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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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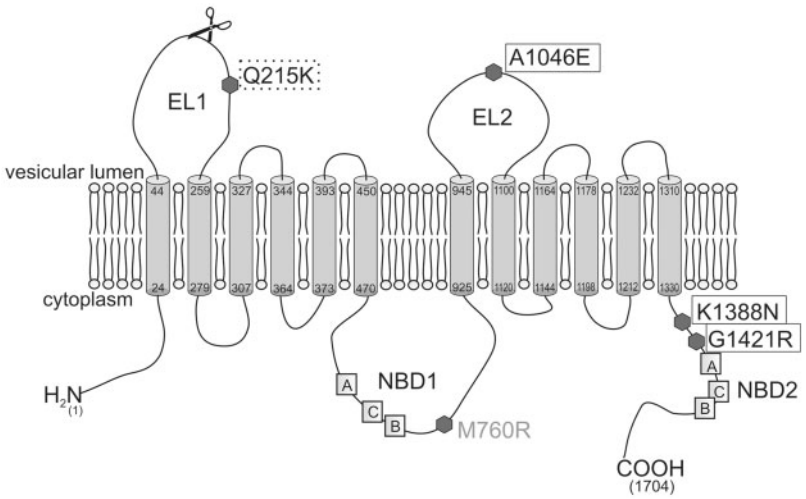

Figure 1. Topology model of ABCA3. Positions of all mutations analyzed in this study are marked. Scissors indicate the cleavage site for processing of the 190 kDa form to the 170 kDa form in post-golgi compartments. Mutations that could be corrected completely are boxed; dashed lines indicate partial 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

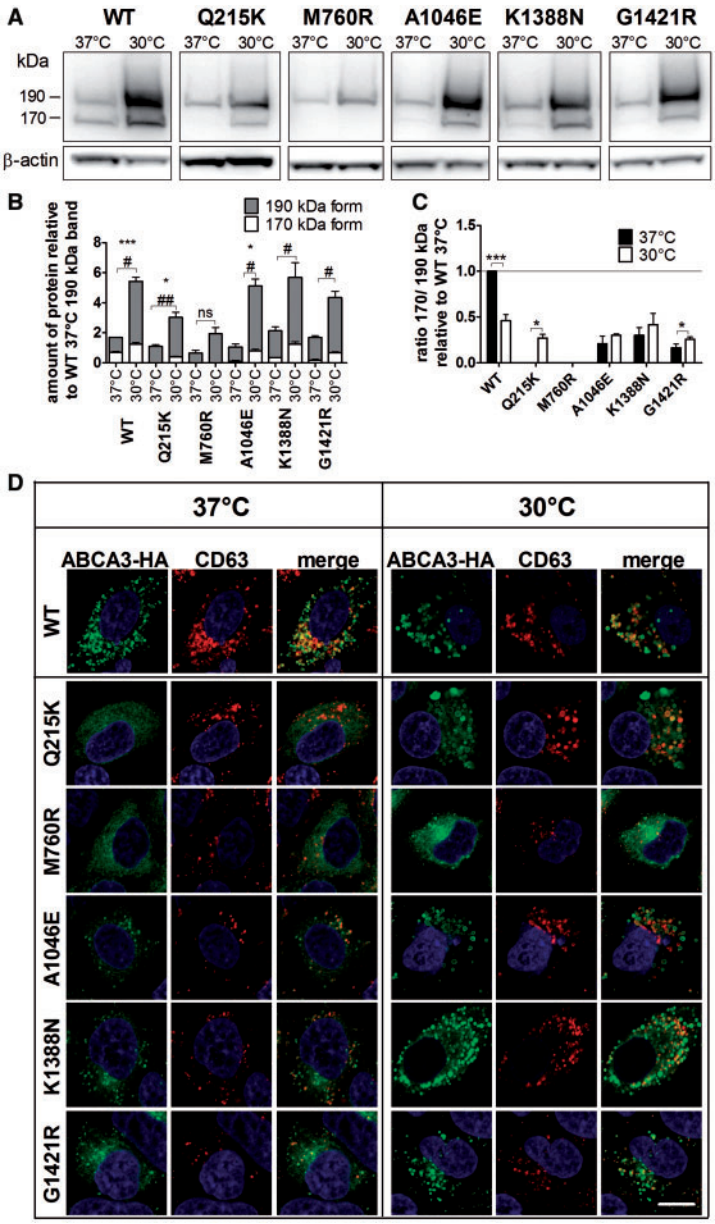

Figure 2. Defects in ABCA3-HA processing caused by mutations are temperature-sensitive. A549 cells stably expressing WT or mutated ABCA3-HA, were grown at either 37°C or 30°C for 48 h and ABCA3-HA protein was analyzed by western blot (**A**). Densitometric quantification of protein amount in each band (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

