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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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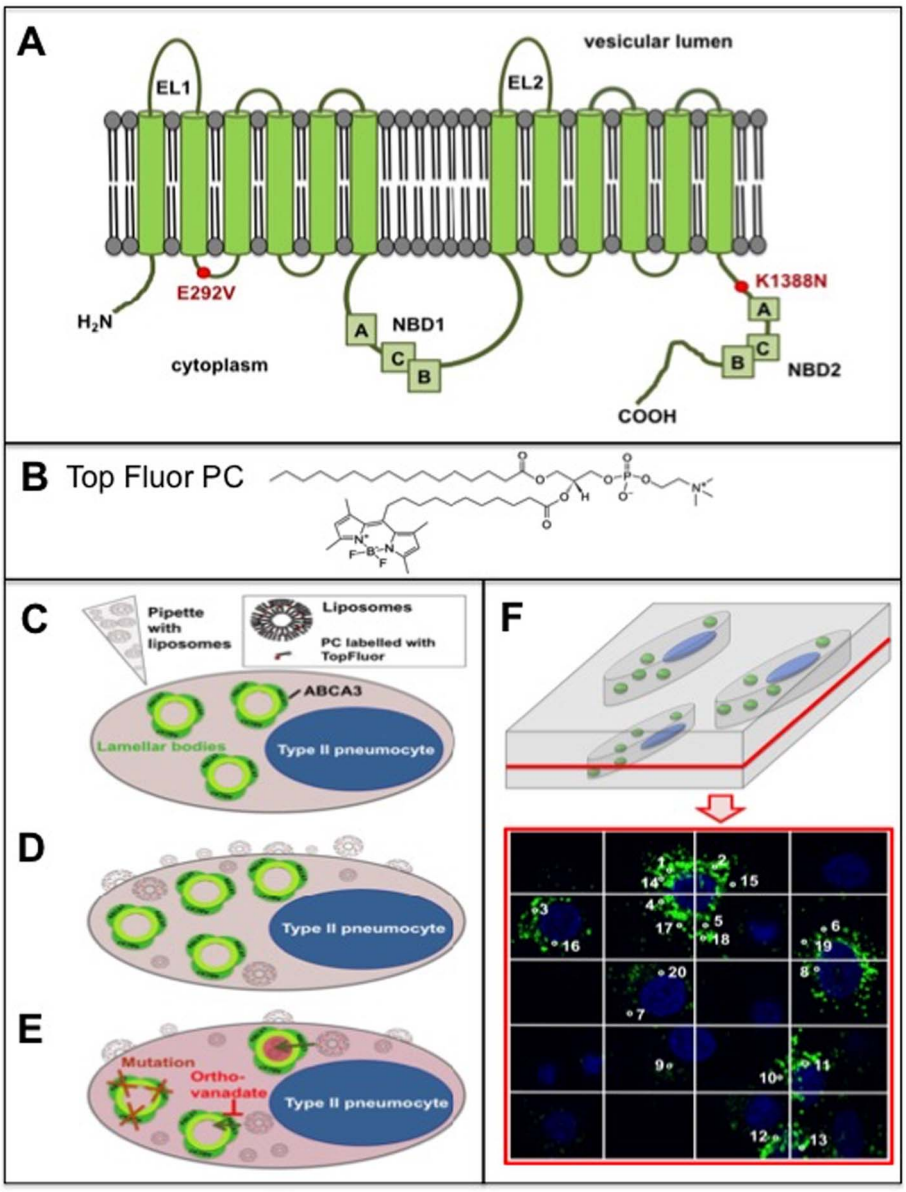

Fig. 1. Schematic overview over the experimental setup.
A) Schematic structure of the ABCA3 transporter with two assessed mutations (E292V and K1388N). The ABCA3 transporter consists of two nucleotide binding domains (NBD1, NBD2), 12 transmembrane domains, two large extracellular loops (EL1, EL2) as 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).

