



Dissecting the impact of SHC-1 inhibitors in enhancing the plasma membrane abundance of the CFTR channel across epithelial cell models

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ABSTRACT

Plasma membrane (PM) ion transport depends on tightly regulated trafficking and activation of ion channels. For the cystic fibrosis transmembrane conductance regulator (CFTR), dysregulation caused by tobacco smoke or chronic inflammation contributes to diseases such as chronic obstructive pulmonary disease (COPD). We previously showed that phosphorylation of CFTR at tyrosine 512 (Y512) by spleen tyrosine kinase promotes its internalization via the MAPK/SHC-1 pathway in CFBE airway cells. Here, we investigated whether this mechanism is conserved in other epithelial models and whether inhibition of SHC-1 increased CFTR abundance at the PM. CFTR surface levels were assessed in CFBE, 16HBE, and Caco-2 cells using biotinylation and immunoblotting after treatment with MEK inhibitor selumetinib, SHC-1 inhibitor idebenone (IDE), or its novel inhibitor 110#3. MAPK activity was evaluated by ERK phosphorylation. We found that MAPK/SHC-1-dependent CFTR internalization was conserved in 16HBE and Caco-2 cells. In CFBE cells, treatment with IDE or 110#3 increased PM CFTR levels, but also unrelated PM proteins (GLUT1 and E-cadherin). No significant effects were observed in 16HBE or Caco-2 cells. These findings suggest CFBE cells may not fully recapitulate endogenous CFTR trafficking and raise the possibility that selective SHC-1/pY512-CFTR inhibitors may modulate CFTR trafficking in COPD and other CFTR-related diseases.

1. Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is an ion channel located at the apical membrane of epithelial cells, where it regulates chloride and bicarbonate secretion [1–3]. In addition, CFTR plays a key role in maintaining luminal pH and epithelial surface hydration [2]. Therefore, the abundance and function of CFTR at the apical membrane are physiologically critical. Its deficiency, whether due to impaired synthesis, defective trafficking, or dysfunctional activity at the plasma membrane (PM), disrupts ion homeostasis and consequently alters the composition and properties of epithelial secretions in organs such as the lung, pancreas, and colon [3]. While CFTR dysfunction is most commonly associated with genetic mutations that cause the autosomal recessive disease cystic fibrosis (CF) [2], functional deficiencies can also be acquired in the context of wild-type (wt) CFTR. External

factors such as tobacco smoke, alcohol, heavy metals, local hypoxia and acidosis, oxidative stress, and inflammation have all been shown to impair CFTR-dependent ion transport [1]. Chronic exposure to these insults has been linked to the pathogenesis of several diseases, including chronic obstructive pulmonary disease (COPD), asthma, and acute pancreatitis [1,3].

In COPD, for instance, tobacco smoke has been shown to impair both the function and apical membrane localization of CFTR in airway epithelial cells, thereby contributing to disease pathophysiology [1, 4–6]. Several mechanisms have been proposed to explain the deleterious effects of cigarette smoke on CFTR, including dysregulation of its transcription, direct inhibition of channel activity by smoke-derived compounds, and enhanced endocytic removal of CFTR chloride channels from the apical PM [4–7].

It is well established that CFTR is primarily removed from the PM via

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clathrin-mediated endocytosis [8]. However, the regulatory cues and signalling pathways that govern this process are not yet fully elucidated. In previous studies, we identified spleen tyrosine kinase (SYK) as a key modulator of CFTR prevalence at the PM. SYK, a cytoplasmic non-receptor tyrosine kinase, phosphorylates CFTR at a specific tyrosine residue (Y512), thereby promoting its internalization from the PM in CFBE airway epithelial cells [9]. Subsequently, we identified that this phosphorylation-dependent internalization was mediated by the adaptor protein Src Homology 2 Domain-Containing-Transforming Protein C1 (SHC-1) [10,11]. The *SHC1* gene encodes three isoforms, among which p52SHC (referred to as SHC-1 in this manuscript) is the most abundant in epithelial cells. Unlike other isoforms with mitochondrial roles, p52SHC is primarily cytoplasmic and acts as an adaptor protein in receptor-mediated signalling. It plays a key role in stimulating the MAPK pathway, particularly through ERK activation, thereby promoting epithelial cell proliferation and survival [12,13]. SHC-1, by recognizing CFTR phosphorylated at Y512 through its phosphotyrosine-binding (PTB) domain, links CFTR internalization in airway epithelial cells to MAPK pathway activation [10,11].

Dysregulation of the MAPK signalling pathway via SHC-1 activity has also been identified as a key contributor to insulin resistance and impaired glucose metabolism in type 2 diabetes (T2D) [14,15]. Upon insulin binding, the insulin receptor (IR) undergoes autophosphorylation at specific tyrosine residues within its cytoplasmic domain, leading to the recruitment of adaptor proteins such as insulin receptor substrate 1 (IRS-1) and SHC-1, which compete for binding to the activated receptor [16]. These adaptor proteins serve as platforms for the activation of distinct downstream signalling cascades: the IRS-1/PI3K/AKT axis, which promotes glucose uptake and anabolic processes including protein, glycogen, and lipid synthesis; and the SHC-1/MAPK pathway, which is primarily associated with cell proliferation and growth. Notably, chronic activation of the SHC-1/MAPK arm has been implicated in the development of insulin resistance in T2D [14,16].

