A molecular mechanistic explanation for the spectrum of cholestatic disease caused by the S320F variant of ABCB4

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Abbreviations: ABC, ATP binding cassette; PC, phosphatidylcholine; PFIC, progressive familial intrahepatic cholestasis; DIC, drug-induced cholestasis; ICP, intrahepatic cholestasis of pregnancy; LPAC, low phospholipid-associated cholelithiasis; CsA, cyclosporin-A.

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Abstract

ABCB4 flops phosphatidylcholine into the bile canaliculus to protect the biliary tree from the detergent activity of bile salts. Homozygous-null *ABCB4* mutations cause the childhood liver disease, progressive familial intrahepatic cholestasis, but cause and effect is less clear with many missense mutations linked to less severe cholestatic diseases. ABCB4^{S320F}, in particular, is described in 13 patients, including in heterozygosity with ABCB4^{A286V}, ABCB4^{A953D} and null mutants, whose symptoms cover the spectrum of cholestatic disease. We sought to define the impact of these mutations on the floppase, explain the link with multiple conditions at the molecular level, and investigate potential for reversal. ABCB4^{S320F}, ABCB4^{A286V} and ABCB4^{A953D} expression was engineered in naïve cultured cells. Floppase expression, localisation and activity were measured by western blot, confocal microscopy and lipid transport assays, respectively. ABCB4^{S320F} was fully-active for floppase activity but expression at the plasma membrane was reduced to 50%. ABCB4^{A286V} expressed and trafficked efficiently but could not flop lipid, and ABCB4^{A953D} expressed poorly and was impaired in floppase activity. Proteasome inhibition stabilised nascent ABCB4^{S320F} and ABCB4^{A953D} but did not improve plasma membrane localisation. Cyclosporin-A improved plasma membrane localisation of both ABCB4^{S320F} and ABCB4^{A953D}, but inhibited floppase activity. *Conclusions:* The level of ABCB4 functionality correlates with, and is the primary determinant of, cholestatic disease severity in these patients. ABCB4^{S320F} homozygosity, with half the normal level of ABCB4, is the tipping point between more benign and potentially fatal cholestasis and makes these patients more acutely sensitive to environmental effects. Cyclosporin-A increased expression of ABCB4^{S320F} and ABCB4^{A953D} suggesting that chemical chaperones could be exploited for therapeutic benefit to usher in a new era of personalised medicine for patients with ABCB4-dependent cholestatic disease.

Bile flow across the canalicular membrane of hepatocytes is dependent on three primary-active transport proteins, the ATP binding cassette (ABC) transporters ABCB11(1, 2) and ABCB4(3) and the P-type ATPase ATP8B1(4) (formerly known as BSEP, MDR3, and FIC1, respectively). The emerging picture is that ABCB4 and ATP8B1 (which functions in complex with its accessory protein CDC50) function to protect the hepatocyte (and cholangiocytes that line the bile ducts) from the deleterious detergent activity of bile salts(5). ABCB4 is thought to flop the membrane lipid phosphatidylcholine (PC) from the inner to the outer leaflet of the canalicular membrane(3, 6, 7), from where it is extracted into the canaliculus by bile salts transported by ABCB11 to form mixed micelles. This reduces the detergent activity of the bile salts(3, 8). In contrast, ATP8B1/CDC50 flips the aminophospholipids, phosphatidylserine, and phosphatidylethanolamine from the outer to the inner leaflet of the membrane(9). This activity is thought to maintain lipid asymmetry of the canalicular membranes of hepatocytes and cholangiocytes, to resist the detergent activity of bile salts(5, 10). All three transporters are known to be critical for bile flow because null mutations in *ATP8B1, ABCB11* and *ABCB4* cause progressive familial intrahepatic cholestasis (PFIC) types 1, 2 and 3, respectively. These monogenetic, autosomal recessive, diseases are characterised by intrahepatic cholestasis within the first year of life which leads to severe growth retardation. Progression to liver failure typically occurs within the first two decades and is often only treatable by orthotopic liver transplantation(7).

Dysfunction of each of the transporters is also considered causative in less severe but nevertheless debilitating complex liver disorders. Of relevance to the current study, *ABCB4* SNPs have been linked to drug-induced cholestasis (DIC(11)), intrahepatic cholestasis of pregnancy (ICP(12)) and low phospholipid-associated cholelithiasis (LPAC(13)). Recently, ABCB4 SNPs have also been suggested to predispose to cholangiocarcinoma(14). In the rare type-3 PFIC the link between mutation and disease is clear, but the majority of cholestatic liver diseases are complex conditions and a causative link with *ABCB4* SNPs that are typically private to individual families is suspected but not proved, with critical evidence at the functional and mechanistic level of protein expression and floppase activity lacking.

Biochemical characterisation of the effect of *ABCB4* SNPs directly has, hitherto, been challenging for several reasons: it is technically difficult to measure translocation of PC from the inner to the outer leaflet of the plasma membrane (this has been achieved previously but interpretation is complicated by the use of fluorescently-tagged PC analogues (6, 15)), and expression of functional PC floppase, transiently *in vitro*, is deleterious to cultured cells(5). Some insight has been obtained by mimicking mutations in the multidrug resistance transporter ABCB1, the closest homologue of ABCB4(16, 17). However, this approach is only relevant if the particular amino acid is conserved in both proteins and performs the same function, ruling out the study of mutations that directly influence PC binding, or responses to hormones of pregnancy, contraceptives and other drugs. Recently, we described the transient expression of active ABCB4 in naïve HEK293T cells by co-expressing it with ATP8B1/CDC50 to maintain the integrity of the plasma membrane(5). This ameliorates the cytotoxic effect of ABCB4 function. We now report the use of this expression system to characterise variant ABCB4 and describe the relationship between genotype and phenotype in a cohort of non-familial patients who share the S320F mutation and who presented with a spectrum of hepatobiliary disease (Table 1).

In the current study, we focus on ABCB4^{S320F} and other mutations (ABCB4^{A286V} and ABCB4^{A953D}) that are found in heterozygosity with ABCB4^{S320F}, and characterised their impact on protein expression, plasma membrane localisation and floppase function. The S320F variant is particularly interesting because it is described in thirteen case studies and is linked with the development of cholestatic disorders which unusually cover the whole spectrum of severity including several ICP, LPAC DIC and PFIC3 cases. The distinct *in vitro* phenotypes correlate with the severity of disease confirming that *ABCB4* genotype is the primary determinant of cholestatic pathophysiology. The phenotypes of ABCB4^{S320F} and ABCB4^{A953D}, but not ABCB4^{A286V}, suggest a translational path to therapeutic intervention.

