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ABCA3 protects alveolar epithelial cells against free cholesterol induced cell death

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ABSTRACT

Diffuse parenchymal lung diseases (DPLDs) are characterized by chronic inflammation and fibrotic remodeling of the interstitial tissue. A small fraction of DPLD cases can be genetically defined by mutations in certain genes, with ABCA3 being the gene most commonly affected. However, the pathomechanisms underlying ABCA3-induced DPLD are far from clear. To investigate whether ABCA3 plays a role in cellular cholesterol homeostasis, phospholipids, free cholesterol, and cholesteryl esters were quantified in cells stably expressing ABCA3 using mass spectrometry. Cellular free cholesterol and lipid droplets were visualized by filipin or oil red staining, respectively. Expression of SREBP regulated genes was measured using qPCR. Cell viability was assessed using the XTT assay. We found that wild type ABCA3 reduces cellular free cholesterol levels, induces the SREBP pathway, and renders cells more resistant to loading with exogenous cholesterol. Moreover, ABCA3 mutations found in patients with DPLD interfere with this protective effect of ABCA3, resulting in free cholesterol induced cell death. We conclude that ABCA3 plays a previously unrecognized role in the regulation of cellular cholesterol levels. Accumulation of free cholesterol as a result of a loss of ABCA3 export function represents a novel pathomechanism in ABCA3-induced DPLD.

1. Introduction

Diffuse parenchymal lung diseases (DPLDs) are a group of more than 200 different disease entities characterized by various degrees of inflammation and fibrosis [1,2]. For the vast majority of DPLDs, the cause is completely unknown and the development of causal therapies is hampered by a lack of knowledge regarding the pathological mechanisms involved. Among the most frequent molecularly defined entities is DPLD associated with mutations in the ABCA3 gene [3]. ABCA3-induced DPLD represents an important molecularly defined prototypic model for DPLD, the exploration of which may lead to more widely generalizable insights. However, the specific molecular mechanisms underlying ABCA3-induced DPLD have not been unraveled.

ABCA3 belongs to the large ABC transporter family and is strongly expressed in lung alveolar epithelial type II cells (ATII cells) where it localizes to the outer membrane of lamellar bodies (LBs) [4,5]. LBs are lysosome-derived lipid-rich organelles which serve in the production, storage and controlled secretion of pulmonary surfactant [4–6]. ABCA3 transports phospholipids and cholesterol into the LB lumen and is essential for the biogenesis of LBs [7,8]. Therefore, its normal function is indispensable for the homeostasis of pulmonary surfactant and normal breathing [9–11]. Mutations in ABCA3 cause a severe respiratory distress syndrome in new-borns that is often associated with fatal outcome [8,12]. However, in many cases, ABCA3 mutations permit longer survival; those patients develop DPLD [13–15].

In the case of surfactant protein C, it has been demonstrated that ER stress and apoptosis of ATII cells arise as a consequence of the accumulation of misfolded protein in the ER, a process that may cause damage to the alveolar epithelium and ultimately lead to fibrosis [16–18]. However, this has not been shown for ABCA3. In addition, not all pathogenic ABCA3 mutations induce protein trafficking/folding defects but rather affect the proteins' lipid transport function. It is therefore obvious to assume that intracellular accumulation of ABCA3 substrates as a result of disturbed ABCA3 transport function plays a role in ABCA3-induced DPLD. The substrate most likely to do harm to the cell would be cholesterol, given that cholesterol is involved in many cellular processes and disturbances of its homeostasis may have strong effects including cell death [19].

Since the importance of ABCA3's cholesterol transport activity for cellular cholesterol homeostasis is unknown, the aim of the present study was to elucidate how ABCA3 modulates cellular cholesterol levels in ATII cells. Our hypothesis was that ABCA3 is needed to maintain physiological cholesterol levels and that ABCA3 dysfunction due to

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1388-1981/

Table 1
Primer sequences for qPCR.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
FDPS[a]	AGCCAAGGAAACAGGATGC	TCCATGATGTCATCTGCCAC
FDFT1	AAAGGGGCAGTGAAGATT	CGGATGGTGGAGATGAT
HMCGR[a]	TCGGTGGCCTCTAGTGAGAT	CATCATGCAGATGGTCAGTG
HMGCS1[b]	CTCTTGGGATGGACGGTATGC	GCTCCAACTCCACCTGTAGG
HPRT1	GCTGACCTGCTGGATTAC	TGCGACCTTGACCATCTT
INSIG1[a]	CGTTCTTGGCTCCCTTGTAT	TCCTTGCTCTCAGAATCGGT
INSIG2[a]	GGGCTGGTCCCAGAAGAT	CAAAGACTGACGCTTCAACG
LDLR	CCACGGCGTCTCTTCCTA	TTGATCTTGGCGGGAGTT
SQLE[a]	TTCTCATCTGAGGTCCATGC	CCACCAGTAAGTGGATGCCT
SREBF1	GCTGCTGACCGACATC	CAAATAGGCCAGGGAAGT
SREBF2[a]	GAGACCATGGAGACCCTCAC	TGCCAGGAAAGGAGCTACAC

[a] Primer sequences were taken from qPrimerDepot (http://primerdepot.nci.nih.gov/).
[b] Primer sequences were taken from PrimerBank (http://pga.mgh.harvard.edu/primerbank/).

mutations results in cholesterol accumulation with detrimental effects on ATII cell viability. Two clinically relevant mutations belonging to different categories were chosen for this study: p.Q215K leads to mistrafficking [20], while p.E292V causes limited functional impairment of ABCA3 transporter function [21]. Based on our findings, we present a novel model to explain how ABCA3 mutations may be related to the pathophysiological features seen in DPLD.

2. Materials and methods

If not indicated otherwise, all chemicals were purchased from Sigma Aldrich (Taufkirchen, Germany).