Recently, we showed that the synthetic ubiquinone analogue idebenone (IDE) selectively attenuates aberrant MAPK pathway activation in models of T2D, thereby restoring insulin sensitivity both *in vitro* and *in vivo* [14]. IDE was found to specifically disrupt the interaction between the PTB domain of SHC-1 and the phosphorylated insulin receptor (p-IR), while leaving the interaction between p-IR and IRS-1 intact. Interference of IDE with the p-IR/SHC-1 interaction reduced MAPK activation and redirected insulin receptor signalling preferentially towards the PI3K/AKT pathway [14,15], promoting glucose uptake via the release of glucose transporter GLUT4-containing endosomes to the PM.

In this study, we sought to investigate whether IDE could also inhibit the PTB-mediated interaction between SHC-1 and SYK-phosphorylated CFTR, as such an effect could hold significant therapeutic relevance for diseases like COPD and CF, where enhancing CFTR stability at the apical membrane is critical for restoring epithelial function and alleviate disease symptoms.

2. Material and methods

2.1. Cell lines and culture

CF patient-derived bronchial epithelial CFBE41o-cells, stably overexpressing wild-type (wt) CFTR (CFBE-wt-CFTR), or stably co-expressing wt-CFTR and the YFP-F46L/H148Q/I152L halide sensor (HS-YFP) were generated and cultured as previously described [10,17]. Healthy individual-derived 16 HBE bronchial epithelial cells (16HBE) were cultured in MEM containing GlutaMAX and supplemented with 10% (v/v) FBS and 100 U/mL penicillin with 100 µg/mL streptomycin, all from Thermo Fisher Scientific (Waltham, MA, USA). Colorectal cancer Caco-2 cell line was maintained in Roswell Park Memorial Institute (RPMI) medium containing GlutaMAX and supplemented with 10% (v/v) FBS, all from Thermo Fisher Scientific. Cells were maintained

at 37 °C with 5% CO₂, and regularly checked for absence of mycoplasma infection by PCR amplification of a 16S ribosomal DNA fragment [primers forward (F) 5' ACTCCTACGGGAGGCAGCA 3' and reverse (R) 5' TGCACCATCTGTCTACTCTGTAAACCTC 3'] from lysates of cells harvested from the culture medium.

2.2. Cell transfection and treatment

Approximately 3x10⁶ CFBE-wt-CFTR cells were reverse transfected with Lipofectamine 2000 from Thermo Fisher Scientific, according to the manufacturer's instructions, in 35-mm dishes with 200 pmol of the indicated siRNAs, siLuc or siSHC. siLuc is an oligonucleotide against luciferase (siLuc, 5'-CGUACGCGAAUACUUCGA) from Eurofins Genomics (Ebersberg, Germany) used as a mock control and siSHC is an oligonucleotide commercial mix used to deplete SHC1 (sc-29480) from Santa Cruz Biotechnology (Dallas, TX, USA). Forty-eight hours after transfection, cells were processed and analysed as indicated.

For drug treatments, cells were incubated 4 h at 37 °C with the vehicle DMSO from Sigma-Aldrich (Saint Louis, MO, USA) or with either idebenone (IDE, Sigma-Aldrich) or compound 110#3 (Buto Corp, Davis, CA) at the final concentrations of 10 µM, 3.3 µM or 1.1 µM. In parallel, selumetinib (sc-364613, Santa Cruz Biotechnology) was used at a final concentration of 10 µM. Stock solutions were prepared 1,000x concentrated in DMSO, to ensure that DMSO concentration during cell treatment did not exceed 0.1% (v/v).

2.3. SDS-PAGE and western blot (WB)

To assess CFTR abundance, samples were resolved on 9% SDS-PAGE mini-gels at 150 V and 4 °C in running buffer [25 mM Tris, 192 mM glycine, 3.5 mM SDS]. Proteins were then transferred onto PVDF membranes at 300 mA for 90 min in ice-cold transfer buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol]. Protein-loaded membranes were blocked with 5% (m/v) non-fat milk in TBS-T buffer [50 mM Tris, 150 mM NaCl (pH 7.6), 0.05% (v/v) Triton X-100] for 1 h at room temperature, except those that were going to be incubated with phospho-antibodies. Membranes were incubated overnight at 4 °C with the following primary antibodies: mouse anti-SHC (sc-967) from Santa Cruz Biotechnology, mouse anti-CFTR clone 596 from Cystic Fibrosis Foundation Therapeutics (Bethesda, MD, USA), mouse anti-E-cadherin from BD Transduction Laboratories (Franklin Lakes, NJ, USA), rabbit anti-GLUT1 (Ab652) from Abcam (Cambridge, UK), mouse anti-α-Tubulin clone B-5-1-2 (T5168), mouse anti-β-Actin clone AC-74 (A2228), mouse anti-U2AF65 clone MC3 (U4758), mouse anti-phospho-ERK1/2 (M8159), and rabbit anti-ERK1/2 (M5670) from Sigma-Aldrich. After three washes with TBS-T, membranes were incubated with secondary antibodies [goat anti-mouse or goat anti-rabbit IgG, both from Bio-Rad (Hercules, CA, USA)] for 1 h at room temperature. Then membranes were washed three times with TBS-T for 10 min and proteins were detected through enhanced chemiluminescence.