Experimental procedures

Mutagenesis

Plasmids pcDNA3-ABCB4, pcDNA3-ABCB4 ^{E558Q}, pCIneo-ATP8B1, and pCIneo-CDC50 were described previously(5). Site-directed mutagenesis to introduce the SNPs c.857C>T (p.A286V), c.959C>T (p.S320F) and c.2858C>A (p.A953D) into the wild-type *ABCB4* cDNA(18) was performed using QuickChange-II (Stratagene, La Jolla, California). Mutagenesis was verified by sequencing of the entire cDNA and promoter region.

Mutagenic Oligonucleotides (5' – 3'):

A286V, GAAAGGTATCAGAAACATTTAGAAAATGTCAAAGAGATTGGAATTAAAAAAGCTATT;

S320F, CTGGCCTTCTGGTATGGATTCACTCTAGTCATATCAAA;

A953D, GCATTTATGTATTTTTCCTATGACGGTTGTTTTCGATTTGGTGCA.

Mammalian Cell Culture

Human embryonic kidney (HEK293T) cells were cultured and transiently transfected as described previously(5). Cells were seeded 24h prior to transfection at a density of 4.6 x 10^4 / cm^2 and triple-transfected with 2.5µg each of ABCB4, ATP8B1 and CDC50 using polyethyleneimine (Sigma, Gillingham UK). Where ATP8B1 and CDC50 were omitted, DNA concentration was maintained constant with 5µg of empty pCIneo. Where applied, MG132 or cyclosporin A (CsA) solubilised in dimethylsulphoxide (DMSO) was added 24h post-transfection. ABCB4 localisation and function was analysed 48h post-transfection.

Western Analysis

Cells were washed twice with PBS and harvested in 150mM NaCl, 20mM HEPES pH 7.4, 1% SDS, 1× EDTA-free complete protease inhibitor cocktail from Roche, 1mM PMSF. Crude protein lysates (1µg) were denatured in Laemmli sample buffer (70℃ for 5 min), separated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane (Millipore, Billerica Massachusetts). Blots were probed with mouse anti-ABCB4 monoclonal P_3II-26 (Sigma, Gillingham UK), diluted 1:2,000 and mouse anti-β-tubulin (SourceBioscience, Nottingham UK) diluted 1:2,000. Primary antibodies were detected using goat anti-mouse secondary antibody, conjugated to horseradish peroxidase diluted 1:2,000 (Santa Cruz, Dallas Texas) and visualised by

enhanced chemiluminescence (GE Healthcare, Amersham UK), or goat anti-mouse, conjugated to IRDye 680 diluted 1:20,000 (LI-COR, Lincoln Nebraska), for visualisation by the Odyssey scanning system. ABCB4 abundance normalised against β-tubulin was analysed using ImageJ software.

Confocal Microscopy

Cells were seeded onto 12mm glass coverslips coated with poly-L-lysine, 24h prior to triple-transfection. Cells were fixed 48h post-transfection in ice-cold 10% acetone (in ethanol) for 20min, blocked in 1% BSA (in PBS) for 1h, and incubated with primary antibodies, mouse anti-ABCB4 (P_3II-26; Sigma, Gillingham UK), and rabbit anti-Na^+/K^+ ATPase (H-300; Santa Cruz, Dallas Texas) at 1:50 dilution in PBS + 1% BSA for 1h. The cells were washed 3x in PBS and incubated with secondary antibody (goat anti-mouse Alexa568 or goat anti-rabbit Alexa488; Invitrogen, Paisley UK), diluted 1:500 in PBS + 1% BSA for 1h. The labelled cells were washed 3x in PBS, mounted on glass slides and imaged using a ZEISS 710 confocal microscope. Images were obtained using a plan-apochromat 63× oil immersion objective with a numerical aperture of 1.4.

Phosphatidylcholine Efflux Assay

PC extraction was measured as described previously(5). Briefly, cells were triple-transfected in poly-L-lysine coated 6-well dishes and fed 2μCi (~24pmol) ^{3}H-choline chloride (PerkinElmer, Waltham Massachusetts) 24h post-transfection. The cells were washed 24h later in pre-warmed DMEM and incubated with 2mM sodium taurocholate hydrate (TC) in DMEM. Media was recovered and cellular lysates prepared. Radionuclide content was measured by liquid scintillation. PC extraction is calculated as radionuclide detected in the media as a percentage of the total (media plus lysate). The data were normalised against PC extraction from cells expressing the non-functional E558Q Walker B mutant of ABCB4.

Results

Expression levels of Variant ABCB4

Mutations were introduced into the *ABCB4* cDNA to mimic the SNPs observed in patients and to encode the A286V, S320F and A953D variant proteins. HEK293T cells were transiently-transfected with either a single plasmid encoding an ABCB4-variant or three plasmids encoding the ABCB4-variant plus ATP8B1 and CDC50 (the pCIneo-ABCB4 plasmids were kept constant at 2.5µg throughout). The transfection conditions were optimised to achieve a confluent monolayer of cells 48hrs post-transfection. Sixty percent of the cells in the population were transfected and 98% of the transfected cells took up all three plasmids (supplementary figure 1). Expression of each variant protein was compared to wild-type ABCB4 and a non-functional mutant carrying a point mutation in the Walker B motif of the first nucleotide-binding domain, ABCB4^{E558Q} (Figure 1). The wild-type transporter expressed poorly in single-transfected cells likely due to the deleterious effect of flopping excess PC into the outer leaflet of the plasma membrane, but expressed well when triple-transfected with ATP8B1/CDC50(5). In contrast, the non-functional Walker B mutant ABCB4^{E558Q} expressed equally well under both conditions. The three variants had distinct expression profiles. ABCB4^{A286V} was similar to the Walker B mutant and expressed to high levels in both the single and triple-transfected cells. ABCB4^{A953D} expressed poorly in both conditions and to only 16.3% of the wild-type level in the triple-transfected cells. ABCB4^{S320F} also expressed to similar levels in the single- and triple-transfected cells, but to 50.1% of the level achieved by the wild-type transporter in the triple-transfected cells.