2.1. Cell culture

A549 cells were obtained from DSMZ (Braunschweig, Germany). Cells were maintained in RPMI 1640 medium (Life technologies, Darmstadt, Germany) supplemented with 10% FBS (Sigma) at 37 °C and 5% CO_2. Stable transfection of A549 cells with *pUB6-ABCA3-WT/Q215K/E292V* vectors was carried out as previously described [22]. For experiments, cells were grown to confluence, trypsinized and seeded at 200,000 cells per 6-well and grown for 48 h except where indicated otherwise. For lipid analysis, cells were washed with PBS twice and lysed with SDS-buffer (0.1% SDS, 1 mM EDTA in 0.1 M Tris pH 7.4) for 15 min at room temperature. Protein concentrations of cell lysates were determined using the BCA assay. Cell viability was assessed by determining cleavage of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-

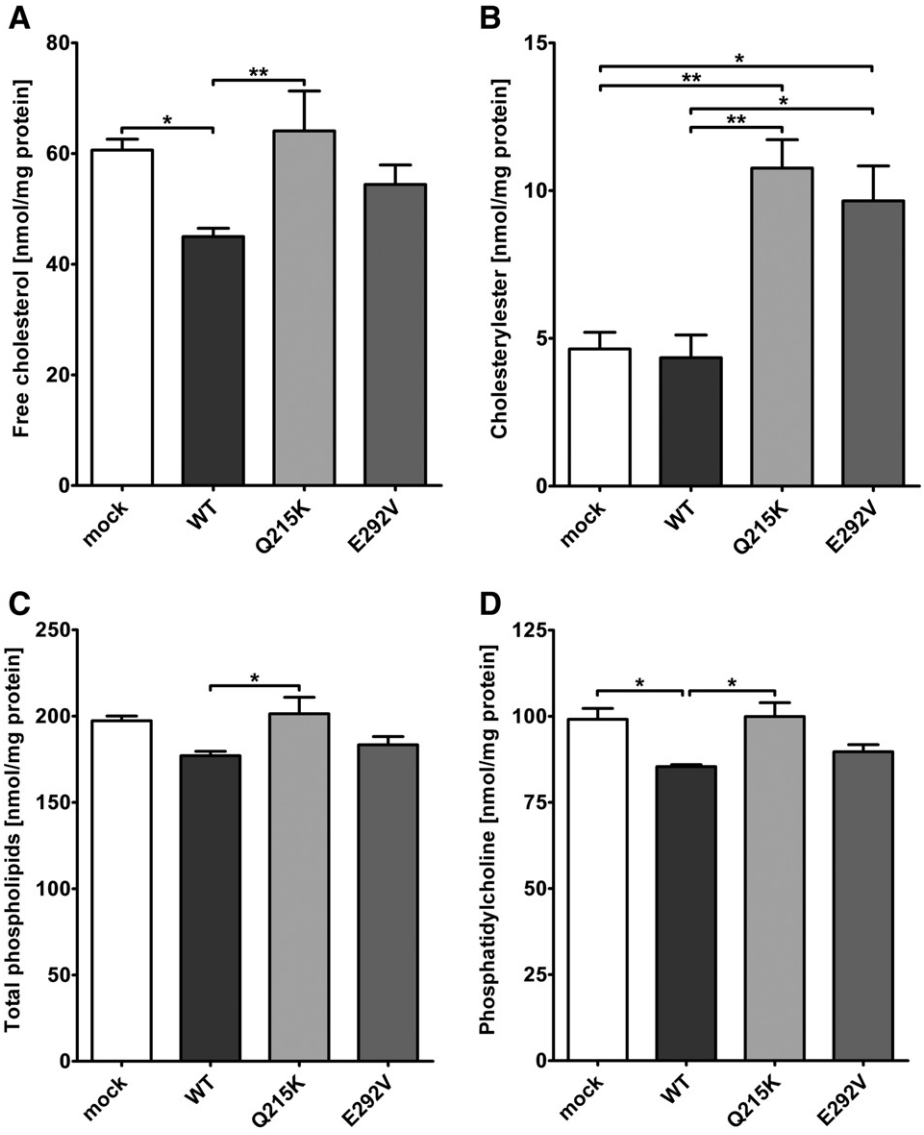

Fig. 1. Lipids in A549 cells as determined by mass spectrometry. Shown are intracellular concentrations of free cholesterol (A), cholesteryl esters (B), total phospholipids (C), and phosphatidylcholine (D). Values are given in nmol/mg protein (n = 4). *P < 0.05, **P < 0.01.

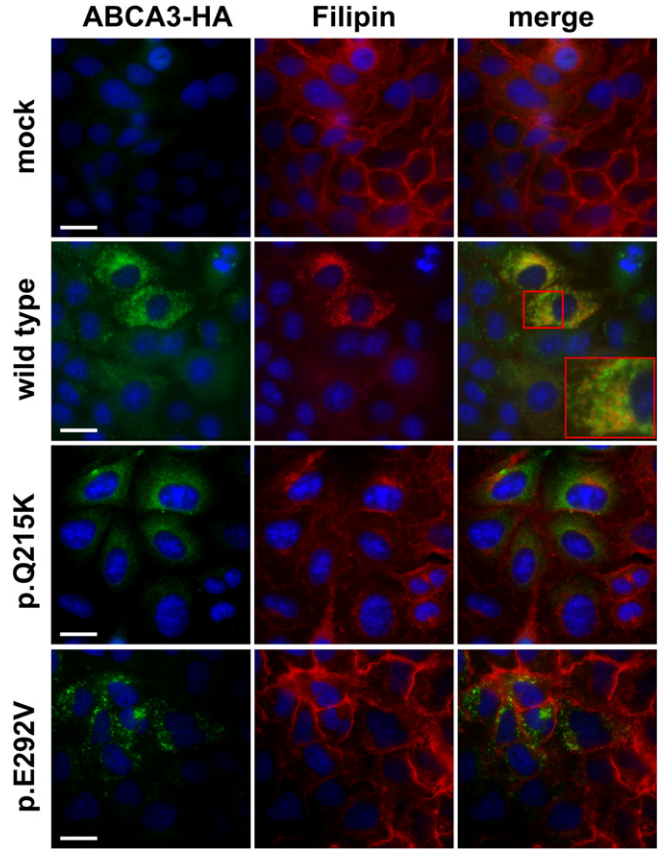

Fig. 2. Modulation of cellular free cholesterol distribution by ABCA3. Free cholesterol was visualized by filipin staining in A549 cells stably transfected with ABCA3-WT, p.Q215K and p.E292V or mock transfected cells. ABCA3-HA signal is shown in green pseudocolor. Scale bars: 10 μm.

tetrazolium-5-carboxyanilide (XTT) in the presence of phenazine methosulfate [23]. For XTT assay, cells were seeded in 96-well plates in RPMI medium without phenol red (Life technologies). Absorbance was measured at 490 nm and 650 nm using a spectrophotometer.

2.2. Triglyceride determination

Cellular triglyceride content was measured using the Triglyceride Quantification Kit (abcam, Cambridge, United Kingdom) according to the manufacturer's instructions.