The ImageJ software (ImageJ 1.53q) from National Institutes of Health (NIH) (Bethesda, MD, USA) was used for densitometric analyses of WB bands, using x-ray films from at least three independent experiments. Band intensities were quantified after applying the 'Remove Background' function, and all images were acquired within the linear detection range and processed using identical parameters within each experiment. The uncropped WB images used in the figures are provided as Supplementary Material.

2.4. Halide-sensitive YFP fluorescence quenching assay

CFTR activity was assessed in CFBE cells stably co-expressing wt-CFTR and a halide-sensitive YFP variant (HS-YFP; F46L/H148Q/I152L), following established protocols [10,18,19]. In brief, cells were treated as specified, rinsed with PBS, and incubated for 15 min in PBS containing 5 µM forskolin (Fsk - ApexBio, Boston, MA, USA), with or without 25 µM

Inh172 (ApexBio), as appropriate. Fluorescence quenching of HS-YFP was monitored by continuously acquiring data every 500 ms for 10 s (baseline), followed by 30 s after the rapid (<1 s) addition of an isotonic PBS solution in which 137 mM NaCl was replaced with NaI (final concentration: 100 mM NaI per well). Fluorescence signals were background-subtracted and normalized to the average baseline intensity measured prior to iodide addition. The rate of halide influx, reflecting CFTR activity [20], was calculated as the initial rate of fluorescence decay (QR) by fitting the data to an exponential decay function using GraphPad Prism v.5.01.

2.5. Cell surface protein biotinylation assays

Sixty-mm dishes were seeded with either 2×10^6 CFBE-wt-CFTR cells, 4×10^6 16HBE cells, or 6×10^6 Caco-2 cells each. Twenty-four hours later cells were treated and incubated as indicated. To determine the steady-state abundance of cell surface proteins, we used non-permeable Sulfo-NHS-SS-Biotin (Santa Cruz Biotechnology) labelling. Briefly, cells were washed three times with ice-cold PBS $+/+$ [PBS (pH 8.0), 0.9 mM CaCl_2 , 0.5 mM MgCl_2] and incubated on ice for 5 min in ice-cold PBS $+/+$ to ensure the arrest of endocytic traffic. Then, cells were incubated on ice for 30 min with 0.5 mg/mL Sulfo-NHS-SS-biotin dissolved in PBS $+/+$. Biotin solution was removed, cells were briefly washed with ice-cold PBS $+/+$ and incubated for 15 min on ice with an ice-cold amine-rich buffer solution [100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.9 mM CaCl_2 , 0.5 mM MgCl_2 , 10 mM glycine, 1% (w/v) BSA] to quench the reaction. Next, cells were again washed three times with ice-cold PBS $+/+$ and lysed in 250 μL PD Buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% glycerol, 1% NP-40, 0.1% SDS] supplemented with protease inhibitors (15 nM aprotinin, 230 nM leupeptin, 100 nM E64, 10 μM EGTA, 150 nM pepstatin, 10 μM PMSF, 10 μM 1,10-phenanthroline; all from Sigma-Aldrich) and with a phosphatase cocktail inhibitor (sc-45065, Santa Cruz Biotechnology). Whole cell lysates were centrifuged at 4°C for 10 min at $10,000 \times g$ and 40 μL of cleared lysates mixed with 40 μL of 2x sample buffer [62.5 mM Tris-HCl (pH 6.8), 3% SDS, 10% glycerol, 196.4 mM dithiothreitol, 0.02% bromophenol blue] for total protein assessment (Input samples). The remaining supernatant was added to streptavidin-coated agarose beads (Sigma-Aldrich) and rotated at 4°C for 1 h. Afterwards, samples were centrifuged at 4°C for 1 min at $3,000 \times g$. Supernatant was discarded and samples were washed four times with a wash buffer solution [100 mM Tris (pH 7.5), 300 mM NaCl, 1% (v/v) Triton X 100]. Beads were dried by buffer aspiration with an insulin needle, then captured biotinylated proteins (Surface samples) were eluted in 20 μL of 2x Sample buffer and analysed along with their respective input samples by WB, as described above.

2.6. MTT-based cell viability assays

To set up the assay, cell density titration curves were first used to ascertain the colorimetric range and the optimal seeding conditions for each of the cell lines used. Final seeding conditions for 96-well plates were: 2×10^4 cells/well for CFBE-wt-CFTR and 16HBE cell lines and 4×10^4 cells/well for Caco-2 cells. Cells were incubated in triplicates for 4 h with fresh media containing either DMSO (vehicle control), the indicated concentrations of idebenone, 110#3, or selumetinib. Medium was replaced and cells were incubated for 20 h at 37°C and 5% CO_2 . Medium was then replaced with 100 μL of fresh medium containing 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich), and cells incubated for 2 h at 37°C . The MTT solution was then removed and 100 μL DMSO (Sigma-Aldrich) were added to each well and cells were incubated at room temperature for 30 min, protected from the light and under soft agitation to dissolve formazan crystals. Absorbance was measured at 570 nm in a Multiscan Ascent spectrophotometric plate reader from Labsystems (Helsinki, Finland).

2.7. Statistical analyses

Statistical analysis was performed using GraphPad Prism v.5.01. Quantitative results reflect the means $\pm$ SD from independent experiments. The values of n reported correspond to independent biological replicates, which provide an adequate basis for statistical analysis. Student's t -tests were used for comparisons between two groups, and one-way ANOVA followed by Dunnett's post-test was applied for multiple-group comparisons. Differences were considered significant when $p < 0.05$.

