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Functional rescue of mutant ABCA3 by correctors and potentiators

vorgelegt von

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Summary

Pulmonary surfactant is a complex mixture of lipids and proteins that lines the alveolar surface to prevent alveolar collapse by reducing the surface tension at the air-liquid interface. Therefore, surfactant plays a critical role for normal gas exchange and lung function. ABCA3, an ABC transporter in alveolar type II (ATII) cells, plays a key role in surfactant homeostasis. Using the energy of ATP hydrolysis by its nucleotide binding domains (NBDs), it translocates surfactant lipids into lamellar bodies (LBs), the storage compartment for surfactant. Mutations in ABCA3 display a common genetic cause for surfactant deficiency-induced respiratory diseases like fatal respiratory distress syndrome in neonates and interstitial lung disease in children and adults. To date no therapy that addresses the underlying cause is available.

In recent years, promising success regarding the pharmacological rescue of mutant CFTR, which is also an ABC transporter, was achieved. Since CFTR and ABCA3 show structural similarity, the aim of this study was to investigate if correctors and potentiators that were shown to rescue CFTR folding and function, respectively, also rescue mutant ABCA3, and to identify possible modulators that might serve as a therapeutic approach in the future. A549 cells, which display a valid model for ATII cells, were stably transfected with hemagglutinin (HA)-tagged wild type or mutant ABCA3. To enable quantification of lipid transport activity of ABCA3, a functional assay was established, in which the transport of fluorescently labeled PC (TopF-PC), the main constituent of surfactant, into ABCA3-HA positive vesicles is assessed.

Evaluation of processing, trafficking, localization and function of the mutant ABCA3 proteins enabled the categorization of mutations as misfolding or functional mutation. Misfolding mutations lead to the retention of the protein in the ER, impeding its correct processing, trafficking, and localization. Functional mutations in contrast do not influence correct processing and trafficking to LBs, but result in impaired lipid transport function, most likely by abolishing ATP binding or hydrolysis.

Temperature sensitivity of mutant protein was shown for four out of five misfolding ABCA3 mutants. The chemical chaperone TMAO and the small molecule correctors C13 and C17 also rescued processing, trafficking, and localization of the same four mutant

proteins. Mutant ABCA3 rescued by C13 and C17 was further shown to be functionally active by quantifying TopF-PC transport.

Furthermore, the effects of the two CFTR potentiators genistein and ivacaftor were evaluated for five functional ABCA3 mutations. The potentiators rescued the diminished lipid transport function of three of the mutants with mutations located in the first NBD of the protein. The remaining two mutants likely impair protein function by other means than impaired ATP binding and hydrolysis and were therefore not rescued by potentiator treatment.

The results presented in this thesis display a first proof that misfolding and functional ABCA3 mutations can be modulated by correctors and potentiators, respectively, providing a potential novel therapeutic option for the treatment of diseases resulting from ABCA3 deficiency.

1 Introduction

1.1 Alveolar cells and surfactant

In the lung, the air is conducted through a highly branched respiratory system leading to approximately 300 million alveolar sacs, which provide an extensive surface for gas exchange (Whitsett et al., 2010). The alveolar surface is mainly covered by two distinct epithelial cell types, alveolar type I (ATI) and type II (ATII) pneumocytes. The flat, non-dividing, squamous ATI cells cover about 90% of the alveolar surface and are important for the gas exchange between the alveolar space and the alveolar capillaries (Crapo et al., 1982). The cuboidal ATII cells cover about 5% of the surface area, are metabolically very active and contain a large number of cell organelles (Fig. 1A). They serve as progenitors of the epithelial cells and are able to transdifferentiate into ATI cells in an event of lung injury (Adamson & Bowden, 1975; Evans et al., 1973). They also play a role in innate immunity and have immunomodulatory functions by secreting anti-inflammatory and antimicrobial substances, chemokines and cytokines. The major function of ATII cells is the production, storage and secretion of pulmonary surfactant (Mason, 2006).

Surfactant, short for surface-active agent, is a complex lipoprotein mixture that lowers the surface tension at the air-liquid interface to prevent alveolar collapse at the end of expiration and thus allows normal gas exchange (Clements, 1957; Pattle, 1955; von Neergaard, 1929). It maintains the alveolar size in the different phases of the respiratory cycle and is important for lung compliance. Furthermore, surfactant is implicated in host defense (Clements, 1977; Wright, 1997, 2005). Surfactant is composed of approximately 90% lipids, mostly phospholipids (PLs) and 10% proteins (Goerke, 1998). The main phospholipid component is phosphatidylcholine (PC), which accounts for about 60-70% of the PLs, and is mainly present in its saturated form as dipalmitoylphosphatidylcholine (DPPC, about 40% of total PC) (Kahn et al., 1995). DPPC is the only surfactant component capable of generating low surface tension during compression (Veldhuizen et al., 1998). Phosphatidylglycerol (PG) displays the second most abundant PL species in surfactant (about 7%). It is important for even spreading of surfactant on the alveolar surface since PC, especially DPPC, has low spreading properties (Akella & Deshpande,

2013). Apart from PC and PG, surfactant also contains low amounts of phosphatidylinositol, phosphatidylethanolamine (PE), sphingomyeline, other PLs and neutral lipids, the most abundant being cholesterol (Akella & Deshpande, 2013; Griese, 1999; Yu et al., 1983). The protein part of surfactant is mainly composed of four different surfactant proteins (SP), SP-A, SP-B, SP-C, and SP-D (Griese, 1999). The hydrophobic proteins SP-B and SP-C are essential for the structural organization of surfactant and play an important role in accelerating the adsorption of PLs at the air-liquid interface, thus contributing to the surface active function of surfactant (Oosterlaken-Dijksterhuis et al., 1991; Wang et al., 1996). Hydrophilic proteins SP-A and SP-D play an important role in innate immunity in the lung. They are able to bind bacteria, fungi and viruses and facilitate their clearance from the lung by mediating phagocytosis and killing by phagocytic cells (Kingma & Whitsett, 2006; Kudo et al., 2004; Lim et al., 1994; Van Iwaarden et al., 1994; Weikert et al., 2000). They further modulate lung inflammation (Madan et al., 1997). SP-D is also involved in regulation of surfactant pool sizes and its reuptake (Ikegami et al., 2000; Ikegami et al., 2005; Korfhagen et al., 1998).

All surfactant components are synthesized, stored, secreted and recycled by ATII cells (Fig. 1). The storage compartment for surfactant inside ATII cells are the lamellar bodies (LBs), specialized lysosome-derived secretory granules with a diameter of 1-2 μm (Weaver et al., 2002). Surfactant PLs are synthesized in the endoplasmic reticulum (ER) and transported to the LBs likely by a non-vesicular transport, since disruption of the Golgi does not affect lipid secretion (Osanai et al., 2001). At the lamellar bodies, PLs are translocated into the lumen by ATP-binding cassette (ABC) transporter A3 (ABCA3, see 1.3.2) (Ban et al., 2007; Mulugeta et al., 2002; Yamano et al., 2001). Inside LBs surfactant lipids are stored as tightly packed concentric membrane lamellae (Weaver et al., 2002), which is dependent on SP-B (Clark et al., 1995; Stahlman et al., 2000). SP-B and SP-C are synthesized in the ER as large precursors. Their proteolytic processing occurs on their route via the Golgi apparatus and multivesicular bodies (MVBs) to the LBs. SP-A and SP-D probably bypass the LBs and are targeted to the plasma membrane via secretory vesicles (Fig. 1B) (Olmeda et al., 2017; Voorhout et al., 1992).

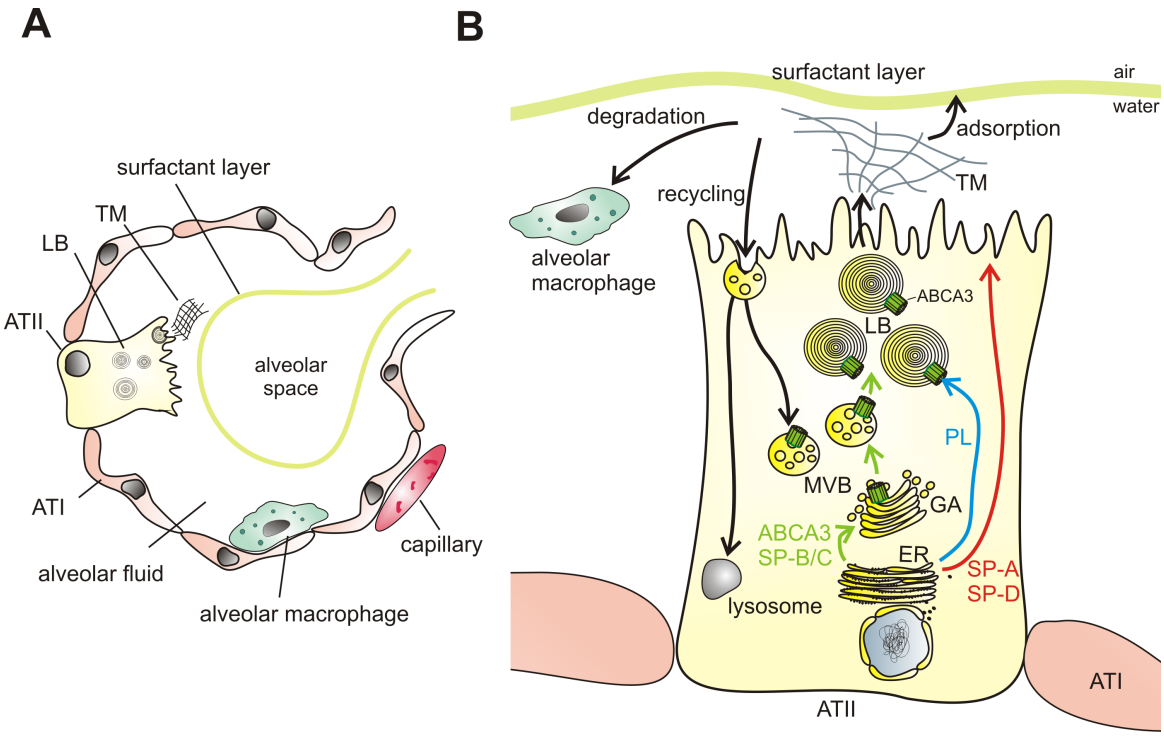

Figure 1: Structure of the alveolus and pulmonary surfactant metabolism. (**A**) Schematic structure of the alveolus. Alveolar sacs are lined by two main cell types: thin squamous alveolar type I (ATI) cells cover about 90% of the surface and are important for gas exchange between alveolar space and the capillaries and form the structure of the alveolar wall; cuboidal ATII cells synthesize, store, secrete and recycle pulmonary surfactant. The storage organelles for surfactant are the lamellar bodies (LB). Secreted surfactant reorganizes as tubular myelin (TM) and its components adsorb to the air-liquid interface to form a surfactant layer. (**B**) Surfactant metabolism. After synthesis of all surfactant components in the endoplasmic reticulum (ER), they are trafficked through the cell via separate pathways. Synthesized phospholipids (PL) are trafficked from the ER directly to the lamellar bodies (LB), where they are translocated into the LB lumen by the lipid transporter ABCA3. ABCA3 and the hydrophobic surfactant proteins (SP) B and C are routed via the Golgi apparatus (GA) and multivesicular bodies (MVB) to the LBs. Inside LBs, surfactant lipids and proteins are stored as tightly packed bilayer membranes. SP-A and D are probably targeted to the plasma membrane by secretory vesicles and bypass storage in LBs. LB contents are secreted into the alveolar fluid via regulated exocytosis and the components rearrange as tubular myelin (TM) and adsorb to the air-liquid interface to form a surfactant layer. Surfactant components can be removed from the surfactant layer and are either cleared by alveolar macrophages or taken up by ATII cells for recycling or degradation in lysosomes. ABCA3, ATP-binding cassette transporter A3, ATI/II, alveolar type I/II cell; ER, endoplasmic reticulum; GA, Golgi apparatus; LB, lamellar body; MVB, multivesicular body; PL, phospholipids; SP, surfactant protein; TM, tubular myelin.

Fusion of LBs with the plasma membrane and the secretion of surfactant into the alveolar space are primarily regulated by mechanical stretching of the alveoli during inspiration (Frick et al., 2004; Wirtz & Dobbs, 1990). After secretion into the fluid lining the alveolar surface, surfactant is re-organized as tubular myelin or vesicular structures

(Goerke, 1998; Nag et al., 1999), facilitating adsorption of PLs to form a functional surfactant film at the air-liquid interface (Griese, 1999).

Maintenance of a functional surfactant film requires removal of surfactant components and incorporation of newly synthesized and secreted components. Surfactant components are therefore either cleared by alveolar macrophages, removed via the mucociliary escalator or are taken up by receptor mediated endocytosis into ATII cells (Gurel et al., 2001; Stern et al., 1986). Internalized components are then recycled via MVBs that fuse with LBs or are targeted for lysosomal degradation (Kalina & Socher, 1990; Perez-Gil & Weaver, 2010) (Fig. 1B).

1.2 Interstitial lung disease

Interstitial lung disease (ILD), also called diffuse parenchymal lung disease (DPLD), represents a group of more than 200 rare, mostly chronic, restrictive pulmonary disorders, which are characterized by abnormal gas exchange often due to fibrotic changes in the interstitium and the alveoli after inflammation, and display significant morbidity and mortality (Fan et al., 2004; Griese et al., 2009). ILD in children (chILD) is very rare with a prevalence of 3.6 cases per million (Dinwiddie et al., 2002) and significantly differs from ILD in adults. ILDs can be distributed into two groups: disorders, which are more prevalent in infancy, and disorders, which occur at all ages (Deutsch et al., 2007).

ChILD often displays with tachypnea, crackles, hypoxemia and diffuse infiltrates on chest radiographs, but signs and symptoms of chILD are mostly unspecific (Fan et al., 2004). ILD can be caused by infections, environmental exposures, autoimmune diseases, or drugs, but most ILDs are idiopathic (Travis et al., 2002). In recent years, genetic disorders that disrupt normal surfactant metabolism have been recognized as an underlying cause of formerly idiopathic ILDs in children and adults. Affected genes include key players of surfactant metabolism like ABCA3, SP-B, SP-C, and thyroid transcription factor-1 (TTF-1), which regulates expression of *ABCA3*, *SP-B* and *SP-C* (Kolla et al., 2007; Stahlman et al., 2007; Turcu et al., 2013). Mutations in the *ABCA3* gene are the most common genetic cause of inherited surfactant diseases (Glasser et al., 2010; Wambach et al., 2012; Wambach et al., 2014) and affected patients present with

variable clinical outcomes ranging from lethal acute respiratory distress syndrome (RDS) in the neonatal period (Shulenin et al., 2004) to late and progressive chronic ILD manifestations in child- and adulthood (Kröner et al., 2017; Wambach et al., 2014).
Treatment of ILDs is mostly unspecific and includes administration of oxygen and mechanical ventilation. Pharmacological treatments include compounds like corticosteroids and hydroxychloroqine, which mainly exert anti-inflammatory effects, even though their efficacy has never been tested in clinical trials due to rarity of the disease (Braun et al., 2015; Bush et al., 2015). If patients do not respond to treatments, lung transplantation may be the last possible option to prolong survival (Eldridge et al., 2017). A causal therapy that addresses the underlying genetic defect is not available.

1.3 The ATP-binding cassette transporter ABCA3

1.3.1 ABC transporters and the ABCA subgroup

Adenosine triphosphate (ATP)-binding cassette (ABC) transporters are one of the largest protein families and are expressed in prokaryotes, plants, fungi, yeast, and animals (Vasiliou et al., 2009). They use the energy of ATP hydrolysis to translocate substrates like lipids, ions, carbohydrates, amino acids, or small proteins across the plasma membrane or intracellular membranes and are thus involved in a range of crucial biochemical and physiological processes (Higgins, 1992; Vasiliou et al., 2009).
ABC transporters share a common architecture containing two transmembrane domains (TMDs), which serve as a passageway for the substrate across the membrane, and two nucleotide-binding domains (NBDs) that bind and hydrolyze ATP to provide the energy required for substrate translocation (Higgins, 1992; Hyde et al., 1990) (Fig. 2). Those four domains are either present in a single protein (full transporter) or the protein contains one NBD and one TMD (half transporter) and assembles as homo- or heterodimers to form a functional transporter (Dean & Allikmets, 1995).
The TMDs vary considerably in their sequence and architecture explained by the heterogeneity of transported substrates (Rees et al., 2009; Saurin & Dassa, 1994). Most ABC transporters exhibit 12 transmembrane helices but their number can range between five and twelve (Rees et al., 2009).

The NBDs of ABC transporters are highly conserved and contain several conserved motifs, like the Walker A motif, or also called P-loop, which is implicated in nucleotide binding, the Walker B motif, the ABC signature motif (LSGGQ, also called C-motif), and the Q-loop (Higgins et al., 1985; Hollenstein et al., 2007; Saraste et al., 1990) (Fig. 2B). The two NBDs are arranged in a head-to-tail orientation so that the Walker A motif of one NBD is facing the LSGGQ motif of the other NBD and vice versa to create two ATP-binding sites (Chen et al., 2003; Dawson & Locher, 2007; Jones & George, 1999; Loo et al., 2002; Smith et al., 2002) (Fig. 2C).

Binding and hydrolysis of ATP at the NBDs leads to conformational changes in the NBDs that are transmitted to the TMDs leading to translocation of the substrate (Locher et al., 2002; Rosenberg et al., 2001). Transmission is achieved via coupling helices at the interface between TMDs and NBDs (Fig. 2 A, C) (Chen et al., 2001; Locher et al., 2002). Those coupling helices share only little sequence identity, but they are present in all ABC transporters, displaying a conserved mechanism of transmission (Hollenstein et al., 2007).

ABC transporters can function as importers or exporters, but importers are only found in prokaryotes (Hollenstein et al., 2007). In mammalians, 49 ABC transporters have been identified and are classified into seven subfamilies, termed ABCA to ABCG, based on their domain organization and sequence homology (Allikmets et al., 1996; Allikmets & Dean, 1998; Dean et al., 2001; Schriml & Dean, 2000).

The subfamily A of ABC transporters is composed of 12 full transporters that are expressed in a variety of different tissues (Annilo et al., 2002; Arnould et al., 2002; Kaminski et al., 2000; Klugbauer & Hofmann, 1996; Luciani et al., 1994; Prades et al., 2002). ABCA transporters are mostly involved in lipid trafficking with their substrates being phospholipids, cholesterol and sphingolipids (Borst & Elferink, 2002; Quazi & Molday, 2011). This subfamily contains the largest ABC proteins with some proteins of more than 2,100 amino acids and more than 200 kDa in size (Prades et al., 2002). ABCA5, ABCA6, ABCA8, ABCA9, and ABCA10 built up a subgroup inside the ABCA subfamily. They share a very high sequence similarity, are clustered on chromosome 17q24 and are generally shorter than the other ABCA members (Arnould et al., 2002).

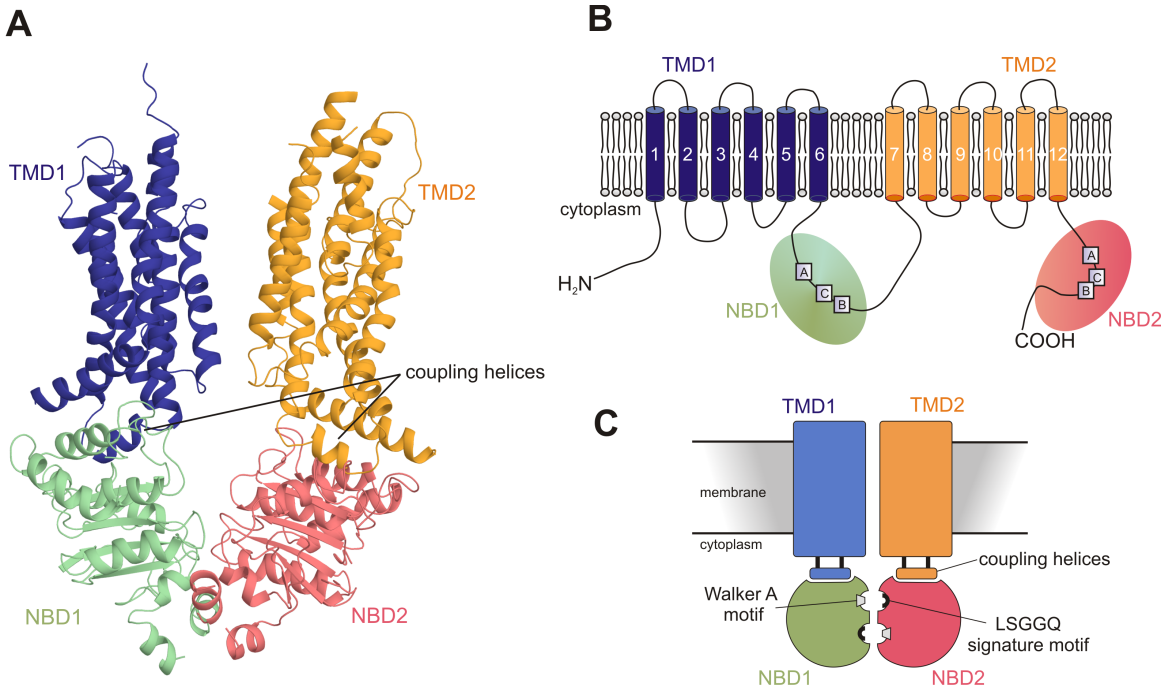

Figure 2: ABC transporter models. (**A**) 3D model of the ABCA3 transporter, showing the two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). The coupling helices transmit conformational changes of the NBDs to the TMDs for substrate translocation. (**B**) 2D model of an ABC transporter showing the transmembrane helices that build a conduit through the cell membrane. NBDs contain the signature Walker A and B motives and a LSGGQ motif, also called C motif. (**C**) Schematic model of ABC transporters showing the dimerization of NBDs in a head-to-tail orientation so that the Walker A motif of one NBD is facing the LSGGQ motif of the other NBD and vice versa.

ABC transporters are involved in a variety of important physiological processes like cholesterol and lipid transport, multidrug resistance, antigen presentation, and ion transport (Abele & Tampé, 2004; Rees et al., 2009). Mutations in ABC transporter genes are thus associated with severe rare genetic diseases. About half of the human ABC transporters from all subfamilies have been identified to play a key role in distinct disorders such as Tangier disease (ABCA1) (Rust et al., 1999), Stargardt disease (ABCA4) (Allikmets et al., 1997), hereditary biliary disease (ABCB4) (de Vree et al., 1998), and cystic fibrosis (ABCC7, CFTR, see 1.4) (Kerem et al., 1989; Riordan et al., 1989).

1.3.2 ABCA3

The 80 kb *ABCA3* gene comprising 33 exons, of which 30 are transcribed, is localized on chromosome 16p13.3. It encodes for a 1704 amino acid protein with a molecular weight of about 190 kDa (Connors et al., 1997; Klugbauer & Hofmann, 1996). ABCA3 is strongly expressed in the lung, but it is also observed in a variety of other tissues including heart, brain, liver, kidney, and pancreas (Stahlman et al., 2007). In the lung, ABCA3 expression is restricted to ATII cells, where it localizes to the outer membrane of LBs to mediate the translocation of surfactant lipids such as PC, PG, phosphatidylserine (PS), and PE into lamellar bodies and is also implicated in cholesterol transport (Ban et al., 2007; Cheong et al., 2006; Cheong et al., 2007; Fitzgerald et al., 2007; Matsumura et al., 2007; Zarbock et al., 2015). ABCA3 thus represents a key player in surfactant homeostasis and LB biogenesis.

The structure of ABCA3 resembles the typical ABC transporter architecture with two TMDs, comprised of six transmembrane helices each, and two NBDs (Fig. 2A, 3). Furthermore, ABCA3 contains a signature-targeting motif (xLxxKN) that routes the protein to post-Golgi sorting vesicles. Since all ABCA transporters (except ABCA10) exhibit this motif but all have different subcellular localizations, subsequent targeting of ABCA3 to the LBs needs further not yet identified signals (Beers et al., 2011). ABCA3 moreover contains two N-linked glycosylation sites at positions N124 and N140, which are important for protein stability (Beers et al., 2013) (Fig. 3).

ABCA3 expression is strongly induced during lung development and peaks shortly before birth (Mulugeta et al., 2002). The ABCA3 promoter contains a glucocorticoid-responsive element; thus ABCA3 expression is induced by glucocorticoids like dexamethasone, which are involved in fetal maturation (Yoshida et al., 2004).

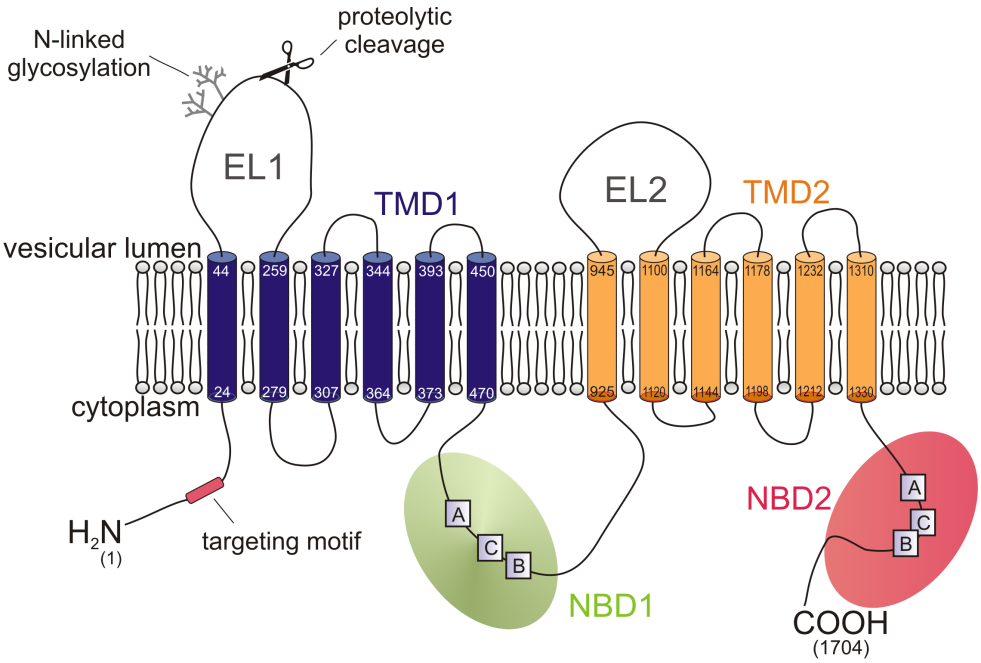

Figure 3: ABCA3 topology. ABCA3 contains two transmembrane domains (TMDs) comprised of six transmembrane helices each, and two nucleotide-binding domains (NBDs) with the signature Walker A and B motives and a C motif. The targeting motif at the N-terminus for routing of the protein to post Golgi sorting vesicles and glycosylation sites at residues 124 and 140 are marked. Scissors mark the proteolytic cleavage site at residue 174. EL: external loop.

After transcription, translation, and translocation to the ER, the ABCA3 protein passes the Golgi apparatus, sorting vesicles, and MVBs on its route to the LBs (Fig. 1B) (Beers et al., 2013; Cheong et al., 2006; Mulugeta et al., 2002; Nagata et al., 2004). During its trafficking through the cell, ABCA3 is glycosylated and the N-terminus is proteolytically cleaved by cathepsins L and B in post-Golgi compartments (Engelbrecht et al., 2010; Hofmann et al., 2016) (Fig. 3). The cleavage of ABCA3 results in the presence of two protein forms in the cell, the 190 kDa full-length protein and a shortened product of about 170 kDa in size (Cheong et al., 2006; Hofmann et al., 2016; Matsumura et al., 2006; Nagata et al., 2004). It is not yet clear if this process is a step of activation or rather degradation of the protein, but presence of the shortened cleavage product might serve as a biomarker for correct trafficking of the protein (Beers & Mulugeta, 2017). In the process of LB secretion, ABCA3 attains the plasma membrane and is subsequently recycled to the LBs or degraded in the lysosomes (Schaller-Bals et al., 2000) (Fig. 1).

1.3.3 ABCA3 mutations

The important role of ABCA3 in normal lung function is underlined by the discovery that bi-allelic ABCA3 deficiency in full-term infants leads to surfactant deficiency and RDS (Brasch et al., 2006; Garmany et al., 2006; Shulenin et al., 2004).

Bronchoalveolar lavage of the patients displayed decreased amounts of PC and PG, reducing the surface activity of surfactant (Garmany et al., 2006; Griese et al., 2015). Affected patients also showed decreased numbers or a complete lack of mature LBs, but displayed electron-dense inclusion organelles with densely packed lipid core structures instead of lamellae (electron-dense bodies) (Edwards et al., 2005; Shulenin et al., 2004). In mouse models, homozygous *Abca3* null mice died shortly after birth due to respiratory distress. They displayed no mature LBs in ATII cells but electron-dense bodies and severely decreased amounts of PC and PG in the pulmonary surfactant, mirroring the findings in ABCA3-deficient infants (Ban et al., 2007; Fitzgerald et al., 2007; Hammel et al., 2007). Heterozygous deletion led to a decrease of PC, PG, PE, and PS in surfactant and less LBs in the ATII cells of the lung (Cheong et al., 2007).

ABCA3 mutations display the most common genetic cause of surfactant related disorders like RDS and ILD and to date, more than 200 mutations have been identified in the human *ABCA3* gene, including nonsense, frameshift, missense, and splice site mutations, insertions, and deletions (Kröner et al., 2017; Wambach et al., 2014). Nonsense and frameshift mutations, which lead to an ABCA3 null phenotype, consistently show poor outcomes and affected infants die shortly after birth. Missense or splice site mutations or insertions/deletions on the other hand are associated with a more chronic phenotype and the age of presentation and clinical outcome vary markedly (Kröner et al., 2017; Wambach et al., 2014). Despite the nature of mutation, other factors can influence the clinical outcomes, including environmental factors, genetic predispositions, and clinical interventions (Wert et al., 2009; Young et al., 2008). Most ABCA3 mutations are unique and restricted to individuals or families and are often carried in compound heterozygosis. Homozygous mutations often result from consanguinity, but uniparental disomy has also been reported (Hamvas et al., 2009; Shulenin et al., 2004).