ABCB4^{S320F} and ABCB4^{A286V} Traffic to the Plasma Membrane while ABCB4^{A953D} is Largely Retained Intracellularly

Triple-transfected cells were fixed, stained and analysed by confocal microscopy to determine whether the variant proteins could traffic to the plasma membrane (Figure 2). Wild-type ABCB4 traffics efficiently to reside in the plasma membrane as shown by the substantial co-localisation with the Na^+/K^+-ATPase. Significant levels of ABCB4^{A286V} and ABCB4^{S320F} were also detected in the plasma membrane.

ABCB4^{A953D}, despite low level of expression, was also detected, but predominantly in the intracellular compartment with only a small fraction at the plasma membrane. This is consistent with the low level of expression of the mature form of ABCB4^{A953D} observed by western analysis.

ABCB4^{S320F} is Fully Active, ABCB4^{A953D} is Partially Active and ABCB4^{A286V} is Inactive for the Efflux of PC

Triple-transfected cells were fed radiolabelled choline to convert into ^{3}H-PC(19). The cells were incubated with taurocholate to extract the flopped PC from the outer leaflet of the plasma membrane. More ^{3}H-PC is extracted from cells that express wild-type ABCB4 than those that express the catalytically inactive ABCB4^{E558Q} (Figure 3). ^{3}H-PC extracted from cells expressing ABCB4^{S320F} was reduced to 49% that of cells expressing wild-type ABCB4, while the level mediated by cells expressing ABCB4^{A286V} or ABCB4^{A953D} was very low. After correction for ABCB4 expression level (Figure 3, inset) the data indicate that ABCB4^{S320F} is fully active as a floppase, while ABCB4^{A286V} is unable to flop PC. ABCB4^{A953D} retains partial functionality with a floppase activity 29.4% that of the wild-type level after correction for the low level of mature protein expressed.

Proteasome Inhibition Causes Accumulation of Immature ABCB4^{S320F} and ABCB4^{A953D} but does not Improve Trafficking to the Plasma Membrane

Membrane proteins that fold inefficiently are targeted for degradation by the proteasome via the endoplasmic reticulum-associated degradation (ERAD) pathway(20, 21). Inhibition of the proteasome could therefore, potentially, slow the rate of degradation and allow the variant proteins to adopt the appropriate fold. As a test of this hypothesis, triple-transfected cells expressing either wild-type ABCB4, ABCB4^{S320F} or ABCB4^{A953D} were treated with the proteasome inhibitor MG132 (10µM) from 24h post-transfection. Western analysis showed that these cells accumulated a smaller molecular weight species of ABCB4 (Figure 4A). This species is not glycosylated and migrates with the same electrophoretic mobility as

deglycosylated ABCB4 (Figure 4B), therefore most likely represents full length, immature protein which accumulates in the ER (Figure 5A, 5B and 5C). Inhibition of the proteasome appears to have little impact on maturation of ABCB4 because there is no increase in abundance of the mature, fully glycosylated protein. These data indicate that ABCB4^{S320F}, ABCB4^{A953D} and also wild-type ABCB4 are subject to ERAD but that inhibition of the proteasome, by itself, does not improve abundance at the plasma membrane *in vitro* and is not likely to improve ABCB4 density in the canalicular membrane of patients.

Cyclosporin-A Improves Folding of ABCB4 but Inhibits PC Efflux

CsA can improve the folding kinetics of the drug efflux pump ABCB1(22). Incubation of triple-transfected cells with CsA 24h post-transfection caused a marked increase in the expression of ABCB4^{S320F} and also wild-type ABCB4 (Figure 4C). Even at low micromolar concentrations of CsA, ABCB4^{S320F} can attain the expression level observed for the wild-type protein in untreated cells. In cells expressing wild-type ABCB4 and ABCB4^{S320F} the improved level of protein expression resulted in increased localisation at the plasma membrane (Figure 5A and 5B, lower panels). The expression level of ABCB4^{A953D} was not increased by CsA (Figure 4C), but the treatment did improve localisation of this variant to the plasma membrane (Figure 5C). Unfortunately, CsA treatment from 24h post-transfection was found to inhibit ABCB4 PC floppase activity (Figure 4D). This was apparent even if the treated cells were washed extensively 48hrs post-transfection and CsA was omitted from the transport buffer.

Discussion

We describe the characterisation of three missense variants of ABCB4 that are linked to a spectrum of cholestatic liver disease in fourteen case studies. The variants were expressed in HEK293T cells which do not polarise but offer two key advantages: they do not express endogenous ABCB4 and they are highly transfectable which is necessary for efficient triple-transfection with ATP8B1/CDC50 to avoid negative selective pressure acting on PC floppase activity. Taurocholate

was added to the cells to extract the ^{3}H-PC flopped by ABCB4 (we showed previously that there is no cellular efflux in the absence of an extracellular acceptor (5)). ABCB4^{A286V} expressed and localised efficiently to the plasma membrane *in vitro* but could not efflux PC. Expression of ABCB4^{S320F} and ABCB4^{A953D} was reduced to 50.1% and 16.3% of the wild-type floppase, respectively, and while ABCB4^{S320F} trafficked to the plasma membrane, a large fraction of ABCB4^{A953D} was retained intracellularly. Efflux of PC from cells expressing ABCB4^{S320F} and ABCB4^{A953D} (respectively, 49% and 5% of the level from cells expressing wild-type ABCB4), which when corrected for floppase abundance at the plasma membrane indicates that ABCB4^{S320F} is fully functional while ABCB4^{A953D} retains only 29.4% floppase activity. The lower density of this functional of ABCB4^{S320F} in the plasma membrane, and its insensitivity to the ATP8B1/CDC50 status of the cells suggests that HEK293T cells have a threshold level of PC floppase activity that they can tolerate. This is not surprising because the lipids of all cells are asymmetrically arranged in the plasma membrane and therefore all cells are likely to have flippases and floppases for specific lipids to maintain asymmetry. We expect that this homeostatic mechanism is capable of withstanding the imbalance caused by ABCB4^{S320F} expression. The *in vitro* data appear consistent with the available expression and immunohistochemistry data from clinical samples (albeit from chronically diseased liver). Patient 5 (Table 1), who is homozygous for ABCB4^{S320F} is reported to express 50% of the normal level of ABCB4(23), and patient 14 who is homozygous for ABCB4^{A953D} shows a marked reduction in expression (although this was not quantified by the authors(24)). This indicates that our *in vitro* system is a good model of the *in vivo* situation and can be used to characterise the impact of missense SNPs on ABCB4 protein expression, localisation and function.