2.3. Lipid analysis using mass spectrometry

For determination using MS, free cholesterol and cholesteryl esters were quantified by electrospray ionization tandem mass spectrometry (ESI-MS/MS) in positive ion mode using a fragment ion of m/z *369* after selective derivatisation of free cholesterol as described previously [24]. Phospholipids were measured according to a published method [25].

2.4. Immunofluorescence/oil-red O staining/filipin staining

Cells grown for 48 h in 8-well chamber slides (ibidi, Martinsried, Germany) were rinsed with PBS and fixed with 3.7% formaldehyde for 20 min at room temperature. Cells were washed with PBS, permeabilized with 0.5% saponin in PBS for 10 min, and incubated with blocking buffer (0.5% saponin, 10% BSA and 5% FBS in PBS) for 30 min. Subsequently, cells were covered with goat-anti-HA-tag antibody (abcam) diluted in blocking buffer and incubated for 1 h at room temperature. After three washes with PBS, cells were incubated with anti-goat Alexa 488 antibody (Life technologies) in blocking buffer for 30 min and again washed with PBS thrice. Lipid droplets were visualized by staining with 0.3% oil red in 36% triethylphosphate for 30 min at room temperature. Free cholesterol was stained using filipin complex. For filipin staining, filipin was added from a 25 mg/ml stock solution in DMSO to the blocking buffer in a final concentration of 50 μg/ml. Nuclei were stained with DAPI or TO-PRO-3 (Life technologies), respectively. Cells were mounted with mounting medium (90% (v/v) glycerol, 2.5% (w/v) 1,4-diazabicyclo[2.2.2]octane, 50 mM Tris, pH 8.0). Immunofluorescent images were recorded on a Zeiss Axiovert microscope using the Axiovision 3.1 software.

2.5. Cholesterol loading

Cholesterol loading of the plasma membrane was performed using β-methyl cyclodextrin (β-MCD) [26]. The molar ratio of β-MCD to cholesterol used was 10:1. Briefly, 10 μmol cholesterol from a stock solution in chloroform was placed in a round bottom flask and the solvent was removed under a stream of nitrogen. 10 ml of a solution of β-MCD in PBS (10 mM) was added. The flask was put in an ultrasonic bath until the cholesterol had dissolved and the resulting solution sterile filtered before being added to cell culture media in a final concentration of 1 mM β-MCD and 100 μM cholesterol, respectively.

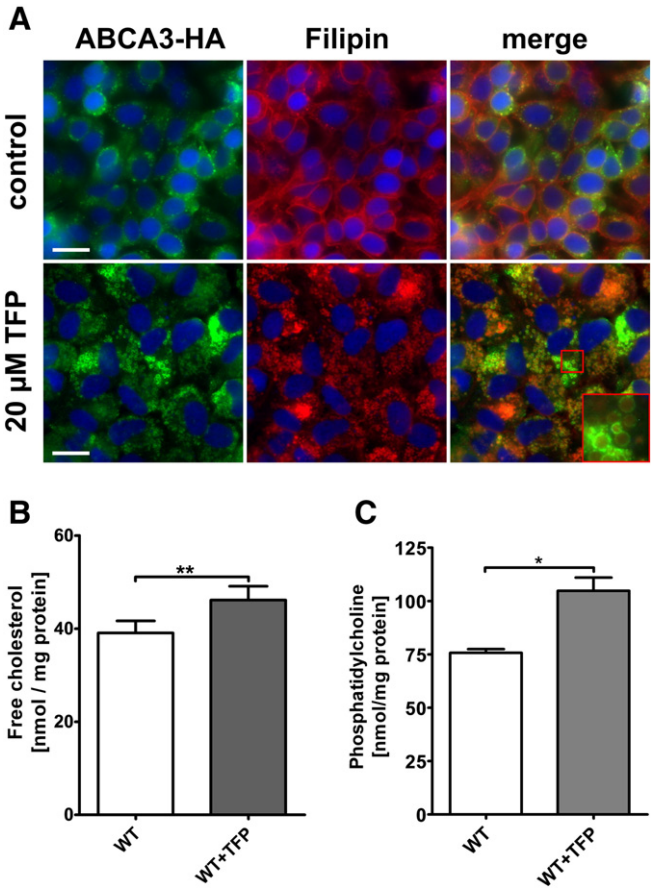

Fig. 3. Effect of exocytosis inhibition in A549 cells expressing ABCA3-WT using trifluoperazine (TFP) on the intracellular distribution and content of free cholesterol and concentration of phosphatidylcholine. (A) Cellular distribution of free cholesterol visualized by filipin staining (red pseudocolor) and ABCA3-HA (green pseudocolor). Cellular content of free cholesterol (B) and phosphatidylcholine (C) is given in nmol/mg protein ($n = 4$). *$P < 0.05$, **$P < 0.01$. Scale bars: 10 μm.

2.6. RNA isolation/cDNA synthesis/quantitative real time PCR

Cells grown to confluence in 6-well plates were washed once with PBS. Cells were harvested and total RNA was isolated with the High Pure RNA Isolation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. RNA concentrations were measured with a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). 1 μg of total RNA was reverse transcribed into cDNA with the Tetro reverse transcription kit (Bioline, Luckenwalde, Germany). Quantitative real-time PCR was carried out using SensiFAST SYBR Hi-ROX Mix (Bioline) on an ABI 7900HT cycler (Applied Biosystems, Darmstadt, Germany). Primer sequences are given in Table 1. HPRT1 was used as housekeeper gene. For analysis of relative changes, data was analyzed according to the $\Delta\Delta C_T$ method [27].

2.7. Statistics

Comparisons of multiple groups were done using one-way repeated measure ANOVAs with Tukey's post hoc test. Results were presented as mean + S.E.M. of a minimum of three different experiments. P-values of less than 0.05 were considered statistically significant. All tests were performed using GraphPad Prism 5.0 (GraphPad Software).

3. Results

3.1. ABCA3 decreases cellular free cholesterol and phosphatidylcholine levels

We determined cellular free cholesterol (FC), cholesteryl esters (CE), total phospholipids, and phosphatidylcholine (PC) in A549 cells expressing HA-tagged ABCA3. A549 cells expressing wild type ABCA3 (ABCA3-WT) showed significantly reduced levels of FC and of PC compared to mock transfected A549 cells and also compared to cells expressing ABCA3–Q215K (Fig. 1A, D). Expression of ABCA3–E292V did neither significantly affect FC nor PC levels. The expression of both mutations resulted in significantly increased levels of CE compared to both, mock and ABCA3-WT transfected cells (Fig. 1B) Cellular levels of total phospholipids were elevated in ABCA3–Q215K cells compared to ABCA3-WT cells (Fig. 1C). Cellular levels of triglycerides were not significantly altered (data not shown).