In vitro studies in A549 cells, a human adenocarcinoma cell line, identified different types of ABCA3 missense mutations (Matsumura et al., 2006). They can result in protein

misfolding, which is recognized by the cell's quality control mechanisms and subsequently leads to ER retention of the mutant protein and its degradation. The protein is not trafficked through the cell and does not reach the LBs (Matsumura et al., 2006; Matsumura et al., 2008; Weichert et al., 2011; Young et al., 2008). Those mutations are termed misfolding mutations and include mutations like L101P or Q215K (Engelbrecht et al., 2010; Matsumura et al., 2006). Mutations in or in close proximity to the NBDs often lead to a functional impairment of ABCA3. The protein indeed reaches the LBs but its ATP binding or hydrolysis function is impaired, resulting in impaired lipid transfer. Those mutations are termed functional mutations and include mutations like N568D or E690K (Matsumura et al., 2006; Matsumura et al., 2008).

1.4 CFTR and cystic fibrosis

Cystic fibrosis transmembrane conductance regulator (CFTR) belongs to the family of ABC transporters (ABCC7), and is the only known member that acts as an anion channel (Anderson et al., 1991a; Bear et al., 1992). The *CFTR* gene is located on chromosome 7q31.2 and encodes for the 1480 amino acid CFTR protein with a molecular weight of 180 kDa (Kerem et al., 1989; Riordan et al., 1989). CFTR is located in the apical membrane of epithelial cells of the lung, liver, pancreas, intestine, reproductive tract, and the sweat glands, where it mediates chloride and bicarbonate transport (Engelhardt et al., 1994; Trezise et al., 1993). Like all ABC transporters, CFTR consists of two TMDs with six transmembrane helices each, forming the channel, and two NBDs that bind and hydrolyze ATP (Higgins, 1992; Riordan et al., 1989). Furthermore, CFTR contains a unique large hydrophilic regulatory (R) domain that is cyclic adenosine monophosphate (cAMP)-dependently phosphorylated by protein kinase A to activate the protein (Anderson et al., 1991b; Chappe et al., 2005; Gregory et al., 1990). Gating of CFTR requires binding of ATP at both binding sites at the NBD interface (Berger et al., 2005). Binding of ATP promotes dimerization of the two NBDs, which leads to conformational changes in the TMDs that subsequently result in channel opening (Csanády et al., 2010; Vergani et al., 2003; Vergani et al., 2005). Experiments showed that ATP in the first ATP binding site is slowly hydrolyzed, while ATP in the second binding site is hydrolyzed

more rapidly (Aleksandrov et al., 2002; Basso et al., 2003), leading to the assumption that hydrolysis of the ATP in the second binding site and release of adenosine diphosphate (ADP) and P_i initializes channel closing (Berger et al., 2005; Carson et al., 1995; Ikuma & Welsh, 2000).

In healthy lungs, the chloride efflux through CFTR channels leads to the establishment of an osmotic gradient that results in water flow into the luminal space to keep the airway surface liquid (ASL) optimally hydrated (Anderson et al., 1991b; Saint-Criq & Gray, 2017). ASL is a thin fluid layer that covers the airway epithelium surface and has important functions in trapping and removing inhaled particles. The volume, pH, and composition of ASL play critical roles for its antimicrobial properties and also for ciliary function of epithelial cells and mucociliary clearance (Abou Alaiwa et al., 2014; Saint-Criq & Gray, 2017; Tarran et al., 2002).

CFTR dysfunction leads to cystic fibrosis (CF), one of the most widespread life-shortening monogenetic diseases with an incidence of about 1 in 2,000 – 3,500 neonates (Kosorok et al., 1996; Southern et al., 2007). Mutations in CFTR result in a lack of chloride efflux accompanied by an increase in sodium absorption mediated by epithelial sodium channels, leading to an imbalance of the osmotic gradient, resulting in ASL dehydration, increased mucus viscosity, and impaired mucociliary transport. This subsequently results in plugging of the small airways, persistent bacterial infections, and chronic inflammation, which are the main causes of morbidity and mortality in patients with CF (Chen et al., 2010; Derichs et al., 2011; Gustafsson et al., 2012; Pezzulo et al., 2012; Quinton, 1983).

1.4.1 CFTR mutation classes

To date more than 2000 mutations in CFTR have been described and can be grouped into six mutation classes (Cystic Fibrosis Mutation Database (CFTR1); Welsh & Smith, 1993; Zielenski & Tsui, 1995). Class I mutations include nonsense, frameshift, and splice site mutations that lead to the total absence of mature CFTR protein. Class II mutations lead to folding and processing defects resulting in retention of the protein in the ER and its degradation instead of trafficking to the cell surface. Class III describes mutations that cause gating defects, meaning the inability of the protein to pump chloride ions even

though it is correctly trafficked to the apical membrane. Mutations of class IV influence channel conductance, i.e. reduced ion flow through the pore. Class V mutations result in reduced protein levels due to alternative splicing or promoter abnormalities that only allow very low levels of normal CFTR mRNA. Class VI mutations decrease CFTR stability at the plasma membrane (Haardt et al., 1999; Highsmith et al., 1997).

Some mutations result in more than one defect and can therefore be grouped into several classes. Mutation classes I-III are generally associated with a more severe phenotype compared to mutations of classes IV-VI (de Gracia et al., 2005; McKone et al., 2006).

Deletion of phenylalanine at position 508 (F508del) in the NBD1 represents the most common CFTR mutation and is found in about 90% of CF patients on at least one allele (Cystic Fibrosis Mutation Database (CFTR1)). The mutation impedes the protein from attaining its native conformation. The misfolded protein is recognized by the cell's quality control system and is retained in the ER und subsequently targeted for proteasomal degradation (Cheng et al., 1990; Meacham et al., 2000; Ward et al., 1995). A small portion of F508del CFTR might be able to escape the control mechanisms and reach the cell surface, but those proteins further display gating defects and decreased stability at the cell surface. F508del is therefore classified into mutation classes II, III, and VI (Dalemans et al., 1991; Denning et al., 1992; Lukacs et al., 1993).

The most common class III mutation and the third most common CFTR mutation in general is a glycine-to-aspartate mutation at position 551 (G551D), which is found in about 4% of CF patients (Cystic Fibrosis Mutation Database (CFTR1)). This mutation is located in the LSGGQ signature motif of the NBD1 of CFTR, which is implicated in ATP binding. The mutation therefore impedes ATP binding and normal CFTR function (Lin et al., 2014).

1.4.2 CFTR modulators

The identification of CFTR, its characterization, and the understanding of its dysfunction in CF were an important step towards the development of drugs that target the underlying cause of the disease. The classification of mutants allows targeting mutations of the same class with the same therapeutic strategy. Compounds that improve protein folding, processing, trafficking, and cell surface expression are called correctors and target class II

mutants. Compounds targeting class III and IV mutants by enhancing CFTR function at the cell surface are called potentiators (Solomon et al., 2015).

In recent years, major breakthroughs were achieved by developing high-throughput screens (HTS) that allow for the fast screening of hundreds of thousands of chemical compounds for the identification of CFTR modulators (Pedemonte et al., 2005; Van Goor et al., 2006). Selected compounds can subsequently be chemically modified (lead optimization) to enhance efficiency and minimize toxicity (Cheng et al., 2007). Using those approaches, one potentiator (called ivacaftor) and one corrector/potentiator combination (lumacaftor/ivacaftor, called Orkambi) were developed that ultimately were approved by the U.S. Food and Drug Administration (FDA) for the treatment of CF (Van Goor et al., 2009; Van Goor et al., 2011; Vertex Pharmaceuticals Inc., 2014, 2015).

1.4.2.1 Potentiators

The first evidence and proof of principle that small molecular compounds enhance CFTR channel activity was the finding that the isoflavone genistein enhances the activity of wild type (WT) and G551D CFTR (Illek et al., 1995; Illek et al., 1999). Genistein (4',5,7-trihydroxyisoflavone) is a phytoestrogen that naturally occurs in soybeans (Dixon & Ferreira, 2002). It affects CFTR channel activity by direct binding to the protein (Moran et al., 2005; Wang et al., 1998; Weinreich et al., 1997). Genistein binds to CFTR at the NBD interface, stabilizing the NBD dimer as well as inhibiting ATP hydrolysis at the second ATP-binding site in NBD2, which controls channel closing. Thereby genistein increases CFTR's open probability P_o by increasing the open time of the channel and decreasing its closed time (Al-Nakkash et al., 2001; Hwang et al., 1997). So far no clinical trials with genistein in CF patients were conducted. In pre-clinical studies, 200 μM genistein rescued the defects of G551D CFTR to 20% of WT level. This concentration might be too high to achieve appropriate blood concentrations (Sohma et al., 2013; Zegarra-Moran et al., 2002).

The development of HTS assays and chemical optimization of lead compounds led to the identification of the potentiator ivacaftor (VX-770) (Van Goor et al., 2009). In recombinant cells and CF patient-derived human bronchial epithelial cells, VX-770 treatment led to an increase of F508del and G551D CFTR activity, measured as increased chloride secretion, apical fluid height, and ciliary beat frequency (Van Goor et al., 2009).

Positive results in clinical studies led to the initial FDA approval of ivacaftor for treatment of patients with the G551D mutation on at least one allele (Accurso et al., 2010; Ramsey et al., 2011) that was later on further extended for eight other mutations (De Boeck et al., 2014). Recently, *in vitro* data on several more mutations was sufficient to extend the approval further, including now 38 different CFTR gating and conductance mutations (Ratner, 2017; Yu et al., 2012).

The precise mechanism of action of VX-770 is not yet understood, but it was shown to directly interact with the CFTR protein to increase the open probability of the CFTR channel by a phosphorylation-dependent, but ATP-independent mechanism (Byrnes et al., 2018; Eckford et al., 2012; Jih & Hwang, 2013).

1.4.2.2 Correctors

Correctors target class II CFTR mutants by restoring folding and trafficking of the protein and thereby increase the amount of CFTR protein that reaches the cell surface. Proof of concept that CFTR can be rescued was delivered by the finding that low temperature incubation of cells, which express F508del-CFTR, rescued the folding defect of the protein and led to its correct trafficking to the plasma membrane (Denning et al., 1992).

Correctors are divided into chemical and pharmacological chaperones and improve the protein folding by either modulating the protein homeostasis or by acting directly on the protein, respectively. Chemical chaperones can further be divided into two subclasses, osmolytes and hydrophobic chaperones (Cortez & Sim, 2014). Osmolytes like trimethylamine N-oxide (TMAO) and glycerol are products of the cell stress response and increase the hydration around the protein and thereby make the protein reduce its relative surface area by tighter packing. This hydrophobic effect stabilizes the protein and thus favors its folded state (Bolen & Baskakov, 2001; Welch & Brown, 1996). Since osmolytes work in an unspecific and colligative way, high concentrations are needed to exert desired effects (Nieddu et al., 2013). Hydrophobic chaperones like 4-phenylbutyric acid (PBA) probably regulate transcription of proteins that are involved in different folding and cell stress processes, for example heat-shock proteins (Wright et al., 2004). PBA, glycerol, and TMAO were shown to aid CFTR protein folding but act in an unspecific way and therefore high concentrations are needed for correction, which

precludes their use in clinical studies (Brown et al., 1996; Fischer et al., 2001; Rubenstein et al., 1997; Rubenstein & Zeitlin, 2000; Sato et al., 1996).

The first two correctors identified by HTS were the bithiazole C4 (also called corr-4a) and the quinazolinole C3 (also called VRT-325). But these two compounds showed low oral bioavailability, low efficiency, and rather high toxicity and were therefore not suitable for clinical use (Loo et al., 2006; Pedemonte et al., 2005).

The corrector VX-809 (lumacaftor) was also identified by HTS and was chemically optimized to enhance its potency and decrease its toxicity. VX-809 showed a high efficacy *in vitro*, especially in primary cultures of bronchial epithelial cells from CF patients harboring the F508del mutation (Van Goor et al., 2011), and is well tolerated in patients (Clancy et al., 2012). Unfortunately, monotherapy with lumacaftor only showed little efficacy in patients homozygous for F508del CFTR mutation (Clancy et al., 2012), explained by the fact that F508del CFTR despite the folding defect also displays impairments in channel gating and protein stability. Therefore a combinational therapy of lumacaftor together with the potentiator ivacaftor (Orkambi) was tested *in vitro* and in clinical studies to target the folding as well as the gating defect of F508del CFTR (Boyle et al., 2014; Van Goor et al., 2011; Wainwright et al., 2015). Since lung function was significantly improved and pulmonary exacerbations were reduced in patients compared to the placebo group, Orkambi was approved by the FDA for use in patients homozygous for F508del CFTR (Vertex Pharmaceuticals Inc., 2015).

The mechanism of action of VX-809 stays elusive. The direct interaction of the compound with CFTR was shown, but the exact binding site is not yet identified (Hudson et al., 2017; Loo et al., 2013; Ren et al., 2013). VX-809 is probably stabilizing the first TMD (Loo et al., 2013; Okiyoneda et al., 2013; Ren et al., 2013), improves its folding (Ren et al., 2013), and stabilizes interactions between the TMDs and the NBDs (Farinha et al., 2013; Loo & Clarke, 2017; Okiyoneda et al., 2013).

2 Aim of the study

Surfactant is a complex mixture of lipids and proteins that adsorbs to the alveolar air-liquid interface to reduce surface tension and thus prevents alveolar collapse at the end of expiration. The lipid transporter ABCA3 plays a key role in surfactant homeostasis. In ATII cells it translocates surfactant lipids into the lumen of LBs, where surfactant is assembled and stored before its exocytosis into the alveolar space. Mutations in ABCA3 display a common genetic cause for diseases like fatal surfactant deficiency-induced respiratory distress in neonates and interstitial lung disease in children and adults, for which currently no causal therapy exists.

Since CFTR and ABCA3 both belong to the ABC transporter family, they share structural similarities. Hence, recent promising advance in the identification of correctors and potentiators to rescue trafficking or function of mutant CFTR, respectively, may provide potential options to rescue mutant ABCA3. The aim of this study was therefore to prove the concept that disease-causing mutant ABCA3 can be modulated by correctors and potentiators *in vitro* and to investigate available options for its functional rescue.

To analyze the effect of modulators on mutant ABCA3, different clinically relevant missense mutations were introduced into a pT2/HB transposon vector containing HA-tagged human ABCA3 by site-directed mutagenesis. Stable expression in A549 cells, an ATII model cell line, was conducted using the Sleeping Beauty transposon system (Geurts, 2003). A sensitive functional assay was established that allows quantification of ABCA3 lipid transport activity. Several mutant ABCA3 proteins were then first characterized in regard to their processing, trafficking, localization, and transport activity. Subsequently, misfolding mutants were subjected to treatment with correctors and functional mutants were analyzed regarding their response to potentiators.

Correction of five misfolding ABCA3 mutants by low temperature, a range of chemical chaperones and several small molecule correctors of CFTR was assessed by quantification of protein processing products in Western blots and correct intracellular protein localization in vesicular structures, which resemble LBs. Furthermore, lipid transport function of rescued ABCA3 was assessed by quantification of transport of fluorescently labeled PC into ABCA3-HA positive vesicles.

Furthermore the effects of CFTR potentiators genistein and ivacaftor on five functionally impaired ABCA3 mutants, two of them in homologous location to two common CFTR mutations, were analyzed in regard to lipid transport function by analyzing the transport of fluorescently labeled PC.

3 Results

3.1 Functional rescue of misfolding ABCA3 mutations by small molecular correctors

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ORIGINAL ARTICLE

Functional rescue of misfolding ABCA3 mutations by small molecular correctors

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Abstract

Adenosine triphosphate (ATP)-binding cassette subfamily A member 3 (ABCA3), a phospholipid transporter in lung lamellar bodies (LBs), is essential for the assembly of pulmonary surfactant and LB biogenesis. Mutations in the ABCA3 gene are an important genetic cause for respiratory distress syndrome in neonates and interstitial lung disease in children and adults, for which there is currently no cure. The aim of this study was to prove that disease causing misfolding ABCA3 mutations can be corrected *in vitro* and to investigate available options for correction. We stably expressed hemagglutinin (HA)-tagged wild-type ABCA3 or variants p.Q215K, p.M760R, p.A1046E, p.K1388N or p.G1421R in A549 cells and assessed correction by quantitation of ABCA3 processing products, their intracellular localization, resembling LB morphological integrity and analysis of functional transport activity. We showed that all mutant proteins except for M760R ABCA3 were rescued by the bithiazole correctors C13 and C17. These variants were also corrected by the chemical chaperone trimethylamine N-oxide and by low temperature. The identification of lead molecules C13 and C17 is an important step toward pharmacotherapy of ABCA3 misfolding-induced lung disease.

Introduction

Surfactant, a mixture of lipids and proteins, prevents the end expiratory collapse of alveolar units and is thereby crucial for normal breathing (1–3). It is synthesized in alveolar type II cells, where the surfactant is stored in lamellar bodies (LBs), a lysosome-derived compartment (4). The transporter adenosine triphosphate (ATP)-binding cassette subfamily A member 3 (ABCA3) localizes to the limiting membrane of LBs and is involved in their biogenesis by transporting surfactant lipids into the lumen of LBs (5–8). ABCA3 consists of two transmembrane domains, each containing six transmembrane helices, and two nucleotide binding domains (NBDs) with ATP-hydrolyzing function (Fig. 1) (9,10). After folding in the endoplasmatic reticulum (ER), ABCA3 is trafficked through golgi and post-golgi compartments, where it is glycosylated and processed, respectively (5,7,11–13). The N-terminus of the 190 kDa protein is proteolytically cleaved by cathepsins L and B, resulting in a shortened 170 kDa form of the protein (12,13). The presence of the cleavage product might serve as a biomarker for correct anterograde post-golgi trafficking of the protein, which enables the processing (14), although it is not clear if this process is a step of maturation or degradation (13,14).

Mutations in ABCA3 may cause respiratory distress syndrome in mature neonates and early death, or chronic interstitial lung disease in children and adults (15,16). To date, there is no treatment targeting such disease causing mutations. Misfolding of ABC transporters due to certain mutations is the underlying cause of many diseases (17) including cystic fibrosis

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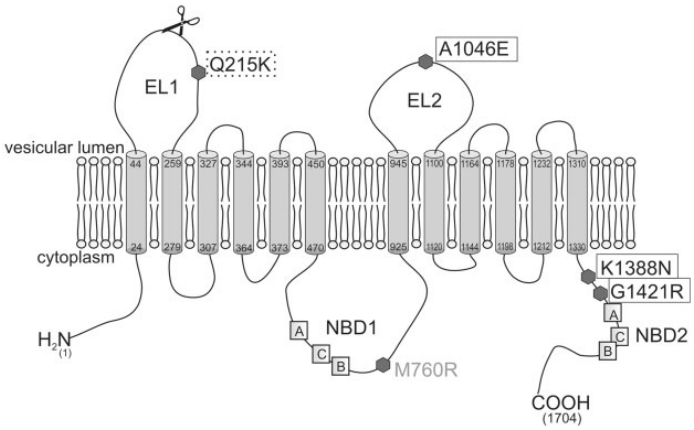

Figure 1. Topology model of ABCA3. Positions of all mutations analyzed in this study are marked. Scissors indicate the cleavage site for processing of the 190 kDa form to the 170 kDa form in post-golgi compartments. Mutations that could be corrected completely are boxed; dashed lines indicate partial correction of Q215K ABCA3. M760R was not susceptible to correction (shown in gray). EL: extracellular loop, NBD: nucleotide binding domain, A: walker A motif, B: walker B motif. C: C motif.

(CF), a disease caused by mutations in the cystic fibrosis conductance regulator (CFTR, *ABCC7*) gene (18). In recent years, small molecular correctors were identified by high throughput screening, which directly bind the mutated CFTR protein, stabilize interaction of its functional domains and restore its folding, intracellular processing, trafficking and function (19–21). The entire development and selection of such correctors were performed using cellular *in vitro* assays, refraining from animal or organ models (20). Following toxicology testing, candidates were successfully tested in humans and thus made rapidly available to patients (22,23).

The goal of this study was to prove the concept that disease causing misfolding *ABCA3* mutations can be corrected *in vitro* and to define the impact of options available to target the protein correctly. We used low temperature, which has been shown to help correct protein folding so proteins reach their final destination (24–28). We also used chemical chaperones, which generally favor a cellular milieu, are not protein specific and were shown to correct multiple ABC transporters (29). Due to structural similarity to CFTR, we also tested small molecular correctors on *ABCA3*. We show that certain mutated and mistrafficked *ABCA3* proteins can be redirected and functionally corrected to wild-type (WT) levels, setting the stage for the development of mutation-group specific drug treatment of *ABCA3* deficiency.

Results

Selection of ABCA3 missense mutations

The *in vitro* mutagenesis model consisted of A549 cells stably expressing HA-tagged WT or mutated *ABCA3* variants. As ER retention of misfolded *ABCA3* proteins interferes with proteolytic processing, the measurement of cleaved and uncleaved ABCA3 products was used as a semi-quantitative marker of mistrafficking (14,30). Five disease-causing mutations known to result in protein misfolding were selected for this study (Fig. 1, Supplementary Material, Table S2). The Q215K and M760R mutations resulted in the complete absence of the post-processing 170 kDa isoform (Fig. 2A, B, 37°C), and the A1046E, K1388N and G1421R variants resulted in a markedly decreased 170/190 kDa ratio (Fig. 2A–C, 37°C).

WT ABCA3-HA protein is localized at the limiting membrane of LBs, seen as lysosome-related organelles displaying vesicle-like structures in A549 cells, co-localizing with the lysosomal marker CD63 (Fig. 2D, 37°C). In contrast, ABCA3-HA proteins containing mutations Q215K or M760R were diffusely distributed in the cell, while proteins harboring A1046E, K1388N or G1421R mutations showed both, small vesicular structures, co-localizing with CD63, accompanied by a diffuse pattern in the cell (Fig. 2D, 37°C).

Low temperature restores processing and subcellular localization of mutant ABCA3-HA proteins

After incubating A549 cells for 48 h at 30°C, the lower 170 kDa form of ABCA3-HA was enriched for WT and all mutated proteins, except M760R ABCA3-HA (Fig. 2A and B). The lower to upper band ratio was slightly increased for all mutated proteins except M760R ABCA3-HA (Fig. 2C). Even at 26°C, no alteration was seen for M760R ABCA3-HA (Supplementary Material, Fig. S1).

Consistent with this, at 30°C, all mutated ABCA3-HA proteins except for M760R ABCA3-HA showed a similar co-localization with lysosomal marker CD63 comparable to WT ABCA3-HA expressing cells, indicating restored LB morphology (Fig. 2D). Since temperature-sensitivity gives a hint if misfolded proteins can be corrected (27,31), our findings suggest that all mutated proteins except M760R ABCA3-HA may be susceptible to correction by small molecules.

The chemical chaperone TMAO restores processing and localization of mutated ABCA3-HA

We tested the chemical chaperones trimethylamine N-oxide (TMAO), dimethylsulfoxid (DMSO), glycerol, 4-phenylbutyric acid (PBA) and suberoylanilide hydroxamic acid (SAHA), effective in other ABC misfolding disorders (summarized in 29). Western blot analysis showed that all chemical chaperones, at the higher concentration tested, led to an unspecific enrichment in total protein, without altering the 170/190 kDa ratio in mutant compared to untreated wild-type cells (Fig. 3A–F, Supplementary Material, Fig. S2). Only TMAO at the highest 200

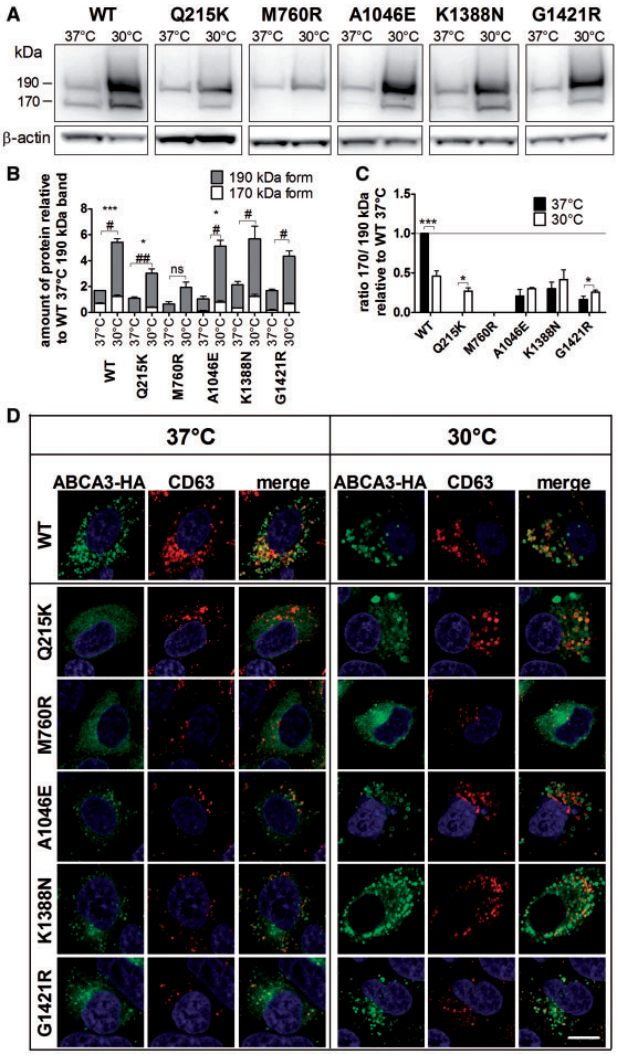

Figure 2. Defects in ABCA3-HA processing caused by mutations are temperature-sensitive. A549 cells stably expressing WT or mutated ABCA3-HA, were grown at either 37°C or 30°C for 48 h and ABCA3-HA protein was analyzed by western blot (**A**). Densitometric quantification of protein amount in each band (190 kDa and 170 kDa) was performed using Image J, with the 190 kDa form of WT protein at 37°C set to 1 (**B**). The ratio of 170/190 kDa form was calculated relative to WT at 37°C (**C**). Confocal microscopy images of cells stained for ABCA3-HA and lysosomal marker CD63 are shown in (**D**). Scale bar represents 10 μm. Results are means + S.E.M. of three independent experiments. */# $p < 0.05$; **/##$p<0.01$; ***/###$p < 0.001$ with * regarding the 190 kDa form and # regarding the 170 kDa form in (B). ns: not significant.

mM concentration was able to significantly increase the 170/190 kDa ratio to a level closer to WT for all mutated proteins except M760R (Fig. 3A–F lower panel). For Q215K, the 170/190 kDa ratio was only partially restored to a level comparable to the less deleterious mutations A1046E, K1388N and G1421R (Fig. 3B).

Analysis of subcellular localization of ABCA3-HA proteins by confocal microscopy confirmed these findings. Only TMAO was able to restore localization of all mutated proteins except Q215K and M760R ABCA3-HA in vesicular-like structures, co-localizing with CD63, comparable to WT protein in untreated cells, resembling intact LB morphology (Supplementary Material, Fig. S3). PBA and SAHA treatment led to a strong accumulation of mutant ABCA3-HA protein in all cells, not showing any vesicular structures or co-localization with CD63 (Supplementary Material, Fig. S3). Treatment with DMSO or glycerol showed no differences compared to untreated cells.

Identification of correctors to restore processing of mutated ABCA3-HA

Cells stably expressing WT ABCA3-HA and mutations were treated with correctors C2, C4, C17, C18 and VX-809 at the commonly used screening concentration of 10 μM. C17 increased the amount of the 170 kDa form of all mutated proteins except M760R ABCA3-HA (Fig. 4A–F upper panel, Supplementary Material, Fig. S4) and led to a significant increase of the 170/190

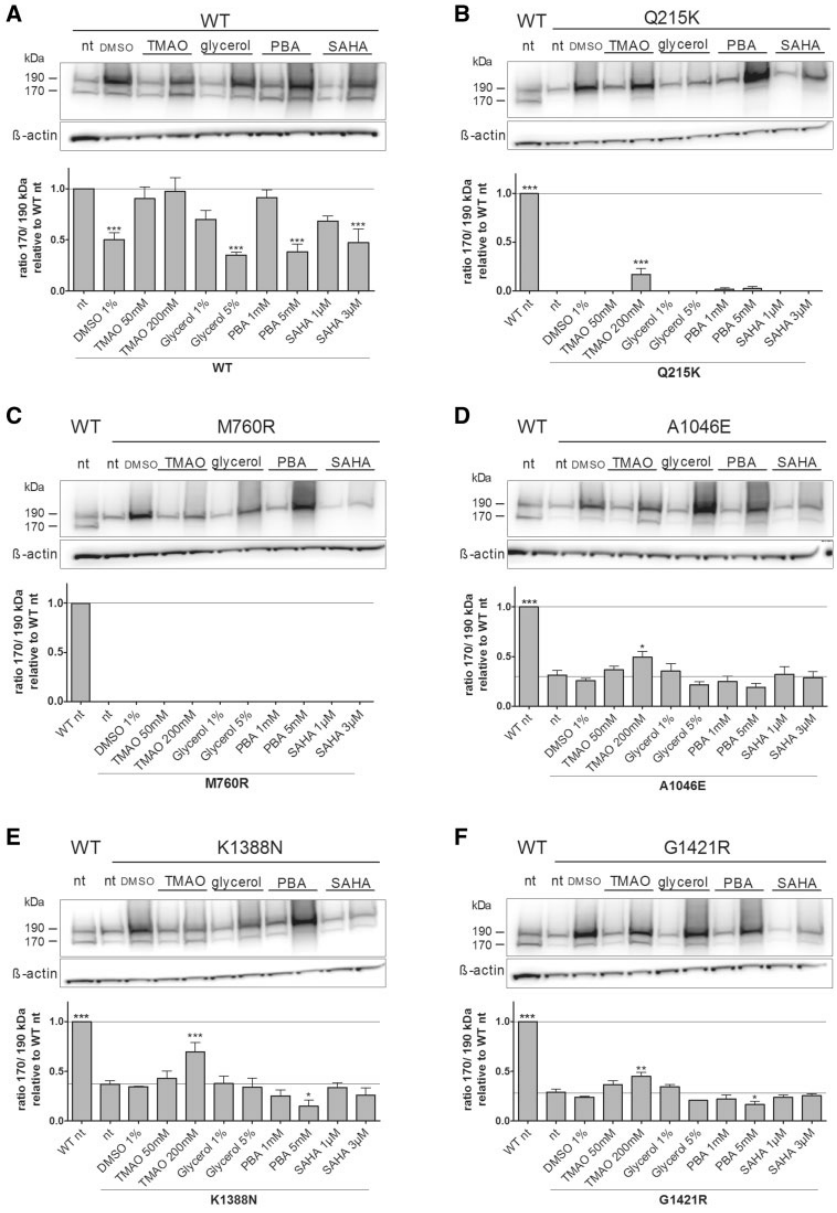

Figure 3. Chemical chaperone TMAO restores processing of ABCA3-HA mutants. A549 cells stably expressing ABCA3-HA WT or mutations were treated with two different concentrations of chemical chaperones for 48 h and ABCA3-HA protein pattern was analyzed by western blot (upper panel). Densitometric quantification of protein amount in each band (190 kDa and 170 kDa, see Supplementary Material, Fig. S2) was performed using Image J and the ratio of 170/190 kDa form was calculated with untreated WT set to 1 (lower panel). (**A**) wild-type ABCA3-HA. (**B**) Q215K ABCA3-HA. (**C**) M760R ABCA3-HA. (**D**) A1046E ABCA3-HA. (**E**) K1388N ABCA3-HA. (**F**) G1421R ABCA3-HA. Results are means + S.E.M. of three independent experiments. $^{*}p<0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ in regard to the untreated control. nt: no treatment.

kDa form ratio of all mutated ABCA3-HA proteins (except M760R ABCA3-HA) toward a WT-like level (Fig. 4A–F, lower panel).