3. Results

3.1. SHC-1 depletion inhibits MAPK pathway activation and increases wt-CFTR abundance at the surface of CFBE-wt-CFTR cells

We previously demonstrated that the adaptor protein SHC-1 is an essential mediator for MAPK-dependent internalization of the wild-type CFTR (wt-CFTR) channel from the plasma membrane (PM) after its phosphorylation by protein kinase SYK at Y512, which is then recognized by the PTB domain of SHC-1 [10]. In the present study, we first compared CFTR PM abundance in CFBE-wt-CFTR cells after either the depletion of endogenous SHC-1 or after pharmacological inhibition of the downstream MAPK pathway using the MEK1/2-selective inhibitor selumetinib. As shown in Fig. 1A, siRNA-mediated depletion of SHC-1 resulted in an approximately 60% reduction in SHC-1 protein levels. This depletion led to a proportional decrease in MAPK pathway activity, as evidenced by reduced levels of phosphorylated ERK1/2 (p -ERK1/2), compared to cells transfected with a control siRNA directed against the ectopic protein luciferase (siLuc). Notably, the extent of p -ERK1/2 inhibition achieved 24 h after SHC-1 depletion in these cells was comparable to that observed following their treatment with 10 μM selumetinib for 4 h (Fig. 1A). Importantly, both SHC-1 depletion and selumetinib treatment led to a similar, approximately 2-fold increase in wt-CFTR abundance at the cells' PM, as assessed by surface protein biotinylation assays (Fig. 1B).

3.2. Idebenone promotes a dose-dependent increase in wt-CFTR surface expression and function, but lacks target specificity

Idebenone (IDE) was previously described as a chemical inhibitor of the interaction between SHC-1's PTB domain and tyrosine phosphorylated insulin receptor, leading to the inhibition of the MAPK signalling branch downstream of this receptor in hepatocytes [14].

We therefore tested whether IDE could reproduce the effect of SHC-1 depletion, increasing wt-CFTR abundance at the PM of CFBE cells. Treatment with different concentrations (10 μM , 3.3 μM and 1.1 μM) of IDE appeared to induce a significant, dose-dependent increase in the amount of CFTR isolated from the cell surface (Fig. 2A), without affecting the overall abundance of the channel in CFBE cells (ratio surface/input quantified in Fig. 2B). In this and all subsequent surface biotinylation experiments, surface CFTR (band C) was primarily normalized to its corresponding input CFTR (band C) in whole cell lysates. This effect was further confirmed using the YFP-quenching assay, a well-established method for assessing CFTR ion transport function [20] (Fig. 2C). In brief, CFBE cells stably expressing wt-CFTR and the halide-sensitive F46L/H148Q/I152L-YFP variant (HS-YFP) were treated with IDE as described above. The rate of YFP fluorescence quenching following extracellular iodide influx was then measured, as it correlates with the number of functional CFTR anion channels correctly localized at the PM [10,11,17,20]. CFTR-specificity of the measurements are demonstrated by the presence of CFTR-specific inhibitor Inh172. A forskolin (Fsk)-induced and dose-dependent increase in CFTR function was observed after IDE treatments (Fig. 2C), consistent with the elevated CFTR abundance at the PM detected by surface protein biotinylation assays (Fig. 2A and B).