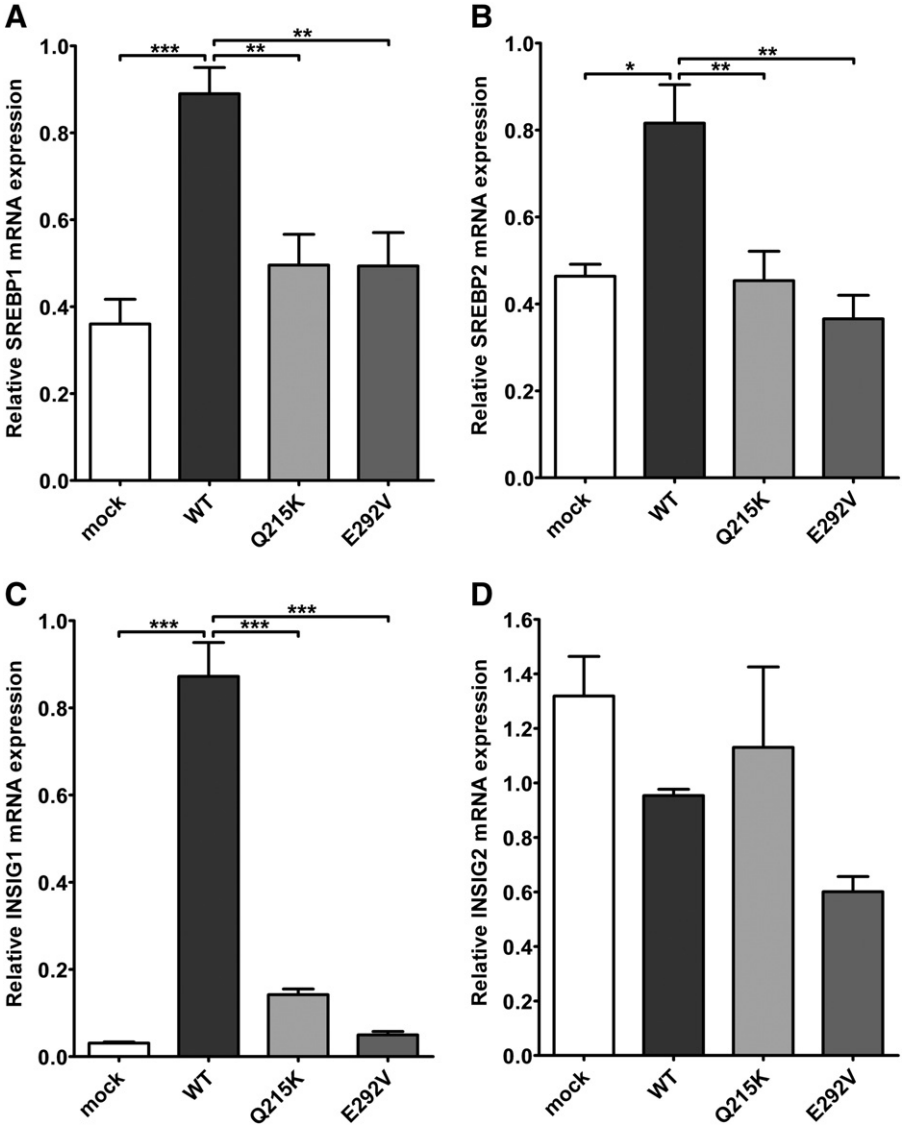

Fig. 4. Expression of wild type ABCA3 induces the SREBP pathway. Shown are mRNA levels of SREBPs and INSIGs in stably transfected A549 ABCA3-WT, ABCA3–Q215K and ABCA3–E292V cells and mock transfected cells. (A) *SREBP1*, (B) *SREBP2*, (C) *INSIG1*, (D) *INSIG2*. Changes are presented relative to ABCA3-WT (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001.

3.2. ABCA3 alters the intracellular distribution of FC

The intracellular distribution of FC was visualized using filipin while ABCA3-HA was detected by immunofluorescence. In concordance with previous studies, expression of wild type ABCA3 results in the formation of large ABCA3-positive vesicles. These vesicles are markedly smaller in ABCA3–E292V cells and completely absent in ABCA3–Q215K cells. Expression of ABCA3-WT resulted in a markedly altered FC pattern compared to mock transfected A549 cells and cells expressing ABCA3 mutations. Abundant cholesterol-loaded vesicles were seen in cells expressing ABCA3-WT, while in mock transfected cells, especially the plasma membrane and intracellular filamentous structures were stained by filipin (Fig. 2). This was also the case in cells expressing ABCA3–Q215K and ABCA3–E292V. In ABCA3-WT cells, ABCA3 colocalized with the cholesterol-loaded vesicles which were surrounded by a circular ABCA3 signal (Fig. 2, red box). The same pattern was also seen when live cells were treated with filipin and is thus not an artifact of fixation (data not shown). In contrast, there was no apparent cholesterol accumulation visible in association with the small ABCA3-positive vesicles observed in cells expressing ABCA3–E292V.

3.3. Inhibition of exocytosis leads to accumulation of FC loaded, ABCA3 positive vesicles

When exocytosis was blocked in A549 cells expressing ABCA3-WT using trifluoperazine (TFP) [28], a marked accumulation of filipin-positive vesicles was observed (Fig. 3A). The vesicles also showed staining for ABCA3 (Fig. 3A, red box) and resembled those seen in untreated cells. The accumulation of vesicles was accompanied by a significant increase in the levels of FC and PC (Fig. 3B+C).