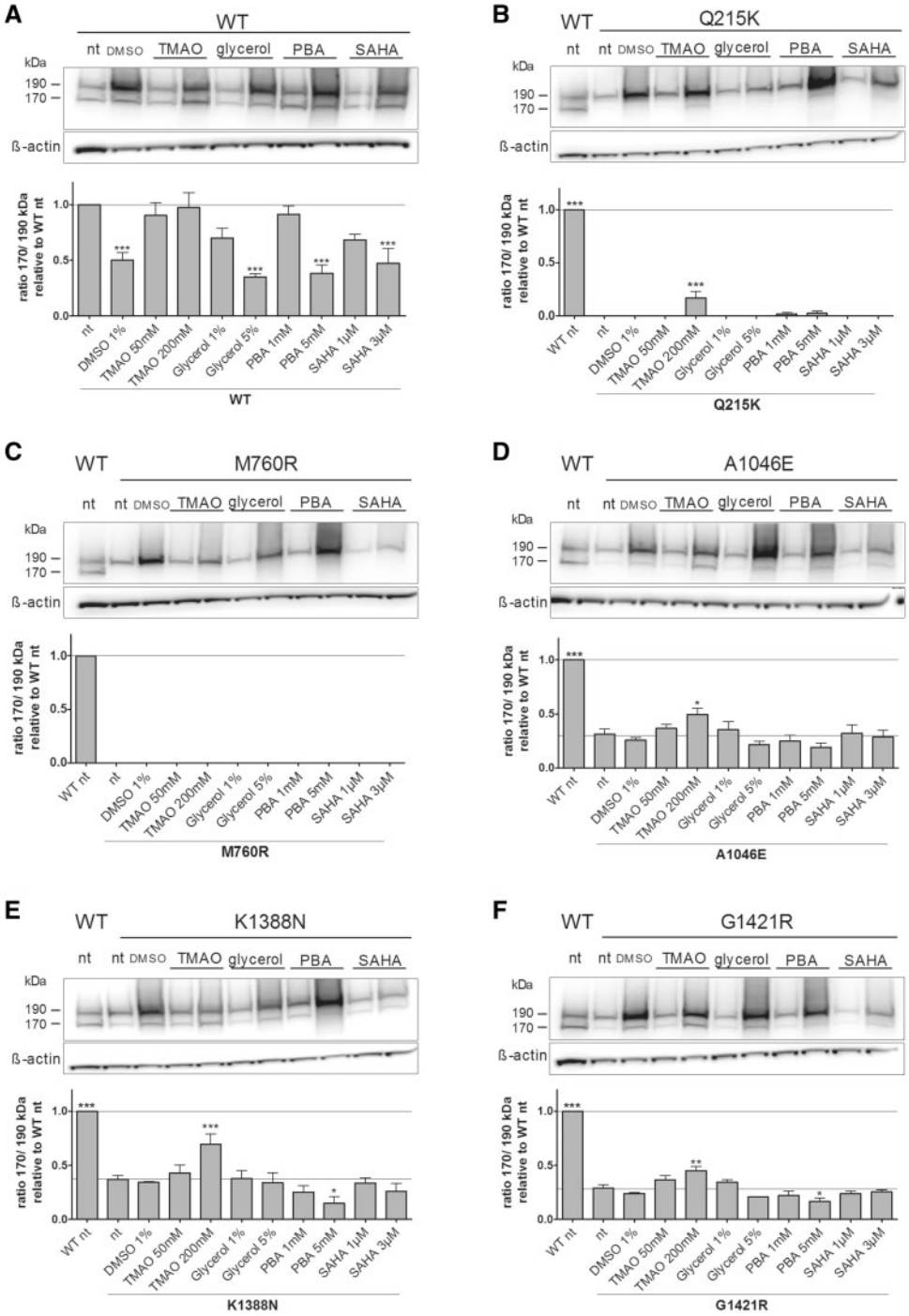

Figure 3. Chemical chaperone TMAO restores processing of ABCA3-HA mutants. A549 cells stably expressing ABCA3-HA WT or mutations were treated with two different concentrations of chemical chaperones for 48 h and ABCA3-HA protein pattern was analyzed by western blot (upper panel). Densitometric quantification 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).

