EC	DC (p-value 1.0)
c.1667T>G	**L556R**	102	0.00	1.000	EC	DC (p-value 1.0)
c.2177C>T	**P726L**	98	0.04	1.000	EC	DC (p-value 1.0)
c.2324C>T	**T775M**	81	0.02	0.999	EC	DC (p-value 0.938)
c.2564A>T	**Q855L**	113	0.02	0.995	EC	DC (p-value 1.0)
c.2860G>A	**G954S**	56	0.05	0.917	EC	DC (p-value 1.0)
c.1954A>G	R652G	125	0.73	0.000	EU	Polymorphism[a] (1.0)

The nonsynonymous *ABCB4* variants in bold character are predicted to be deleterious, according to the following methods of analysis:
Grantham: values range from 5 to 215. Low values (<50) indicate chemical similarity and high values (>50) indicate more radical differences.
SIFT (Sorting Intolerant From Tolerant): values range from 0 to 1. Values close to 0 are less tolerated, whereas those near 1 are better tolerated substitutions.
PolyPhen 2 predicts impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations. Values range from 0 to 1. Values close to 0 are better tolerated substitutions considered as benign whereas those near 1 are predicted to be damaging.
EC/EU classifies non synonymous variants as evolutionary conserved (EC) or unconserved (EU) based on sequence alignment analysis.