3.4. ABCA3 induces expression of SREBP-regulated genes

To further dissect the fate of FC in A549 cells expressing ABCA3-WT, we analyzed expression of genes coding for ER cholesterol sensors. Expression of both sterol-regulatory proteins SREBP1 and SREBP2 was significantly up-regulated in cells expressing ABCA3-WT compared to mock transfected cells, indicating reduced ER cholesterol in ABCA3-WT cells (Fig. 4A+B). INSIG1 and INSIG2 play an important role in the SREBP regulation mechanism and *INSIG1* is regulated by SREBPs while *INSIG2* is expressed constitutively. Consistent with SREBP activation,

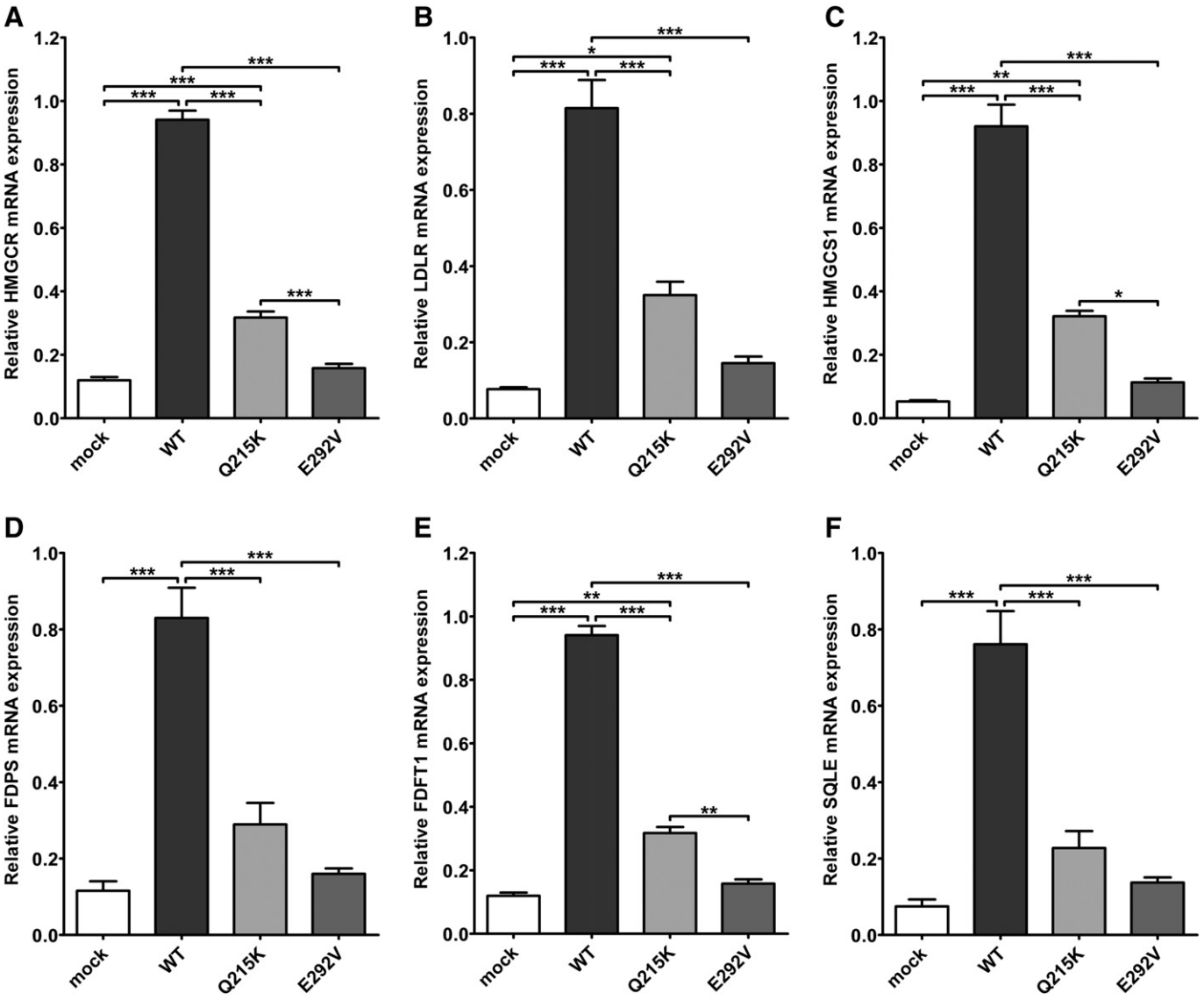

Fig. 5. Expression of wild type ABCA3 induces genes involved in cholesterol synthesis and uptake. Shown are mRNA levels of SREBP target genes in stably transfected A549 ABCA3-WT, ABCA3–Q215K and ABCA3–E292V cells and mock transfected cells. (A) HMGCR, (B) LDLR, (C) HMGCS1, (D) FDPS, (E) FDFT1, and (F) SQLE. Changes are presented relative to ABCA3-WT ($n = 6$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

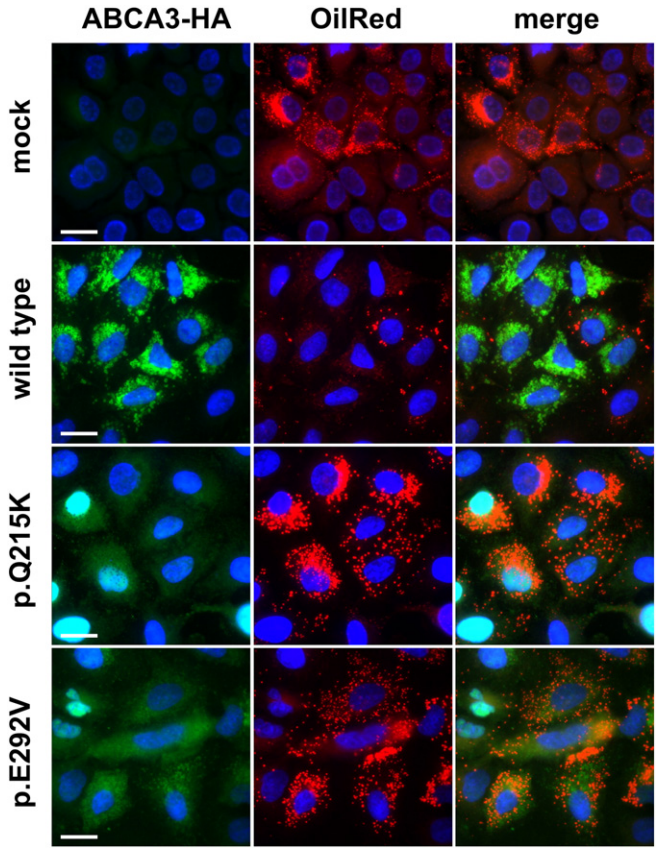

Fig. 6. Expression of wild type ABCA3 reduces cellular lipid droplet content. Lipid droplets in stably transfected A549 ABCA3-WT, p.Q215K and p.E292V cells and mock transfected cells were visualized by Oil Red O staining and fluorescence microscopy. Cells were co-stained with immunofluorescent HA-tag-antibody to label overexpressed ABCA3 protein (green pseudocolor). Scale bars: 10 μm.