The *in vitro* phenotype of the variant floppases correlates with the severity of disease confirming that ABCB4 genotype is the primary determinant of cholestatic pathophysiology in this cohort. The ABCB4^{S320F} variant is common to the first thirteen patients described in Table 1. In patients 1 and 2(25), ABCB4^{S320F} is heterozygous with either ABCB4^{Y279X} (which, truncated to only a quarter of the polypeptide, would be unable to form a functional ABC transporter(26)) or the non-active ABCB4^{A286V}, and therefore ABCB4^{S320F} would be the only functional ABCB4 PC floppase present in the canalicular membrane. Extrapolating from the *in vitro*

data indicates that less than one quarter of the normal activity of the PC floppase is unable to prevent damage to canalicular membranes and results in the severe childhood disease PFIC3. The critical effect of the level of ABCB4 expression and function is also supported by the two siblings described by Poupon *et al.*,(27) (patients 3 and 4; Table 1), who are heterozygous for ABCB4^{S320F} and ABCB4^{A953D} and so are likely to have only 27% of the PC floppase activity of a normal individual. These patients presented with LPAC characterised by cholesterol gallstones which precipitate from the bile due to the low solubility of cholesterol in mixed micelles that are low in PC(13). Unusually for LPAC patients, they developed PFIC3 in the third decade of life, suggesting that even a small increase in PC-floppase activity (compared to patients 1 and 2) can delay the onset of this progressive disease.

In cases 10 – 13 (Table 1), ABCB4^{S320F} is heterozygous with the wild-type allele and these patients present with LPAC, DIC or ICP(28-30). In DIC and ICP, the cholestasis is induced and limited by drugs (the contraceptive pill and fertility treatment in the two cases described) or pregnancy hormones which are thought to inhibit the transporters at the canalicular membrane directly(31) and/or reduce their levels of expression by direct inhibition of the farnesoid-X receptor(32). The allelic combination of patients 10 to 13 (where ABCB4^{S320F} is combined with the wild-type allele) is likely to encode three quarters of the normal level of ABCB4 floppase activity, which we would argue is sufficient to protect against childhood PFIC3 but insufficient to prevent LPAC, DIC or ICP. It has been suggested previously that relation of genotype to phenotype in patients 11 to 13 may be complicated by additional variation at the *ABCB11* locus. The ABCB11^{V444A} variant is reported to express poorly compared to the wild-type allele both *in vitro* and *in vivo*(30, 33) and is likely, therefore, to reduce bile salt efflux across the canalicular membrane. Population studies have identified ABCB11^{V444A} as a risk factor for several forms of cholestasis(30, 34-37). However, reduced bile salt efflux would be likely to reduce the toxicity of the bile in the canaliculae and therefore may be expected to alleviate the pathology of disease in patients with low levels of ABCB4. Patient 11 is homozygous for the wild-type *ABCB11* allele, patient 12 is heterozygous and patient 13 is homozygous for ABCB11^{V444A}, but all present with biliary conditions of similar severity suggesting that the ABCB11 genotype is of little relevance in these cases.

The tipping point for development of PFIC3 in childhood, would appear to be half the normal level of functional ABCB4. Patients 5 to 9 are homozygous for ABCB4^{S320F} and so would be expected to express half the normal level of ABCB4. These patients presented with the full spectrum of cholestatic disease, including LPAC (patient 6)(38), ICP (patients 7 and 9)(13, 39), DIC and ICP (patient 8)(13), and one patient (patient 5)(23) who presented with PFIC3 aged 8. Patient 5 responded to treatment with the less toxic bile acid, ursodeoxycholate, but developed liver fibrosis and gallstones by age 10. At the tipping point of ABCB4 functionality, these patients are likely to be more acutely sensitive to environmental affects such as therapeutic drug intake, hormones and diet which may modulate liver disease severity. A similar threshold effect has also been postulated for rodent models of ABCB4-dependent cholestatic disease(40).

None of these SNPs are close to an exon/intron boundary and while an effect on message stability cannot be ruled out, the close correlation of the *in vitro* cell biological data with patient symptoms suggest that the phenotypic effects are mediated at the protein level. This implies that relatively subtle changes in ABCB4 abundance and activity can affect bile composition and the progression of disease. The corollary, that relatively subtle improvement in PC flopping is likely to have significant therapeutic benefit, make ABCB4 an attractive target for intervention. The defects associated with ABCB4^{S320F} and ABCB4^{A953D} but not ABCB4^{A286V} may be correctable by agents that improve ABCB4 protein level at the canalicular membrane. Treatment with CsA produced a marked improvement in abundance and plasma membrane localisation of both wild-type ABCB4 and ABCB4^{S320F}, and also in the localisation of ABCB4^{A953D} at the plasma membrane, but it was found to inhibit PC floppase activity. CsA is thought to act as a chemical chaperone for the drug efflux pump ABCB1 by binding directly to the nascent protein and improving folding kinetics by induced fit(22). The effects of CsA on the abundance, localisation and floppase activity suggests a similarly direct interaction with ABCB4. Despite the inhibitory effect, the CsA data provide proof-of-principle that the density of ABCB4^{S320F} and ABCB4^{A953D} at the plasma membrane can be manipulated, and that there is scope for development of drugs to target increased expression of the PC floppase at the canalicular membrane. 127 non-synonymous SNPs in *ABCB4* have been identified to date (http://abcmutations.hegelab.org). *In vitro* characterisation will

be key to stratify these missense variants of ABCB4 to clarify the relationship between genotype and phenotype, and to identify those disease-causing variants that could be targeted for therapeutic intervention.

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Patient	1st allele	2nd allele	Sex	Additional Mutations	Disease	Reference
1	S320F	Y279X	F	NK	PFIC3	Degiorgio *et al*., (25)
2	S320F	A286V	F	NK	LPAC to PFIC3	Degiorgio *et al*., (25)
3[a]	S320F	A953D	M	ABCB11-V444A$^{+/-}$	LPAC to PFIC3	Poupon *et al*., (27)
4[a]	S320F	A953D	F	ABCB11-V444A$^{+/-}$	LPAC to PFIC3	Poupon *et al*., (27)
5	S320F	S320F	M	NK	PFIC3	Colombo *et al*., (23)
6	S320F	S320F	NK	NK	LPAC	Rosmorduc *et al.,* (38)
7	S320F	S320F	F	NK	ICP	Rosmorduc *et al*., (13)
8	S320F	S320F	F	NK	DIC[c] and ICP	Rosmorduc *et al*., (13)
9	S320F	S320F	F	NK	ICP	Pauli-Magnus *et al*., (41)
10	S320F	WT	F	NK	ICP	Bacq *et al*., (28)
11[b]	S320F	WT	F	ABCB11-wt$^{+/+}$	LPAC and ICP	Zimmer *et al*., (29)
12[b]	S320F	WT	F	ABCB11-V444A$^{+/-}$	LPAC	Zimmer *et al*., (29)
13	S320F	WT	F	ABCB11-V444A$^{+/+}$	DIC[c] and ICP	Keitel *et al*., (30)
14	A953D	A953D	NK	NK	PFIC3	Keitel *et al*., (24)

Table 1. Missense mutations in 14 patients with cholestatic liver disease who carry ABCB4^{S320F} and related variants.