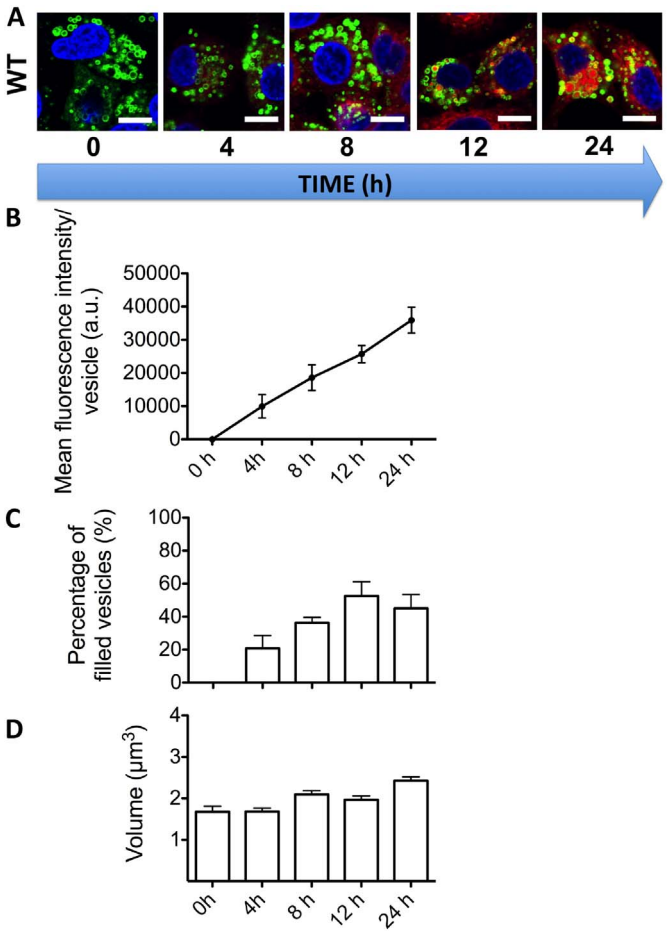

Fig. 2. Time-dependent lipid uptake.
A) Immunofluorescence staining of ABCA3-HA wild type protein in A549 cells, after treatment with liposomes containing TopF-PC for 0, 4, 8, 12 and 24 h. Scale: 10 µm.
B) Fluorescence intensity increase per vesicle during a period of 24 h measured with the Fiji plugin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

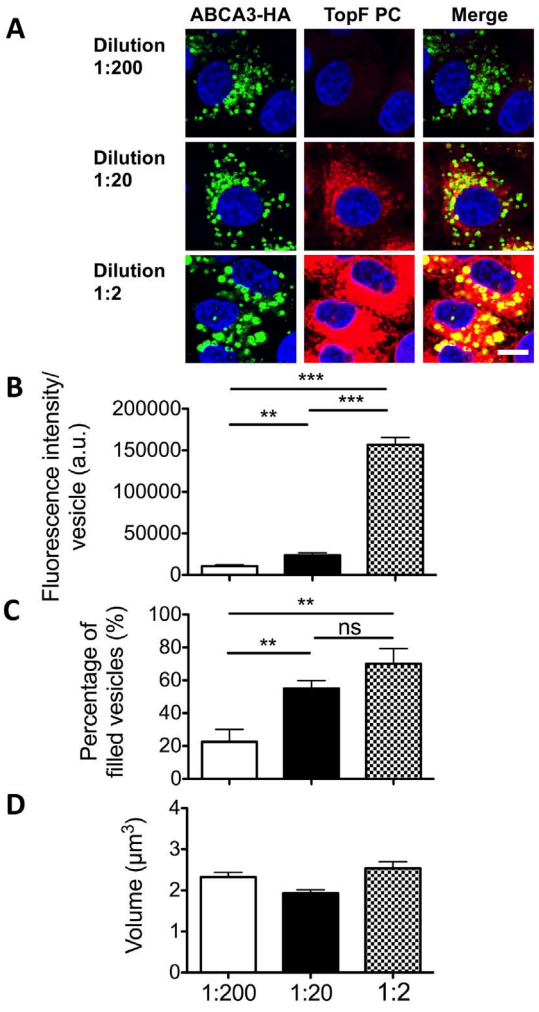

Fig. 3. Concentration dependency of lipid uptake.
A) Confocal fluorescence images with immunofluorescent staining of HA-tagged ABCA3-wild type protein included in vesicles and with different liposome concentrations containing TopF-PC solved in OptiMEM media (1 part of liposomes and 200 parts of OptiMEM, 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