we found marked up-regulation of *INSIG1* in ABCA3-WT cells while for *INSIG2*, no significant change of expression was noticed (Fig. 4C+D). Also several other SREBP target genes were found to be up-regulated in cells expressing ABCA3-WT. Among them were genes involved in the de novo biosynthesis of cholesterol, such as HMG-CoA synthase (*HMGCS1*), HMG-CoA reductase (*HMGCR*), which is the rate-controlling enzyme of the pathway, farnesyl pyrophosphate synthase (*FDPS*), squalene synthase (*FDFT1*), and squalene epoxidase (*SQLE*), and also the LDL receptor (*LDLR*) (Fig. 5). Unlike expression of ABCA3-WT, expression of mutated ABCA3 failed to induce expression of SREBPs and INSIG1; mRNA levels of all three proteins were similar to mock controls in cells expressing either ABCA3–Q215K or ABCA3–E292V (Fig. 4A–C). Consistent with absent SREBP induction, the SREBP target genes we analyzed showed significantly lower expression in cells expressing mutated ABCA3 when compared to ABCA3-WT expressing cells (Fig. 5).

3.5. Cells expressing mutated ABCA3 accumulate excessive cholesteryl esters in lipid droplets

Since the expression of mutant ABCA3 resulted in significantly increased cellular levels of CE, intracellular distribution of CE (and other neutral lipids) was visualized using oil red staining. While abundant lipid droplets were seen in cells expressing ABCA3–Q215K and ABCA3–E292V and also in mock transfected cells, they were scarcely present in ABCA3-WT expressing cells (Fig. 6). It is worthy of mention that lipid droplets seemed to be smaller in size in mock transfected cells compared to cells expressing mutant ABCA3.

3.6. ABCA3 protects cells from FC-induced cytotoxicity

Since ATII cells are challenged by exogenous cholesterol when taking up surfactant by endocytosis, we asked whether ABCA3 mutations would interfere with the cells' capacity to deal with excess FC. To analyze the effects of exogenous FC on cells expressing wild type and mutated ABCA3, cellular plasma membranes were loaded with FC using β-MCD and subsequently stained with filipin. As a result of cholesterol loading, filipin staining was markedly more intense in all cells (Fig. 7). ABCA3-WT expressing cells were filled with filipin positive vesicles; FC staining again colocalized with the ABCA3 signal. These vesicles were hardly found in ABCA3–E292V cells where the strongest staining was observed at the plasma membrane. In cells expressing ABCA3–Q215K and also in mock transfected cells, a change of cell morphology and membrane blebbing was noticed when compared to untreated control cells, indicating cell death (Fig. 7, arrowheads).

To assess the effect of cholesterol loading on cell viability, a XTT assay was performed. While cell viability was almost unchanged in cells expressing wild type ABCA3 and only a slight reduction was seen in ABCA3 p.E292V and mock transfected cells, viability was reduced significantly in ABCA3–Q215K cells (Fig. 8A). In the case of ABCA3–Q215K and mock transfected cells, the effect of cholesterol loading on cell viability was partly reversed when cells were simultaneously treated with U18666A (10 μM) which inhibits egress of cholesterol from late endosomes (Fig. 8B). Treatment of cells with β-MCD alone did not significantly alter cell viability (data not shown).

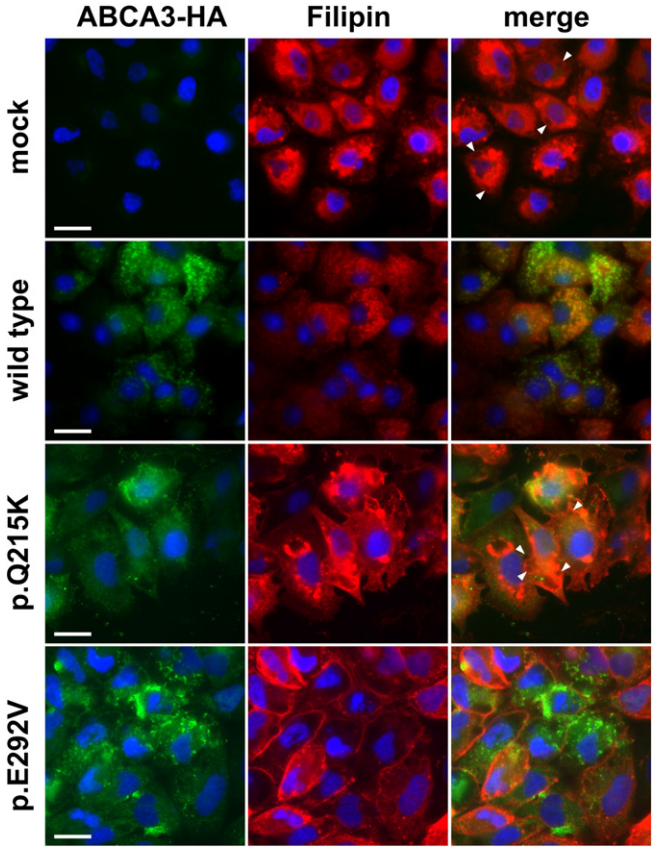

Fig. 7. Effect of free cholesterol loading on A549 cells. Cells expressing ABCA3-WT, p.Q215K and p.E292V and mock transfected cells were treated with free cholesterol-β-methylcyclodextrin complex. Free cholesterol was then visualized by filipin and ABCA3 was co-stained with anti HA-tag antibody green (pseudocolor). White arrowheads indicate membrane blebbing. Scale bars: 10 μm.