Patient 12 is nulligravid and also carries a SNP in the FXR gene which results in the missense mutation M1V and lowers the abundance of FXR by two thirds(42). The bile acid sensor FXR controls transcription of ABCB4 and ABCB11 therefore low levels of FXR in patient 12(29) is likely to lower ABCB4 abundance further, but in parallel with ABCB11. [a,b]Siblings. [c]Drugs in these cases refer to prescription of the contraceptive pill or progesterone fertility treatment. NK: not known.

Figure Legends

Figure 1. Analysis of Expression of ABCB4 missense variants

(A) Western blot of the expression of wild-type and variant ABCB4 in HEK293T cells in the absence and presence of ATP8B1/CDC50 as indicated (black vertical line indicates samples were run on the same gel but were non-contiguous). The Walker B mutant E558Q was used as a control for expression of a non-functional protein. (B) Expression levels of the mature 160kDa form of the ABCB4 variants in different biological replicates were normalised to wild-type ABCB4 expression expressed contemporaneously in the absence of ATP8B1/CDC50. Mean expression level +/- sem are plotted. Statistical analysis was by unpaired Student's t-test ($n \geq 3$; ***$P < 0.005$; ns, not significantly different).

Figure 2. Trafficking of variant ABCB4 to the plasma membrane.

Wild-type and variant ABCB4 were expressed in HEK293T cells in the presence of ATP8B1/CDC50. Cells were fixed, permeabilised and stained for ABCB4 (magenta) and the plasma membrane protein, Na+/K+-ATPase (green). Nuclei were stained with DAPI (blue) and the cells imaged by confocal microscopy. Scale bar denotes 20 μm

Figure 3. Phosphatidylcholine efflux by ABCB4 variants in the presence of ATP8B1/CDC50.

PC floppase activity was tested in HEK293T cells expressing wild-type ABCB4 (WT), ABCB4^{S320F}, ABCB4^{A286V}, or ABCB4^{A953D} in the presence of ATP8B1/CDC50. ^{3}H-PC extracted in the presence of 2mM TC was calculated as the percentage of total cellular radioactivity after subtraction of the background level from cells expressing the Walker B mutant ABCB4^{E558Q}. The data were analysed by Student's t-test ($n \geq 3$; *$P < 0.01$, ***$P < 0.005$). Inset

shows percentage mean PC floppase activity after correction for ABCB4 variant mean expression levels.

Figure 4. Effects of Potential Modulators of ABCB4 expression and function

(A) Cells were triple-transfected to express wild-type ABCB4 (WT), $ABCB4^{S320F}$ or $ABCB4^{A953D}$. Treatment with the proteasome inhibitor MG132 (10μM) 24h post-transfection induced accumulation of a smaller molecular weight form of the floppase, detected by western analysis. (B) Whole-cell lysates treated with PNGase F shows that deglycosylated mature protein migrates with the same mobility as the smaller molecular weight form produced by treatment with MG132 (loading was adjusted to ensure similar levels of variant and wild-type protein). (C) Representative gels and bar graph showing that treatment of triple transfected cells with CsA 24h post-transfection reproducibly causes the mature wild-type ABCB4 and $ABCB4^{S320F}$ to accumulate, but has no effect on the abundance of $ABCB4^{A953D}$. Expression levels of the mature protein are normalised against β-tubulin expression. (D) CsA inhibits the PC floppase activity of wild-type ABCB4. Triple-transfected cells were incubated with 0-1μM CsA 24h post-transfection for the indicated times. The media was washed from the cells and replaced with fresh media containing TC. Data were analysed by Student's t-test ($n \geq 3$; *$P < 0.01$, ***$P < 0.005$). Black vertical line(s) on panels B and C indicate that samples were run on the same gel but were non-contiguous.

Figure 5. Effect of MG132 and CsA on ABCB4 Expression and Trafficking

(A) Wild-type ABCB4. (B) $ABCB4^{S320F}$. (C) $ABCB4^{A953D}$. Cells were cultured on glass coverslips, triple-transfected and treated 24h post-transfection with the proteasome inhibitor MG132 (10μM) or the chemical chaperone CsA (1μM) as indicated. The cells were fixed, stained and imaged by confocal microscopy 48h post-transfection. Staining as per Figure 2. Scale bar, 20μm.