MutationTaster evaluates disease causing potential of sequence alterations: DC, disease causing mutation. [a]Allele frequency in the general population is 0.1056 with 1117 healthy homozygotes for a total of 12790 allele counts.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in
Q855L
G954S
P726L
S346I
F357L
T775M
T175A
N-ter
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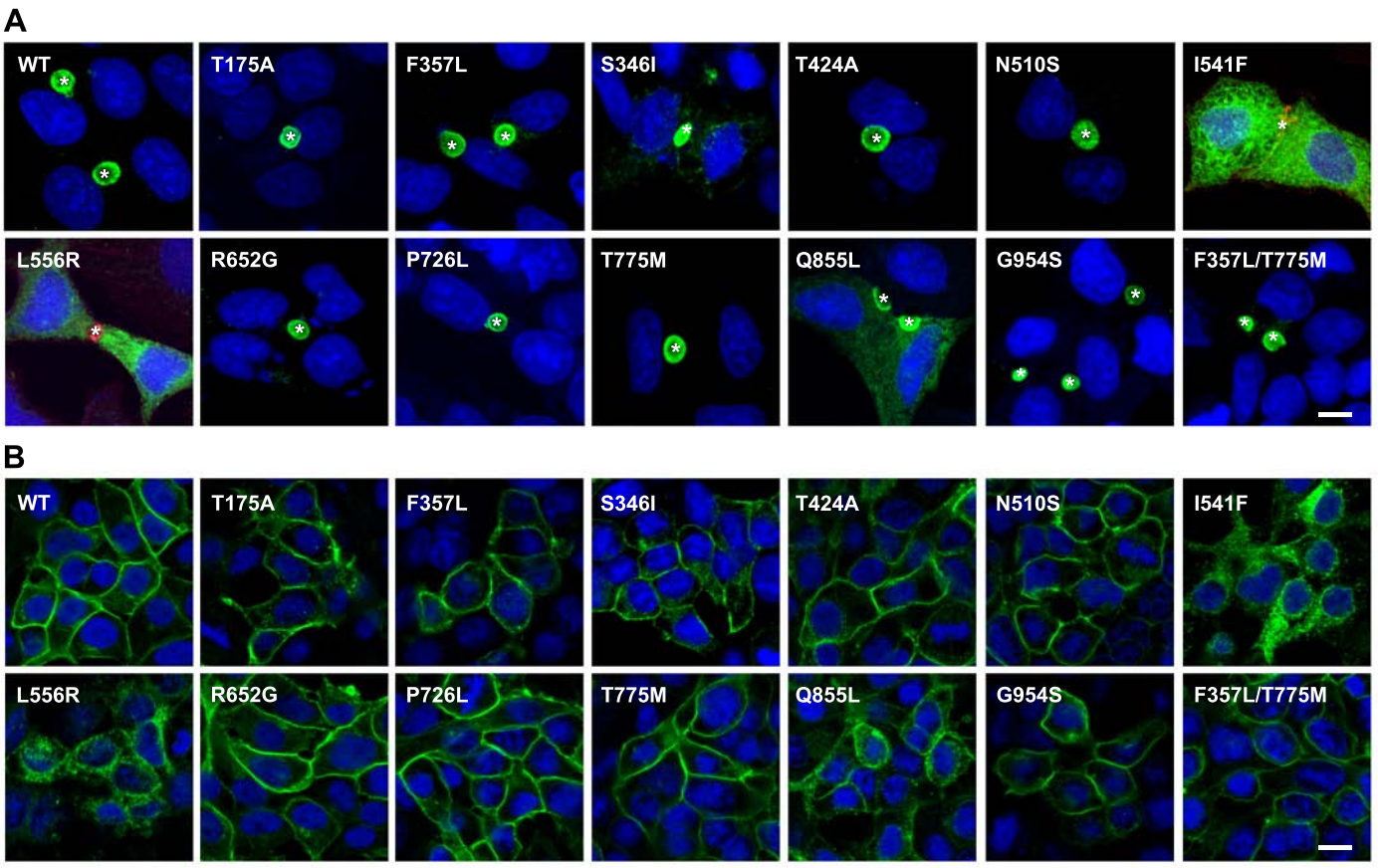
R652G
C-ter
L556R
I541F
N510S
A
B
S
A
B
S

Figure 1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T424A
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I541F
L556R
R652G
P726L
T775M
Q855L
G954S
F357L/T775M
B
WT
T175A
F357L
S346I