Next, we tested correctors C13 and C14, which are analogues of C17 (Supplementary Material, Table S1). C13, similar to C17, led to an increase in the amount of the 170 kDa form and the 170/190 kDa form ratio of all mutated proteins except M760R ABCA3-HA (Fig. 4A–F, Supplementary Material, Fig. S4). Interestingly, the ratio was not increased in cells expressing the ABCA3-HA mutation A1046E, because the amount of the upper band was also highly increased by C13 treatment (Fig. 4D). C14 was able to increase the amount of 170 kDa form (Supplementary Material, Fig. S4) and the ratio of processed 170 kDa to unprocessed 190 kDa form for K1388N and G1421R ABCA3-HA (Fig. 4E and F).

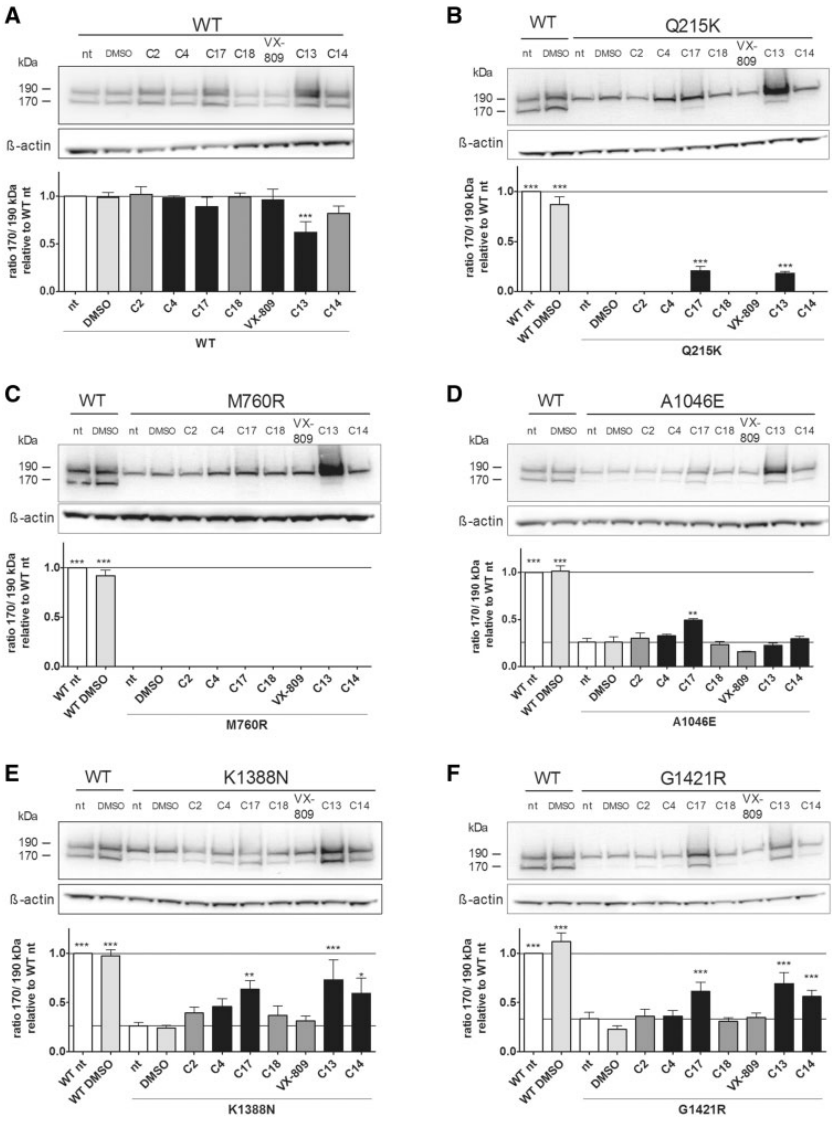

Figure 4. CFTR correctors restore processing of ABCA3-HA mutants. A549 cells stably expressing ABCA3-HA WT or mutations were treated with 10 μM of correctors for 48 h and ABCA3-HA protein pattern was analyzed by western blot (upper panel). Densitometric quantification of protein amount in each band (190 kDa and 170 kDa, see Supplementary Material, Fig. S4) was performed using Image J and the ratio of 170/190 kDa form was calculated with untreated WT set to 1 (lower panel). (**A**) wild-type ABCA3-HA. (**B**) Q215K ABCA3-HA. (**C**) M760R ABCA3-HA. (**D**) A1046E ABCA3-HA. (**E**) K1388N ABCA3-HA. (**F**) G1421R ABCA3-HA. Results are means + S.E.M. of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$' $^{***}p < 0.001$ in regard to the DMSO vehicle control. nt: no treatment.

To test combinations of correctors coming from different pharmacological classes, we selected Q215K ABCA3-HA, a mutation where correction was least efficient. Combining C13 or C17 with correctors C18 or VX-809 had no additive effects (Supplementary Material, Fig. S5).

Correctors C17 and C13 are most potent and restore subcellular distribution of mutated ABCA3-HA

As C17 and C13 were the most potent correctors, they were used for further experiments. Effects of higher concentrations were tested (Supplementary Material, Fig. S6), but due to severe decrease of cell viability at higher concentrations (Supplementary Material, Fig. S7), 10 μM of correctors were used in all further experiments.

Upon C13 or C17 treatment, all mutated ABCA3-HA proteins except M760R ABCA3-HA displayed a vesicle-like distribution in the cell, co-localizing with CD63, comparable to the pattern in cells expressing WT ABCA3-HA (Fig. 5). Only Q215K ABCA3-HA in addition showed remaining diffuse distribution in the cell. Interestingly, upon C13 treatment A1046E ABCA3-HA protein was also apparent in vesicular structures, even though it did not lead to an increase of the 170/190 kDa ratio in western blot

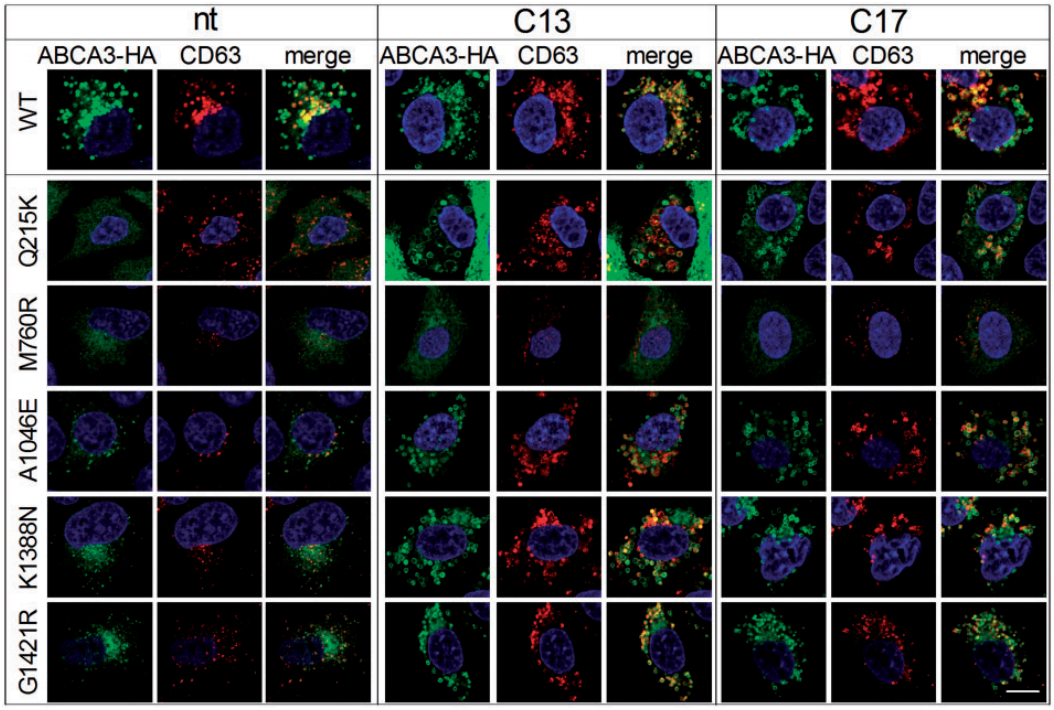

Figure 5. Correctors C17 and C13 restore subcellular localization of ABCA3-HA mutants. A549 cells stably expressing ABCA3-HA WT or mutations were treated with 10 μM of C13 or C17 for 48 h and stained for ABCA3-HA and lysosomal marker CD63. nt: no treatment; scale bar represents 10 μm.

(Fig. 4D). Taken together, these results show that correctors C13 and C17 were able to restore proper processing, trafficking and subcellular localization of all mutated ABCA3-HA proteins tested, except M760R ABCA3-HA.

Transport of TopF-labeled PC into ABCA3-HA positive vesicles after corrector treatment

Transport of TopFluor-labeled phosphatidylcholine (TopF-PC) serves as a functional assay for ABCA3 activity (32). C17 treatment led to an increase of TopF-PC in all analyzed ABCA3-HA-positive vesicles in cells expressing either WT or mutated ABCA3-HA and also if only filled vesicles were taken into account (Fig. 6A and B). Furthermore, the portion of filled vesicles was increased in Q215K, A1046E and G1421R ABCA3-HA expressing cells and the volume of ABCA3-HA-positive vesicles was increased in all cells, including WT ABCA3-HA cells (Fig. 6C and D). As a control for active ATP-dependent transport of TopF-PC, we used ortho-vanadate to inhibit ATPase function (32) and no transport of TopF-PC into ABCA3-HA vesicles was detected (Supplementary Material, Fig. S8).

C13 treatment led to an increase of TopF-PC in all measured vesicles in all cells with the exception of G1421R ABCA3-HA expressing cells (Fig. 6A). If only filled vesicles were taken into account, C13 increased the amount of TopF-PC in ABCA3-HA positive vesicles similar to WT-like levels in cells expressing Q215K, A1046E and K1388N ABCA3-HA (Fig. 6B). The portion of filled vesicles was increased in Q215K, A1046E and G1421R ABCA3-HA expressing cells (Fig. 6C). The volume of ABCA3-HA positive vesicles was increased in all cells after C13 treatment, including WT ABCA3-HA expressing cells (Fig. 6D). Representative pictures of these findings are shown in Figure 6E and Supplementary Material, Fig. S9.

Discussion

Missense mutations in ABCA3 can lead to misfolding and mistrafficking of the protein, resulting in the absence of ABCA3 from LBs, defective LB structure and complete loss of phospholipid transport function (Fig. 7). In this study, we proved that clinically relevant misfolding mutations in ABCA3 can be corrected *in vitro*. Our results showed that four of the five analyzed variants were temperature-sensitive and were corrected by chemical chaperone TMAO and correctors C13 and C17. Correction was assessed as restored N-terminal processing, localization of the protein at the limiting membrane of lysosome-related organelles resembling LBs, their morphological intactness and restoration of the phospholipid transport function (Fig. 7).

Four of the five investigated ABCA3 mutations, located in different domains of the protein, were responsive to low temperature, all of which restoring collocation to the LB limiting membrane after 30°C incubation except for M760R. It is postulated, that temperature-sensitivity gives a hint whether mutated proteins are susceptible to corrector treatment (27,31). Our data are in agreement with this hypothesis, as M760R ABCA3-HA was also not corrected by the chemical chaperones or correctors tested.

In CF, small molecular correctors for CFTR (ABCC7) were recently identified by high throughput *in vitro* assays followed by lead optimization and clinical studies. Novel disease-modifying treatments were made available for CF patients in a

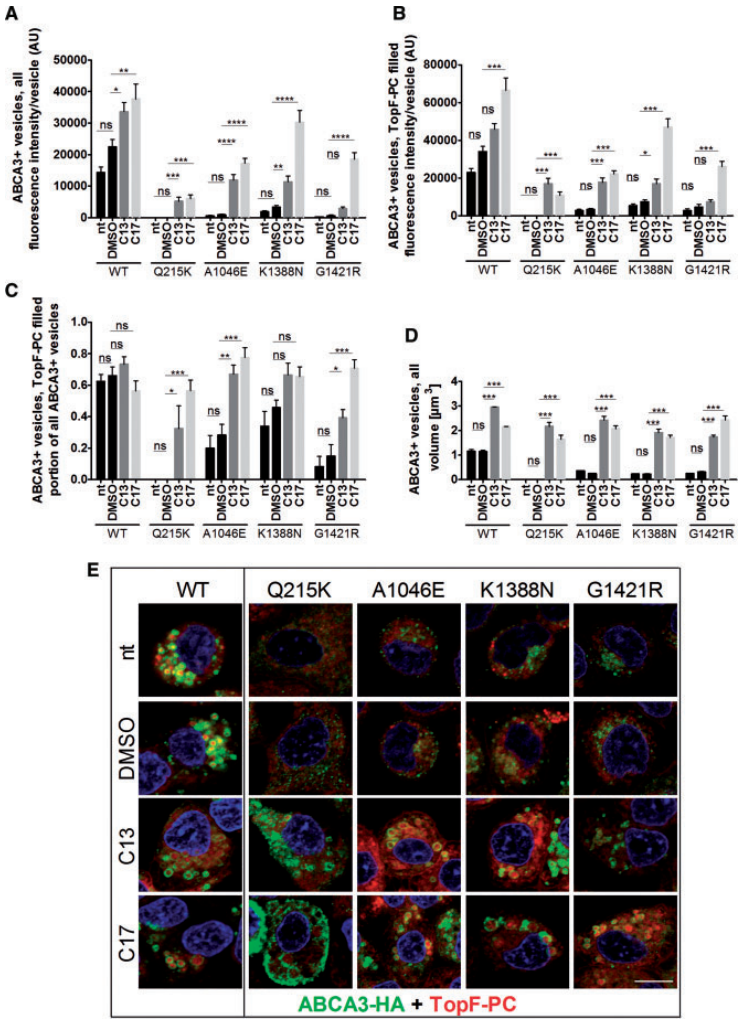

Figure 6. Corrector treatment increases transport of TopFluor-labeled PC into ABCA3-HA positive vesicles. After treatment with 10 µM C13 or C17 for 24 h, cells were incubated with liposomes containing TopFluor-conjugated phosphatidylcholine (TopF-PC) and treated with correctors for another 24 h. After fixation, cells were stained for ABCA3-HA and (**A**) the fluorescence intensity in all analyzed ABCA3-HA positive (ABCA3+) vesicles, (**B**) the fluorescence intensity in only TopF-PC-filled vesicles, (**C**) the portion of TopF-PC-filled vesicles and (**D**) the volume of ABCA3-HA positive vesicles were measured using Fiji (Image J). (**E**) Representative pictures of the experiment, see also Supplementary Material Fig. S9. Scale bar represents 10 µm. Pseudo colors were used to stay consistent with former experiments. Three independent experiments were performed in total. Results are means + S.E.M. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p<0.0001$. nt: no treatment.

very short time (23,33). These correctors act protein specific and at low doses (34) but their precise mechanism of action is poorly understood. Some correctors like VX-809 were shown to bind the CFTR protein directly and stabilize the domain interactions to promote the native folding conformation (35). Other correctors might mitigate the interaction of mutated CFTR with the proteostasis machinery, preventing protein retention and degradation (36).

We tested seven correctors with different mechanisms of action, including the compound VX-809 that was recently approved by the Food and Drug Administration (FDA) for CF treatment (Vertex press release, http://www.businesswire.com/news/home/20150702005760/en/; date last accessed January 12, 2018). Class II correctors (C4, C17, C13 and C14, all bithiazoles) stabilize the NBD2 of CFTR and its interfaces with other protein domains (19). For the tested ABCA3 mutations, only class II correctors were able to correct the misfolded proteins, indicated by restoration of processing and intracellular localization, with C13 and C17 being the most potent ones.

The phospholipid transport activity of ABCA3 variants was comparable to WT ABCA3 after C13 treatment or even higher than WT levels after C17 treatment. These results indicate that the mutations investigated here lead to a misfolding defect and do not additionally impair the phospholipid transport function of the protein. All tested mutations are localized in extra- or intracellular loops of the protein but not directly in the NBDs (Fig. 1). Therefore,

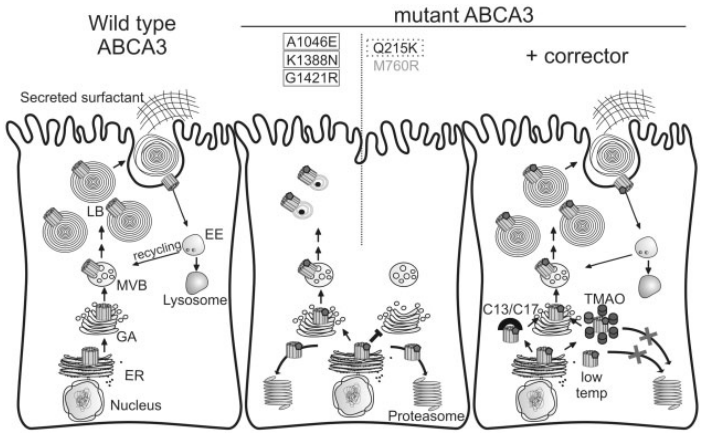

Figure 7. Intracellular trafficking pathways of wild-type, mutant and corrected ABCA3. *Left – Wild-type ABCA3*. After its synthesis and translocation to the ER, properly folded ABCA3 is routed via the golgi apparatus (GA) through post-golgi compartments like sorting vesicles, multivesicular bodies (MVB) and composite bodies, where N-terminal processing of the 190 kDa protein to a 170 kDa form takes place, to the limiting membrane of lamellar bodies (LB). When the content of LBs is released via regulated exocytosis, ABCA3 stays in the plasma membrane and is recycled or degraded in lysosomes. EE: early endosome. *Middle – mutant ABCA3*. ABCA3 mutations like Q215K and M760R lead to misfolded protein that is unable to escape the ER and is targeted for degradation in the proteasome. For mutations A1046E, K1388N, and G1421R, a small portion of the ABCA3 protein can escape the ER, undergoes regular trafficking and processing, and is located at the limiting membrane of LBs, which are smaller and fewer than in wild-type cells. *Right. Corrected mutant ABCA3*. Low temperature, chemical chaperone TMAO, and correctors C13 and C17 correct the misfolding defect of mutant ABCA3 (except for M760R, gray) and restore trafficking, processing, and morphological features of LBs. Correctors bind directly to the protein, whereas TMAO acts as an osmolyte and low temperature might slow down degradation of the mutant protein. Mutations that could be corrected completely are boxed; dashed lines indicate partial correction of Q215K ABCA3.

it is likely that the NBDs, i.e. the ATP-hydrolyzing domains, are not directly affected by the mutations. The correctors also had an impact on WT ABCA3-HA and increased the amount of ABCA3-HA protein, the amount of TopF-PC per vesicle and the volume of ABCA3-HA positive vesicles. This suggests that a certain portion of WT ABCA3 is also degraded, comparable to CFTR where about 70% of newly synthesized protein is degraded due to high quality control (37). The correctors probably increase the availability of WT ABCA3-HA thus increasing TopF-PC transport into ABCA3-HA positive vesicles.

It is important to note that correction of Q215K ABCA3-HA was not as effective as that of the other mutant proteins. Only a small portion of the cells was susceptible to correction, resulting in fewer vesicles in fewer cells analyzed. Since only a portion of protein was corrected, the lipid transport function is lower than in WT ABCA3-HA expressing cells. In this case, an additional treatment with potentiators might be beneficial to restore ABCA3 function further like shown for CFTR mutations (38). C13 and C17 were also shown to rescue ATP8B1, a member of the P-type cation transport ATPase family, lacking homology to CFTR. These finding suggest a more general molecular mechanism of action of these compounds, probably by modulating the proteostasis machinery.

Class I correctors that stabilize interactions between NBD1 and intracellular loops 1 and 4 of CFTR (C18, VX-809) (19) did not show any correction for the tested ABCA3 mutations. Unfortunately, VX-809 (lumacaftor), which is an approved drug for CF, showed no effect on ABCA3 mutations tested. This might be due to its optimization for CFTR (34,39–41). However, VX-809 was shown to correct other proteins like mutant ABCA4, but mutations were located in the NBD1 of the protein that shows high similarity to CFTR (42). Interestingly, a combination of classes I and II correctors that additively enhanced efficacy of correction in CFTR (19) did not enhance the efficacy of ABCA3 correction.

We explored a range of chemical chaperones, previously shown to correct other misfolding defective ABC transporters (29). Chemical chaperones can be divided into two subclasses, osmolytes and hydrophobic chaperones. Osmolytes include DMSO, TMAO and glycerol. They sequester water molecules and thereby leave a hydrophobic environment around the protein, favoring its folded state to decrease exposure of the hydrophilic backbone to the hydrophobic surroundings (43). We show that only TMAO was able to restore processing of the mutant proteins and their intracellular localization. TMAO was only effective at 200 mM, a concentration that precludes its use *in vivo* or in clinical studies (44). Hydrophobic chaperones like PBA and SAHA regulate transcription of proteins that are involved in different folding processes, like heat-shock proteins (45,46). They were shown to be less toxic than osmolytes (47), but unfortunately were not able to correct ABCA3 processing or trafficking. In contrast, they led to a heavy intracellular accumulation of ABCA3-HA protein. They act as histone deacetylase inhibitors, which were shown to transcriptionally activate Cytomegalovirus (CMV) promoters (48). It is likely that the CMV promoter, which controls ABCA3-HA expression in the designed vector, was stimulated by PBA or SAHA treatment, thereby increasing the expression of mutated misfolded ABCA3-HA that accumulates in the cell.

In this study, we used the A549 cell model stably expressing clinically relevant mutations. A potential limitation of such an approach may be that the impact of patient-specific other genetic or environmental influences (49) cannot readily be assessed. The corrector response in patients may be difficult to predict. Corrector activity was shown to also be influenced by cell background (50). In future studies, this can be overcome by the use of patient-specific primary cell cultures or induced pluripotent stem (iPS) cells.

As ABCA3 mutations are all rare and without mutational hot spots in the *ABCA3* gene, patient populations are too small to

conduct clinical trials on individual mutations. Thus, *in vitro* identification of groups of mutations that can be targeted by the same modulator is highly warranted. In this regard the FDA just recently announced, that '*in vitro* assay data could potentially be used in place of additional small clinical trials when seeking to expand [treatments that target specific mutations] to other population subsets', referring to ivacaftor, a CFTR potentiator (51). The next steps toward clinical trials comprise chemical optimization of the correctors identified to enhance their specificity to ABCA3 and lower potential toxicity. Furthermore, libraries of compounds should be screened for other possible correctors and experiments should be performed in patient-derived cells or iPS cells.

The results presented here show that misfolding mutations in ABCA3 can be corrected *in vitro*. This is a proof of principle and a first step toward the development of pharmacological therapies for diseases caused by ABCA3 misfolding, for which currently no treatment is available.

Materials and Methods

Chemical chaperones and correctors

Correctors C2, C4, C13, C14, C17 and C18 were obtained from Cystic Fibrosis Foundation Therapeutics (Bethesda, Maryland, USA). VX-809 was purchased from Sellekchem (Munich, Germany). Supplementary Material, Table S1 shows their full chemical names. PBA, TMAO, DMSO and SAHA were purchased from Sigma Aldrich (Taufkirchen, Germany). Glycerol was obtained from Merck Millipore (Darmstadt, Germany).

Cell culture

A549 cells were obtained from the German Collection of Microorganisms (DSMZ, Braunschweig, Germany) and maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Life technologies, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS, Sigma) at 37°C and 5% CO_2.

Plasmids

A pT2/HB transposon vector (Addgene, Cambridge; plasmid#26557) was generated, containing hABCA3 cDNA (NM_001089) with corresponding CMV promoter elements fused to a C-terminal HA-tag and puromycin resistance gene, as described before (52). Single point mutations p.Q215K (c.643C > A), p.M760R (c.2279T > G), p.A1046E (c.3137C > A), p.K1388N (c.4164G > C) and p.G1421R (c.4261G > A) were introduced into the vector using the Q5® site-directed mutagenesis kit (NEB, Massachusetts, USA). Primer sequences are given in the Supplementary Materials and Methods section.

Transfection and generation of stable cell clones

Transfection of A549 cells according to the sleeping beauty transposon system (53) and generation of stable cell clones were performed as described earlier (52).

Protein isolation and western blotting

A549 cells were lysed in radioimmunoprecipitation assay buffer [0.15 M sodium chloride, 1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, 5 mM ethylene diamine tetraacetic acid (EDTA), 50 mM Tris (pH 8)] (all from Sigma, except EDTA from GE Healthcare, Buckinghamshire, UK, Tris from Merck Millipore), supplemented with complete protease inhibitor (Roche, Mannheim, Germany). Protein concentrations were determined using the Pierce BCA protein assay (Thermo Fisher Scientific, Waltham, Massachusetts, USA). 15 or 20 μg of total protein were separated on NuPage Mini 3–8% Tris-Acetate gels (Invitrogen, Waltham, Massachusetts, USA) and subsequently transferred to a polyvinylidenfluorid membrane (Merck Millipore). The membrane was probed with rat anti-HA monoclonal antibody (Roche) followed by incubation with rabbit anti-rat IgG (H + L) HRP secondary antibody (Southern Biotechs, Birmingham, Alabama, USA). β-Actin (Santa Cruz, Dallas, Texas, USA) probing served as a loading control. Detection was performed using SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Densitometric analysis was performed using Image J software.

Immunofluorescence staining and confocal microscopy

A549 cells expressing ABCA3-HA were fixed with 4% paraformaldehyde (Merck Millipore) and permeabilized with 0.5% TritonX-100 (Sigma). To block unspecific binding sites, cells were incubated in blocking solution [3% Bovine serum albumin (BSA, Sigma) and 10% FBS in PBS]. To detect ABCA3-HA protein localization, cells were incubated with Anti-HA antibody (Sigma) and Anti-CD63 antibody (Abcam, Cambridge, UK) and according AlexaFluor secondary antibodies (Life technologies). Nuclei were stained with 0.1 μg/ml 4′, 6-diamidin-2-phenylindol (DAPI, Life technologies). Subsequently, cells were covered in mounting medium [90% glycerol in PBS and 2% 1, 4-diazabicyclo[2.2.2]octane (DABCO, Merck Millipore)]. Images were obtained using a ZEISS LSM 800 with ZEN 2 blue edition software.

Viability assay

Cells were treated with different concentrations of correctors in phenol red free RPMI medium + 10% FBS. Cell viability was assessed by quantification of the specific cleavage of yellow XTT tetrazolium salt (Sigma) to orange formazan in the presence of phenazine methosulfate (PMS, Sigma). Absorbance at 450 nm was measured using a spectrophotometer.

TopFluor-PC transport quantification

Surfactant-like liposomes were prepared and transport of TopF-PC into HA-positive vesicles was quantified as described before (32). In short, A549 cells expressing WT or mutant ABCA3-HA were pre-treated with correctors for 24 h. After labeling the cells with TopF-PC containing liposomes (1:20 diluted in OptiMEM, Thermo Fisher Scientific), cells were incubated with medium containing correctors for another 24 h. To stop the lipid uptake, cells were covered with 5% BSA (in PBS) for 30 min at 4°C for removal of labeled lipids adherent to the cell membrane. Cells were fixed, permeabilized with saponine (Carl Roth GmBH, Karlsruhe, Germany) and stained for HA-tag. Microscopy, fluorescence analysis and acquisition of vesicle volume and percentage of filled vesicles was performed as described previously (32) using a confocal laser-scanning microscope (LSM 800, ZEISS with ZEN 2 blue edition software) and the modified Fiji (Image J) plugin "Particle_in_Cell-3D" (54).

Statistical analysis

Comparison of two groups was performed using *t*-test. Comparisons of multiple groups were done using one-way analysis of variance with Dunnet's post hoc test to compare to the untreated or vehicle-treated control.

Results were plotted as means + S.E.M. *P*-values < 0.05 were considered statistically significant. All tests were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, USA).