Figure 1

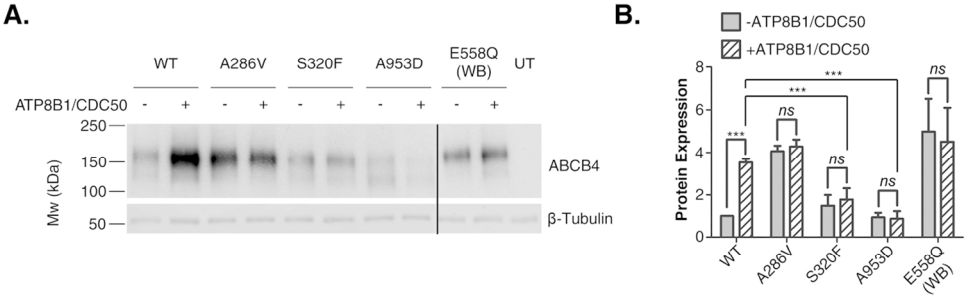

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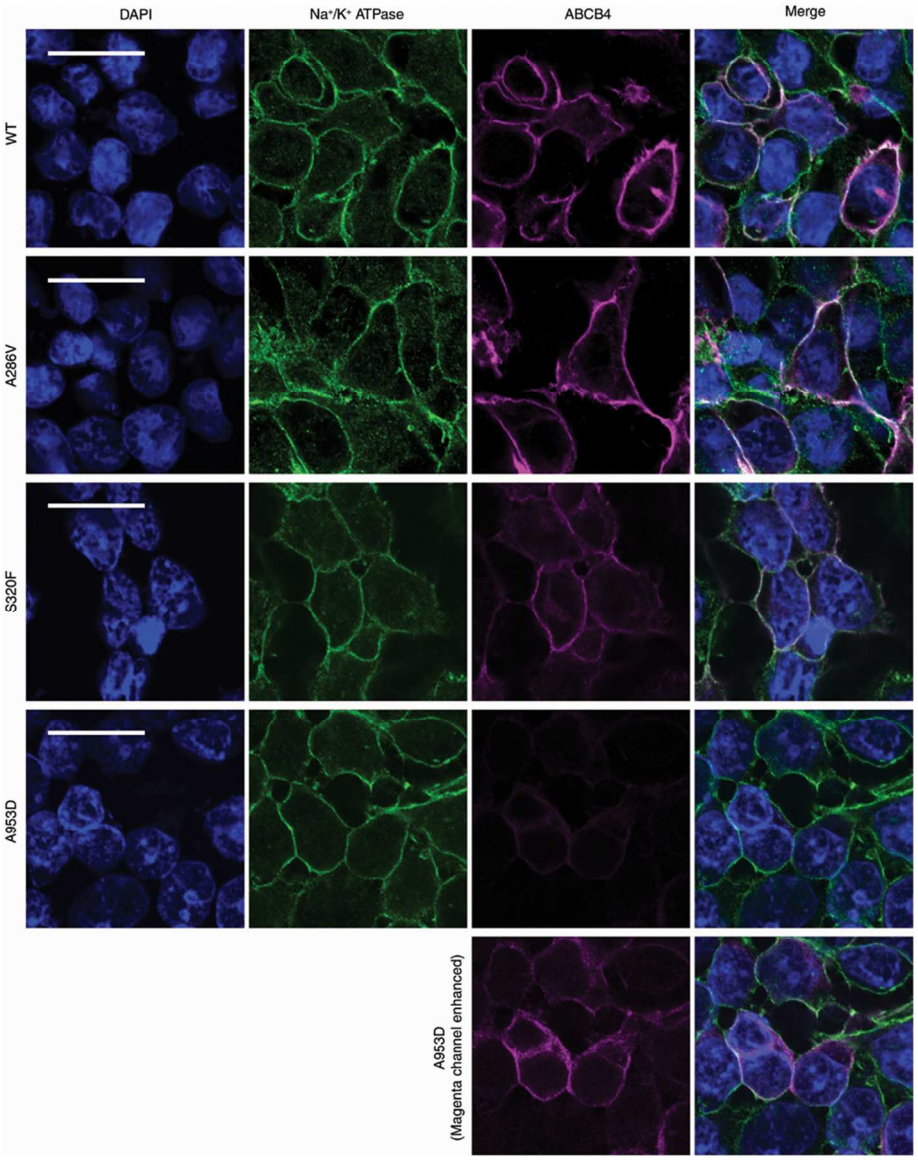
Figure 2



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Wild-type and variant ABCB4 were expressed in HEK293T cells in the presence of ATP8B1/CDC50. Cells were fixed, permeabilised and stained for ABCB4 (magenta) and the plasma membrane protein, Na+/K+-ATPase (green). Nuclei were stained with DAPI (blue) and the cells imaged by confocal microscopy. Scale bar denotes 20 μm.

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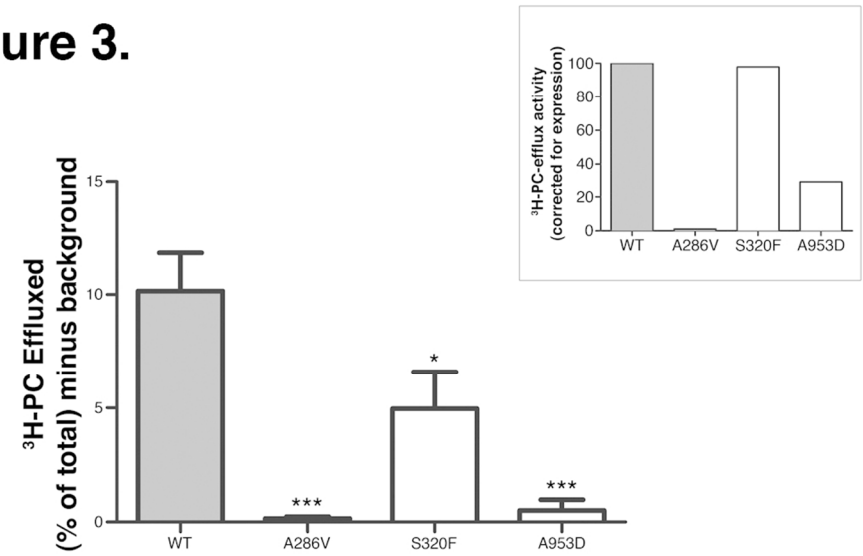
Figure 3.



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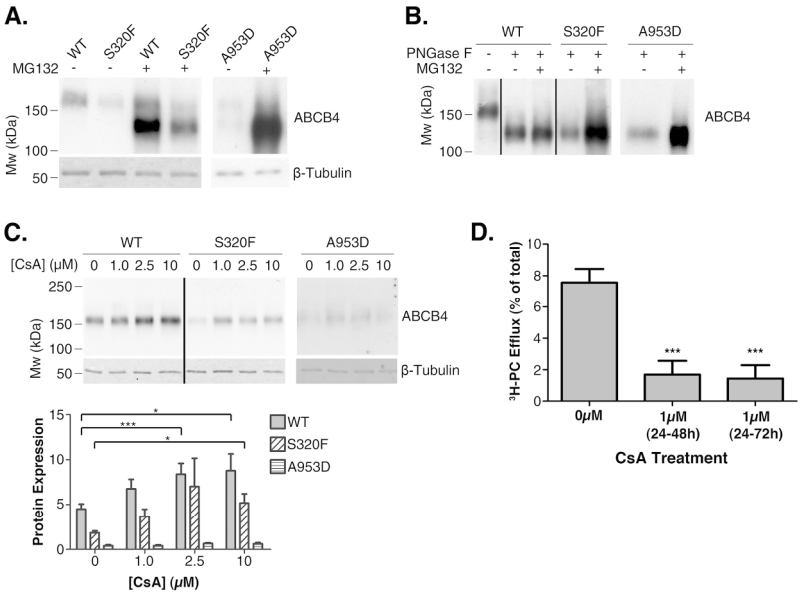