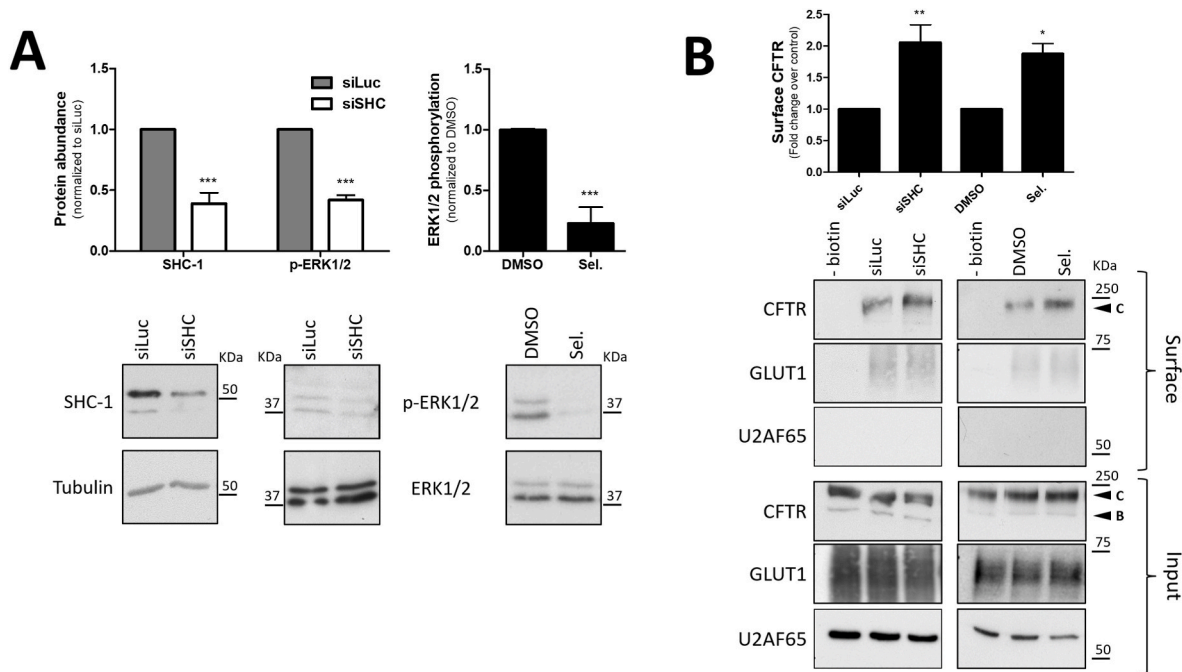


Fig. 1. SHC-1-dependent activation of the MAPK pathway modulates CFTR protein abundance at the plasma membrane. CFBE-wt-CFTR cells were transfected with either control siRNA (siLuc) or SHC-1-targeting siRNA (siSHC), or treated for 4 h with vehicle (DMSO) or 10 μ M of the MEK inhibitor selumetinib (Sel.). (A) The steady-state levels of SHC-1, relative to tubulin, and of phosphorylated ERK1/2 (p-ERK1/2), relative to total ERK1/2, were analysed by Western blot (representative blots shown below) and quantified by densitometric analysis (graphs above). (B) CFTR abundance at the plasma membrane (PM) was assessed by surface protein biotinylation followed by streptavidin pull-down and immunoblotting for CFTR. GLUT1 was used as a constitutively expressed PM marker for the biotinylated fraction of PM proteins. U2AF65 was used as a control for intracellular protein contamination. “- biotin” corresponds to control cells that underwent the entire biotinylation protocol but were incubated with PBS lacking the biotin reagent. Graph bars represent means $\pm$ SD of $n = 4$ (siRNAs) or $n = 3$ (Sel.) independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by unpaired two-tailed t -test.

However, in contrast to the effects observed following SHC-1 depletion or selumetinib treatment, IDE treatment resulted in concomitant changes in cell surface levels of another membrane protein, GLUT1, an independent, constitutively expressed PM marker deliberately included to rigorously determine whether IDE-induced changes were CFTR-specific or instead reflected generalized alterations in integral membrane protein abundance (Fig. 2A). Upon normalization to GLUT1, the densitometric analysis of CFTR levels at the PM were not significantly different from those observed under control (DMSO) conditions (Fig. 2D). Unlike GLUT4, for which PM abundance is directly regulated by insulin, GLUT1 is generally not involved in the insulin response. However, insulin signalling has been known to influence GLUT1 expression at the transcriptional level through pathways such as PI3K/AKT and mTOR [21,22]. Thus, as an additional control, we examined the effects of IDE treatment on the PM levels of E-cadherin, a cell-cell adhesion protein not involved in insulin-regulated glucose transport. Similar to GLUT1, E-cadherin levels at the PM increased in response to IDE treatment, showing changes comparable to those observed for both GLUT1 and CFTR (Fig. 2A and E). These results suggested that, unlike SHC-1 depletion or selumetinib treatment, IDE does not specifically enhance CFTR abundance at the PM, but instead appears to unspecifically increase the levels of integral PM proteins.

3.3. IDE can reduce the viability of CFBE cells but in a MAPK pathway-independent manner

The MAPK/ERK signalling pathway that can be stimulated via SHC-1 plays a critical role in regulating epithelial cell viability by modulating key processes such as cell survival, proliferation, and apoptosis [23]. To investigate whether IDE impacts this pathway, we compared the effects on ERK1/2 phosphorylation of a 4-h treatment with IDE (10–1.1 μ M range) to those with 10 μ M selumetinib. In addition, we measured CFBE

cell viability 20 h post-treatment. As expected, selumetinib treatment markedly reduced ERK1/2 phosphorylation (Fig. 3A) and led to a significant decrease in cell viability (Fig. 3B). In contrast, IDE treatment did not alter ERK1/2 phosphorylation at any concentration tested (Fig. 3A). Nonetheless, at the highest concentration, IDE also reduced CFBE cell viability (Fig. 3B). Since this effect occurred without changes in ERK1/2 activity, it suggests that IDE impairs viability through a MAPK-independent mechanism, potentially linked to the observed general dysregulation of membrane protein abundance.

3.4. 110#3 induces a less pronounced, MAPK-independent increase in CFTR PM abundance

To address the apparent lack of specificity of IDE in regulating CFTR PM abundance in CFBE cells, we examined the effects after treatment with compound 110#3, a novel SHC-1 inhibitor with improved specificity for disrupting the SHC-1/p-IR interaction and better pharmacokinetic properties [24]. Interestingly, although some enrichment of CFTR PM levels was observed, 110#3 effects were unexpectedly less pronounced, reaching statistical significance only at 10 μ M (Fig. 4A and B), and treatment led to comparable changes in both GLUT1 and E-cadherin cell surface levels (Fig. 4C). Additionally, and again in contrast to selumetinib treatment, 110#3 did not affect ERK1/2 phosphorylation at any concentration tested (Fig. 4D). These findings suggest that in comparison to IDE, the improved specificity of 110#3 for disrupting the SHC-1/p-IR interaction resulted in a reduced ability to increase the PM abundance of CFTR, as well as of other membrane proteins. This reinforces the reasoning that the upregulation of CFTR at the PM observed with IDE may arise from non-specific mechanisms, rather than its interference with the SHC-1/p-CFTR interaction or the modulation of MAPK signalling in CFBE airway cells, and is therefore distinct from the described SHC-1/p-IR signalling.