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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Supplements

Supplemental material and methods

Primer

Site-directed mutagenesis was performed with the following primers (mutated nucleotides are underlined):

Q215K-for: 5'-CCTGGCCGTGAAGCATGCTGT-3'
Q215K-rev: 5'-AAGCCTTCCCGGATGTACC-3'

M760R-for: 5'-GGCTATCACAGGACGCTGGTG-3'
M760R-rev: 5'-GGCACCGTATTTCTGCTTG-3'

A1046E-for: 5'-AACAACCAGGAGTACCACTCTC-3'
A1046E-rev: 5'- GAACAAGGCGTTGACGAC-3'

K1388N-for: 5'-AGCTCTCCAACGTGTACGAGC-3'
K1388N-rev: 5'-CCTTGATAATCAGAGGTGTG-3'

G1421R-for: 5'-CAATGGAGCCAGGAAGACCACGAC-3'
G1421R-rev: 5'-AAGCCCAGCAGGCCGAAG -3'

Sanger sequencing verified all resulting vector constructs. The sequence analysis and alignment was performed using Clone Manager Suite (Version 6.00).

Supplemental figures and tables

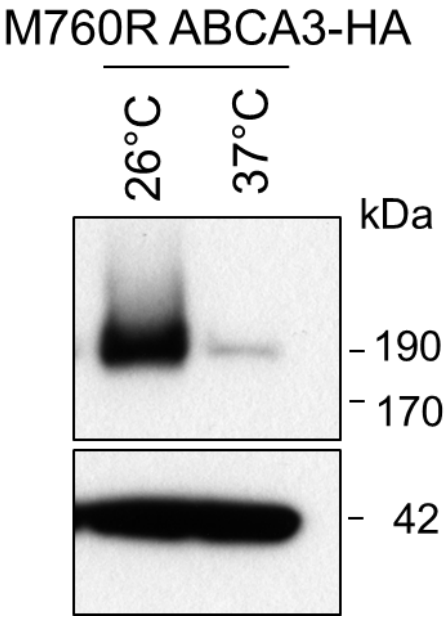


Figure S1: M760R ABCA3-HA processing defect is not temperature-sensitive. A549 cells stably expressing M760R ABCA3-HA were cultured at either 26°C or 37°C for 48 hours and protein pattern was analyzed by Western blot.

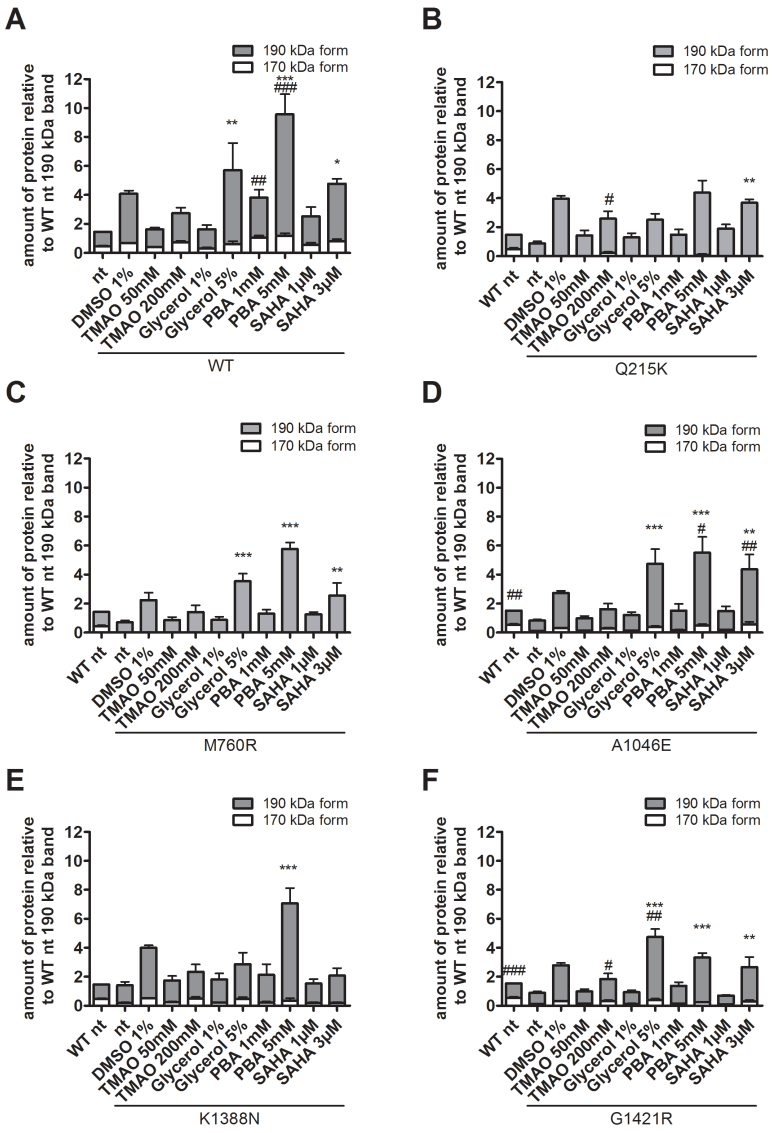


Figure S2: Chemical chaperone TMAO restores processing of ABCA3-HA mutants. A549 cells stably expressing ABCA3-HA WT or mutations were treated with two different concentrations of chemical chaperones for 48 hours and ABCA3-HA protein pattern was analyzed by Western blot (see fig. 3). Densitometric quantification of protein amount in each band (190 kDa and 170 kDa) was performed using Image J.

A) wild type ABCA3-HA

B) Q215K ABCA3-HA

C) M760R ABCA3-HA

D) A1046E ABCA3-HA

E) K1388N ABCA3-HA

F) G1421R ABCA3-HA

Results are means + S.E.M. of three independent experiments. */# $p<0.05$ **/##$p<0.01$ ***/###$p<0.001$ in regard to the DMSO vehicle control with * regarding the 190 kDa and # regarding the 170 kDa form. nt: no treatment.

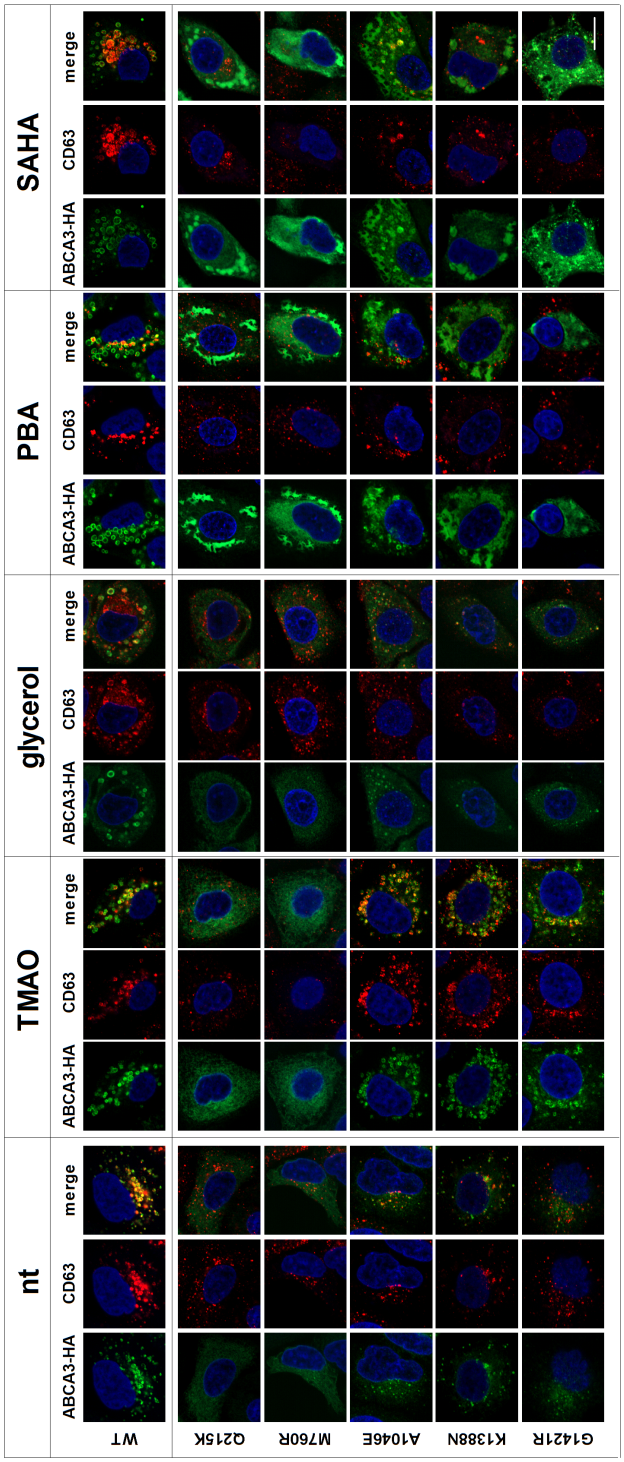


Figure S3: **Chemical chaperone TMAO restores subcellular localization of ABCA3-HA mutants**. A549 cells stably expressing ABCA3-HA WT or mutations were treated with the higher concentration of chemical chaperones from fig. 3 for 48 hours and stained for ABCA3-HA and lysosomal marker CD63. nt: no treatment; scale bar represents 10 µm.

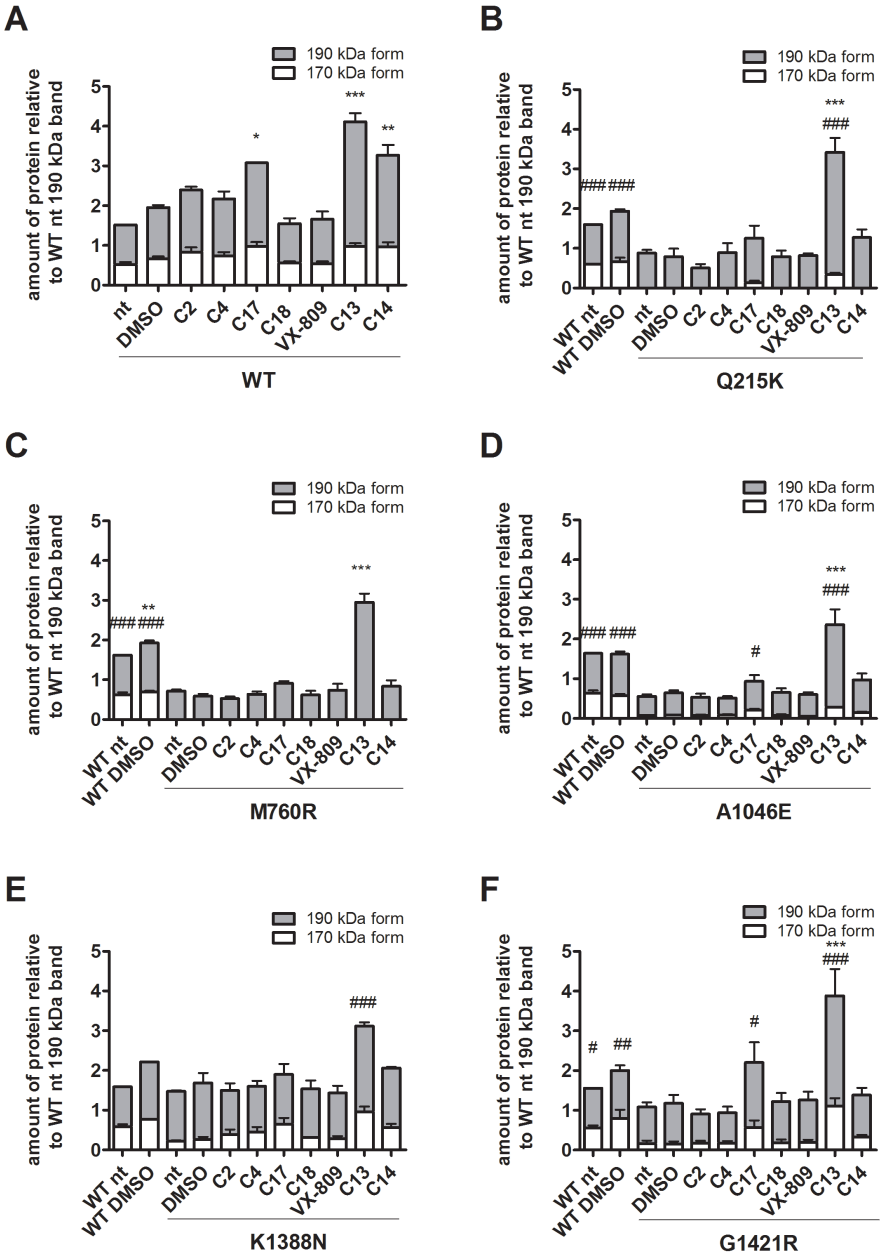


Figure S4: CFTR correctors restore processing of ABCA3-HA mutants. A549 cells stably expressing ABCA3-HA WT or mutations were treated with 10 µM of correctors for 48 hours and ABCA3-HA protein pattern was analyzed by Western blot (see fig. 5). Densitometric quantification of protein amount in each band (190 kDa and 170 kDa) was performed using Image J.

A) wild type

B) Q215K ABCA3-HA

C) M760R ABCA3-HA

D) A1046E ABCA3-HA

E) K1388N ABCA3-HA

F) G1421R ABCA3-HA

Results are means + S.E.M. of three independent experiments. */# $p<0.05$ **/##$p<0.01$ ***/###$p<0.001$ in regard to the DMSO vehicle control with * regarding the 190 kDa and # regarding the 170 kDa form. nt: no treatment.

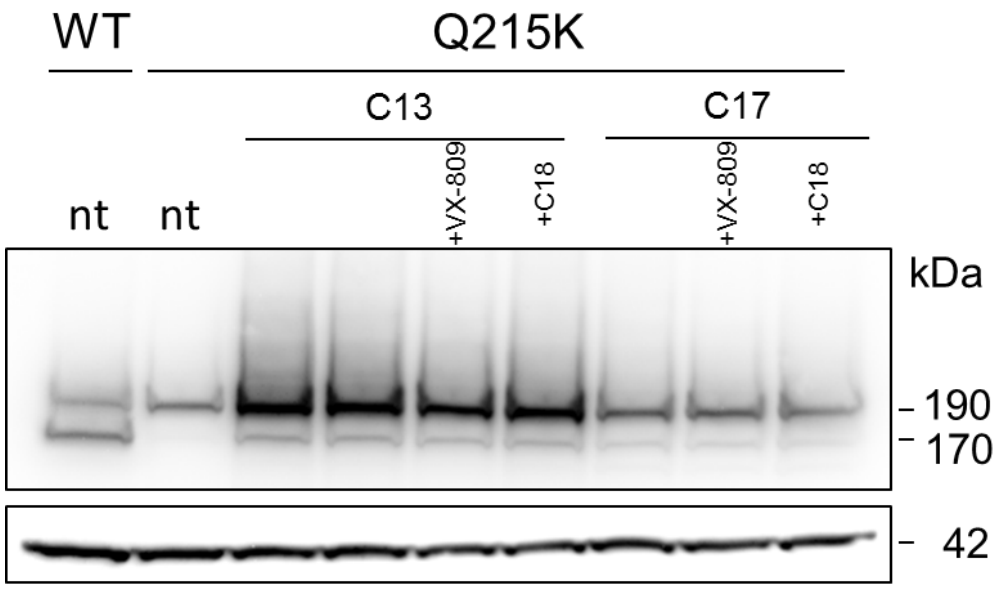


Figure S5: Combination of correctors from different classes has no additive effects. A549 cells expressing Q215K ABCA3-HA were treated with combinations of C13 or C17 with either VX-809 or C18 for 48 hours and ABCA3-HA protein pattern was analyzed by Western blot.

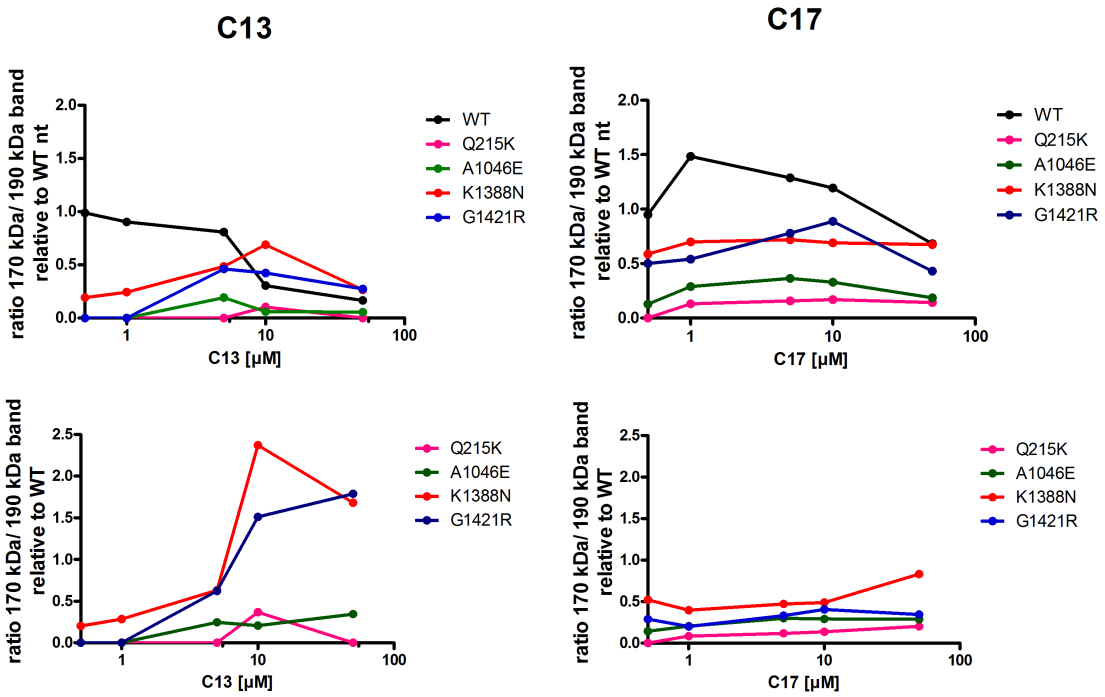
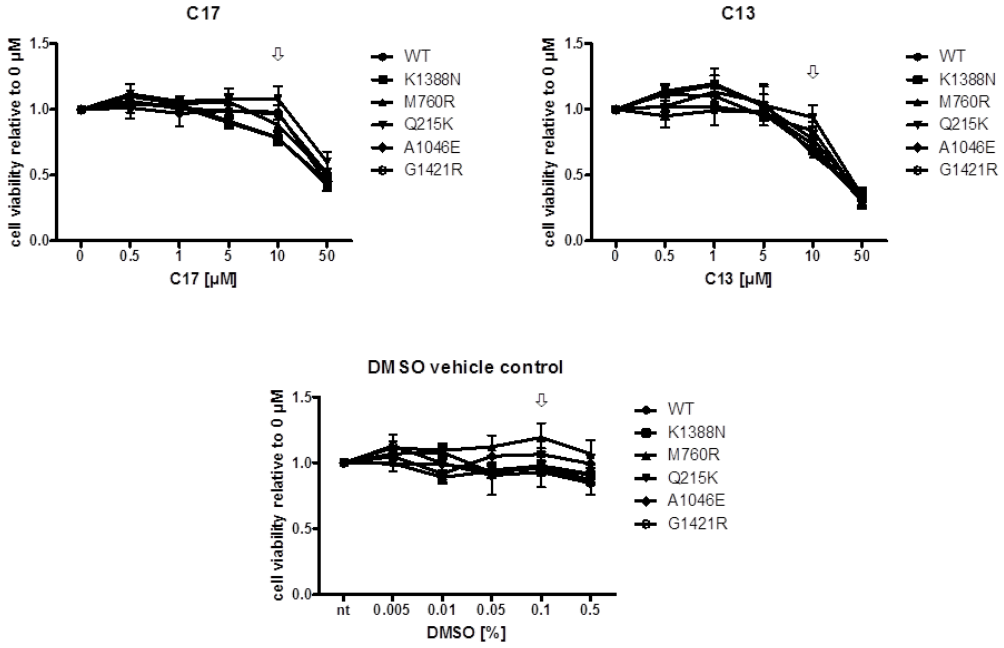


Figure S6: Effectiveness of different corrector concentrations. A549 cells stably expressing WT or mutated ABCA3-HA were treated with increasing concentrations of C13 and C17. After 48 hours cells were harvested, lysed, and proteins were separated for Western blot. For each condition the 170/190 kDa ratio was calculated after densitometric analysis using ImageJ.



	C17		C13		DMSO		
	10µM	50µM	10µM	50µM	0.01%	0.1%	0.5%
WT	ns	***	*	***	*	ns	*
Q215K	ns	***	ns	***	ns	ns	ns
M760R	ns	***	*	***	ns	ns	ns
A1046E	**	***	**	***	ns	ns	ns
K1388N	**	***	*	***	ns	ns	ns
G1421R	ns	***	ns	***	ns	ns	ns

Figure S7: Effects of C13 and C17 on cell viability.

A549 cells stably expressing WT or mutated ABCA3-HA were treated with increasing concentrations of C13, C17 or the vehicle DMSO. After 48 hours, cell viability was measured using XTT assay. The arrow indicates the concentration used in all experiments (10 µM). Results are means + S.E.M. of three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

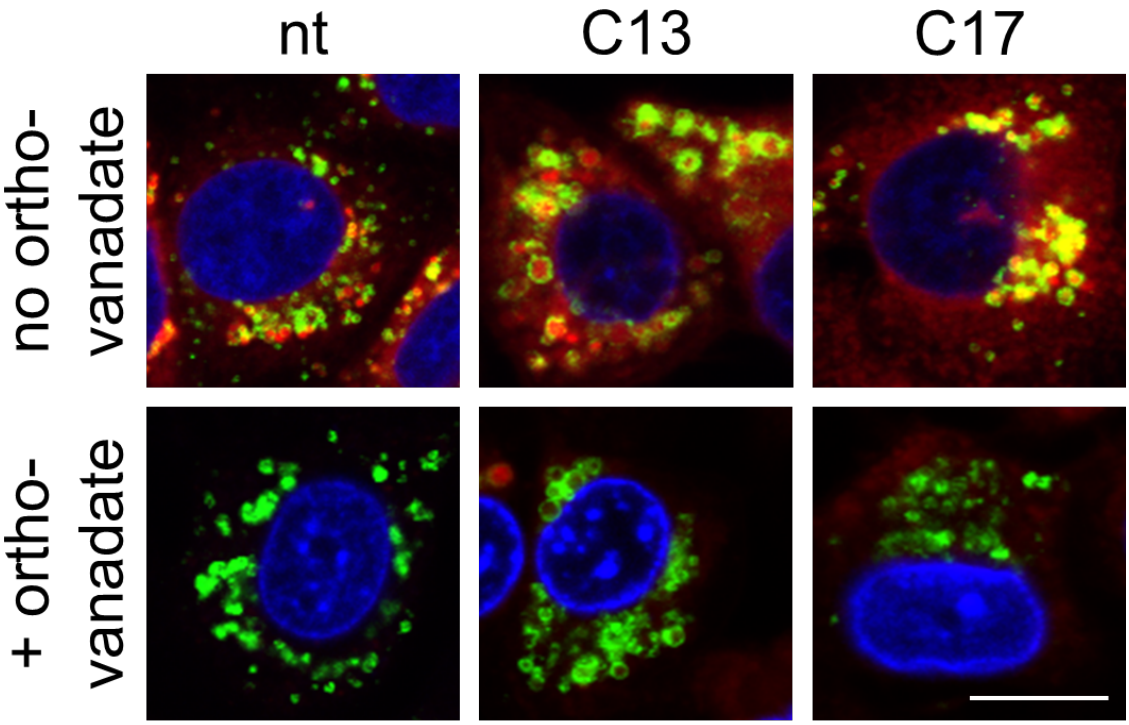


Figure S8: **Ortho-vanadate inhibits ABCA3-dependent TopF-PC transport into ABCA3-HA positive vesicles.** WT ABCA3-HA expressing A549 cells were treated with TopF-PC (red) containing liposomes for 24 hours and cultured with or without 12.5 mM ortho-vanadate for 22 h. ABCA3-HA proteins (green) were stained by immunofluorescence. Scale bar represents 10 µm.

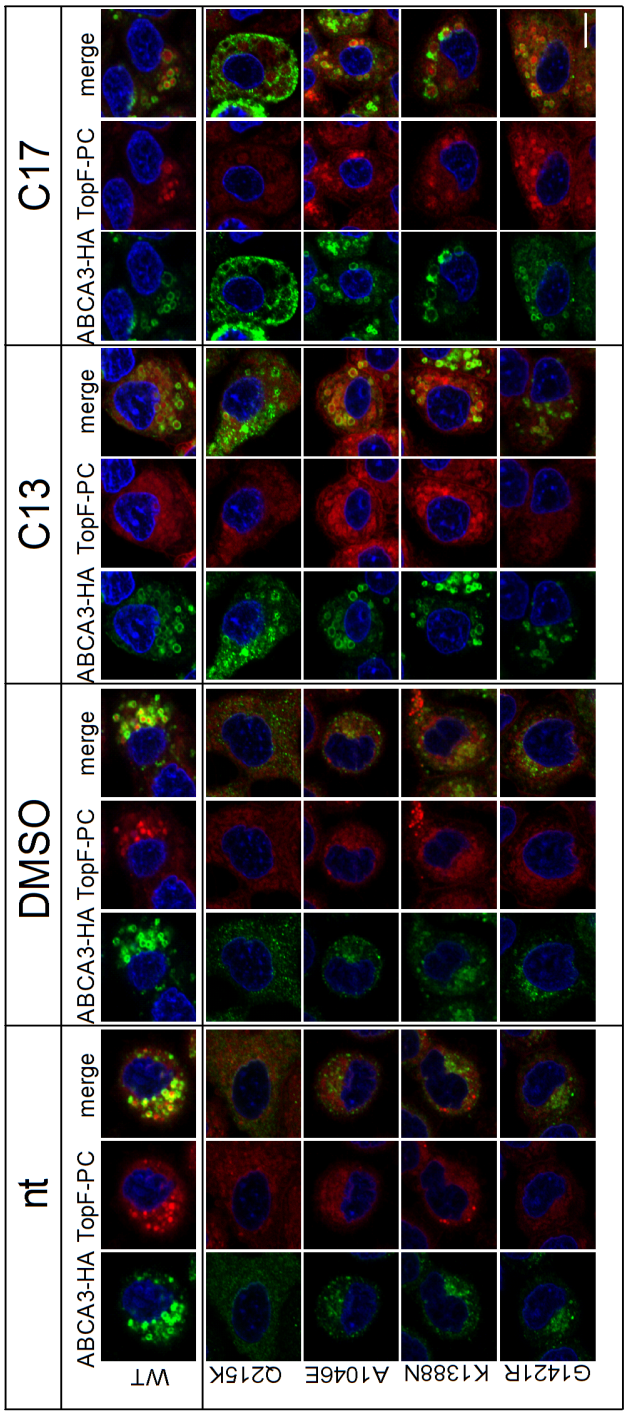
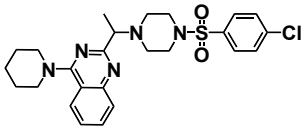
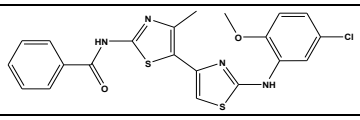
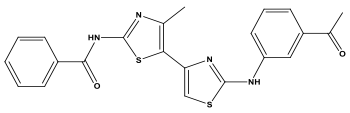
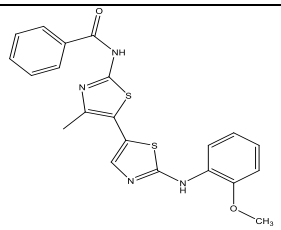
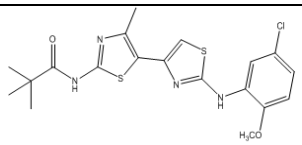
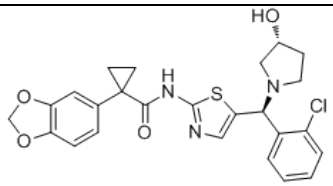
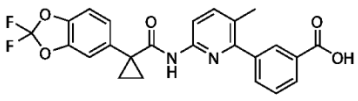


Figure S9: Corrector treatment increases transport of TopF-PC into ABCA3-HA positive vesicles. A549 cells stably expressing ABCA3-HA WT or mutations were treated with 10 µM of correctors C13 or C17 and labeled with TopF-PC. After 24 hours cells were fixed and stained for ABCA3-HA. Scale bar represents 10 µm. nt: no treatment.

Supplemental table 1: **Utilized small molecular correctors from CF foundation with full chemical names**. Structures of the compounds were adopted from the CF foundation website (https://www.cff.org/Research/Researcher-Resources/Tools-and-Resources/CFTR-Chemical-Compound-Program/).

ID	Chemical name	Chemical structure
C2	2-{1-[4-(4-Chloro-benzensulfonyl)-piperazin-1-yl]-ethyl}-4-piperidin-1-yl-quinazoline	
C4	N-[2-(5-Chloro-2-methoxy-phenylamino)-4'-methyl-[4,5']bithiazolyl-2'-yl]-benzamide	
C13	N-(2-(3-acetylphenylamino)-4'-methyl-4,5'-bithiazol-2'-yl)benzamide	
C14	N-(2'-(2-methoxyphenylamino)-4-methyl-5,5'-bithiazol-2-yl)benzamide	
C17	N-(2-(5-chloro-2-methoxyphenylamino)-4'-methyl-4,5'-bithiazol-2'-yl)pivalamide	
C18	1-(benzo[d][1,3]dioxol-5-yl)-N-(5-((S)-(2-chlorophenyl)((R)-3-hydroxypyrrolidin-1-yl)methyl)thiazol-2-yl)cyclopropanecarboxamide	
VX-809	3-(6-(1-(2,2-difluorobenzo[d][1,3]dioxol-5-yl)cyclopropanecarboxamido)-3-methylpyridin-2-yl)benzoic acid	

Supplemental table 2: **Overview of analyzed ABCA3 variants**. Allele frequency was obtained from ExAC (1). aa, amino acid.