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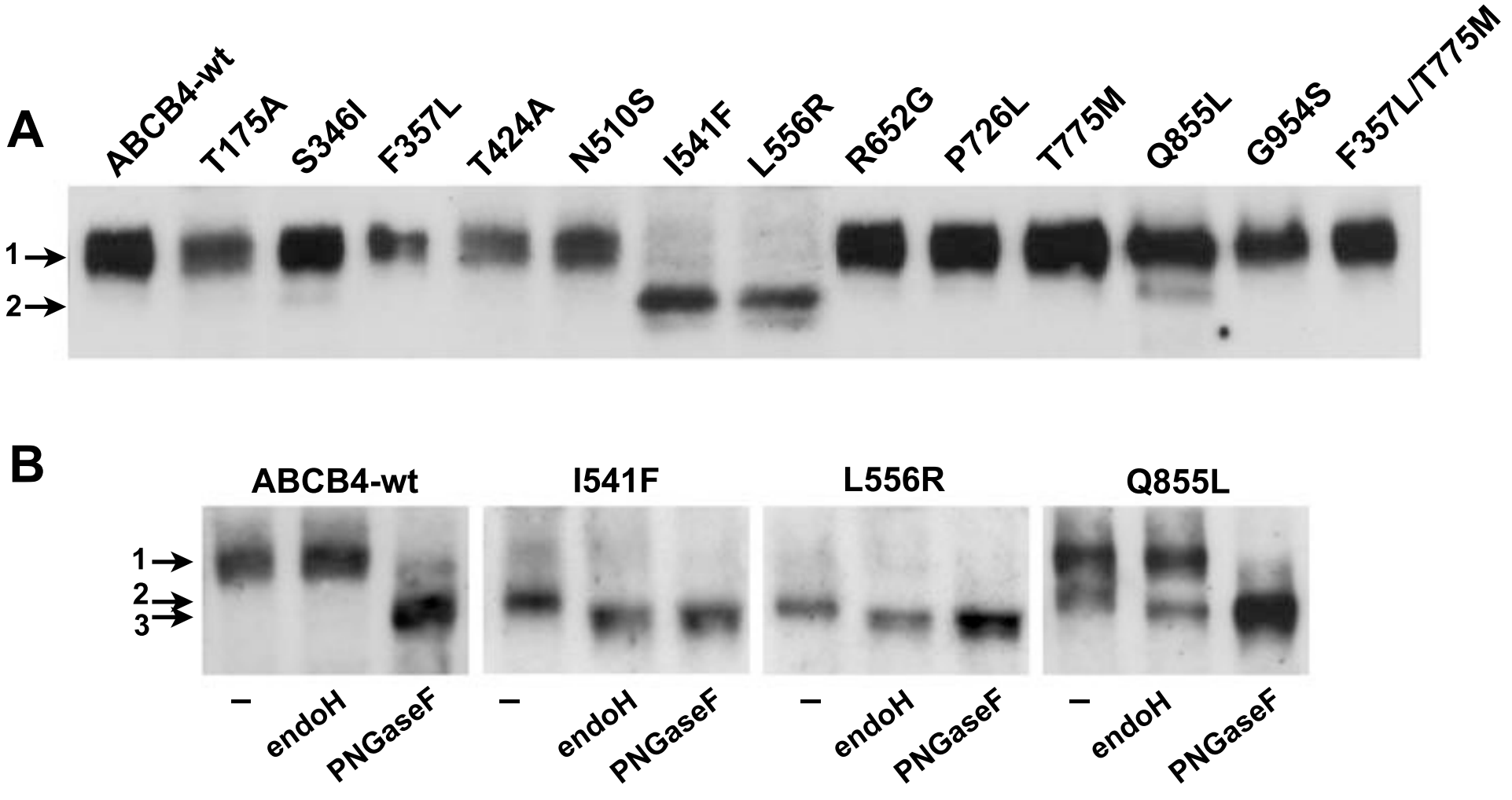
T775M
Q855L
G954S
F357L/T775M

Figure 2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G954S
F357L/T775M
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2
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ABCB4-wt
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L556R
Q855L
1
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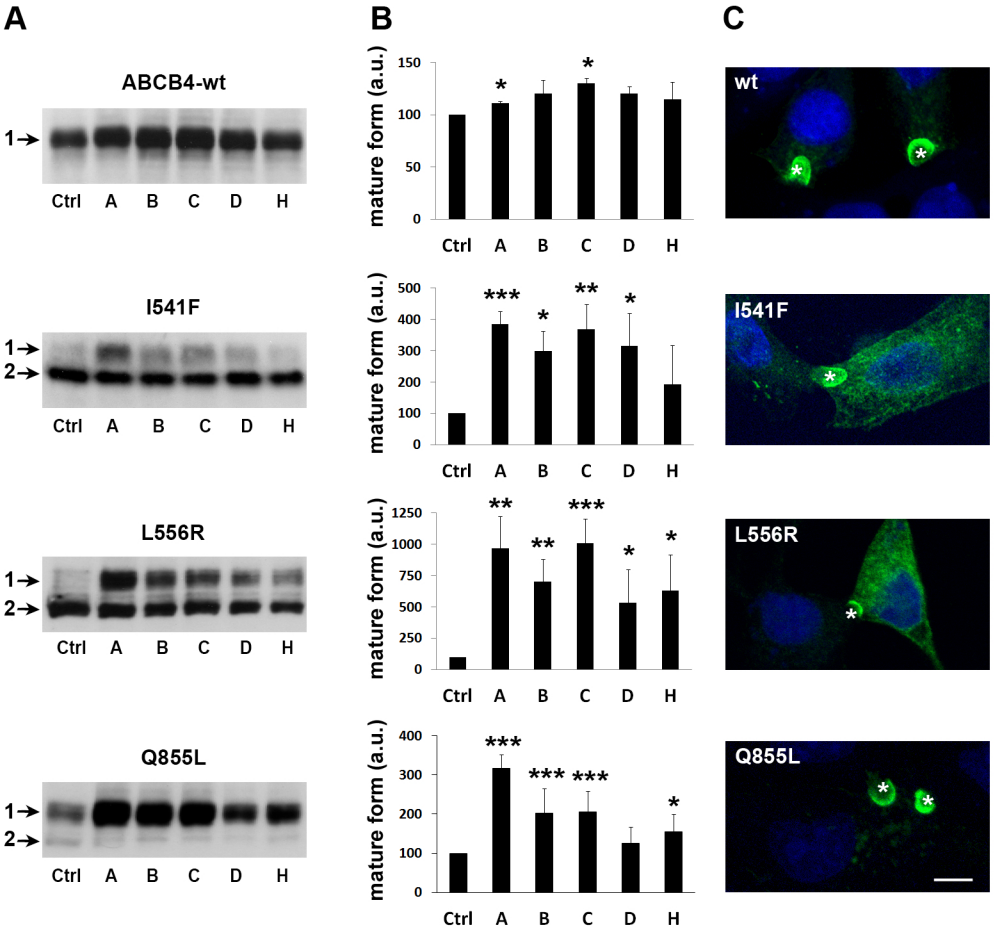
endoH
PNGaseF

Figure 3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I541F
L556R
1250
1000
750
500
250
0
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L556R