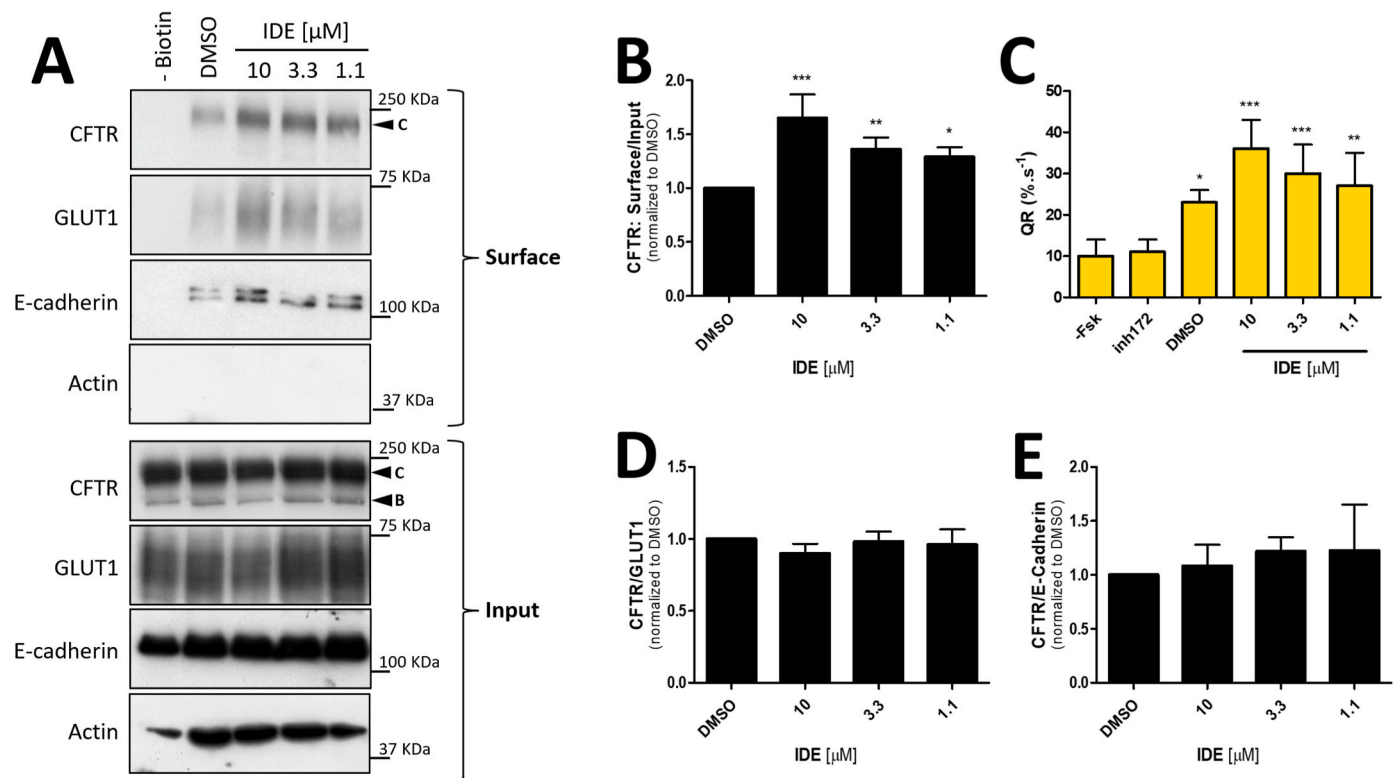


Fig. 2. IDE treatment increases CFTR abundance and function at the cell surface, but the effect is not specific to CFTR. CFBE-wt-CFTR cells were treated for 4 h with either vehicle (DMSO) or increasing concentrations of idebenone (IDE, 1.1–10 μ M). (A) Plasma membrane (PM) protein fractions (Surface; upper panels) and total cell lysates (Input; lower panels) were analysed by immunoblotting following surface protein biotinylation (“-Biotin”: vehicle-treated cells with no biotin reagent during surface labelling; Actin was used as a control for intracellular protein contamination). All surface and input panels derive from the same membranes, respectively, and all lanes within each panel come from the same exposure. (B) CFTR band intensity at the PM was quantified relatively to total CFTR levels in input lysates. (C) For ion transport measurements, CFBE cells stably expressing wild-type CFTR and the halide-sensitive YFP variant (HS-YFP – F46L/H148Q/I152L) were treated as described in (A), or with 25 μ M Inh172. CFTR ion transport activity was stimulated with 5 μ M forskolin (Fsk) [10,18,19]. The graph shows the rate of YFP fluorescence quenching upon iodide (100 mM) addition, reflecting CFTR-mediated halide transport. (D) Surface CFTR levels were quantified relatively to GLUT1 abundance in the biotinylated (PM) fraction. (E) Quantification of CFTR signals at the PM relative to E-cadherin signals. Graph bars represent means $\pm$ SD of $n = 5$ independent experiments (biotinylation) and $n = 3$ (HS-YFP assay). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA followed by Dunn’s post-tests.