Sequence variation of ABCA3 protein	p.**Q215K**	**p.M760R**	**p.A1046E**	**p.K1388N**	**p.G1421R**
Allele frequency in healthy population	<0.000001%	0.00082%	n.a.	0.00082%	0.00001651%
Clinical outcome	lethal	probably lethal, lung transplant	lethal	lethal	lethal
Genetic analysis	Homozygous c.643C>A	Compound heteroz. c.2279T>G (p.M760R); c.622C>T (p.R208W)	Compound heteroz. c.3137C>A (p.A1046E); c.4360-1G>C (Del Ex29), c.4012G>A (p.A1338T)	Homozygous c.4164G>C	Compound heteroz. c.4261G>A (p.G1421R), c.577C>T (p.P193S)
Prediction (mutation taster)	Disease causing, aa sequence changed, protein features affected, splice site changes	Disease causing, aa sequence changed, protein features affected	Disease causing, aa sequence changed, protein features affected, splice site changes	Disease causing, aa sequence changed, protein features affected, splice site changes	Disease causing, aa sequence changed, protein features affected, splice site changes
References	(2)	(3)	(2)	(2, 4)	(2)

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3.2 Potentiation of ABCA3 lipid transport function by ivacaftor and genistein

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ORIGINAL ARTICLE

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Potentiation of ABCA3 lipid transport function by ivacaftor and genistein

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Abstract

ABCA3 is a phospholipid transporter implicated in pulmonary surfactant homoeostasis and localized at the limiting membrane of lamellar bodies, the storage compartment for surfactant in alveolar type II cells. Mutations in ABCA3 display a common genetic cause for diseases caused by surfactant deficiency like respiratory distress in neonates and interstitial lung disease in children and adults, for which currently no causal therapy exists. In this study, we investigated the effects of ivacaftor and genistein, two potentiators of the cystic fibrosis transmembrane conductance regulator (CFTR), on ABCA3-specific lipid transport function. Wild-type (WT) and functional ABCA3 mutations N568D, F629L, G667R, T1114M and L1580P were stably expressed in A549 cells. Three-dimensional modelling predicted functional impairment for all five mutants that was confirmed by in vitro experiments (all <14% of WT functional activity). Treatment with potentiators rescued the mutants N568D (up to 114% of WT), F629L (up to 47% of WT), and G667R (up to 60% of WT), the latter variation needing higher concentrations of genistein, showing reduced affinity of the potentiator to the mutant protein. Our results present a first proof that functional ABCA3 mutations are rescued by CFTR potentiators, making them a potential therapeutical option for patients suffering from surfactant deficiency due to ABCA3 mutations.

KEYWORDS
ABCA3, CFTR potentiators, genistein, interstitial lung disease, ivacaftor

1 | INTRODUCTION

Pulmonary surfactant is a lipoprotein complex that lines the alveolar spaces and is synthesized, stored and secreted by alveolar type II (ATII) cells. Surfactant is crucial for normal breathing, its main function is to lower the surface tension at the air-liquid interface to prevent end-expiratory collapse of alveolar units.[1-4] The storage compartments for surfactant are the lysosome-derived lamellar bodies (LBs). Adenosine triphosphate (ATP)-binding cassette subfamily A member 3 (ABCA3), a lipid transporter involved in surfactant homoeostasis, is localized at the outer membrane of lamellar bodies.[5-8] Like all ABC transporters it is composed of two transmembrane domains (TMDs) that form a pore and two nucleotide-binding domains (NBDs) that bind and hydrolyse ATP to generate the energy to transport surfactant lipids into the lumen of LBs.[9,10]

Phosphatidylcholine (PC) is the most abundant lipid species in human pulmonary surfactant[11] and was shown to be transported by ABCA3.[6] We recently established a functional assay to quantify the lipid transport function of ABCA3 by assessing the fluorescence intensity of TopFluor-labeled PC (TopF-PC) inside ABCA3-positive

vesicles that resemble LBs in A549 cells.[12] The overall transport activity, expressed as the fluorescence intensity per vesicle in all measured vesicles is thereby composed of three different parameters: the volume of the vesicles, the portion of filled vesicles and the fluorescence intensity in filled vesicles.

Mutations in ABCA3 lead to surfactant deficiency and pulmonary diseases like fatal respiratory distress in newborns or chronic interstitial lung disease in children (chILD) and adults.[13,14] To date, no causal therapies exist to treat patients suffering from lung diseases due to ABCA3 mutations. It is therefore a major task to identify pharmacological modulators for ABCA3 that would allow to treat those diseases.

In cystic fibrosis, a pulmonary disease caused by mutations in the ABC transporter cystic fibrosis transmembrane conductance regulator (CFTR, ABCC7),[15,16] compounds have successfully been developed, which partially or completely correct the molecular defect in a mutation-specific manner. Misfolding mutations, like the most frequent variation F508del, that lead to impaired processing and trafficking through the cell due to ER retention can be targeted by so called correctors that increase the delivery of CFTR to the cell surface.[17-20] Functional mutations, like the third-most frequent CFTR variation G551D, that display impaired function but correct processing and localization, can be rescued by potentiators.[17,20-22] CFTR potentiators ivacaftor (IVA) and genistein (GEN) lead to an increase in CFTR transport activity at the cell surface by enhancing its open probability (P_0).

For ABCA3 we showed that a treatment with correctors rescued processing, trafficking, localization and function of misfolding mutations.[23] Since a lot of ABCA3 mutations are classified as functional mutations, the main goal of this study was to evaluate the effect of potentiators on the lipid transport function of those mutations. Therefore we analysed three well-described functional ABCA3 mutants, namely N568D in NBD1, T1114M in TMD2 and L1580P in NBD2.[24-26] We additionally analysed F629L and G667R variations, which were homologous to positions F508 and G550 in CFTR respectively (Figure 1A and B). G667R is located in the NBD1 conserved ABC signature motif, similarly affected in G551D in CFTR. It was selected because a rare variant has been described in humans in this position.[27]

All five mutants showed severely impaired lipid transport function that was rescued by treatment with the CFTR potentiators ivacaftor (IVA) and genistein (GEN) in mutants N568D, F629L and G667R. The results presented here might pave the way for mutation-group specific treatment of pulmonary diseases caused by ABCA3 mutations.

2 | MATERIALS AND METHODS

2.1 | Molecular modelling

Structural models of wild-type and mutant ABCA3 were build using phyre2 protein modelling webserver.[28] The model of ABCA3 full-length in its unbound conformation was build based on the 4.1Å electron microscopy structure of human ABCA1 (pdbid: 5XJY).[29] Solely the NBD1-2 and the TMD1-2 were kept in the final model. These regions have an identity of 46% with the template, ensuring an accurate prediction of ABCA3 structure. The model of the NBD1-NBD2-ATP was modelled based on the 3.25Å crystallography structure of bacterial MacB dimer bound to ATP (pdbid: 5LJ7).[30] The NBD1 and NBD2 of ABCA3 have identities of 30% and 22% respectively to the template NBD domain of MacB. Mutations in the TMD (T1114M) or of the TMD/NBD interface (F629L) were modelled on full-length ABCA3 while mutations close to the ATP binding sites (N568D, G667R and L1580P) were modelled on the NBD1-NBD2-ATP assembly.

2.2 | Sequence analysis

Protein sequence alignment between ABCA3 and CFTR was performed with the UniProt sequence alignment online tool.[31] F629 and G668 were identified to correspond to F508 and G551 in CFTR (Figure 1B). Variations F629L and G667R were identified using the Exome Aggregation Consortium (ExAc) Browser,[27] with G667 corresponding to G550 in CFTR since no mutation was listed for position G668 in ABCA3.

Conservation analysis was performed with ConSurf Server.[32] Initial sequence selection was performed on the Uniref90 database using an E-value threshold of 0.0001. Within this pool, the best 137 ABCA3 sequences were manually selected. Final alignment was performed with the MAFFT-L-INS-i method and scoring was calculated using the Bayesian method.

2.3 | Potentiators

Ivacaftor (VX-770, IVA) was purchased from Sellekchem (Munich, Germany). Genistein (GEN) was purchased from Sigma Aldrich (Taufkirchen, Germany). Both substances were dissolved in dimethyl sulphoxide (DMSO, Sigma).

2.4 | Cell culture

A549 cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and cultured in RPMI 1640 medium (Life technologies, Darmstadt, Germany) supplemented with 10% foetal bovine serum (FBS, Sigma) at 37°C and 5% CO_2.

2.5 | Plasmids

A pT2/HB transposon vector (Addgene, Cambridge, plasmid#26557) was generated, containing hABCA3 cDNA (NM_001089) with corresponding CMV promoter elements fused to a C-terminal HA-tag and puromycin resistance gene, as described before.[33] Single point mutations p.N568D (c.1702 A > G), p.F629L (c.1887 C > G), p.G667R (c.1999 G > A), p.T1114M (c.3341 C > T), and p.L1580P (c.4739 T > C) were introduced into the vector using the Q5®

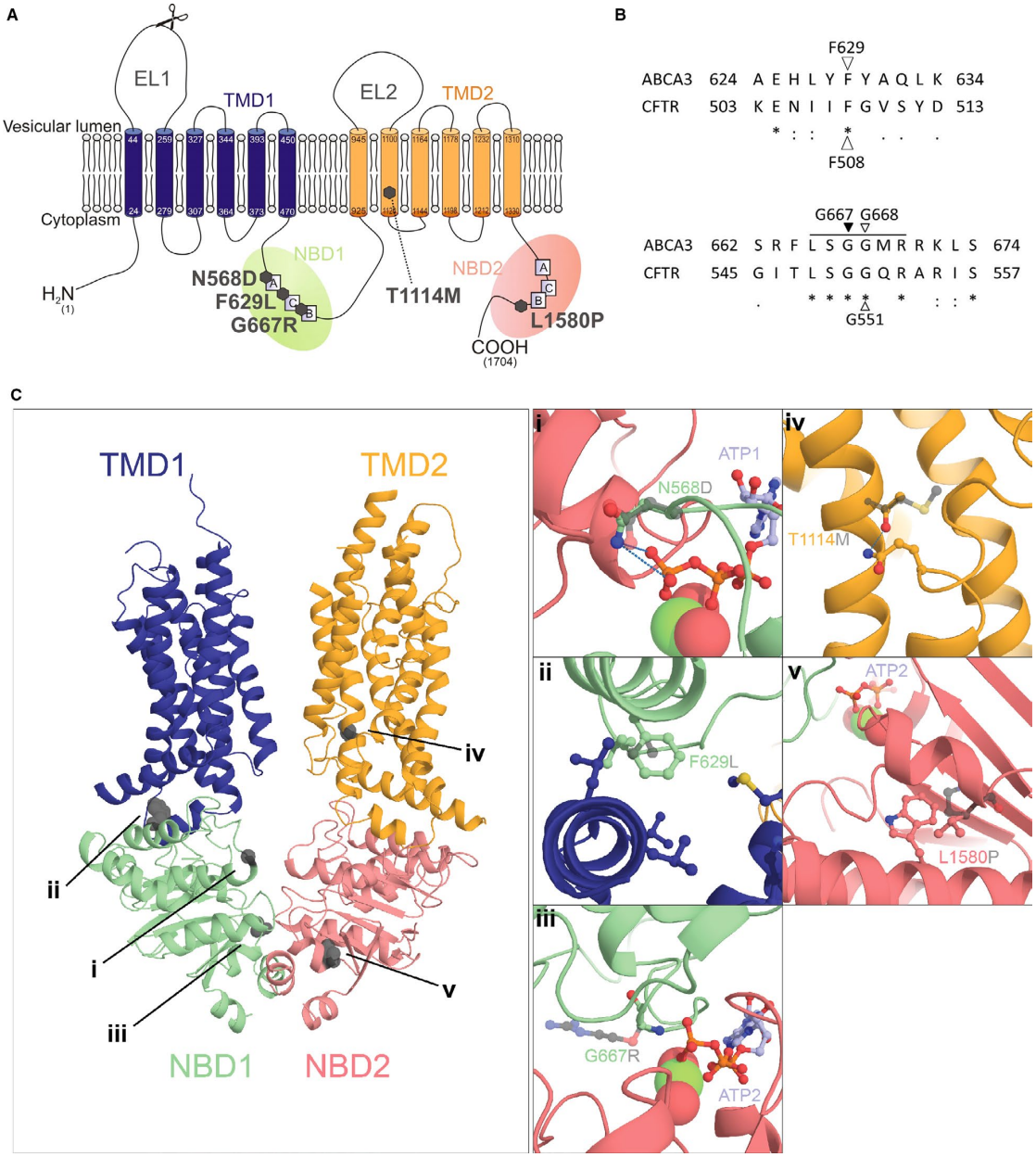

FIGURE 1 Localization and molecular consequences of five functional ABCA3 mutations. A, Two-dimensional (2D) topology model of ABCA3 with marked positions of the five analysed functional mutations. Scissors mark cleavage site for processing of the 190 to 170 kDa form. EL: external loop, A: Walker A motif, B: Walker B motif, C: C motif. B, Sequence alignment between ABCA3 and cystic fibrosis transmembrane conductance regulator (CFTR) to identify amino acids homologous to F508 and G551 in CFTR. Variations F629L and G667R were identified using the Exome Aggregation Consortium (ExAc) Browser,[27] with G667 corresponding to G550 in CFTR since no mutation was listed for position G668 in ABCA3. A solid line indicates the conserved ABC signature motif (LSGGQ). C, Full-length 3D model of ABCA3 with detailed pictures of location and consequences of the five functional ABCA3 mutations (i) N568D, (ii) F629L, (iii) G667R, (iv) T1114M, (v) L1580P. Mutated residues are represented as grey spheres in the full-length model. In the detailed pictures, the side chains of the wild-type residues are represented in full sticks and the substituting residues are shown in transparent grey sticks at each mutated position. Other residues of interest and ATP are also represented in stick when required; likely hydrogen bonds of interest are represented as blue dotted lines. ATP: adenosine triphosphate; NBD: nucleotide-binding site; TMD: transmembrane domain

site-directed mutagenesis kit (NEB, Massachusetts, United States). Primer sequences are given in the Supporting Information Materials and Methods Section.

2.6 | Transfection and generation of stable cell clones

Transfection of A549 cells using the sleeping beauty transposon system[34] and generation of stable cell clones were performed as described before.[33]

2.7 | Protein isolation and Western blotting

A549 cells were lysed in radioimmunoprecipitation assay (RIPA) buffer [0.15 mol/L sodium chloride, 1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, 5 mmol/L ethylene diamine tetraacetic acid (EDTA), 50 mmol/L Tris (pH 8)] (Sigma, EDTA from GE Healthcare, Buckinghamshire, UK, Tris from Merck Millipore, Darmstadt, Germany), supplemented with complete protease inhibitor (Roche, Mannheim, Germany). Protein concentrations were measured using the Pierce BCA protein assay (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 15 µg protein was separated on NuPage Mini 3-8% Tris-Acetate gels (Invitrogen, Waltham, Massachusetts, USA) and subsequently transferred to a polyvinylidene fluoride (PVDF) membrane (Merck Millipore). For probing of ABCA3-HA, rat anti-HA monoclonal antibody (Roche) and rabbit anti-rat IgG (H + L) HRP secondary antibody (Southern Biotechs, Birmingham, AL) were used. β-Actin (Santa Cruz, Dallas, TX) probing served as a loading control. SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) was used for detection. Densitometric analysis was performed with Image J software.

2.8 | Immunofluorescence staining and confocal microscopy

For immunofluorescent stainings, cells were seeded in ibiTreat slides (ibidi, Martinsried, Germany). Cells were fixed with 4% paraformaldehyde (Merck Millipore) and permeabilized with 0.5% TritonX-100 (Sigma). Cells were incubated with blocking solution [3% bovine serum albumin (BSA, Sigma) and 10% FBS in PBS] to block unspecific binding sites. ABCA3-HA protein and CD63 were probed with anti-HA (Sigma) and anti-CD63 antibody (abcam, Cambridge, UK), and according AlexaFluor secondary antibodies (life technologies). Nuclei were stained by incubation with 0.1 µg/ml 4′,6-diamidin-2-phenylindol (DAPI, life technologies). Subsequently, cells were covered in mounting medium [90% glycerin in PBS and 2% 1,4-diazabicyclo[2.2.2]octane (DABCO, Sigma)] and images were acquired using a ZEISS LSM 800 with ZEN 2 blue edition software.

2.9 | TopFluor-PC transport quantification

Surfactant-like liposomes were prepared and transport of TopFluor-labeled phosphatidylcholine (TopF-PC) into HA-positive vesicles was quantified as described before.[12] In short, TopF-PC containing liposomes (1:20 diluted in OptiMEM, Thermo Fisher Scientific) were offered to the cells expressing WT or mutant ABCA3-HA for 30 minutes at 4°C. After two hours at 37°C, cells were treated with potentiators or DMSO as a vehicle control for 24 hours. Then cells were covered with 5% BSA (in PBS) for 30 minutes at 4°C for removal of residual labelled lipids adherent to the cell membrane. Cells were fixed, permeabilized with saponin (Carl Roth GmBH, Karlsruhe, Germany) and stained for HA-tag. Microscopy, fluorescence analysis and quantification of vesicle volume and percentage of filled vesicles were performed as described previously[12] using a confocal laser-scanning microscope (LSM 800, ZEISS with ZEN 2 blue edition software) and the modified Fiji (Image J) plugin "Particle_in_Cell-3D".[35]

2.10 | Statistical analysis

Data are shown as means ± SEM. Statistical significance among means was calculated using one-way ANOVA with Dunnet's post hoc test to compare to the WT or DMSO vehicle-treated control. $P < 0.05$ was considered significant.

3 | RESULTS

3.1 | ABCA3 mutations are predicted to be functional mutations according to an ABCA3 3D model

Three-dimensional structure modelling was performed and molecular consequences of all five mutations were consistently predicted to impair function of the ABCA3 lipid transport activity (Figure 1). These observations are indicated for each mutation below.

The residue N568 is located in the conserved Walker A motif. Its side chain is directly involved in the binding of the third phosphate group of ATP. The loss of the side chain amine induced by the N568D mutation likely prevents this interaction and reduces or completely prevents ATP binding to ABCA3 (Figure 1C,i).

Residue F629, homologous to F508 in CFTR (Figure 1B), is very well conserved and is located in the NBD1 at a hydrophobic pocket, making interface with the transverse helices of TMD1. Substitution of a phenylalanine by a leucine prevents some hydrophobic interactions and may hinder the allosteric transmission of conformational changes to the TMD following ATP hydrolysis (Figure 1C,ii).

G667R is located in the conserved ABC signature motif (LSGGQ) (Figure 1B) implicated in ATP binding at the NDB1/NBD2 interface. In the ATP-bound form G667 is in close proximity to phosphate groups 2 and 3 of the ATP molecule. A substitution to an arginine adds a large side chain that cannot be accommodated and prevents ATP binding (Figure 1C,iii).

Residue T1114 is located in the second helix of the TMD2, far from the NBDs (Figure 1C,iv). It likely forms a hydrogen bond with the conserved residue Q929 in the loop following the first transverse helix of the TMD2. Its mutation to a methionine precludes this hydrogen bonding and might prevent conformational changes of the

TMD2 happening upon ATP hydrolysis in the NBDs and thereby reduce the transport activity of the protein.

Residue L1580 is located in a highly conserved helix following the H-loop. Its mutation to a proline, incompatible with helical secondary structure, has likely a strong impact on the conformation of this region and of the close H-loop that interacts with ATP and the NBD1 (Figure 1C,v).

In summary, based on these data from 3D modelling, we expected functional impairment of all five mutants, making them possible targets for treatment with potentiators to rescue the functional defect.

3.2 | Functional ABCA3 mutants display correct subcellular localization and processing but impaired lipid transport function in A549 cells

WT ABCA3-HA and all five mutant proteins were stably expressed in A549 cells and showed vesicular structures that resemble LBs and co-localized with the lysosomal marker CD63, demonstrating their correct protein localization (Figure 2A). However, the vesicles formed by all mutant proteins were significantly smaller than those in WT-ABCA3 expressing cells (Figure 2B).

N-terminal cleavage of ABCA3 in post-Golgi compartments resulting in the presence of two products of about 190 and 170 kDa,[36,37] serves as a marker for correct protein trafficking.[23,37,38] All five mutant proteins showed both processing products in Western blots (Figure 2C) with a ratio of 170 to 190 kDa form not significantly different from the WT protein, indicating correct processing and trafficking through the cell (Figure 2D).

Despite normal processing and localization, all five mutants exhibited a strong decrease in lipid transport activity compared to WT as predicted from the 3D model (Figure 3A and E, no treatment). Volume of the analysed vesicles, the portion of filled vesicles and the fluorescence intensity in filled vesicles were diminished for all mutants compared to WT, resulting in a transport activity of 14% of WT in N568D and T1114M mutants, 12% activity of WT in F629L and G667R mutants and 10% of WT lipid transport activity in L1580P mutant (Figure 3A-D, no treatment).

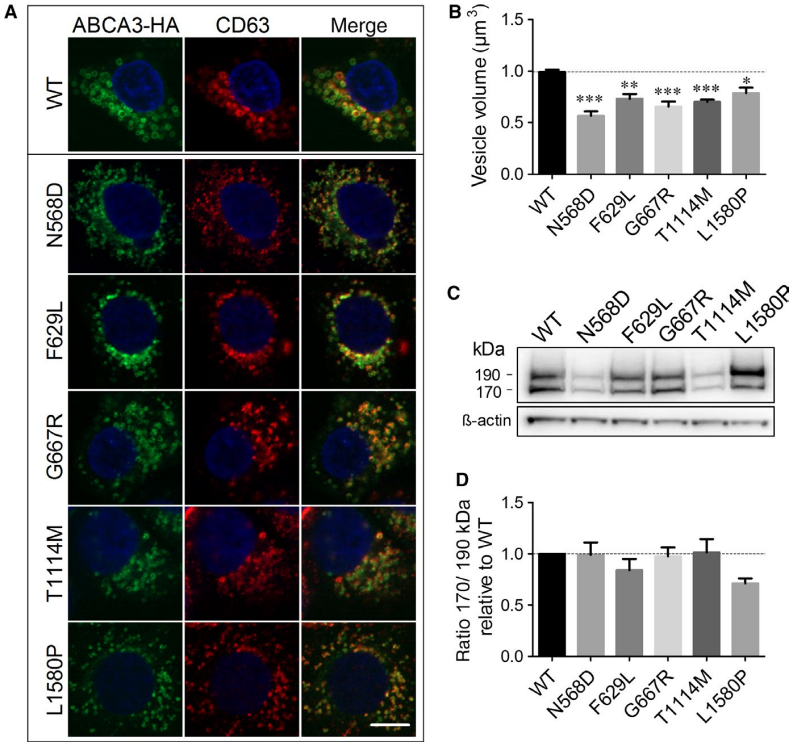

FIGURE 2 Subcellular localization and protein processing is not affected by functional mutations. A, Subcellular localization of ABCA3 wild-type (WT) and mutants shown by immunofluorescence and confocal microscopy. A549 cells stably expressing ABCA3 WT or mutants were fixed, permeabilized and stained for ABCA3-HA and lysosomal marker CD63. All proteins are localized in vesicular structures resembling lamellar bodies, co-localizing with CD63. Scale bar represents 10 µm. B, Volume of ABCA3-HA-positive vesicles in A549 cells stably expressing WT and mutant protein. All analysed mutations led to significantly smaller vesicles compared to WT ABCA3 in A549 cells. Results are taken from the functional assay shown in Fig. 3D and are given as means ± SEM of three independent experiments. $^{*}P < 0.05$ $^{**}P < 0.01$ $^{***}P < 0.001$ compared to WT. C, Western blot analysis of WT and mutant ABCA3. Molecular masses are indicated on the left, β-actin served as a loading control. D, Quantification of the Western blot shown in (C). Densitometric quantification of protein amount was performed with ImageJ. The ratio of 170/190 kDa processing form serves as a marker for correct processing. Mutants do not show different ratios from WT, indicating correct trafficking and processing in the cell. Results are means ± SEM of five independent Western blots

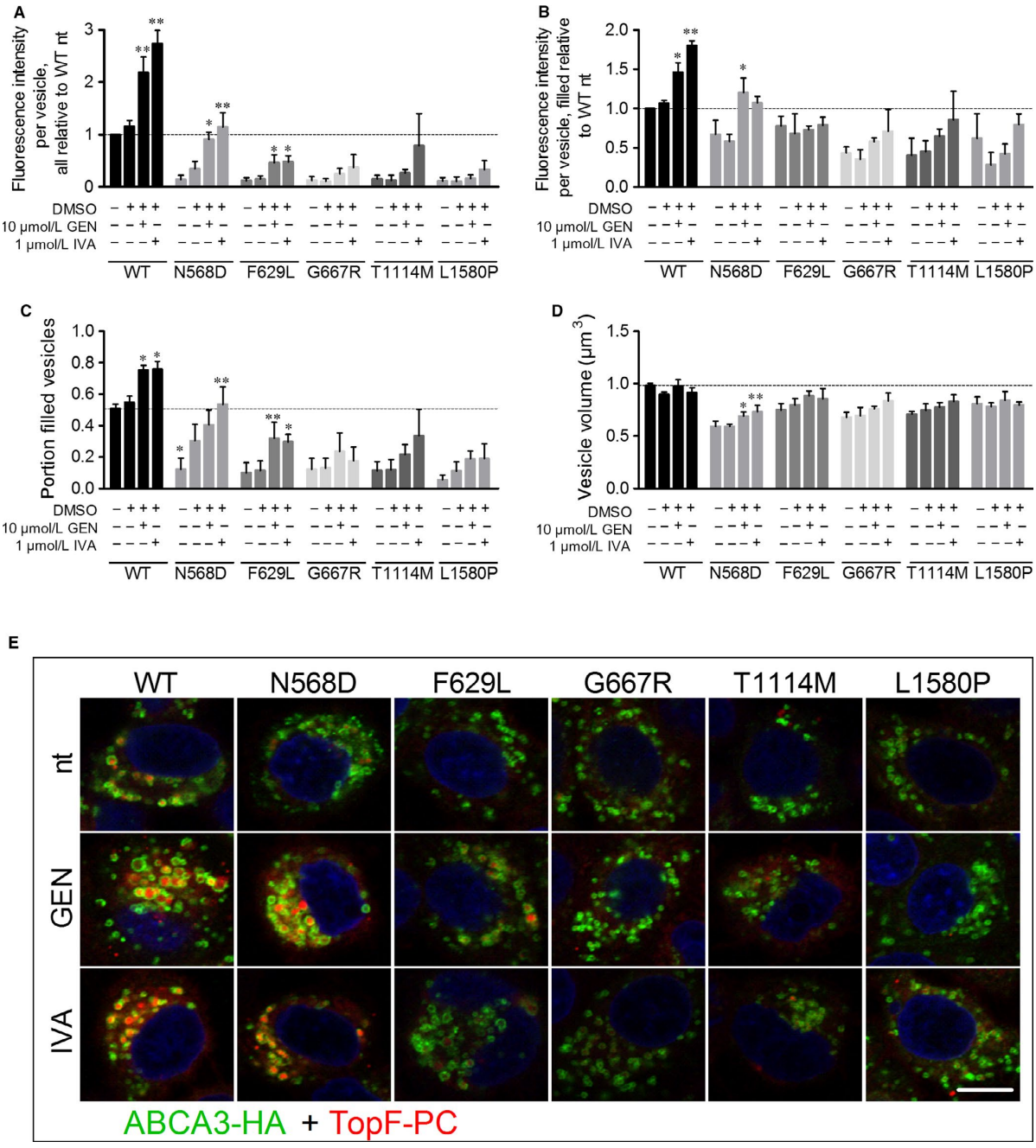

FIGURE 3 Transport of TopF-labeled phosphatidylcholine (TopF-PC) is increased by potentiators ivacaftor and genistein. A549 cells expressing wild-type (WT) or mutant ABCA3 were incubated with liposomes containing TopF-PC and treated with potentiators for 24 h. After fixation and staining for ABCA3-HA, confocal microscopy pictures were obtained to measure: A, TopF-PC fluorescence intensity per vesicles in all analysed ABCA3-HA-positive vesicles relative to WT nt, B, TopF-PC fluorescence intensity in only TopF-PC-filled ABCA3-HA-positive vesicles relative to WT nt, C, Portion of TopF-PC-filled vesicles and D, Volume of all analysed ABCA3-HA-positive vesicles. E, Representative pictures of the experiment. Scale bar represents 10 µm. Pseudo colours were used to stay consistent with former experiments. Results are means ± SEM of three independent experiments. $^{*}P < 0.05$ $^{**}P < 0.01$ compared to dimethyl sulphoxide (DMSO) vehicle controls. nt: no treatment; GEN: genistein, IVA: ivacaftor; TopF-PC: TopFluor-labeled phosphatidylcholine

3.3 | CFTR potentiators ivacaftor and genistein improve WT ABCA3 transport function and rescue functional defects of some ABCA3 mutants

In WT ABCA3-HA expressing cells, treatment with 1 μmol/L ivacaftor or 10 μmol/L genistein led to a 2.7- and twofold increase of lipid transport activity, respectively, resulting from an increase in the portion of filled vesicles and the fluorescence intensity in filled vesicles (Figure 3).

In N568D expressing cells, potentiator treatment led to a drastic elevation of lipid transport activity from 14% of WT activity to 114% in presence of ivacaftor and 90% with genistein, resulting from an increase in vesicle volume, portion of filled vesicles and fluorescence intensity in filled vesicles to a WT level (Figure 3).

Lipid transport activity of F629L mutant was increased to 47% of WT activity by ivacaftor and 46% by genistein treatment, due to a significant increase of the portion of filled vesicles (Figure 3A and C).