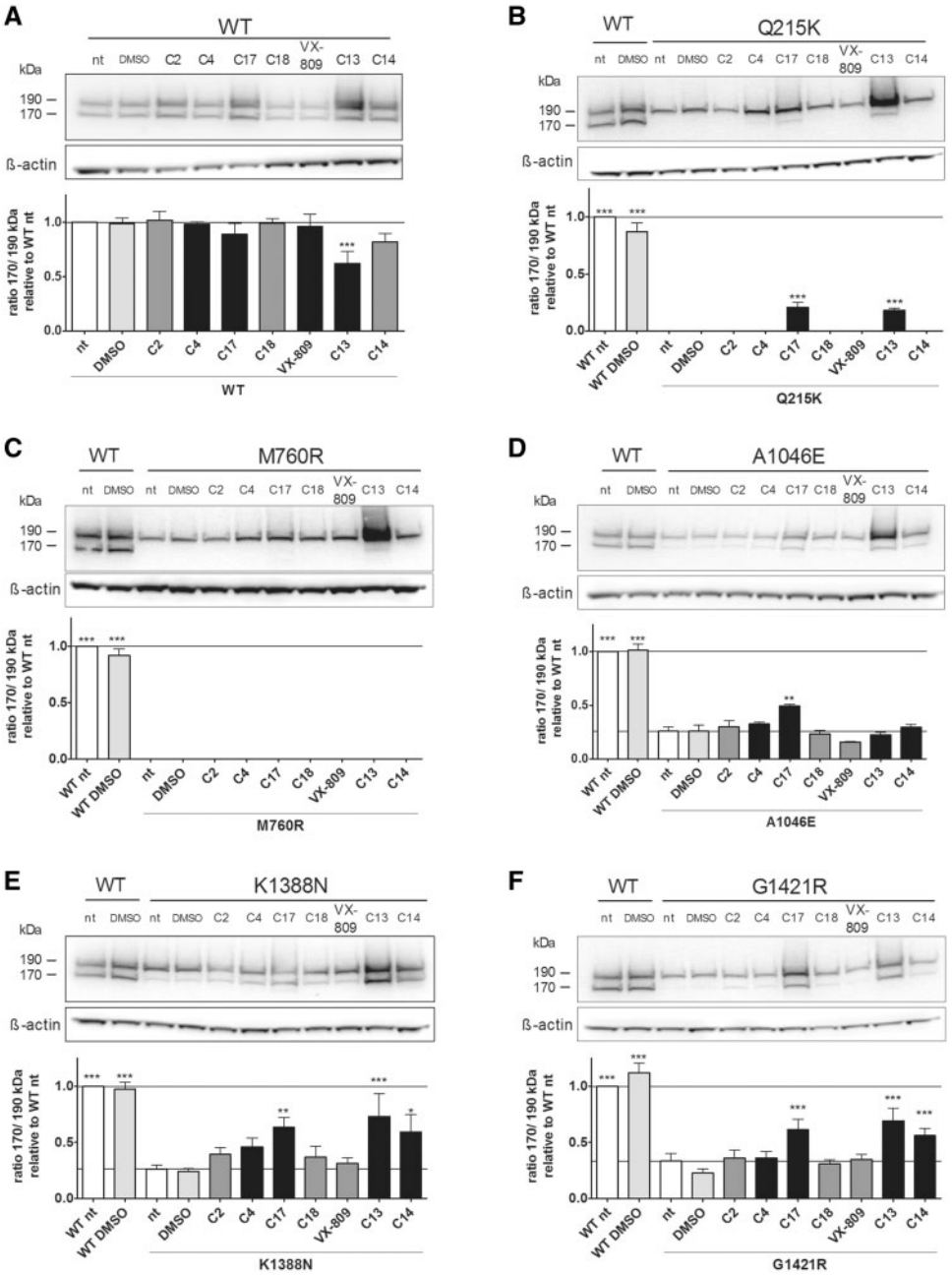

Figure 4. CFTR correctors restore processing of ABCA3-HA mutants. A549 cells stably expressing ABCA3-HA WT or mutations were treated with 10 μM of correctors for 48 h and ABCA3-HA protein pattern was analyzed by western blot (upper panel). Densitometric quantification of protein amount in each band (190 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