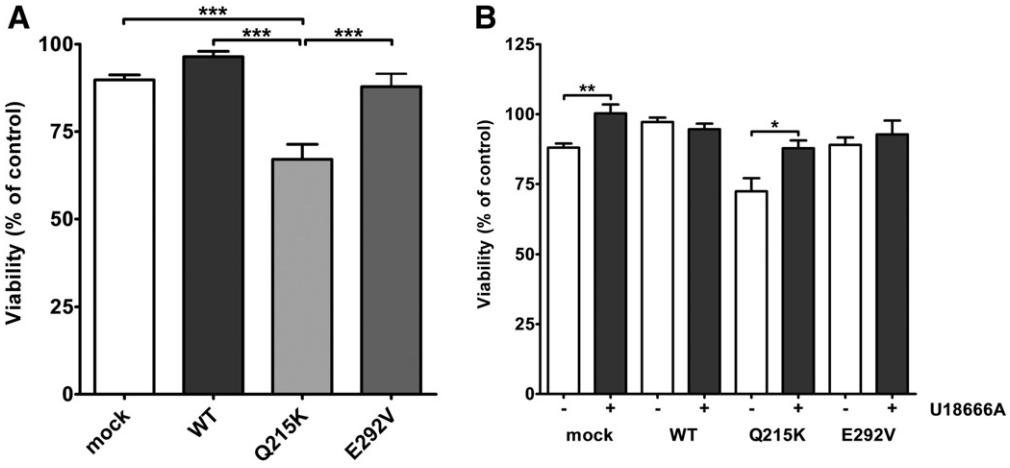

Fig. 8. Effect of free cholesterol loading on cell viability. (A) Cells expressing ABCA3-WT, p.Q215K and p.E292V and mock transfected cells were treated with free cholesterol–β-methylcyclodextrin complex before cell viability was assessed by XTT assay. (B) The same experiment was performed in the presence (+) of absence (−) of the cholesterol trafficking inhibitor U18666A. Viabilities are given as percent of wild type (n = 6). *P < 0.05, ***P < 0.001.

4. Discussion

In the current study, we show that ABCA3 reduces cellular levels of FC by acting as a FC exporter. Furthermore, we show that ABCA3 mutations affect the cells' capability to deal with exogenous cholesterol: while in cells expressing wild type ABCA3, FC loading had little effect on cell viability, FC loading proved to be toxic especially to cells expressing the mistrafficking mutation p.Q215K. Thus, ABCA3 protects cells from free cholesterol toxicity and interference by mutations abrogates this protective effect. Increased vulnerability to FC toxicity represents a novel mechanism by which ABCA3 mutations can damage ATII cells and ultimately cause DPLD.

We propose that ABCA3 facilitates lowering of FC levels by removing FC from the cell. This is in concordance with the known transport of FC by ABCA3 into LB during LB biogenesis [8]. LB FC is secreted into the alveolar space together with surfactant proteins and phospholipids when LBs fuse with the plasma membrane. In addition, several lines of evidence point to ABCA3 acting as a cholesterol exporter: 1) expression of ABCA3 results in reduced levels of ER cholesterol as shown by increased SREBP activity; 2) filipin staining showed cholesterol to be

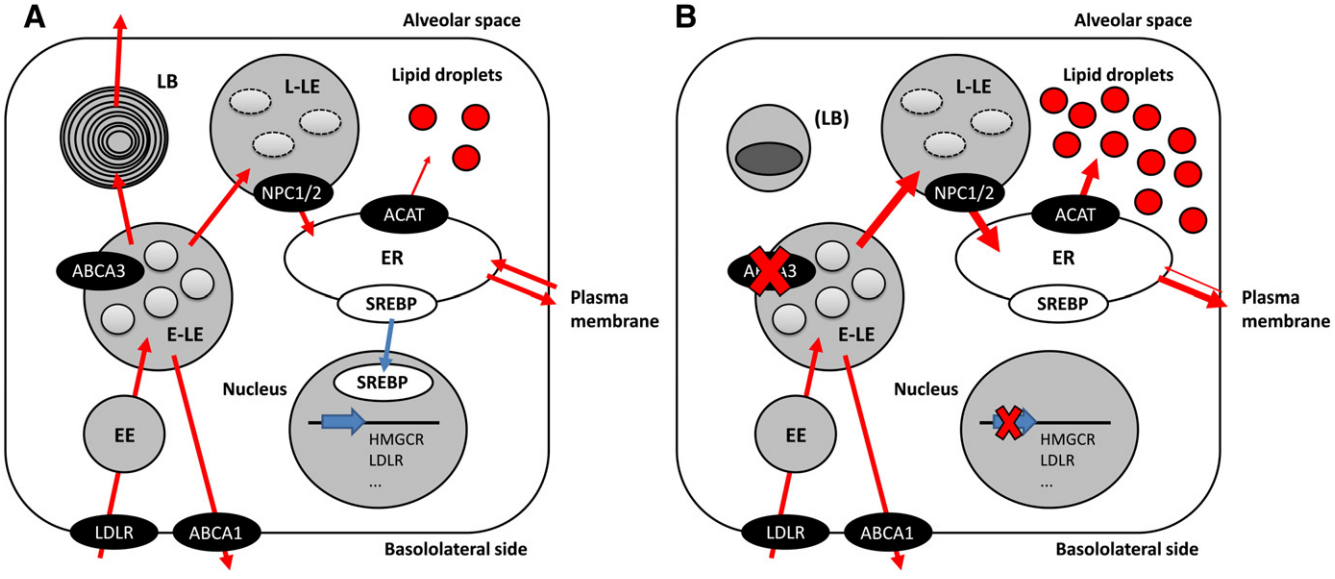

Fig. 9. Role of ABCA3 in cellular cholesterol trafficking. Red arrows indicate cholesterol flux through cellular compartments. A) Physiological state: cholesterol is either taken up from circulation by the LDL receptor or from the alveolar space together with surfactant phospholipids. It is currently unknown whether this uptake is receptor-mediated and which receptor is responsible. Cholesteryl esters from LDL are cleaved to free cholesterol as they reach the late endosomes (LE). Cholesterol can be removed from the early endosomal (EE) compartment by the plasma membrane ABC transporter ABCA1 and loaded onto HDL particles. Once in the LE, cholesterol can enter two routes: the first is facilitated by ABCA3 which resides in a subpopulation of LE termed "early late endosomes" (E-LE) and transports cholesterol along with phospholipids and thereby forms intralumenal vesicles characteristic for LE. From the latter, lamellar bodies (LB) originate and upon their fusion with the plasma membrane, cholesterol is removed from the cell as a component of surfactant. The second route for cholesterol export from LE is mediated by the Niemann-Pick proteins NPC1 and NPC2 which reside in a second subpopulation of LE called "late late endosomes" (L-LE) and leads to the ER. Once in the ER, cholesterol may be re-esterified by Acyl-CoA-Acyltransferase (ACAT) and stored into lipid droplets together with triglycerides. The cholesterol sensor SREBP resides in ER membranes and if their cholesterol content is low, translocates to the nucleus where it activates transcription of genes associated with cholesterol biosynthesis and uptake (*HMGCR*, *LDLR*, etc.). From the ER, cholesterol can also reach the plasma membrane which represents the largest cholesterol pool in most cells. B) Pathological state induced by dysfunction of ABCA3 caused by mutations: ABCA3 transport function is lost or reduced substantially. As a result, less LB are formed which are also smaller in size and lack multilamellar structure. Instead, they acquire a fried-egg like appearance typical for ABCA3 mutations. Due to absent ABCA3-dependent transport, more cholesterol is routed to the ER via the NPC-dependent pathway. An elevated ER cholesterol level has several consequences: 1) more cholesterol is esterified by ACAT and more lipid droplets are formed; 2) more cholesterol is trafficked to the plasma membrane; and 3) SREBP cannot leave the ER and thus, expression of its target genes is suppressed. If ER cholesterol levels remain high for a longer period of time, apoptosis of the cells will ultimately occur. Due to increased cholesterol levels in the endosomal–lysosomal system, basolateral ABCA1-dependent cholesterol export can be increased.