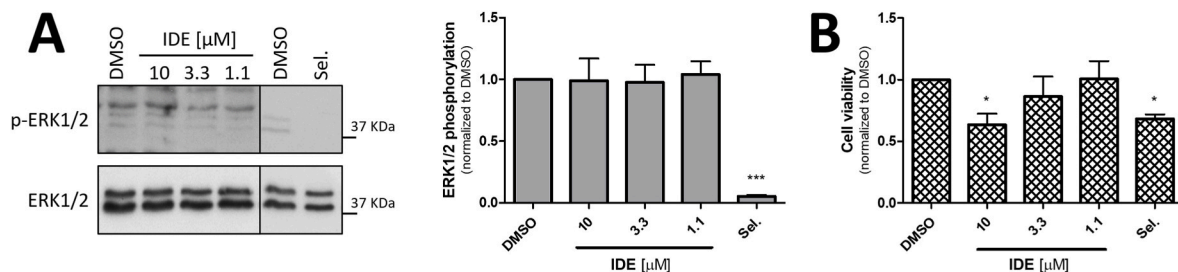


Fig. 3. At higher concentrations, IDE reduces CFBE cell viability but not through inhibition of MAPK signalling. CFBE-wt-CFTR cells were treated for 4 h with vehicle (DMSO), increasing concentrations of idebenone (IDE, 1.1–10 μ M), or 10 μ M of the MEK inhibitor selumetinib (Sel.). (A) MAPK pathway activity was assessed by Western blot analysis of phosphorylated ERK1/2 (p-ERK1/2) relative to total ERK1/2. Representative blots are shown (left), and band intensities were quantified by densitometry (right). (B) Cell viability was measured 20 h post-treatment using the MTT assay. Graph bars represent means $\pm$ SD of $n = 4$ independent experiments (p-ERK1/2) and $n = 3$ (MTT). $p < 0.05$, *** $p < 0.001$ by one-way ANOVA followed by Dunn’s post-test. IDE-treated samples shown in each panel come from the same blotting membrane. Selumetinib experiments were performed in parallel under equivalent conditions, also using DMSO (vehicle) as control, but the lysates were analysed on different membranes. When images from different membranes are presented together, similar exposure levels were selected for visualization, while all lanes within each membrane were acquired and processed using identical settings.

Despite their widespread use in CF drug research, CFBE-CFTR cells were generated by stable transduction of CFTR cDNA [25] followed by clonal selection for high protein expression, which may have yielded clones with dysregulated endocytic trafficking [26]. To better assess the relevance of IDE and 110#3 in regulating CFTR PM abundance, we next evaluated their effects in cell models that endogenously express CFTR and therefore better preserve native regulatory pathways and

context-dependent interactions. For this purpose, we used 16HBE airway epithelial cells and Caco-2 colorectal cells that do not show the massive CFTR overexpression detected in CFBE cells (Fig. 4E).