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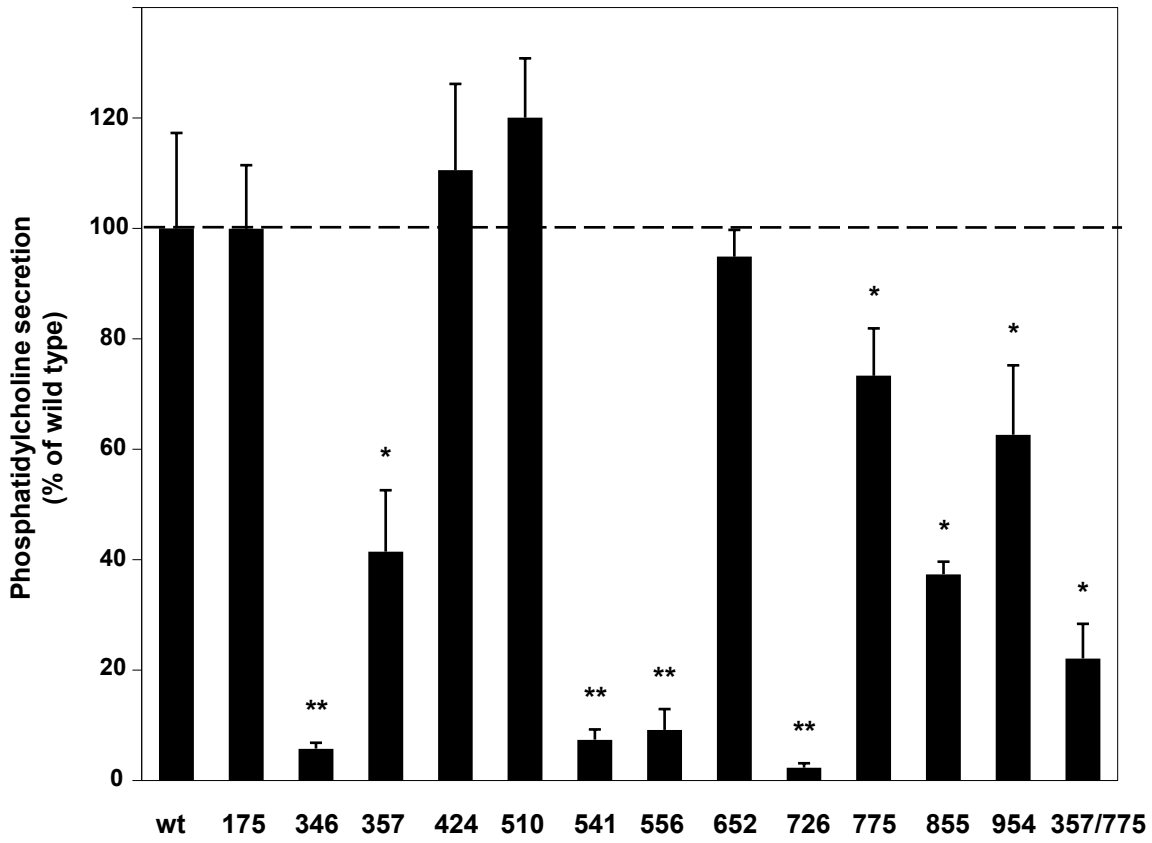
***
*
Q855L

Figure 4


Phosphatidylcholine secretion
(% of wild type)
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20
40
60
80
100
120
**
*
**
**
**
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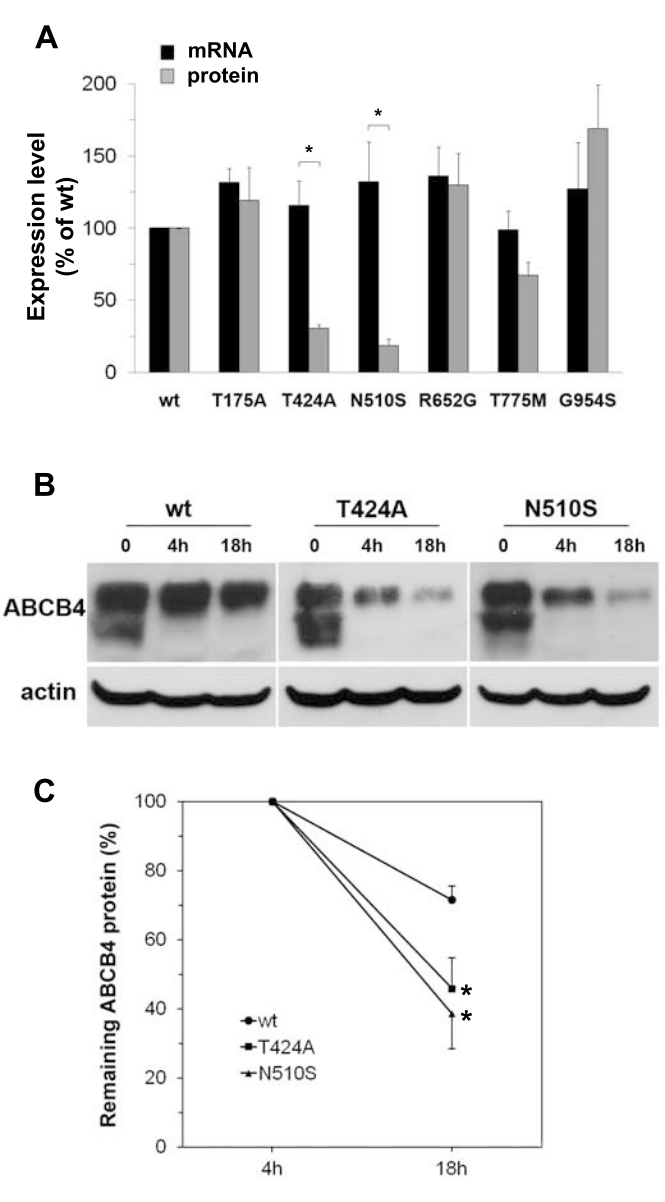
855
954
357/775

Figure 5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B
wt
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N510S
0
4h
18h
ABCB4
actin
C
Remaining ABCB4 protein (%)
100
80
60
40
20
0
wt
T424A
N510S
*
*
4h
18h

Figure 6