concentrated in ABCA3-positive intracellular vesicles; and 3) inhibition of exocytosis using TFP resulted in a marked accumulation of cholesterol-laden, ABCA3-positive vesicles. Regarding the identity of these vesicles, van der Kant et al. recently showed that ABCA3 localizes to subset of late endosomes (LE) they termed "early late endosomes" [29]. In concordance with our findings, the authors suggest that ABCA3 decreases LE lipid load by loading FC and possibly other lipids onto HDL or pulmonary surfactant lipoprotein particles.

Based on these findings, we put forward the hypothesis that by mobilizing cholesterol from the endosomal–lysosomal compartment, ABCA3 reduces flux of cholesterol through the NPC-dependent pathway to the ER. Diminished ER cholesterol level in turn activates SREBPs and decreases acyl-coenzyme A: cholesterol acyltransferase (ACAT) activity and storage of cholesteryl esters in the form of lipid droplets. If ABCA3 function is disturbed by mutations, more FC remains in the endosomal–lysosomal compartment and is eventually trafficked to the ER where SREBP activity is repressed and ACAT and lipid droplet formation are activated in an attempt to decrease FC levels. These proposed mechanisms are summarized in Fig. 9.

Handling of FC is of critical importance for ATII cells since these cells play a major role in clearance of inactivated surfactant [30]. Uptake of surfactant results in cholesterol loading of the endosomal compartment and without proper ABCA3 function, excess cholesterol can cause damage to the cell. Especially elevated ER cholesterol levels can have detrimental effects. For example in macrophages, cholesterol loading of the ER was reported to deplete ER calcium stores which induces the unfolded protein response (UPR), ultimately leading to the activation of caspase 3 and apoptosis [31]. The assumption that ER cholesterol loading is involved in the toxic effects of FC in cells expressing mutated ABCA3 is supported by the following observations: 1) diminished SREBP activity as an indicator of high ER cholesterol and 2) dependence on intact FC trafficking to the ER as evidenced by alleviation of FC toxicity by U18666A which inhibits egress of FC from lysosomes. The latter phenomenon can also be observed in macrophages [32]. Apart from ER cholesterol loading, continuously elevated FC levels can cause damage to the cell in several other ways. For example, the ratio of FC to phospholipid determines the fluidity of membranes; if it rises above a physiological level, loss of membrane fluidity can result in dysfunction of integral membrane proteins like ACAT [19]. Macrophages adapt to increasing FC by increasing phospholipid synthesis, especially of phosphatidylcholine [33]. The same mechanism seems to be at work in A549 cells where we observed increased levels of phospholipids and phosphatidylcholine in particular.

Here we also delineate differentially the effect of two clinically relevant ABCA3 mutations, i.e. p.Q215K and p.E292V. While p.Q215K was associated with severe respiratory distress and early death [34], p.E292V seems to permit longer survival [3,13]. Concordantly, our studies support earlier results that ABCA3–E292V function is moderately impaired [21]. Cell viability after FC loading was only slightly reduced compared to wild type ABCA3, pointing to considerable residual cholesterol transport activity of this mutated protein. In contrast, expression of ABCA3–Q215K resulted in reduced viability not only compared to wild type ABCA3 but also compared to mock transfected cells. Since endogenous ABCA3 is present in A549 cells [4], this finding supports a dominant negative effect of this mistrafficking mutation.

While our own findings and previous in vitro studies support a role for ABCA3 in cholesterol homeostasis [35], the evidence from in vivo models is not clear. Although prominent alterations of lung phospholipid content were found in Abca3$^{-/-}$ mice, no significant change in cholesterol levels was noted [36,37]. However, Besnard et al. reported reduced expression of cholesterol biosynthesis genes in mice harboring conditional knockout of Abca3 at nine months of age [38]. Although the authors did not report direct measurement of cholesterol, reduced cholesterol biosynthesis is possibly a consequence of cholesterol accumulation in these mice. As it is the case with lung disease in Abca1$^{-/-}$ and Abcg1$^{-/-}$ mice [39,40], it is very likely that the effects of disturbed cholesterol homeostasis due to ABCA3 defects only become evident after a considerable time period.

There are some important limitations to our study. First, in our study, the plasma membrane and endosomes are loaded with cholesterol using β-MCD. This procedure may not adequately mimic the uptake of cholesterol from the alveolar space. It is therefore possible that β-MCD produces endosomal FC levels that exceed those found in ATII cells challenged by uptake of surfactant. However, even doubling the concentration of β-MCD-cholesterol did not reduce viability of cells expressing wild type ABCA3 (data not shown), indicating that FC levels were well inside the range that can be handled by ABCA3. Second, although widely used, there are concerns regarding the suitability of A549 cells as a model for AT II cells and confirmation of our results is clearly required in a more in vivo equivalent setting. Third, there is still no definitive proof that cholesterol is actively transported by ABCA3. It may be that ABCA3-mediated PC transport creates a sink where cholesterol passively accumulates [8]. Our results may also be explained by a secondary effect of ABCA3 mutations on cellular cholesterol homeostasis, for example by compensatory regulation of other transport proteins.

Taken together, we show that ABCA3 protects cells from FC toxicity due to uptake of exogenous cholesterol by facilitating FC removal from the cell. Mutations in ABCA3 can diminish this export function of the transporter and reduce cellular resistance to FC, leading to increased cell death. Death of alveolar cells due to FC toxicity will compromise the function of the alveolar epithelium and trigger tissue remodeling which may result in the development of fibrosis. Based on these novel insights, we identified intracellular cholesterol trafficking as a promising target for therapeutic intervention in ABCA3-induced DPLD.

Transparency document

The Transparency document associated with this article can be found, in online version.

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