For all other mutant proteins, a slight yet not significant increase of lipid transport activity upon potentiator treatment was detected.

Potentiators did not influence the processing of ABCA3 assessed by Western blotting (Figure S1). To further address specificity of their effects, potentiators were also tested in A549 cells expressing misfolding mutants Q215K and K1388N. Neither their processing and thus nor their function was affected (Figure S2), confirming exclusive effects of potentiators on functional mutations.

To further rule out a potential influence of the lower protein expression of N568D ABCA3-HA on the results obtained, we additionally tested another cell clone (N568D-2) with a higher ABCA3-HA expression than in WT ABCA3-HA cells and confirmed the results reported above (Figure S3).

3.4 | G667R mutant displayed lower affinity to genistein and was rescued by increased concentrations

Genistein was shown to bind to the LSGGQ signature motif in CFTR.[39-41] Since G667R is located in this motif in ABCA3 (Figure 1B), we suggested a decreased affinity of genistein to the mutant protein that might be overcome by higher concentrations of the potentiator.

About 50 μmol/L of genistein led to a 2.8-fold increase of lipid transport function in WT-ABCA3 expressing cells resulting from an increase in fluorescence intensity in filled vesicles and the portion of filled vesicles, whereas 100 μmol/L genistein did not increase the lipid transport (Figure 4A and B).

In G667R expressing cells, 100 μmol/L genistein led to a significant increase in lipid transport to 60% of WT function. Assessing filled vesicles only, this concentration increased lipid transport to a WT level (Figure 4A and B) and also the portion of filled vesicles and the volume were slightly also not significantly increased (Figure 4C and B).

In L1580P ABCA3 expressing cells, no significant effect of genistein at increased concentrations was detected.

In our experimental setup, concentrations of 5 μmol/L ivacaftor reduced the viability of the cells (data not shown), so no analysis of higher concentrations of ivacaftor on lipid transport in G667R ABCA3 expressing cells was possible. Viability assays showed the toxicity of ivacaftor for the cells (Figure S4).

4 | DISCUSSION

Functional impairment of ABCA3 due to mutations may lead to fatal or chronic disturbances of ATII cells and surfactant homoeostasis resulting in pulmonary diseases like neonatal respiratory distress syndrome and chronic interstitial lung disease. In the present study, we showed impaired phospholipid transport function of ABCA3 due to distinct disease causing functional mutations that can be rescued by the CFTR potentiators ivacaftor and genistein for mutations located in the NBD1 of the protein.

The functional defect displayed by mutants N568D and F629L was successfully rescued by 1 μmol/L ivacaftor or 10 μmol/L genistein. For mutant G667R, 100 μmol/L genistein was sufficient to yield a significant increase in lipid transport function. Like described before, mutation N568D led to a functional impairment of the ABCA3 protein with only 14% of WT transport function despite correct processing and localization.[25] Furthermore, the two mutants F629L and G667R also showed functional impairment with 12% of WT activity but normal processing and localization and were therefore also classified as functional mutations. Ivacaftor and genistein treatment elevated transport activity of WT ABCA3 by 2.7- and twofold, of N568D mutant up to 114% and 90% of WT function, respectively, and 46% and 47% for F629L mutant.

Since genistein is presumably binding in the LSGGQ motif of NBD1,[41] where the mutation G667R is located, the affinity of the potentiator to the mutant protein is likely to be lowered like shown for G551D in CFTR.[39,40] For WT ABCA3, genistein treatment exerted potentiating effects up to a concentration of 50 μmol/L and inhibitory effects at higher concentrations resulting in a bell-shaped dose-response relation like also shown for CFTR.[39,42,43] This is explained by the assumption of two binding sites for genistein, one high-affinity site activating the protein and a second low affinity site exerting an inhibitory effect.[41,43] In G667R ABCA3 expressing cells, on the other hand, only 100 μmol/L genistein yielded a significant increase in lipid transport activity to a level of 60% of WT function. Therefore the dose-response curve was shifted to the right compared to WT ABCA3, indeed indicating a reduced binding of genistein. Lowered affinity of potentiators to CFTR protein harbouring the G551D mutation was also shown for various other potentiator compounds including ivacaftor.[22,44,45] In our cell model, higher concentrations of ivacaftor reduced the viability of the cells and we could not evaluate their effects on ABCA3 activity. In the TopF-PC transport assay, a concentration of 5 μmol/L ivacaftor impaired cell viability, impeding evaluation of lipid transport. In such experiments, the cells are

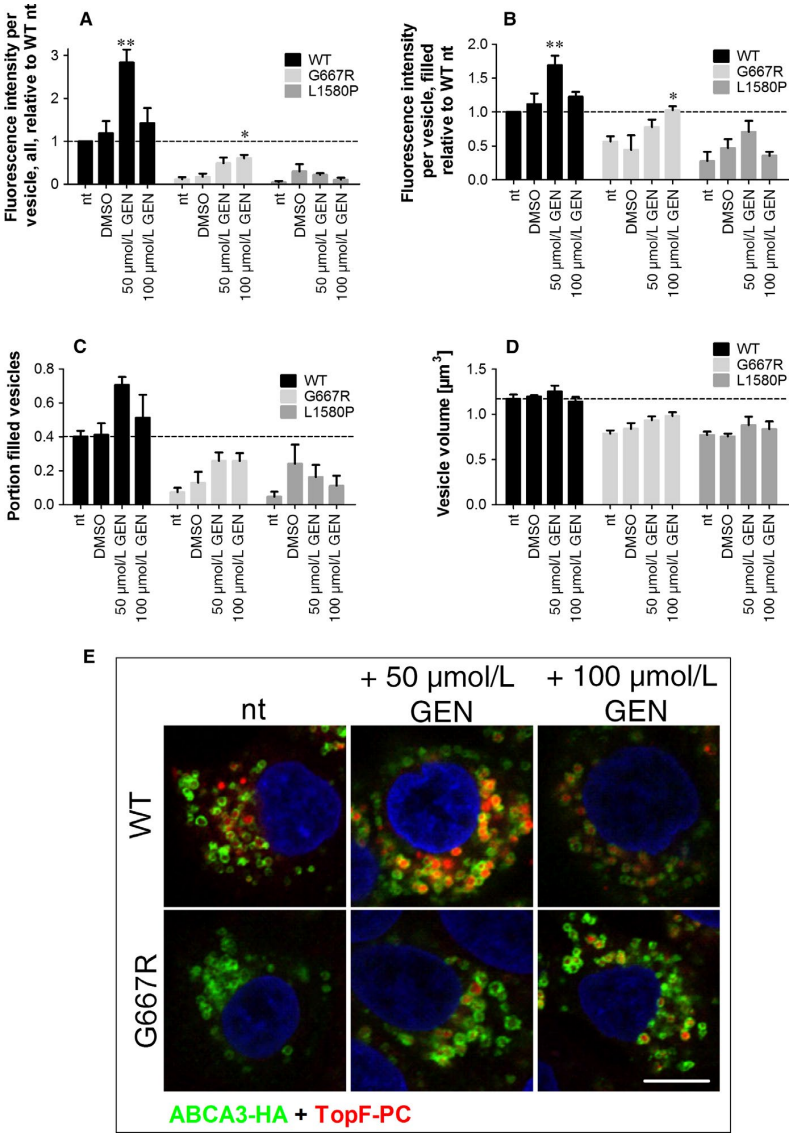

FIGURE 4 G667R ABCA3 mutant is rescued by increased concentrations of genistein. A549 cells expressing wild-type (WT) or mutant ABCA3 were incubated with liposomes containing TopFluor-conjugated PC (TopF-PC) and treated with 50 or 100 µmol/L genistein (GEN) for 24 h. After fixation and staining for ABCA3-HA, confocal microscopy pictures were obtained to measure: A, TopF-PC fluorescence intensity per vesicles in all analysed ABCA3-HA-positive vesicles relative to WT nt, B, TopF-PC fluorescence intensity in only TopF-PC-filled ABCA3-HA-positive vesicles relative to WT nt, C, Portion of TopF-PC-filled vesicles, and D, Volume of all analysed ABCA3-HA-positive vesicles. E, Representative pictures of the experiment. Scale bar represents 10 µm. Pseudo colours were used to stay consistent with former experiments. Results are means ± SEM of three independent experiments. $^{*}P < 0.05$ $^{**}P < 0.01$ compared to dimethyl sulphoxide (DMSO) vehicle controls. nt: no treatment; GEN: genistein, TopF-PC: TopFluor-labeled phosphatidylcholine

incubated at 4°C and in serum-reduced medium, so that treatment with ivacaftor probably adds an additional stressor to the cells. Cell type-specific toxicity may be related to differences in cellular uptake of the drug.[46]

Impaired function of T1114M and L1580P mutants was not rescued by potentiator treatment. We recorded a lipid transport function of 14% and 10% of WT function for T1114M and L1580P as reported before.[25,26] For mutation T1114M, Matsumura *et al* assessed a rather moderate impairment of 52% of WT ATP hydrolysis function but showed a decreased lipid transport function not different from untransfected cells.[26]

The residue T1114 is located in the TMD, where it likely ensures the transmission of conformational changes triggered by NBD dimerization to the TMDs and the extracellular domain, required to translocate the substrate. Mutation of this threonine to methionine likely decouples NBD dimerization and substrate translocation, explaining the lack of effect induced by potentiators that stabilize the NBD dimer formation to enhance transport function and activity. This is further supported by the fact that ivacaftor was also ineffective to rescue the L927P CFTR mutant (T1114 is homologous to L935 in CFTR), which is also located in the eighth transmembrane helix and is implicated in conformational changes necessary to open the channel.[47,48] Furthermore, ivacaftor did not overcome impaired PC secretion activity in a TMD mutant of ABCB4.[49]

Residue L1580 is not directly located in the ATP binding site, however its mutation to a proline most likely breaks the helix, in which it is located. This will affect the upstream H-loop, which is also implicated in NBD dimerization and ATP binding. In addition to

preventing the ATP-induced NBD dimerization, it is possible that the change in conformation might actively prevent the mutated protein to reach the active state even in presence of potentiators, explaining its non-responsiveness even at high concentrations.

Furthermore, since ivacaftor was chemically adjusted to specifically act on CFTR[22] it might only exert effects on regions of ABCA3 that show very high homology to CFTR, like the NBD1, which might explain exclusive effects on mutations located in this domain.

In this study, we used the A549 cell model stably expressing WT and mutant ABCA3. A limitation of this approach is the current inability to predict the effect of potentiators in patients. On the one hand, there is a lack of information on influences of the patient-specific genetic and environmental background. On the other hand, the impact of overexpression of ABCA3 is unknown. In future studies, those limitations might be overcome by the use of patient-specific primary cell cultures or induced pluripotent stem (iPS) cells. The optimal model would utilize patient-derived alveolar epithelial type II cells, which are not readily available due to rarity of the patients and difficulties to access the terminal area of the lungs.

Nevertheless, the A549 model is a valuable tool to identify groups of mutations that can be targeted by the same modulator. Similar to cystic fibrosis, where in vitro studies on Fisher rat thyroid cells expressing rare CFTR mutants were sufficient for the approval of ivacaftor for 23 rare CFTR mutations without need of patient data from clinical trials.[48,50] Our functional assay using TopF-PC reliably reproduced lipid transport and ATPase activity studies of the mutant proteins performed by Matsumura et al[25,26] (Table S1) and also replicated dose-response relations of genistein in CFTR,[39,42,43] making it suitable for high-throughput screens to identify other substances that act as potentiators for ABCA3.

Here we showed that some functional ABCA3 mutations were rescued by the potentiators genistein and ivacaftor. This provides a proof of principle and a first step for the development of pharmacological therapies for interstitial lung diseases caused by ABCA3 mutations, for which currently no treatment is available.

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CONFLICT OF INTEREST

The authors confirm that there are no conflict of interest.

AUTHORS CONTRIBUTION

MG, SK designed the study; SK, YL, MF performed the research; FD, MS performed the 3D modelling and analysed putative consequences of mutations; SK, MG analysed the data and wrote the manuscript. All authors read and approved the final manuscript.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supporting Information

Supplemental material and methods

Primer

Site-directed mutagenesis was performed with the following primers (mutated nucleotides are underlined):

N568D forward: 5'-GCTGGGCCACGACGGTGCCGG-3'

N568D reverse: 5'-AGGACGGTGATCTGTCCCTCGTACAG-3'

F629L forward: 5'-ACCTTTATTTGTACGCCCAGC-3'

F629L reverse: 5'-GCTCTGCGACTGTCAAGT-3'

G667R forward: 5'-CTTCCTGAGCAGGGGCATGAG-3'

G667R reverse: 5'-CGGCTCCGTGAGTTCCAC-3'

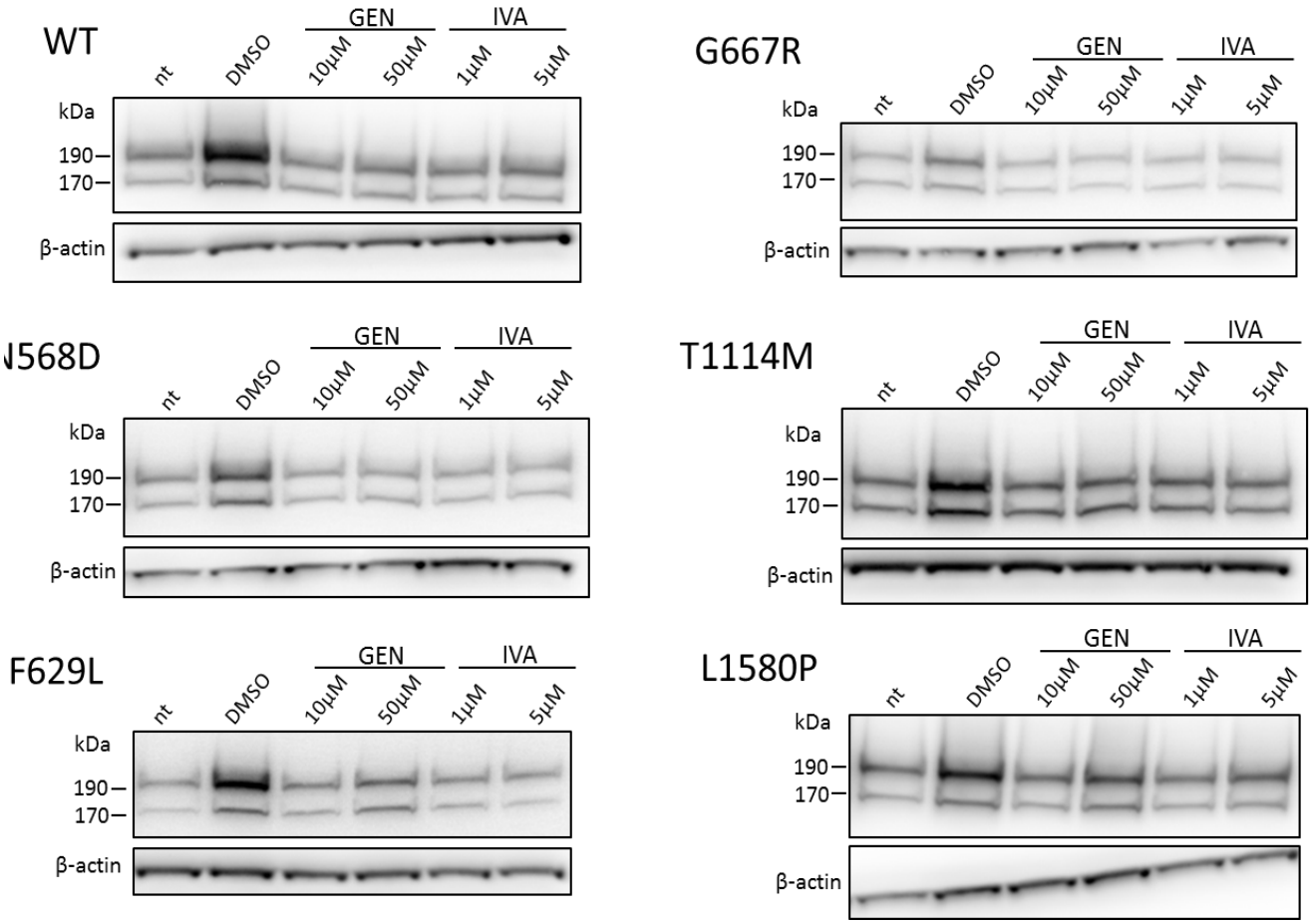
T1114M forward: 5'-TTGGCCAGCATGTTCTCCATC-3'

T1114M reverse: 5'-GAATGCCATGGCGAAGAG-3'

L1580P forward: 5'-TGTGAGGCCCCGTGCACCCGG-3'

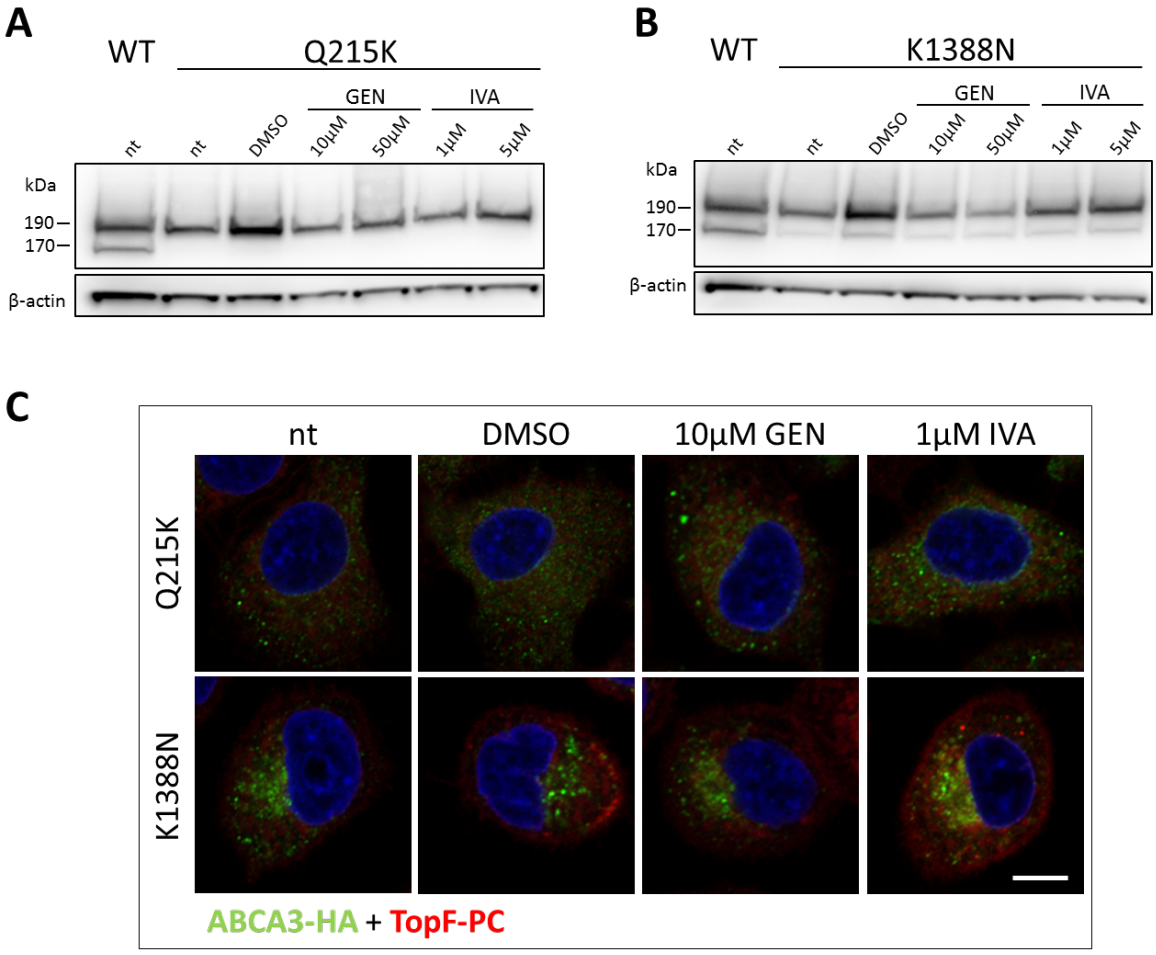
L1580P reverse: 5'-CTCCTCCATGCTGTGGGAGG-3'

Resulting constructs were verified via Sanger sequencing using Clone Manager Suite (Version 6.00).



Supplemental figure S1: Potentiator treatment is not affecting protein processing.

Western blot analysis of WT ABCA3-HA and all five mutants after potentiator treatment. Treatment with potentiators genistein (GEN) and ivacaftor (IVA) had no effect on protein processing. Molecular masses are indicated on the left, β-actin served as a loading control.

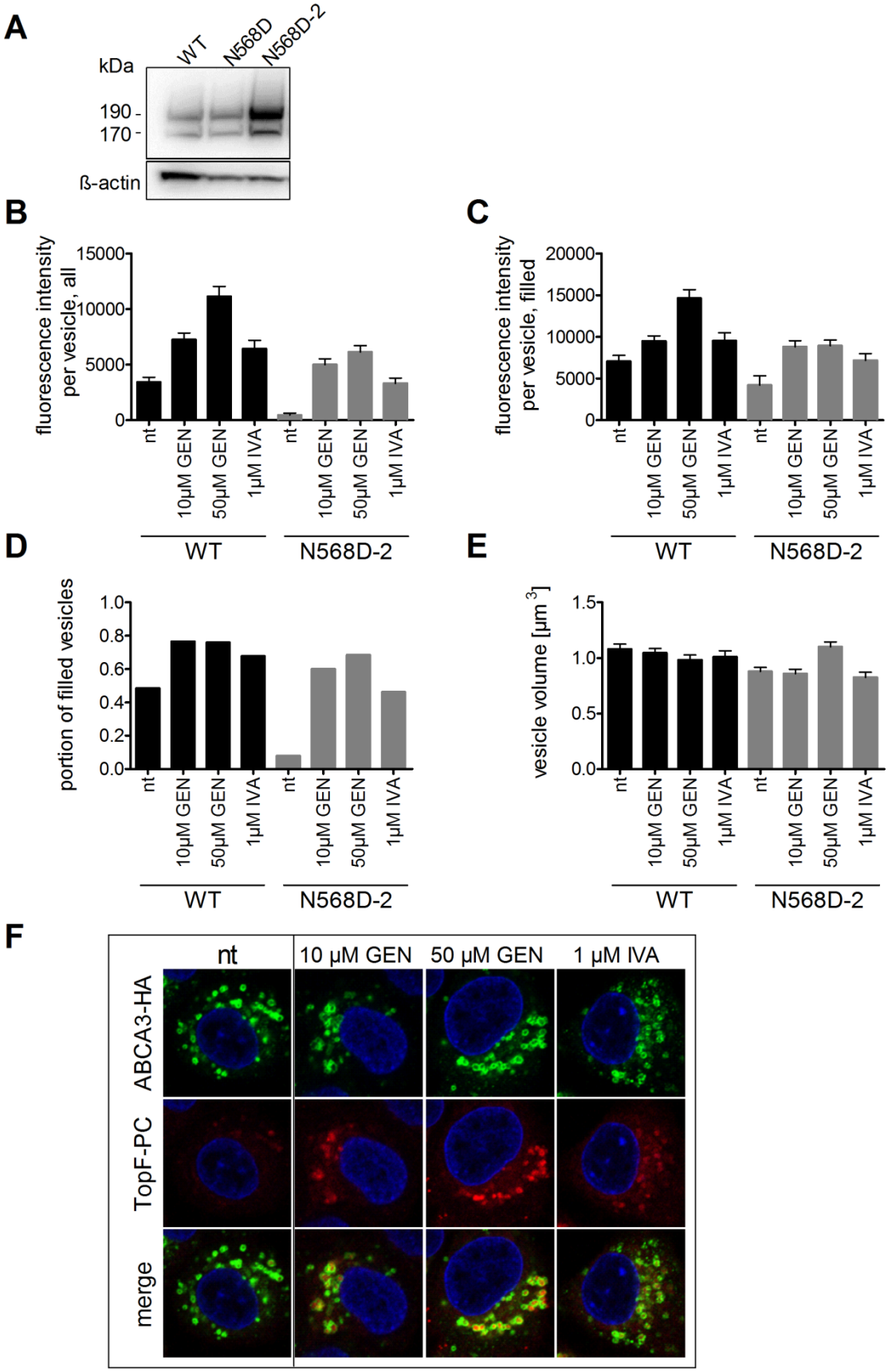


Supplemental figure S2: Potentiators have no effects on misfolding mutations.

A) Western blot analysis of Q215K- and K1388N-ABCA3. Impaired processing of misfolding mutations is not affected by potentiator treatment. Molecular masses are indicated on the left, β-actin served as a loading control.

B) Representative pictures obtained by confocal microscopy. Potentiator treatment does not affect localization of Q215K- or K1388N-ABCA3-HA. Therefore no lipid transport can be assessed. Scale bar represents 10 µm.

GEN: genistein; IVA: ivacaftor


A
WT
N568D
N568D-2
kDa
190
170
ß-actin
B
fluorescence intensity per vesicle, all
15000
10000
5000
0
nt
10µM GEN
50µM GEN
1µM IVA
WT
N568D-2
C
fluorescence intensity per vesicle, filled
20000
15000
10000
5000
0
D
portion of filled vesicles
1.0
0.8
0.6
0.4
0.2
0.0
E
vesicle volume [µm-3]
1.5
1.0
0.5
0.0
F
nt
10 µM GEN
50 µM GEN
1 µM IVA
ABCA3-HA
TopF-PC
merge

Supplemental figure S3: Transport of TopF-labeled PC in N568D-2 mutant is increased upon potentiator treatment.

A) Western blot analysis of WT ABCA3-HA and two different clones of mutant N568D ABCA3-HA. Mutant N568D-2 shows much higher protein expression than WT and N568D mutant. Molecular masses are indicated on the left, β-actin served as a loading control.

A549 cells expressing WT or mutant ABCA3 were incubated with liposomes containing TopFluor conjugated PC (TopF-PC) and treated with potentiators genistein (GEN) or ivacaftor (IVA) for 24 hours. After fixation and staining for ABCA3-HA, confocal microscopy pictures were obtained to measure

B) TopF-PC fluorescence intensity per vesicle in all analyzed ABCA3-HA positive vesicles,

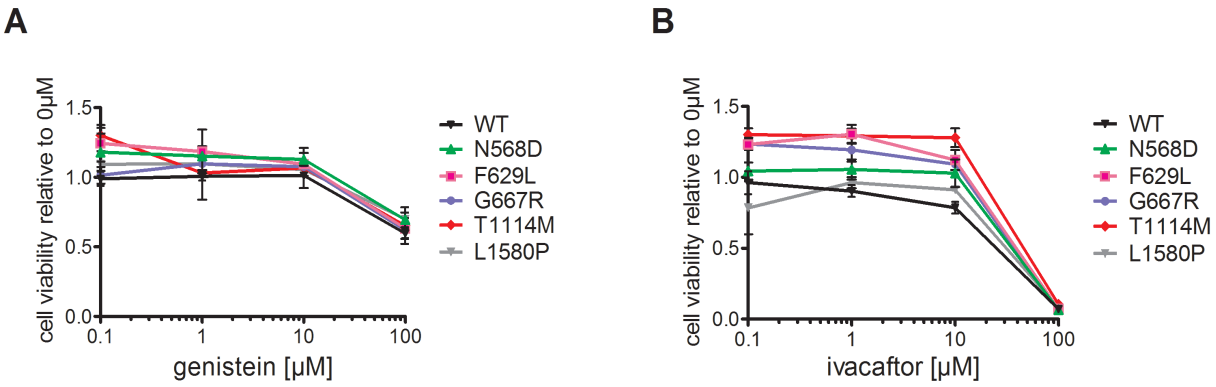
C) TopF fluorescence intensity in only filled ABCA3-HA positive vesicles,

D) Portion of filled vesicles, and

E) Volume of all analyzed ABCA3-HA positive vesicles.

F) Representative pictures of the experiment showing N568D-2 ABCA3-HA mutant. Scale bar represents 10 µm. Pseudo colors were used to stay consistent with former experiments.

Results are means ± S.E.M. of 120 analyzed vesicles. nt: no treatment; GEN: genistein; IVA: ivacaftor; TopF-PC: TopFluor-labeled phosphatidylcholine



Supplemental figure S4: Cell viability upon potentiator treatment.

A549 cells stably expressing WT or mutant ABCA3-HA were treated with increasing concentrations of potentiators genistein (GEN) and ivacaftor (IVA) for 24 hours. Cell viability was assessed by quantification of the specific cleavage of yellow XTT tetrazolium salt (Sigma) to orange formazan in the presence of phenazine methosulfate (PMS, Sigma). Absorbance at 450 nm was measured using a spectrophotometer.

Supplemental table 1: Overview of potentiator effects on WT and mutant ABCA3. Results of functional assays performed in this study are summarized and compared to results obtained before. nt: no treatment.

		Fluorescence intensity in all vesicles relative to WT		
		nt		
	ATPase activity % WT *	nt	Genistein (10µM)	Ivacaftor (1µM)
WT	100	100	**217 ± 30**	**272 ± 25**
N568D	13	14 ± 8	**90 ± 14**	**114 ± 26**
F629L	-	12 ± 5	**46 ± 15**	**47 ± 11**
G667R	-	12 ± 7	**60 ± 8**$^{\times}$	37 ± 24
T1114M	52†	14 ± 7	26 ± 7	78 ± 61
L1580P	9	10 ± 7	16 ± 6	33 ± 16

bold: significant change compared to DMSO vehicle control

*: measurements by Matsumura *et al*. (1, 2)

†: lipid transport function not different from untransfected cells (without ABCA3)

×: treatment with 100µM genistein

References

(1) Matsumura, Y., Ban, N., Ueda, K. and Inagaki, N. (2006) Characterization and classification of ATP-binding cassette transporter ABCA3 mutants in fatal surfactant deficiency. *J. Biol. Chem.*, **281**, 34503-34514.