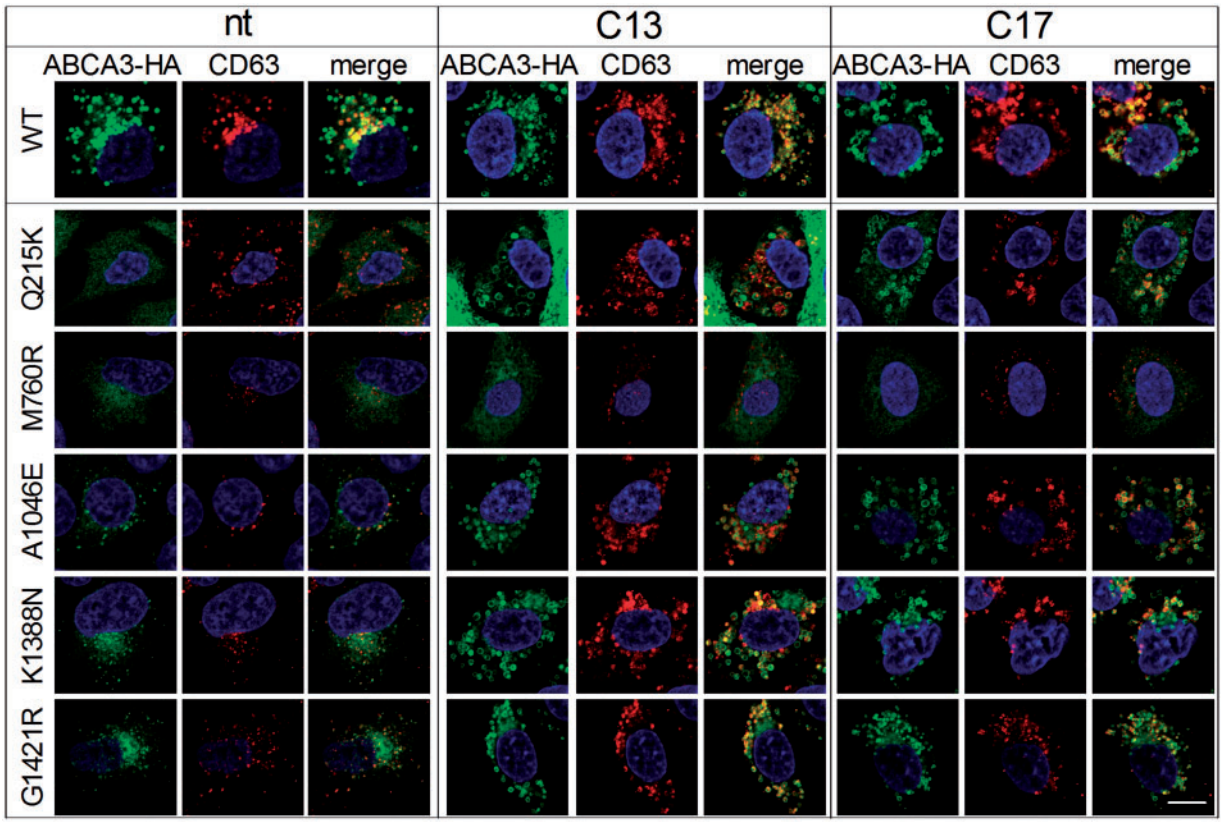

Figure 5. Correctors C17 and C13 restore subcellular localization of ABCA3-HA mutants. A549 cells stably expressing ABCA3-HA WT or mutations were treated with 10 μM of C13 or C17 for 48 h and stained for ABCA3-HA and lysosomal marker CD63. nt: no treatment; scale bar represents 10 μm.