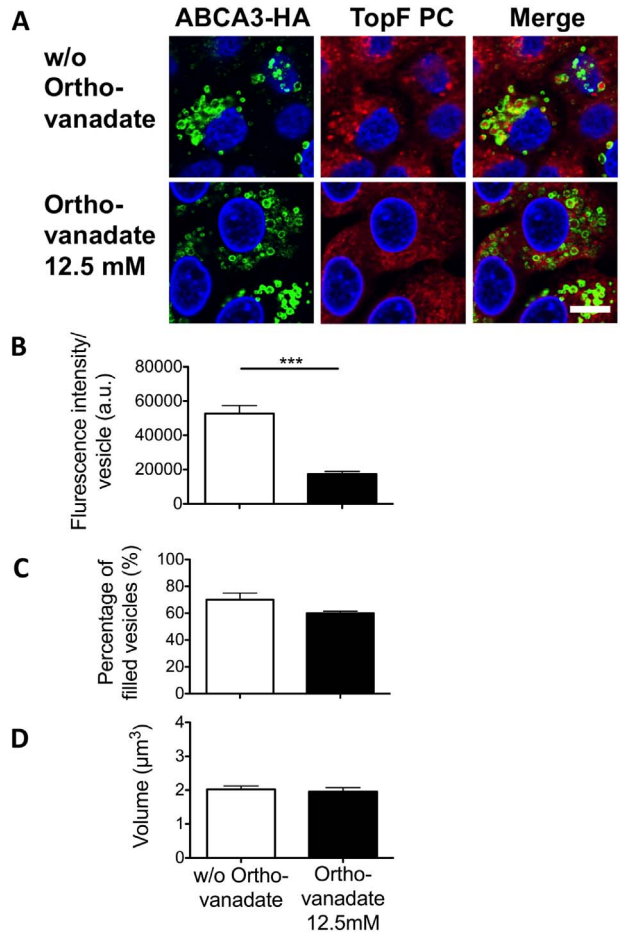

Fig. 4. ABCA3 specific lipid uptake.
A) Fluorescence images of ABCA3-wild type cells treated with TopF-PC containing liposomes for 24 h and with/without 12.5 mM ortho-vanadate for 22 h. ABCA3-HA proteins were stained immunofluorescently.
B) Fluorescence intensity per vesicle measured with Fiji plugin 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.

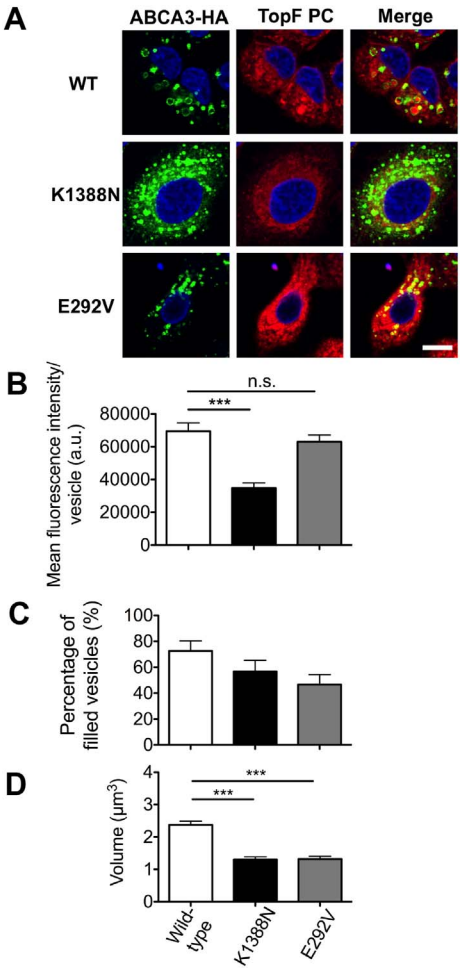

Fig. 5. Effects of mutations within the ABCA3-gene on the transport of the ABCA3 protein and volume of ABCA3-dependent vesicles.
A) Immunofluorescent staining of HA-tagged ABCA3-wild type, ABCA3-K1388N and ABCA3-E292V proteins after cells were treated with TopF-PC containing liposomes for 24 h.
B) Fluorescence 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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