(2) Matsumura, Y., Ban, N. and Inagaki, N. (2008) Aberrant catalytic cycle and impaired lipid transport into intracellular vesicles in ABCA3 mutants associated with nonfatal pediatric interstitial lung disease. *Am. J. Physiol. Lung Cell Mol. Physiol.*, **295**, L698-707.

3.3 Quantification of volume and lipid filling of intracellular vesicles carrying the ABCA3 transporter

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Quantification of volume and lipid filling of intracellular vesicles carrying the ABCA3 transporter

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ABSTRACT

The ABCA3 lipid transporter is located in the limiting membrane of lamellar bodies (LBs) in type-II-pneumocytes. Mutations within the ABCA3 gene may functionally impair the transporter, causing lung diseases in newborns, children and adults. Assays to quantify volume and lipid filling of the LBs on the level of the vesicular structures and thereby assess the function of ABCA3 are still lacking. In the present study human influenza haemagglutinin- (HA-) tagged wild type and mutant ABCA3 proteins were stably expressed in lung A549 cells. Fluorescently-labelled TopFluor phosphatidylcholine (TopF-PC) incorporated in surfactant-like liposomes was delivered to the cells and visualized by confocal microscopy. Subsequently, a comprehensive image analysis method was applied to quantify volume and fluorescence intensity of TopF-PC in ABCA3-HA-positive vesicles. TopF-PC accumulated within the vesicles in a time and concentration-dependent manner, whereas the volume remained unchanged, suggesting active transport into preformed ABCA3 containing vesicles. Furthermore, this finding was supported by a decrease of the fluorescence intensity within the vesicles when either the ATPase of the transporter was inhibited by vanadate, or when a disease-causing mutation (K1388N) close to the ABCA3-nucleotide binding domain 2 was introduced. Conversely, a mutation (E292V) located in the first cytoplasmic loop of ABCA3 did not significantly affect lipid transport, but rather resulted in smaller vesicles. In addition to these findings, the assay used in this work for analysing the PC-lipid transport into ABCA3 positive vesicles will be useful to screen for compounds susceptible to restore function in mutated ABCA3 protein.

1. Introduction

Pulmonary surfactant is needed in the lungs to reduce surface tension and prevent alveolar collapse during expiration. It is composed of phospholipids, especially phosphatidylcholine (PC) and phosphatidylglycerol, along with minor amounts of phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine, sphingomyelin and surfactant proteins [1,2]. Surfactant is accumulated and stored in the lysosome-derived lamellar bodies (LBs) in type-II-pneumocytes. LBs are eventually exocytosed into the alveolar space, and the surfactant spreads along the air-liquid interface [3].

ATP-binding cassette (ABC)-transporters actively transport a wide variety of substrates across different membranes. ABCA3, which belongs to the class of full ABC transporters consists of 1704 amino acids with two nucleotide-binding domains for ATP hydrolysis and two membrane-spanning domains (Fig. 1A) [4,5,6].

In the lungs, ABCA3 is located in alveolar type II epithelial cells at the limiting membrane of the lamellar body, a type II cell-specific organelle related to lysosomes [7]. It is needed for the biogenesis of LBs and functions as an intracellular transmembrane transporter which carries lipids from the cytosol into the LBs and thereby generates pulmonary surfactant [8]. Although the molecular composition of the surfactant lipids is believed to reflect the transport specificities of ABCA3, little is yet known about its transport activities and specificities. ABCA3 likely transports PC and phosphatidylglycerol [9]. This is in agreement with ex-vivo data from children with ABCA3 deficiency, showing a depletion in PC and phosphatidylglycerol transport leading to depletion in alveolar surfactant [10].

In patients, ABCA3 deficiency and loss of function mutations lead to lethal respiratory distress syndrome in neonates. In addition, less

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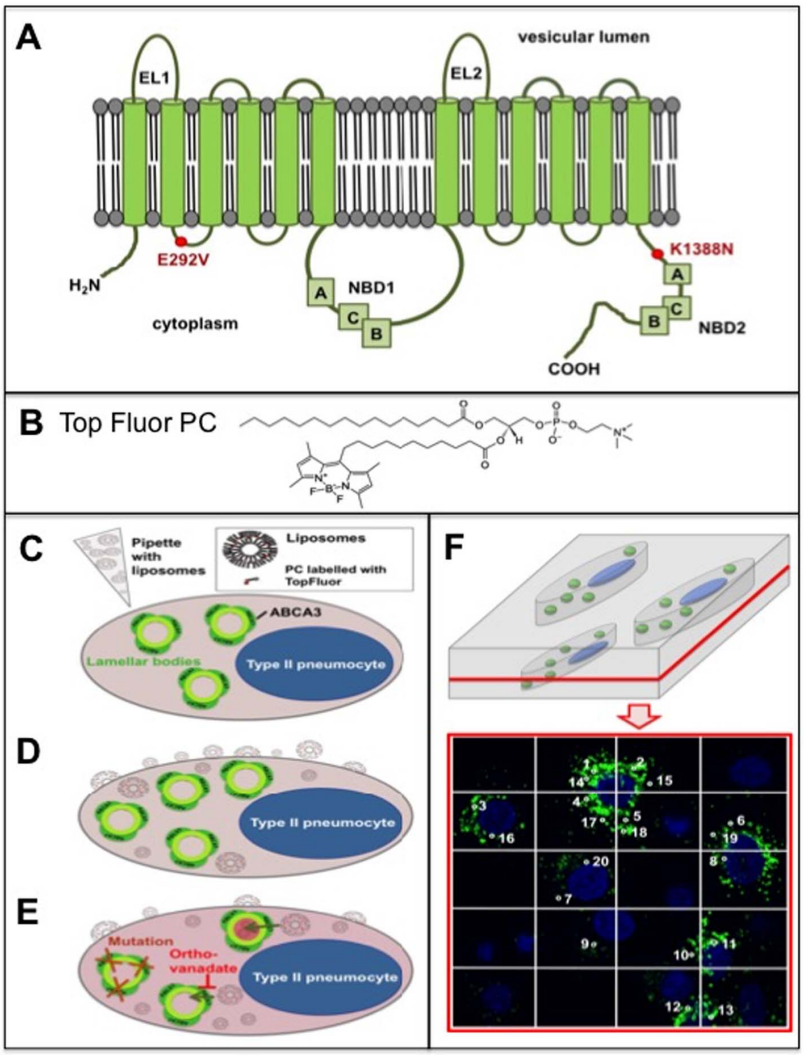

Fig. 1. Schematic overview over the experimental setup.
A) Schematic structure of the ABCA3 transporter with two assessed mutations (E292V and K1388N). The ABCA3 transporter consists of two nucleotide binding domains (NBD1, NBD2), 12 transmembrane domains, two large extracellular loops (EL1, EL2) as well as a Walker A motif (A), a Walker B motif (B) and a Walker C signature motif (C), which is unique to ABCA transporters. Illustration adapted from [33].
B) Structural formula of Top Fluor Phosphatidylcholine. Copyright and permission for using by Avanti Polar Lipids Inc. (https://avantilipids.com/images/structures/810281s.gif, downloaded 2017-08-06).
C–E) Scheme for experimental setup. C illustrates cell treatment with TopF-containing liposomes and liposome accumulation on the cell surface before incorporation into the cell, D displays the ABCA3-dependent lipid uptake from the cytosol into the LBs and E depicts the accumulation of liposomes in the ABCA3-positive vesicles. The lipid transport via ABCA3 can be inhibited by the ATPase inhibitor ortho-vanadate or by a mutation affecting the nucleotide binding domain.
F) Scheme for vesicular selection. From each Z-stack, 20 ABCA3-containing vesicles were chosen with a particular pattern containing 20 fields.

damaging mutations lead to chronic interstitial lung diseases in children and adults [11,12]. To date more than two hundred different ABCA3 mutations from patients suffering from respiratory symptoms have been described [13]. Aberrantly formed LBs may represent a biomarker for decreased or loss of ABCA3-function as for example the clinically relevant mutations K1388N and E292V, which are classified as functional mutations [14,15,16]. Unfortunately until now only methods assessing the whole cells were performed, as subcellular fractioning and direct isolation of aberrantly formed LBs due to decreased ABCA3 function is technically challenging.

In contrast to other ABC transporters which are localized at the cellular plasma membrane and therefore can be easily assessed, ABCA3 is localized intracellularly in membranes of LBs. Thus, it is very difficult to remove and measure substrates transported by ABCA3. Over the past years different cellular assays were established to assess ABCA3 function of non-mutated protein. For example, several groups used sucrose fractionation of intracellular compartments, biochemical lipid analysis and electron microscopy to show that ABCA3 mediates the uptake of choline-phospholipids into the vesicular structures, and is needed for LB biogenesis [9,17]. Cheong et al. showed that silencing of ABCA3 with small interfering RNA reduces the uptake of PC into the ABCA3 + cells and therefore concluded that PC was a substrate of ABCA3 [18]. Using confocal microscopy and analysis with fluorescent NBD-labelled lipid analogs of PC and phosphatidylethanolamine for visualization of the uptake these authors studied cellular dysfunction secondary to ABCA3 mutations [17,19]. To further characterize cellular dysfunction of ABCA3 mutations, alterations of the transporters spatial distribution in the cells were monitored by immunostaining [20,14]. Others assessed the ABCA3 transporting activity indirectly using its ability to sequester and detoxify doxorubicin, other cytotoxic drugs or imatinib into the lysosomal compartment [21,22,23,24,25].

Whereas these experiments analyzed many aspects of normal and mutated ABCA3, the actual lipid transport on the level of the vesicular lamellar body structures has not been assessed yet. Here, we established a method to quantify the uptake of fluorescently labelled PC specifically into ABCA3 positive vesicles in a human cellular model stably expressing wild type and mutant ABCA3 proteins.

2. Methods

2.1. Generation of stable cell clones and treatment protocols

Cells were cultured and stable cell clones were generated as previously described [14].

Surfactant-like liposomes were prepared by mixing 1 μmol TopF-PC, 1.67 μmol egg-phosphatidylcholine, 2.33 μmol 16:0 phosphatidylcholine, 0.67 μmol phosphatidylglycerol (all from Avanti Polar Lipids, Alabaster, USA) and 1 μmol cholesterol (Sigma, Taufkirchen, Germany). Chloroform was evaporated under a stream of N_2 and the dried lipids were redissolved in PBS (Sigma). The solution was placed in a ultrasonic bath for 30 min at 50 °C to generate small liposomes and centrifuged at 1000*g* for 20 min to remove disposals. Before cell treatment, liposomes were solved in OptiMEM (ThermoFisher, Waltham, USA), a modification of Eagle's Minimum Essential Media, buffered with HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and sodium bicarbonate with a 50% reduced serum content, at the ratio of 1:2, 1:20 or 1:200 for dose-response experiments. A ratio of 1:20 was used for all the other experiments. The liposomes were unilamellar and had a size of about 100 nm and a polydispersity index (PdI) of 0.4 (n = 3 determinations), which was assessed with a Malvern laser particle analyser (Zetasizer, Malvern Instruments GmbH, Herrenberg, Germany). ABCA3-WT and -variant cells were disseminated in μ-slides with 8 independent wells (IBIDI, Martinsried, Germany) and incubated at 37 °C with 5% CO_2 for 24 h. Afterwards, cells were incubated for 15 min at 4 °C, medium was replaced by the liposome-OptiMEM mixture and cells were incubated at 4 °C for 30 min. Subsequently, the liposome mixture was replaced by pre-warmed OptiMEM and cells were incubated for different time periods at 37 °C with 5% CO_2. To stop the uptake, OptiMEM was removed and the cells were incubated with 5% bovine serum albumin (BSA, Sigma) solved in PBS at 4 °C for 30 min which removes labelled lipids adherent to the outer membrane. Finally, cells were fixed with 3.7% formaldehyde for 20 min and treated with 0.1% glycine for 10 min.

Whenever necessary, cells were treated with 12.5 mM ortho-vanadate (Sigma) to block the ATPase activity of ABCA3 2 h after cell labelling with the liposomes. All experiments were repeated at least 3 times to exclude coincidental occurrences.

Instead of mock-transfected cells, which would not exhibit LBs, comprising vesicular selection, WT-ABCA3-HA transfected cells were used as controls.

2.2. Immunostaining

Fixed cells were permeabilized with 0.5% saponine (Karl Roth, Karlsruhe, Germany) for 10 min and afterwards incubated in blocking solution containing 3% BSA and 10% FBS for 30 min. Subsequently, the cells were treated with the first antibody against the HA-tag (rat anti HA; Roche, Mannheim, Germany). Before and after treating the cells with the secondary antibody Alexa 555 goat anti-rat (Roche), cells were washed three times with PBS, then incubated with DAPI for 10 min and afterwards covered with mounting medium (90% Glycerine and 20% DABCO in PBS).

2.3. Microscopy

Cells in at least two chambers were exposed to the same conditions. From each of these two chambers three Z-stacks with 0.4 μm height and 123.02 μm length and width were imaged using a Leica confocal microscope with a 405 Diode, an Argon and a HeNe 543 laser. All confocal images were acquired with the same conditions of laser intensity, gain, offset and pinhole width. For observation of the TopF-PC lipids, the filter set included an excitation filter of 488 nm; for observation of the Alexa 555 antibody an excitation filter of 543 nm; and for DAPI an excitation filter of 405 nm.

2.4. Fluorescence analysis with the Fiji-Plugin "Particle_in_Cell-3D"

From each Z-stack, 20 ABCA3 + vesicles were chosen with a particular pattern containing 20 fields (Fig. 1F). To ensure an unbiased selection, first ABCA3 + vesicles were randomly selected using the signal from 543 nm excitation. Next, associated lipids were visualized at 488 nm and the fluorescence intensity within the vesicles was measured using the Fiji-Plugin "Particle_in_Cell-3D" [26], an image analysis method developed to quantify the cellular uptake of fluorescently-labelled targets. The Plugin, originally designed for the analysis of single cells, was customized to allow the selection and analysis of multiple vesicles. The quantification of lipids was performed using the Routine 4 of Particle_in_Cell-3D. Here, lipids within vesicles were automatically selected in the image and analyzed accordingly.

2.5. Vesicle volume

The diameter of the previously selected ABCA3 + vesicles was measured by using the Fiji software and the volume was determined by considering spherical vesicles (i.e. V = 4/3π ∗ (d/2)ˆ3).

2.6. Percentage of filled vesicles

To assess the amount of ABCA3-containing vesicles filled with fluorescently-labelled lipids, a percentage of filled vesicles per analyzed stack was measured. The average result was calculated by combining the stack results.

2.7. Statistical analysis

Whereas the images illustrated the data obtained, quantitative assessment of lipid content of ABCA3 + vesicles analysis is crucial for the approach presented. From all filled ABCA3-containing vesicles, the mean and the standard error of the fluorescence intensity was determined. For comparison of multiple groups, one-way repeated measure ANOVAs with Dunett's multiple comparison tests was done. Comparison of two groups was calculated by using the Student's *t*-test.

3. Results

3.1. Time and concentration dependence of lipid uptake is specific for ABCA3 + vesicles

To localize the lipid uptake, ABCA3 + wild type vesicles were labelled by immunostaining against HA (green, Fig. 2A). With time and exposure to red-labelled PC, the fluorescence intensity in the cells and in the ABCA3 + vesicles increased. Quantification of PC in the ABCA3 + vesicles, which is proportional to the amount of lipids within the LBs, linearly increased with time for up to 24 h (Fig. 2B). The percentage of filled vesicles followed this trend and increased accordingly to the amount of lipids per vesicle (Fig. 2C). Interestingly, the mean volume of the vesicles remained basically the same (Fig. 2D).

Fig. 3 shows that the higher the concentration of PC, the more efficiently it was transported into the LBs (Fig. 3A–C), without detectable changes in the mean volume of the vesicles (Fig. 3D). Based on these experiments we chose the dilution of 1:20 for further experiments.

3.2. ABCA3 specific lipid uptake

To confirm that the lipid uptake was an active ABCA3-dependent transport, and that it did not proceed by passive diffusion, cells were treated with ortho-vanadate (Fig. 4A). Ortho-vanadate inhibits the ATPase activity of ABC-transporters [27]. After 22 h of treatment with ortho-vanadate the fluorescence intensity and the amount of filled vesicles were significantly decreased, whereas the volume of the vesicles stayed the same (Fig. 4B–D).

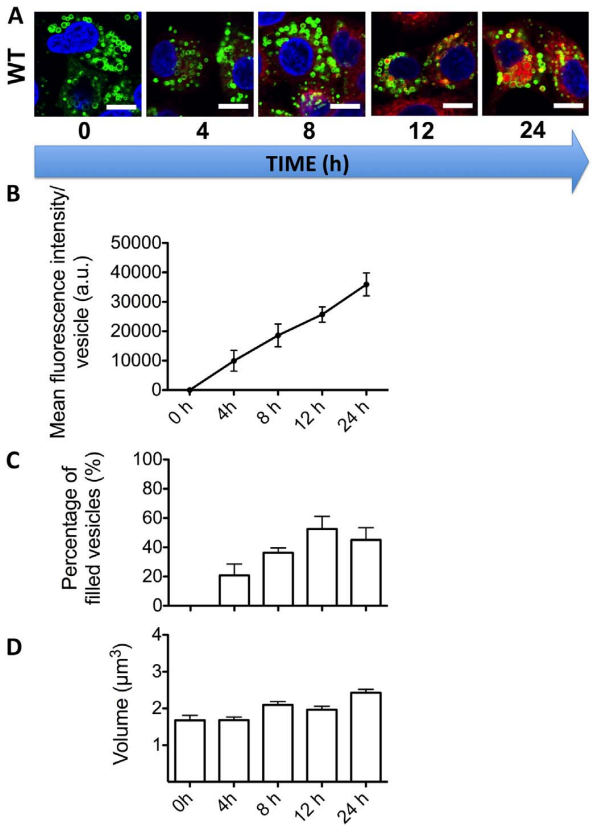

Fig. 2. Time-dependent lipid uptake.
A) Immunofluorescence staining of ABCA3-HA wild type protein in A549 cells, after treatment with liposomes containing TopF-PC for 0, 4, 8, 12 and 24 h. Scale: 10 µm.
B) Fluorescence intensity increase per vesicle during a period of 24 h measured with the Fiji plugin Particle_in_Cell-3D.
C) Percentage of ABCA3-wild type positive cells containing TopF-PC.
D) Volume of ABCA3-positive vesicles in μm^3 during a period of 24 h, calculated based on the diameter measured with Fiji.

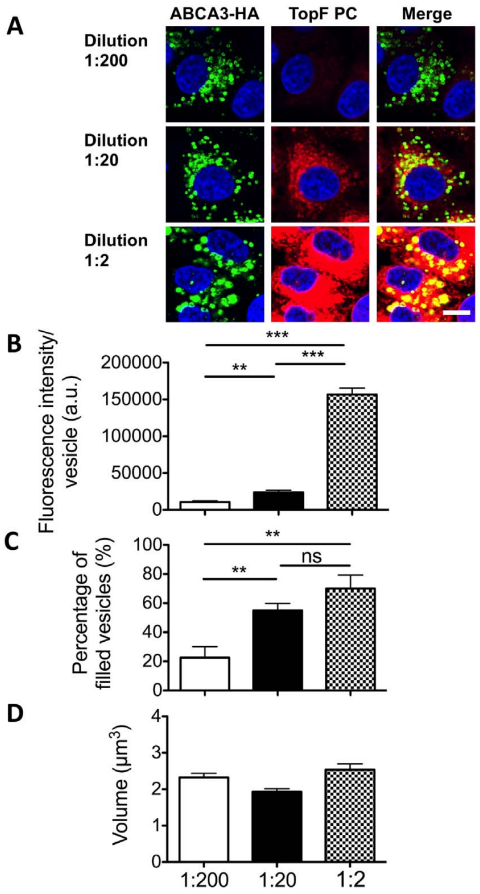

Fig. 3. Concentration dependency of lipid uptake.
A) Confocal fluorescence images with immunofluorescent staining of HA-tagged ABCA3-wild type protein included in vesicles and with different liposome concentrations containing TopF-PC solved in OptiMEM media (1 part of liposomes and 200 parts of OptiMEM, 1 part of liposomes and 20 parts of OptiMEM, which is the normally used mixing ratio, and 1 part of liposomes and only 2 parts of OptiMEM) after 24 h of treatment. Scale: 10 µm.
B) Fluorescence intensity per ABCA3-wild type vesicle measured with the Fiji Plugin Particle_in_Cell-3D after 24 h of treatment (SEM, *** $P < 0.001$, ** $P < 0.01$).
C) Percentage of ABCA3-wild type positive vesicles containing TopF-PC (SEM, ** $P < 0.01$).
D) Volume of ABCA3-positive vesicles after treatment with different liposome concentrations for 24 h of incubation measurement.

3.3. Mutation dependent effects on the ABCA3-dependent PC-transport

The ABCA3-variant K1388N, which is located close to the NBD2 domain of the ABCA3 transporter (Fig. 1A) had a profound impact on the lipid transport of the ABCA3 protein (Fig. 5A). Remarkably, the fluorescence intensity of the ABCA3 + vesicles and the percentage of filled vesicles, as well as their volume were significantly decreased (Fig. 5B–D). Vesicle volume and content were reduced by about 50%. For comparison, we used another ABCA3-variant, E292V. This is located in a loop combining two transmembrane domains, but not nearby a NBD domain (Fig. 1A). The E292V mutation also reduced the volume of the LBs (Fig. 5D), indicating untoward effects in lamellar body genesis, reducing the percentage of filled vesicles in comparison to the wild type (Fig. 5C). However, it did not significantly affect the PC transport function of the ABCA3-transporter when compared to the wild type (Fig. 5B).

4. Discussion

In this study we developed a sensitive assay to quantify the uptake of PC, the major pulmonary surfactant phospholipid, into ABCA3-containing intracellular vesicles. The lipid-specific uptake was observed to be time and dose-dependent and could be blocked by manipulation of the ABCA3 transporter, i.e. inhibition of its ATPase activity by a chemical or by introducing a disease-causing mutation into its ATP-binding domain.

Using confocal microscopy and A549 cells stably expressing ABCA3-WT, we demonstrated that the fluorescently conjugated PC TopF-PC was endocytosed by A549 cells as described previously [28] and enriched in ABCA3 labelled vesicles. We previously demonstrated that the ABCA3-containing vesicles are equivalent to LBs, as ABCA3 co-localizes with CD63, a marker for late endosomes and LBs [14]. Using TopF-PC had the huge advantage over NBD-PC as the fluorescent signal of the label is less sensitive to bleaching, whereas the accumulation of the label in LBs is similar [29,19,30]. By using this approach it was possible to detect small lipid amounts in the vesicles with confocal microscopy. After 4 h of incubation with the lipids the fluorescence intensity was already measurable with the Fiji plugin, which was consistent with the visualization in the confocal images. Even small differences in the fluorescence intensity between the different points of time were detectable. Nagata et al. stated that the ATPase activity of ABCA3 is

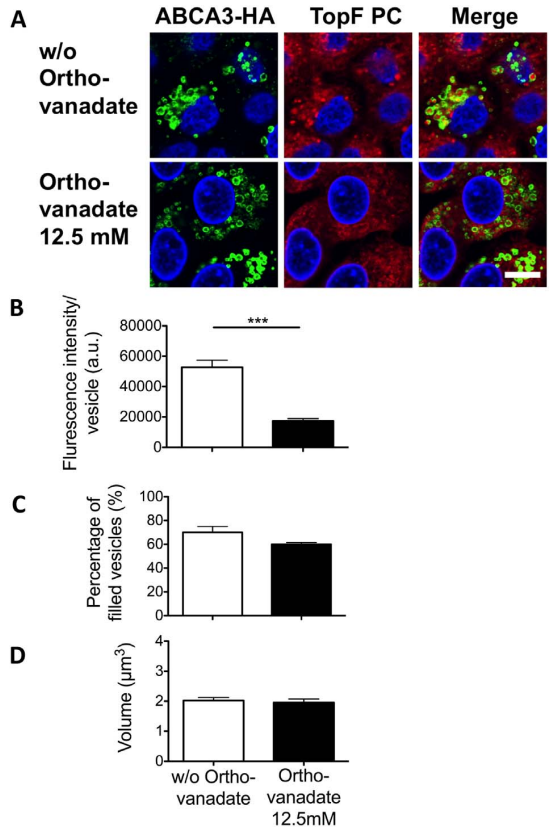

Fig. 4. ABCA3 specific lipid uptake.
A) Fluorescence images of ABCA3-wild type cells treated with TopF-PC containing liposomes for 24 h and with/without 12.5 mM ortho-vanadate for 22 h. ABCA3-HA proteins were stained immunofluorescently.
B) Fluorescence intensity per vesicle measured with Fiji plugin Particle_in_Cell-3D. Ortho-vanadate-treated (12.5 mM) or untreated cells when analyzed after incubation with TopF-PC containing liposomes (SEM, *** $P < 0.001$).
C) Percentage of ABCA3-positive vesicles affiliating TopF-PC after treatment with ortho-vanadate in comparison to untreated cells.
D) Volume of ABCA3-dependent vesicles in cells treated or not treated with ortho-vanadate.

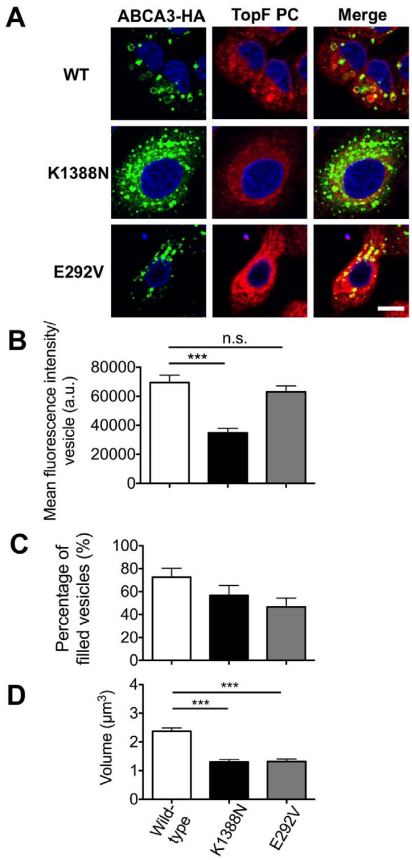

Fig. 5. Effects of mutations within the ABCA3-gene on the transport of the ABCA3 protein and volume of ABCA3-dependent vesicles.
A) Immunofluorescent staining of HA-tagged ABCA3-wild type, ABCA3-K1388N and ABCA3-E292V proteins after cells were treated with TopF-PC containing liposomes for 24 h.
B) Fluorescence intensity per vesicle in a.u. measured with the Fiji plugin Particle_in_Cell-3D after 24 h of incubation (SEM, *** $P < 0.001$).
C) Comparison of the percentage of fluorescent lipid uptake into ABCA3-positive vesicles carrying different ABCA3-mutations and ABCA3-wild type.
D) Vesicle volume of ABCA3-dependent vesicles of different ABCA3 variants in measured with Fiji (SEM, *** $P < 0.001$).

induced by lipids provided in the cytosol [17]. Our results support that statement as we observed a positive correlation between the amount of lipids provided to the cells and the lipids taken up into the vesicles.

In order to show that the lipids did not diffuse passively into the vesicular structures, the cells were treated with ortho-vanadate, which blocks the nucleotide binding domain of the ABC transporters. To make sure that only the ABCA3 dependent lipid-transport was influenced by the vanadate but not the lipid uptake into the cells, vanadate was added 2 h after the lipids were placed on the cells. After 2 h of incubation, lipids could be found in the cytosol but not in the LBs (data not shown). Lipid uptake into ABCA3 positive vesicles decreased significantly in the cells treated with ortho-vanadate, whereas the volume of the organelles remained the same. This confirmed an active ABCA3-dependent transport of the labelled lipids into the vesicles. However, it should be taken into consideration that ortho-vanadate does not exclusively inhibit the ATPase activity of the ABCA3 transporter, but rather of all ATPases. As there is no known ABCA3 inhibitor specifically affecting function and not expression (as siRNA does), inhibiting ABCA3 by ortho-vanadate was the approach of choice. Thus, indirect effects on ABCA3 cannot be excluded, as the specificity of the transport is derived from the direct and sole observation of ABCA3 stained organelles. In another approach to demonstrate specificity of the observed transport, we selected cells stably transfected with a mutation very close to the nucleotide binding domain (K1388N) of the ABCA3 transporter; such data should additionally support the critical role of an intact ATP-binding domain for phospholipid transport into the vesicles. Cells stably expressing ABCA3-K1388N were able to form vesicles, but these vesicles were significantly smaller and the lipid amount within them was significantly lower than in the cells expressing ABCA3-WT. Therefore, we show that phospholipid transport into the vesicles was not only dependent on the integrity of the ABCA3 protein and LB generation, but also on the phospholipid transport function and LB filling.

Interestingly, a mutation in the first cytoplasmatic loop of the transporter (E292V) did not significantly affect the amount of lipid accumulation per ABCA3-containing vesicle. However, in those cells the volume of the vesicles and the percentage of filled vesicles were smaller. This implicates that the LB formation and overall transport activity of cells carrying the E292V mutation were impaired, but lipid transport function appeared normal. These results are in accordance with the previous exploration by Matsumura et al. who as well revealed only a moderately preserved lipid transport in cells expressing the

ABCA3-E292V mutation [15]. Therefore, mutations like E292V within the protein loop seem to influence the building of the vesicles but not the ATP-dependent transport of lipids, for which the NBD domain plays a central role. This is also in agreement with a milder clinical presentation of some patients carrying the E292V mutation, who are prone to develop interstitial lung disease but have a higher life expectancy in comparison with K1388N patients [11,14].

A major strength of the method presented here is that it allows the quantification of PC transport by the ABCA3 transporters into the vesicular organelles carrying this transporter in a cellular system. As there is no standard reference, we cannot prove the results with another method. In our system, ABCA3 is almost exclusively expressed intracellularly, and cannot be detected on the cell surface in sufficient quantity by immune stains. This precludes the easy development of high-throughput assays to directly measure transporter activity, as for other ABC transporters [31]. Similarly, the direct assessment of the transporter's dependency on the different molecular lipid species is not possible with this set-up. An alternative approach may be ABCA3 expression in the outer membrane of yeast [32] which could be a good goal for the future.