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Figure 5A

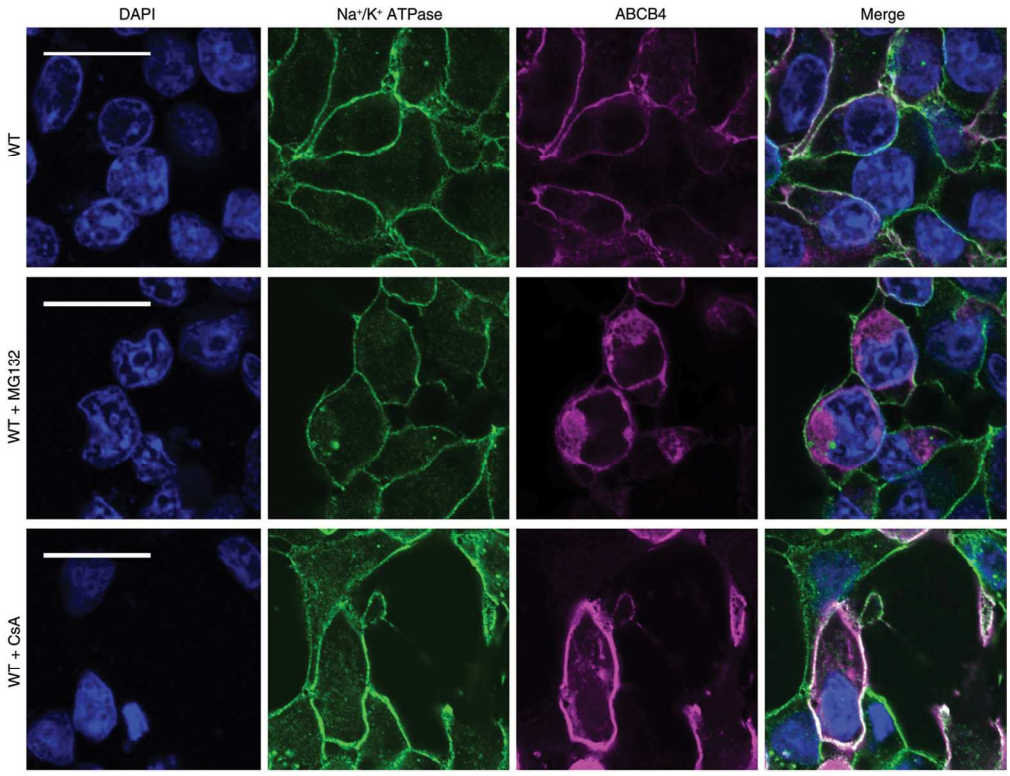

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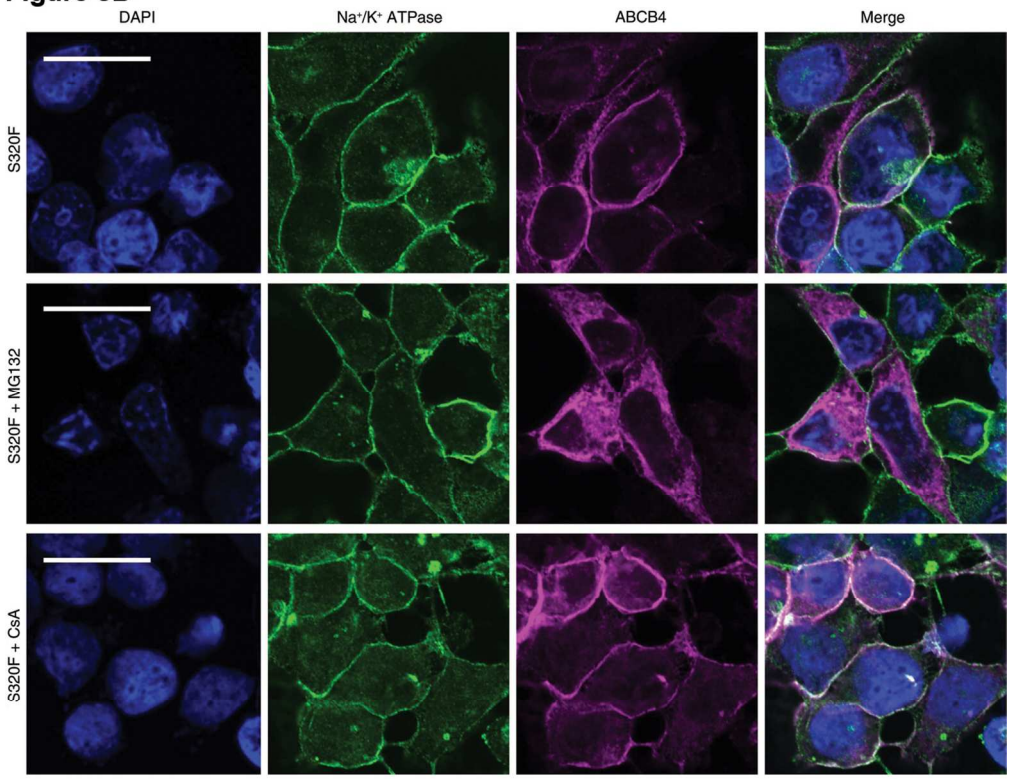
Figure 5B