(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

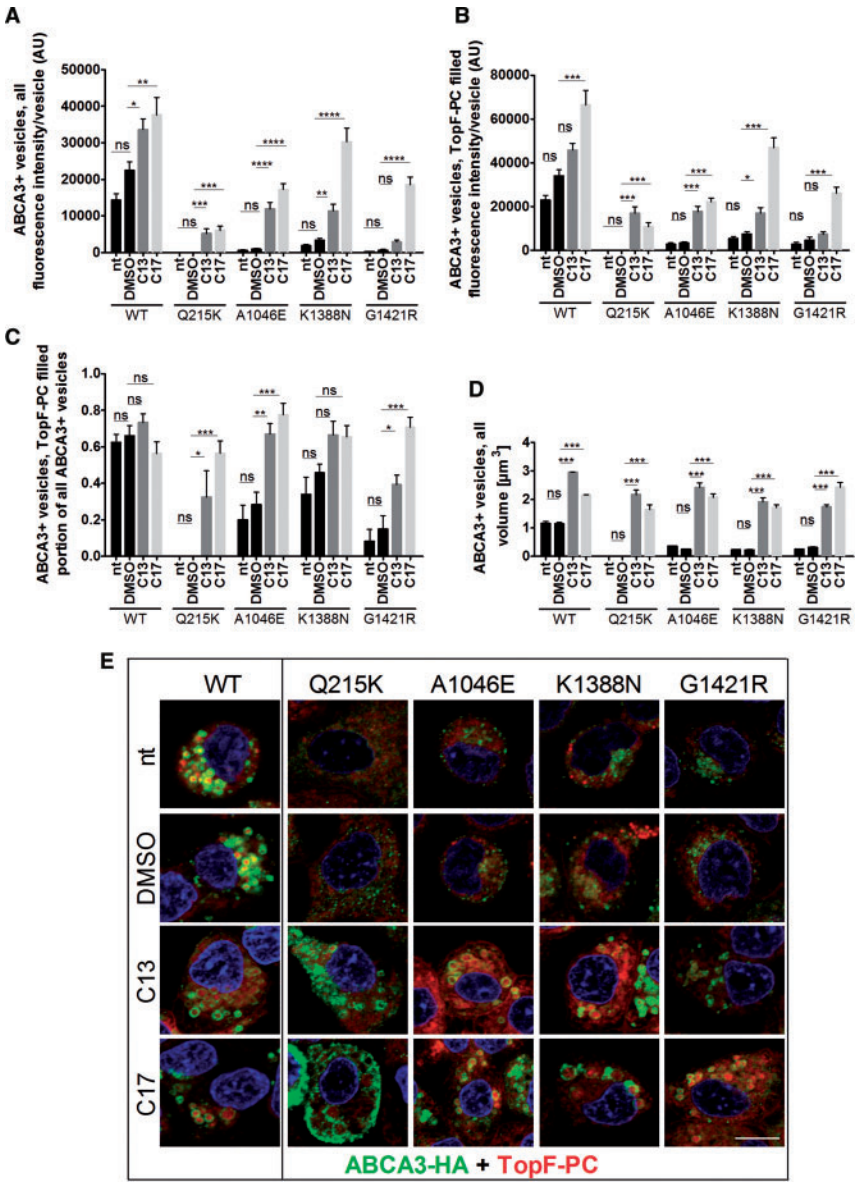

Figure 6. Corrector treatment increases transport of TopFluor-labeled PC into ABCA3-HA positive vesicles. After treatment with 10 µM C13 or C17 for 24 h, cells were incubated with liposomes containing TopFluor-conjugated phosphatidylcholine (TopF-PC) and treated with correctors for another 24 h. After 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,

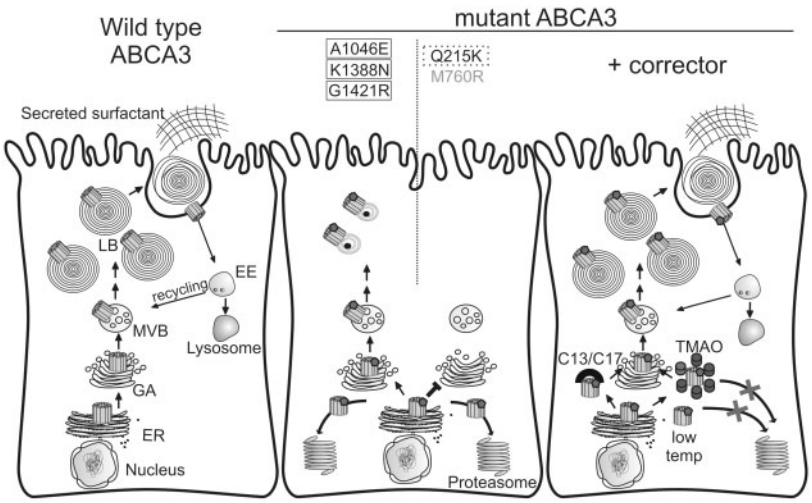

Figure 7. Intracellular trafficking pathways of wild-type, mutant and corrected ABCA3. *Left – Wild-type ABCA3.* After its synthesis and translocation to the ER, properly folded ABCA3 is routed via the golgi apparatus (GA) through post-golgi compartments like sorting vesicles, multivesicular bodies (MVB) 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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