Thanks to this novel method it will be possible to search for inhibitors or activators of wild type or mutated ABCA3 protein. Such molecules could be used as potential candidates to explore treatment options for patients with ABCA3 induced lung disease [12]. In this context expression of the transporter in a stable cellular model is of advantage for comparison and detailed study, as many different mutations occur in the many rare patients, unfortunately lacking mutational hot spots. Taken together, we present a method which quantifies specific PC transport into ABCA3 + vesicles. This enables to directly assess ABCA3 transport function and to screen for therapeutic modulators of ABCA3 activity.

Transparency document

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

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4 Discussion

ABCA3 is a key player in alveolar surfactant homeostasis in ATII cells since it transports surfactant lipids into LBs, the storage compartment of surfactant. Mutations in ABCA3 display the most common genetic cause for lung diseases like fatal respiratory distress syndrome in newborns and chronic interstitial lung disease in children and adults. To date no causal therapies are available for patients suffering from those diseases due to ABCA3 deficiency. In this study, five clinically relevant ABCA3 misfolding mutations and five functional mutations were analyzed to prove the concept of modulation of mutant ABCA3 *in vitro* and to identify possible correctors and potentiators for ABCA3 (summary of the results is presented in Table 1). Four of the five misfolding mutants were temperature-sensitive and were corrected by the chemical chaperone TMAO and by CFTR corrector compounds C13 and C17. The correction was determined as restored N-terminal processing of the protein, which indicates its Golgi apparatus passage, restored localization at the limiting LB membrane, and restored lipid transport function. Furthermore, impaired lipid transport function of three of the five analyzed functional ABCA3 mutations was rescued by the known CFTR potentiator genistein and the FDA approved compound ivacaftor.

4.1 Identification of correctors for ABCA3

Incubation of baby hamster kidney cells at low temperature was shown to lead to a so called cold-shock response, which causes down-regulation of proteins, which are involved in protein biosynthesis and degradation, and up-regulation of proteins involved in folding, maturation, and trafficking (Gomes-Alves et al., 2009). This explains the increase in ABCA3 protein amount in the cells after incubation at 30°C and the mechanism of action of correcting misfolded ABCA3 by low temperature. Incubation of the cells at 30°C led to rescued trafficking, processing, and localization of all tested misfolding ABCA3 mutants except for M760R. This mutation might therefore lead to a more severe folding defect, also shown by the fact that cells expressing M760R ABCA3

Table 1: Results of functional rescue of ten different clinically relevant ABCA3 mutants. Shown is a short description about the severity of the phenotype in the patient, the *in vitro* classification as a misfolding or functional mutation, resulting in treatment with either correctors or potentiators, respectively. Results from the functional assay, in which transport of TopF-labeled PC into ABCA3-HA-positive vesicles was assessed, are expressed as percent of WT activity, which was set to 100%. Bold numbers indicate significant changes compared to no treatment. ExAC, Exome Aggregation Consortium; GEN, genistein; ILD, interstitial lung disease; IVA, ivacaftor; nt, no treatment; pILD: pediatric ILD; RDS, respiratory distress syndrome; ^{x}: treatment with 100 µM GEN (G667R).

ABCA3 mutation	**Genotype and Severity phenotype** (clinic)	**Mutation class** (*in vitro*)	**Fluorescence intensity in all vesicles relative to WT nt [%](*in vitro*)**			**Reference**
			nt	**C13**	**C17**	
WT	-	-	100	**235±20**	**263±33**	
Q215K	Homozygous, lethal RDS	misfolding	0	**36±8**	**42±8**	(Brasch et al., 2006)
M760R	Compound heterozygous (R208W), respiratory failure	misfolding	0	0	0	(Doan et al., 2008)
A1046E	Compound heterozygous (DelEx29, A1338T), lethal RDS	misfolding	4±1	**83±12**	**120±10**	(Kröner et al., 2017)
K1388N	Homozygous, lethal RDS	misfolding	13±2	**80±12**	**212±25**	(Kröner et al., 2017; Wittmann et al., 2016)
G1421R	Compound heterozygous (P193S), lethal RDS	misfolding/ functional?	1±0.7	20±3	**130±15**	(Kröner et al., 2017)
			nt	**GEN**	**IVA**	
WT	-	-	100	**217±30**	**272±25**	
N568D	No mutation identified on one allele, lethal RDS	functional	14±8	**90±14**	**114±26**	(Matsumura et al., 2006; Shulenin et al., 2004)
F629L	Homolog to F508 in CFTR	functional	12±5	**46±15**	**47±11**	ExAC (Karczewski et al., 2017)
G667R	Homolog to G550 in CFTR	functional	12±7	**60±8**^{x}	37±24	ExAC (Karczewski et al., 2017)
T1114M	Compound heterozygous (E292V), pILD	functional	14±7	26±7	78±61	(Bullard et al., 2005; Doan et al., 2008; Matsumura et al., 2008)
L1580P	Compound heterozygous (4552insT), lethal RDS	functional	10±7	16±6	33±16	(Matsumura et al., 2006; Shulenin et al., 2004)

completely lack the processed 170 kDa form, while most of the other mutant proteins show a reduced abundance of the processed form. Q215K ABCA3, which also shows no detectable processing, is only partially rescued. Since temperature sensitivity was described as an indicator if proteins are susceptible to correction (Brown et al., 1997; Gautherot et al., 2012), and M760R was not corrected by any of the tested compounds, temperature sensitivity might also serve as marker for susceptibility to correction of mutant ABCA3.

All tested chemical chaperones in this study - like lowered growth temperature - increased the amount of ABCA3 protein in the cells. This is probably explained by the fact that osmolytes are products of the cell stress response and therefore stabilize misfolded proteins and reduce their degradation (Brown et al., 1997; Wang & Bolen, 1997). The amount of WT ABCA3 is also increased after treatment, since newly synthesized WT protein is probably also in parts degraded due to high quality control, like shown for CFTR where a remarkable portion of protein is degraded before maturation (Lukacs et al., 1994). PBA and suberanilohydroxamic acid (SAHA) might also induce expression of ABCA3 since in this experimental setting ABCA3 is controlled by a Cytomegalovirus (CMV) promoter and these compounds were shown to act as histone deacetylase inhibitors that transcriptionally activate the CMV promoter (Kusaczuk et al., 2015; Lai et al., 2010; Lea & Tulsyan, 1995). Indeed, in immunofluorescent stainings heavy accumulation of mutant ABCA3 in the cytoplasm was observed upon PBA and SAHA treatment. Of the tested chemical chaperones, only TMAO was able to restore processing, trafficking, and localization of mutant ABCA3. Despite increasing ABCA3 protein abundance, only TMAO did alter the ratio of 170 kDa to 190 kDa form of mutant protein, which indicates correct processing and trafficking through the cell, which is further confirmed by correct localization at the LBs. It stays elusive why TMAO did rescue mutant ABCA3 but other chemical chaperones failed in doing so. Bandyopadhyay *et al*. described that other osmolytes like glycerol were able to rescue different ‘cell death and differentiation protein’ mutants than TMAO and TMAO-sensitive mutants were mostly unsusceptible to glycerol (Bandyopadhyay et al., 2012). Furthermore, mutant α1-antitrypsin was rescued by glycerol and PBA but not by TMAO (Burrows et al., 2000) and mutant γD-crystallin was shown to be rescued by PBA but not

by TMAO, DMSO, or glycerol (Gong et al., 2010). Those findings suggest different mechanisms of action of the compounds that are still not fully understood. Furthermore, molecular defects resulting from distinct mutations might be different even though they all result in misfolding. This indicates that different ABCA3 mutations might be rescued by other chemical chaperones.

Since osmolytes act in an unspecific and colligative mode, high concentrations are needed for their effects. In a mouse model about 50% of mice died after injection of multiple doses of TMAO, which resulted in a serum concentration of about 50 mM (Bai et al., 1998). TMAO corrected misfolded ABCA3 at concentrations of 200 mM, which precludes its use in clinical trials due to toxicity. More specific compounds that were identified as correctors for CFTR rescued mutant ABCA3 in much lower concentrations (10 μM), the most potent compounds being C13 and C17, which restored processing, trafficking, and localization of ABCA3, and further restored its lipid transport function, shown by the transport of TopF-PC into ABCA3-HA-positive vesicles, which resemble LBs.

Fluorescence intensity in filled WT ABCA3-HA positive vesicles was nearly threefold increased upon C17 treatment. C13 treatment did not significantly increase fluorescence intensity in only filled vesicles. Furthermore, function of A1046E and K1388N ABCA3 is rescued to about 80% of WT activity upon C13 treatment, and up to 120% and 212% of WT activity after treatment with C17 (Table 1). This might indicate a dual function of C17 as a corrector and a potentiator. This hypothesis is further underlined by the fact that fluorescence intensity in vesicles of G1421R ABCA3 expressing cells was only increased compared to DMSO treated cells when treated with C17 but not after C13 treatment. Residue G1421 is located close to the conserved Walker A motif of the second NBD. Introduction of the much larger side chain of arginine (R) might hinder ATP binding, explaining the low lipid transport activity even after correction of processing and trafficking by C13 (Fig. 4). This mutant therefore might display an additive functional defect that is rescued by the potential dual function of C17.

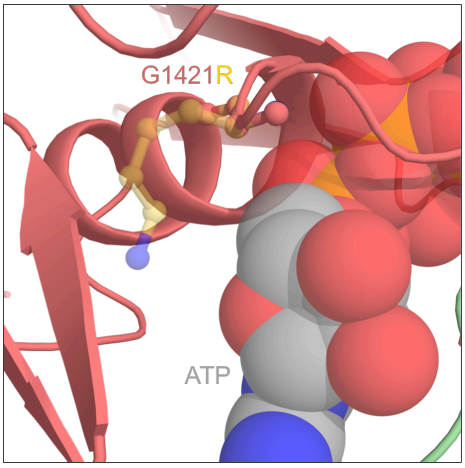

Figure 4: Three-dimensional modeling of the G1421R mutation in ABCA3. The side chain of the wild type residue is represented in full red sticks and the substituting residue is shown in transparent yellow sticks at the mutated position. Residue G1421 is located close to the Walker A motif of the second NBD. Introduction of the larger side chain of Arginine (R) likely hinders ATP binding.

Unfortunately, VX-809, which is approved by the FDA for the treatment of CF, did not correct mutant ABCA3, probably due to its chemical optimization to specifically act on CFTR (Loo et al., 2013; Ren et al., 2013; Solomon et al., 2015; Van Goor et al., 2011). In this study, only the bithiazoles C13, C14, and C17 led to correction of the tested mutants. C13 and C17 were also shown to rescue ATP8B1, a member of the P-type cation transport ATPase family, lacking homology to CFTR, suggesting a more general mechanism of action not restricted to ABC transporters (van der Woerd et al., 2016).

C13 and C17 might serve as lead compounds for correctors of ABCA3 and chemical lead optimization might be the next step in the development of a therapeutic drug. Alternatively, identification of approved compounds that are able to correct misfolding ABCA3 mutants displays a faster and cheaper approach to provide a possible treatment for patients suffering from ABCA3 deficiency. Our group therefore established a HTS based on the results presented here. In cooperation with the Assay Development and Screening Platform (ADSP) of Helmholtz Center Munich, a high-throughput setting was established that automatically seeds cells in a 384-well format, treats the cells with compound libraries, fixes and stains the cells. A microscope is automatically obtaining pictures and its software was trained to recognize WT-like ABCA3-HA vesicles. Several FDA approved compounds were identified in a first screen that were validated by the here presented low-throughput approaches (unpublished data, not shown). Identified compounds might display a therapeutic option for patients when used in drug repurposing initiatives. Drug repurposing harbors the big advantage that preclinical and clinical trials are completed and information about activity and safety of the compounds is already

available, resulting in decreased time for development and approval, reduced costs, and higher success rates compared to the procedure of developing new compounds (DiMasi, 2013).

4.2 Identification of potentiators for ABCA3

To analyze potentiation of impaired ABCA3 lipid transport activity, three ABCA3 mutants, which were already extensively characterized, were analyzed (Matsumura et al., 2006; Matsumura et al., 2007; Matsumura et al., 2008). Furthermore, two ABCA3 mutations with homologous location to the most common and third most common CFTR mutants, F508del and G551D, were analyzed. The identified mutations F629L and G667R also resulted in correct processing and subcellular localization of the protein but impaired lipid transport function, classifying them as functional mutations.

N568D and F629L ABCA3 were potentiated by low concentrations of ivacaftor (1 μM) and genistein (10 μM). Function of the G667R mutant was rescued by 100 μM genistein. The potentiators did not influence processing of the proteins and therefore had no effect on two misfolding mutants. The residue G667 is located in the ATP-binding LSGGQ motif. Genistein was shown to bind to this region in CFTR (Moran & Zegarra-Moran, 2005). A mutation at this location might therefore not only reduce ATP but also genistein binding (Moran et al., 2005; Zegarra-Moran et al., 2002). WT ABCA3 was potentiated by 50 μM genistein, but 100 μM genistein exerted inhibitory effects, resulting in a bell-shaped dose-response curve, similar to CFTR (Illek et al., 1995; Wang et al., 1998; Zegarra-Moran et al., 2002). This is explained by the assumption of two binding sites for genistein, one high affinity site activating the protein and a second low affinity site exerting inhibitory effects (Moran & Zegarra-Moran, 2005; Wang et al., 1998). For G667R ABCA3 the dose-response curve was shifted to the right compared to WT ABCA3 since only 100 μM genistein induced a significant increase in lipid transport activity, indeed indicating a reduced binding of genistein. Decreased binding of various potentiators including ivacaftor was shown for G551D CFTR (Cai et al., 2006; Ma et al., 2002; Van Goor et al., 2009). In our experimental setup higher concentrations of ivacaftor were toxic for the cells impeding their evaluation on G667R ABCA3 lipid

transport function. It stays elusive why this compound that was shown to be safe in clinical trials exerts high toxicity in our cell model, but it might be explained by cell-type-specific uptake and accumulation of the drug (Lei et al., 2011).

Impaired lipid transport function of T1114M and L1580P ABCA3 was not rescued by ivacaftor or genistein. Mutation of the threonine at position 1114 to methionine likely decouples NBD dimerization and substrate translocation. Since potentiators stabilize the NBD dimer formation to enhance transport function and activity, no effect was seen for the T1114M mutation. In CFTR, ivacaftor was also ineffective in overcoming the defects introduced by the mutation L927P, which is like T1114M located in the eighth transmenbrane helix of the protein (T1114M in ABCA3 corresponds to L935 in CFTR) and is implicated in conformational changes necessary to open the channel (Van Goor et al., 2014; Zhang et al., 2018). Furthermore, ivacaftor did not overcome impaired PC secretion activity in a TMD mutant of ABCB4 (Delaunay et al., 2017).

Mutation of leucine to a proline at position 1580 might break the helix it is located in, which might influence the adjacent H-loop involved in ATP binding and NBD dimerization. The mutation might prevent the protein to reach an active state even in the presence of potentiators, explaining why the lipid transport function of the protein is not enhanced by ivacaftor or genistein.

Another explanation might be that ivacaftor, like VX-809, was chemically refined to specifically work on CFTR. This might explain why in this study the compound only potentiated mutants with mutations located in the first NBD, which shows high homology to CFTR. Delaunay *et al*., who investigated the effects of ivacaftor on mutant ABCB4 and only found it to be active for mutations located in the NBDs, drew the same conclusion (Delaunay et al., 2017).

Ivacaftor might display a future therapeutic option for patients suffering from surfactant deficiency diseases due to ABCA3 mutations. Furthermore, the data presented here proves the principle of potentiation of function-defective mutant ABCA3 und thus might serve as a basis for the establishment of an HTS to identify more compounds that can modulate ABCA3 function. Based on the HTS that our group already established to screen for correctors, the TopF-PC transport assay could be used in the HTS setting to

automatically quantify the fluorescence intensity accumulated in automatically recognized vesicles.

4.3 Transport of TopFluor-labeled PC as a functional assay for ABCA3

Here, a functional assay to monitor the lipid transport activity of ABCA3 in A549 cells was established, which was employed to identify correctors and potentiators for ABCA3. In this assay, liposomes containing TopFluor-labeled PC (TopF-PC) besides other unlabeled lipids found in surfactant are offered to the cells and the accumulation of TopFluor fluorescence inside ABCA3-HA positive vesicles after 24 hours is assessed. The fluorescence intensity per vesicle in all measured vesicles, which is influenced by the volume of the vesicles, the portion of filled vesicles, and the fluorescence intensity in filled vesicles, represents the overall lipid transport activity of ABCA3. The transport of TopF-PC was shown to be dose and time dependent and its specific transport by ABCA3 was shown by abrogated accumulation in the vesicles after treatment with the ATPase inhibitor orthovanadate. In other studies so far, lipid transport by ABCA3 could only be quantified by analyzing lipid contents in whole cell lysates or density gradient fractions, or by ATPase assays (Matsumura et al., 2006; Matsumura et al., 2008; Wambach et al., 2016), but the actual transport into ABCA3-HA positive vesicles could not be analyzed. The ATPase activity is not necessarily an equivalent of lipid transport activity, if the NBDs and therefore ATP binding and hydrolysis are unaffected by mutations. In the case of the T1114M mutant for example, Matsumura *et al*. assessed a rather moderately decreased ATP activity but severely decreased lipid transport function, indicated by decreased choline-phospholipid contents in sucrose gradient fractions of post nuclear supernatants of the cells (Matsumura et al., 2008). In this study, results obtained by Matsumura *et al*. for the lipid transport activity of mutants N568D, T1114M, and L1580P were successfully reproduced (Matsumura et al., 2006; Matsumura et al., 2007; Matsumura et al., 2008). Furthermore, dose-response relations of genistein on WT and G667R ABCA3 mirror results obtained for WT and G551D CFTR (Illek et al., 1995; Wang et al., 1998; Zegarra-Moran et al., 2002), confirming the use of the employed TopF-PC transport assay as a reliable tool to quantify ABCA3 function.

With the transport of TopF-PC the recycling pathway of surfactant lipids is addressed, since liposomes, resembling surfactant, are offered the cells through the medium. Our lab now established a different approach using the choline analogue propargylcholine, which was shown to be inserted into cellular choline-containing lipids and is easily visualized by a click reaction to conjugate an azido fluorophore to its three-carbon propargyl group (Aharoni et al., 2016; Jao et al., 2009; Paper et al., 2018). Using this approach, the *de novo* synthesis of surfactant lipids and their transport into ABCA3-HA vesicles can be assessed using the same software tool for quantification utilized here (Li et al., 2019). Furthermore, PLs containing propargylcholine mimic natural lipids much better than TopF-PC where the fluophore might influence biophysical and biochemical properties of PC by changing its size, and might affect transport by the cells and by ABCA3. However, in a study utilizing TopFluor-labeled Ceramide-1-phosphate (C1P), the authors were able to rule out such influences and identified TopFluor-C1P as a reliable mimetic of C1P (Shirey et al., 2016). An advantage of the TopF-PC over propargylcholine-PC is its possible use in live-cell imaging in future studies (Modzel et al., 2017).

PC is the main constituent of pulmonary surfactant. This is why TopF-PC was chosen to assess ABCA3 transport activity in this study. But ABCA3 was shown to also transport PG, PS, PE, and cholesterol (Ban et al., 2007; Cheong et al., 2006; Cheong et al., 2007; Fitzgerald et al., 2007; Matsumura et al., 2007; Zarbock et al., 2015). Therefore, to elucidate full ABCA3 transport function, future studies might benefit from mass spectrometry measurements of lipids from isolated LBs.

4.4 A549 cells as a model for alveolar type II cells

Immortal cell lines display several advantages like easy cultivation, reproducibility of experiments, and nearly unlimited supply. Furthermore, their cultivation is low-cost, the cells can maintain their phenotype through a long cultivation period, and the genetic manipulation and stimulation are easy to be conducted. In this study the A549 cell line was used, which was isolated from a lung carcinoma patient in 1973 (Giard et al., 1973) and was characterized as a suitable model for ATII cells (Nardone & Andrews, 1979; Shapiro et al., 1978). A549 cells express ABCA3, but the cells used in this study showed

nearly undetectable levels of intrinsic ABCA3 mRNA and no detectable protein in Western blots in mock control transfected cells (Wittmann et al., 2016). The effects of the intrinsic ABCA3 are therefore negligible, since all experiments specifically concentrated on introduced HA-tagged ABCA3. The key role of ATII cells is the production, storage, and secretion of surfactant. The A549 cells utilized here form lamellar bodies that can be displayed by electron microscopy only after stable transfection with wild type ABCA3-HA but not in mock control transfected cells (Schindlbeck et al., 2018; Wittmann et al., 2016). Furthermore, the TopF-PC transport assay confirmed ABCA3-dependent surfactant lipid transport into ABCA3-positive vesicles. Therefore, the A549 cell model displays a valuable tool to analyze ABCA3 function and dysfunction and to group mutations for identification of suitable modulators.

Nevertheless, this approach harbors several limitations, like the current inability to predict the effect of tested ABCA3 modulators in patients due to a lack of information about the influence of the patient-specific genetic and environmental background. The CFTR corrector VX-809 for example showed high efficacy *in vitro* for F508del CFTR but only exerted moderate effects in CF patients homozygous for F508del CFTR (Clancy et al., 2012).

The optimal *in vitro* model to analyze ABCA3 modulators would utilize patient-derived ATII cells, but those cells are not readily available due to rarity of the patients and difficulties to access the terminal area of the lungs. Furthermore, it was shown that primary ATII cells spontaneously differentiate into ATI cells during one to two weeks of cultivation (Fuchs et al., 2003). Those limitations might be overcome by the use of recently developed models for ATII cells, which include the use of embryonic stem cells (Spitalieri et al., 2011), mesenchymal stem cells (Cerrada et al., 2014), ATII progenitor cells (Fujino et al., 2010), or induced pluripotent stem cells (Ghaedi et al., 2013). But these model systems are still not completely characterized and display several technical difficulties, which limit their widespread use (Cooper et al., 2016).

Therefore, the use of A549 cells displays a suitable model system to prove the concept of modulation of ABCA3 by correctors and potentiators like done in this study. In cystic fibrosis for example, *in vitro* studies on Fisher rat thyroid cells were sufficient for the

extended approval of the potentiator ivacaftor for 23 rare CFTR mutants, so that no additional clinical trials had to be conducted (Ratner, 2017; Van Goor et al., 2014).

4.5 Structural model of ABCA3 and ABCA3 mutation classes

To better understand molecular consequences of mutations, a structural full-length and a NBD model of WT and mutant ABCA3 were built in this study using the electron microscopy structure of ABCA1 and the crystallography structure of bacterial MacB dimer as templates, respectively (Crow et al., 2017; Qian et al., 2017). To date, a reliable model from crystallographic structure of ABCA3 is still lacking since generation of crystal structures is especially challenging for multi-membrane-spanning proteins. They display a hydrophobic surface and therefore require the use of detergents to be extracted from the cell membranes. Furthermore, they are usually flexible and unstable (Carpenter et al., 2008).

The model employed here allows insight into defects that were shown in experiments, but it cannot be used to predict them. The model is built on similarities to other proteins and is therefore not accurate in all regions. The extracellular domains between ABCA1 and ABCA3 for example do not align well, consequently, interpretation of mutations located in those domains is difficult. The analysis of mutations in the NBDs is more precise since this region is well conserved in all proteins. Furthermore, the model can help to find explanations why modulators do not rescue impairments caused by certain mutations like in the case of potentiators and the mutations T1114M and L1580P.

The model might also help to further categorize mutations into different mutation classes. To date ABCA3 mutations are mostly classified as nonsense, misfolding, or functional mutations (Matsumura et al., 2006; Wambach et al., 2014), and some genotype-phenotype correlations have been demonstrated (Kröner et al., 2017; Wambach et al., 2014), but many ABCA3 mutations still remain unclassified or result in several defects. For example ABCA3 harboring the mutations R208W, R288K, or G964D shows normal protein processing, localization, and lipid transport function, but predisposes for the development of ILD by yet undefined mechanisms (Schindlbeck et al., 2018). Impairments in protein stability or enhanced turnover might be implicated and should

further be investigated. Furthermore, subclasses of functional mutations could be useful, taking into consideration if ATP binding and hydrolysis is affected or if impaired signal transduction to TMDs might hinder the protein's transport function. Those impairments due to mutations might be identified with the help of the 3D model.

A classification system analogous to the one used for CFTR mutations would facilitate the identification of novel mutation class specific therapeutics for ABCA3-related lung diseases. So far no treatment options for class V and VI CFTR mutations have been proposed, but advances in CF research might have direct impact on ABCA3 and other proteins implicated in rare genetic diseases. Ataluren for example was shown to rescue class I CFTR mutants by suppressing premature stop codons (Ryan, 2014). Unfortunately, ataluren did not rescue the R1561X ABCA3 mutant (data not shown), but different compounds should be tested. Furthermore, in future studies combinations of different approaches should be tested, for example G1421R ABCA3 might profit from combinations of correctors and potentiators to rescue both, its folding and lipid transport function.

4.6 Conclusion

In the present thesis, misfolded mutant ABCA3 was rescued by incubation at low temperature, the chemical chaperone TMAO, and the CFTR correctors C13 and C17. The latter might in future studies be further optimized to minimize toxicity and enhance efficiency of the compounds to provide a safe new pharmacological therapy for ABCA3 deficiency. Alternatively, the results presented here served as a basis for the establishment of a high-throughput screen to identify FDA approved compounds that might provide a new therapeutic option for patients suffering from ABCA3 deficiency in the near future. Furthermore, ivacaftor, which is already approved as a potentiator for the treatment of CF, was shown to potentiate ABCA3 function and might be investigated in repurposing initiatives. In conclusion, this study provides the proof of concept that mutant ABCA3 can be rescued pharmacologically and the identification of correctors and potentiators for defective ABCA3 pave the way for the development of novel mutation group specific pharmacological strategies to treat ABCA3 deficiency.

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6 Appendix

6.1 List of abbreviations

ABC	ATP-binding cassette
ABCA3	ATP-binding cassette subfamily A member 3
ADP	Adenosine diphosphate
ASL	Airway surface liquid
ATI/II	Alveolar type I/II cell
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
C1P	Ceramide-1-phosphate
cAMP	Cyclic adenosine monophosphate
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
chILD	Childhood interstitial lung disease
CMV	Cytomegalovirus
DABCO	1,4-Diazabicyclo[2.2.2]octane
DAPI	4′,6-Diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DPLD	Diffuse parenchymal lung disease
DPPC	Dipalmytoylphosphatidylcholine
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EL	External loop
ER	Endoplasmic reticulum
FBS	Fetal bovin serum
FDA	U.S. Food and Drug Administration
GA	Golgi apparatus
GEN	Genistein (4’,5,7-trihydroxyisoflavone)
HA	Hemagglutinin
HDACi	Histone deacetylase inhibitor

HRP	Horseradish peroxidase
HTS	High-throughput screen
IgG	Immunoglobulin G
ILD	Interstitial lung disease
iPS cell	Induced pluripotent stem cell
IVA	Ivacaftor
LB	Lamellar body
MacB	Macrolide export ATP-binding/permease protein MacB
MVB	Multivesicular body
NBD	Nucleotide binding domain
ns	Not significant
nt	No treatment
OptiMEM	Serum reduced Eagle's Minimum Essential Medium
P_O	Open probability
PBA	4-phenylbutyric acid
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PL	Phospholipids
PMS	Phenazine methosulfate
PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride
R domain	Regulatory domain
RDS	Respiratory distress syndrome
RIPA buffer	Radioimmunoprecipitation assay buffer
RPMI	Roswell Park Memorial Institute medium
SAHA	Suberanilohydroxamic acid
SM	Sphingomyeline
SP-A/-B/-C/-D	Surfactant protein A/B/C/D

TM	Tubular myeline
TMAO	Trimethylamine N-oxide
TMD	Transmembrane domain
TopF	TopFluor
TopF-PC	TopFluor-labeled phosphatidylcholine
Tris	Tris(hydroxymethyl)aminomethane
TTF-1	Thyroid transcription factor-1
WT	Wild type
XTT	2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

6.2 Declaration of contribution

Contributions to "Functional rescue of misfolding ABCA3 mutations by small molecular correctors"

The study was designed and planned by Matthias Griese and me. I performed all experiments, prepared all figures and wrote the manuscript. A part of the stable cell lines were provided by Ulrike Schindlbeck and Thomas Wittmann. Maria Forstner, Jacqueline Harfst and I established remaining stable cell clones. Stefanie Höppner helped establishing the functional assay that was employed in this study by me. Further I was responsible for submission, revision, and resubmission of the manuscript.

Contributions to "Potentiation of ABCA3 lipid transport function by ivacaftor and genistein"

The study was planned and designed by Matthias Griese and me. I conducted all site-directed mutagenesis experiments. I established all stable cell lines and conducted screens to choose single cell clones with the help of Yang Li and Maria Forstner. I performed all experiments, prepared all figures and wrote the manuscript. 3D modeling was performed with the help of Florent Delhommel and Michael Sattler. Further I was responsible for submission, revision, and resubmission of the manuscript.

Contributions to "Quantification of volume and lipid filling of intracellular vesicles carrying the ABCA3 transporter"

The study was planned and designed by Matthias Griese, Ralf Zarbock and Stefanie Höppner. I helped with cell culture work and procedures of the experiments. Further I discussed results with Stefanie Höppner, proofread the final manuscript and helped with the revision of the manuscript.

Susanna Kinting Prof. Dr. Matthias Griese

6.3 Statutory declaration and statement

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbstständig und ohne unerlaubte Hilfe angefertigt wurde.

Ich erkläre hiermit, dass die Dissertation weder ganz noch in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist und dass ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

München, den 03.01.2020

Susanna Kinting