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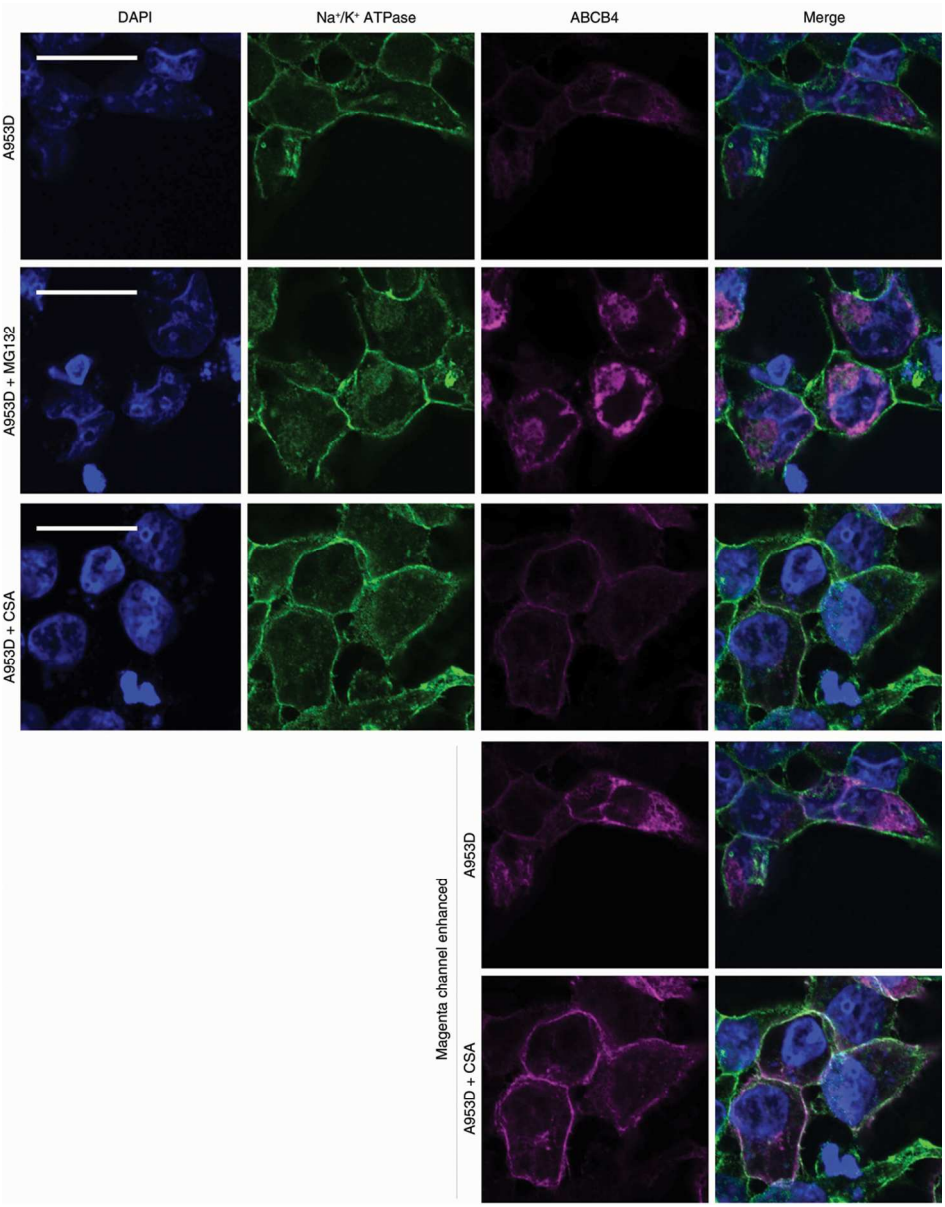

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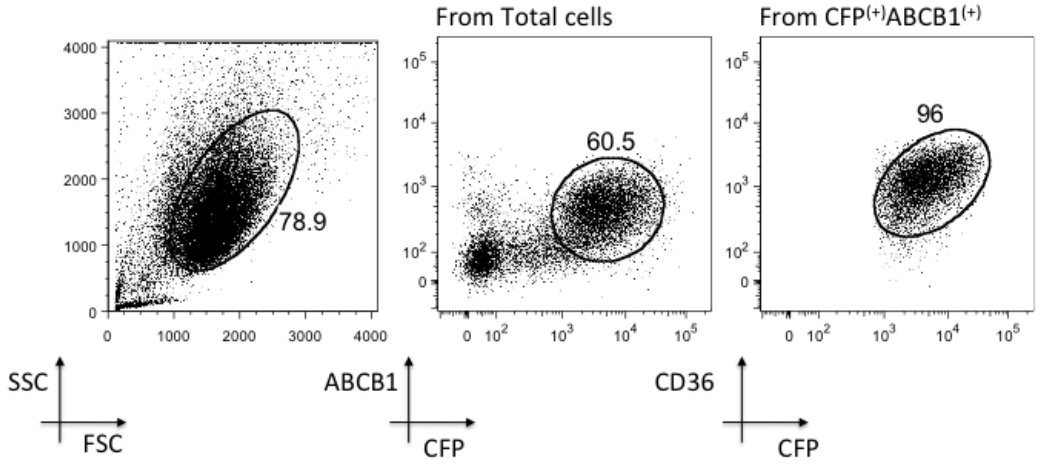
Supplementary data



S1 – Efficiency of plasmid uptake for triple transfection

In order to quantify how many cells take up one, two or all three plasmids in our triple transfection protocol, cells were transfected with plasmids encoding the human scavenger receptor CD36 and the human multidrug resistance pump ABCB1 (both of which can be detected by monoclonal antibodies that recognize extracellular epitopes) and free cyan fluorescent protein (CFP). Live cells were gated according to side and forward scatter reflecting normal cell size and granularity, respectively (left hand panel), then for recombinant protein expression. In the example shown, ABCB1 and CFP expression are detected in the middle panel. Double positive cells were then gated and assessed for expression of the third recombinant protein. In the example shown, the ABCB1 and CFP positive cells were gated and assessed for expression of CD36 (right hand panel). The data show that 60% of the cells in the population were transfected and expressed both ABCB1 and CFP. Of these 96% also expressed CD36. We conclude therefore that if a cell is competent to take up one plasmid, it will take up all three.

Materials and Methods:

HEK293T ells were triple transfected using the PEI transfection reagent, as described in the main materials and methods. Forty-eight hours post transfection the cells were harvested, washed in FACS buffer (PBS and 1% fatty acid-free BSA) and resuspended at $1x10^7$cells per ml. A saturating concentration (2µg) of mouse anti-CD36 monoclonal antibody mAb1258 was added to 50µl of cells and incubated for 30 minutes on ice. The cells were centrifuged at 400g for 1 minute at 4°C and resuspended in 1ml FACS buffer. The wash step was repeated twice more and the cells resuspended in 50µl of FACS buffer. A saturating concentration (4µg) of goat, anti-mouse IgG secondary antibody conjugated to Alexafluor 647 (Invitrogen) was added to the cells and incubated in the dark for 30 mins on ice. The cells were recovered by centrifugation and washed as before, and then resuspended in 50µl FACS buffer. To detect ABCB1, 0.5µg PE-conjugated anti-ABCB1 monoclonal antibody UIC2 (Immunotech) was added to the cells and incubated in the dark, on ice, for 30 mins. Cells were recovered and washed as above, then resuspended in 1ml FACS buffer. Flow cytometry was used to measure cellular fluorescence. 10,000 cells of normal size

and granularity were analyzed for CFP, CD36 and ABCB1 surface expression. The data was analysed by FlowJo (Treestar).