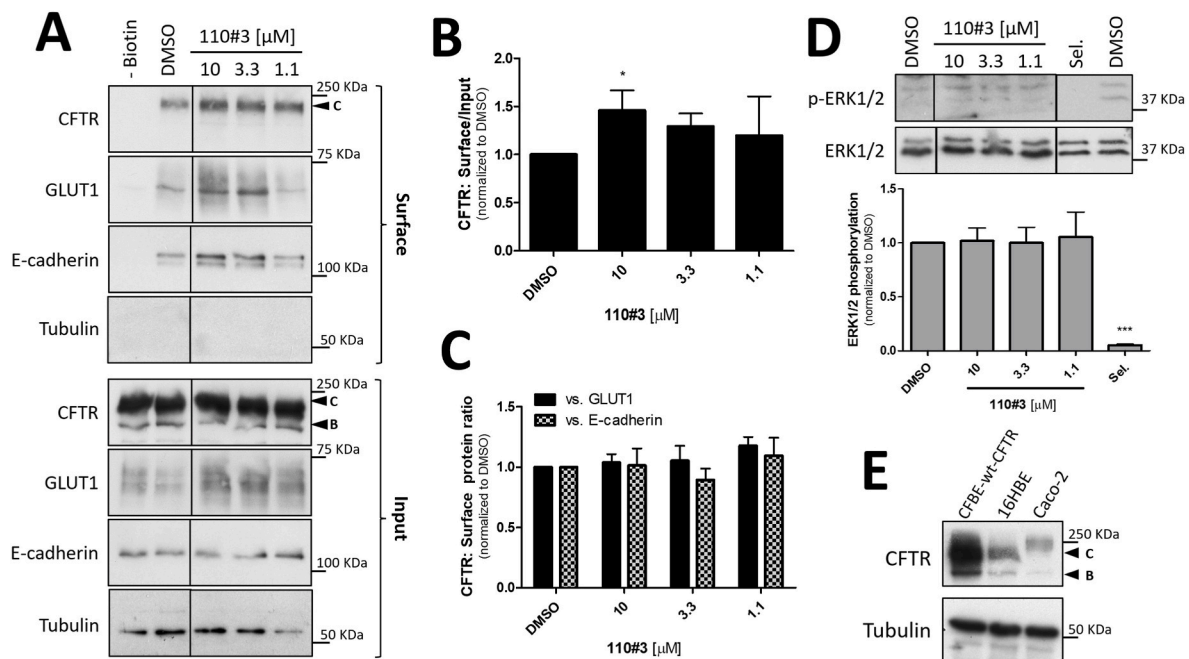


Fig. 4. Compound 110#3 modestly increases CFTR plasma membrane levels in a non-specific and MAPK-independent manner. CFBE-wt-CFTR cells were treated for 4 h with vehicle (DMSO), increasing concentrations of 110#3 (1.1–10 μ M), or 10 μ M of the MEK inhibitor selumetinib (Sel.), as indicated. (A) Surface (biotinylated) and total (input) protein fractions were analysed by immunoblotting to evaluate CFTR abundance at the plasma membrane (Surface; upper panels) and in total cell lysates (Input; lower panels). “-Biotin”, correspond to vehicle-treated cells exposed to no biotin reagent during surface labelling. Tubulin was used as a control for intracellular protein contamination. (B) Surface CFTR signal was quantified relatively to total CFTR in the corresponding input samples. (C) Surface CFTR levels were quantified relatively to either GLUT1 or E-cadherin signals in the biotinylated fraction to assess response specificity. (D) MAPK pathway activity was assessed via Western blot analysis of phosphorylated ERK1/2 (p-ERK1/2) relative to total ERK1/2. Representative blots (top) and densitometric quantification (bottom) are shown. (E) Comparative CFTR protein abundance in CFBE-wt-CFTR cells versus epithelial cell lines with endogenous CFTR expression (16HBE and Caco-2). Graph bars represent means $\pm$ SD of $n = 4$ independent experiments. $p < 0.05$, *** $p < 0.001$ by one-way ANOVA followed by Dunnett's post-test. All 110#3 treatment conditions within each experiment were obtained from the same exposure of the same blotting membrane. Selumetinib experiments were performed in parallel under equivalent conditions, also using DMSO (vehicle) as control, but the lysates were analysed on different membranes. Comparable exposure levels were chosen across membranes for the representative figure display, and identical acquisition and processing parameters were applied to all lanes within each membrane.

3.5. IDE treatment fails to enhance CFTR PM abundance or suppress MAPK signalling in 16HBE airway epithelial cells

In contrast to CFBE cells, treatment of 16HBE cells with IDE at the 10–1.1 μ M dose range produced no significant change in the PM abundance of either CFTR or GLUT1 (Fig. 5A–C). However, similar to what was observed in CFBE cells, treatment with 10 μ M of selumetinib induced a significant, over 2-fold increase in CFTR but not of GLUT1 abundance at the cells' surface (Fig. 5A–C). Consistently, while IDE failed to reduce ERK1/2 phosphorylation at any of the used dosages, selumetinib treatment drastically suppressed the levels of phosphorylated ERK1/2 in these cells. These findings reinforce the previously described association between MAPK pathway inhibition and the selective enhancement of CFTR retention at the PM, independent of the airway epithelial cell model used [10,11]. Additionally and in contrast to CFBE cells, treatment with 10 μ M IDE did not affect the viability of 16HBE cells, whereas selumetinib still caused a slight but significant reduction (Fig. 5E). These findings further support the hypothesis that the effects of IDE on membrane protein abundance and cell viability in CFBE cells are likely mediated by mechanisms unrelated to SHC-1 interference or MAPK pathway modulation, and may reflect a cell line-specific response to the drug. To further investigate this hypothesis, we next tested these drugs on a cell line from a different tissue origin.

3.6. IDE treatment did not alter CFTR surface levels, MAPK pathway activity or the viability of Caco-2 colorectal cells

Colon is a tissue in which CFTR function has been used for diagnostic purpose [27,28]. Caco-2 cells are a widely accepted *in vitro* model of

colon epithelial cells due to their ability to spontaneously differentiate in culture into polarized monolayers that exhibit key features of intestinal enterocytes [29–31]. Moreover, these cells express endogenous levels of functional wt-CFTR [17,32,33] comparable to those of 16HBE cells (Fig. 4E).

Notably, Caco-2 cells showed neither any significant change in CFTR surface abundance (Fig. 6A–C) nor in ERK1/2 phosphorylation (Fig. 6D) when treated with IDE across the tested dose range, consistent with the effects observed in 16HBE cells. In contrast, selumetinib treatment led to a marked, roughly 2-fold increase in CFTR levels at the PM (Fig. 6A–C) and a strong suppression of ERK1/2 phosphorylation (Fig. 6D), again paralleling the response seen in 16HBE cells. However, although IDE also did not significantly affect Caco-2 cell viability, selumetinib caused a more pronounced decrease in their viability (Fig. 6E), likely reflecting the reliance of these adenocarcinoma-derived cells on MAPK signalling for survival and sustained proliferation [34]. Altogether, these findings further support the hypothesis that IDE treatment exerts an unspecific effect on PM protein retention and cell viability in CFBE cells, not mediated by SHC-1 or MAPK activity, and distinct from the described SHC-1-PTB/p-IR signalling.

3.7. Compound 110#3 has no effect on CFTR PM levels in 16HBE and Caco-2 cells

Consistent with the effects observed for IDE, treatment of 16HBE cells with compound 110#3 across the 10–1.1 μ M concentration range elicited no significant changes in the abundance of CFTR or GLUT1 at the PM (Fig. 7A). As expected, comparable results were also obtained in Caco-2 cells, with CFTR and GLUT1 levels remaining unaltered in both

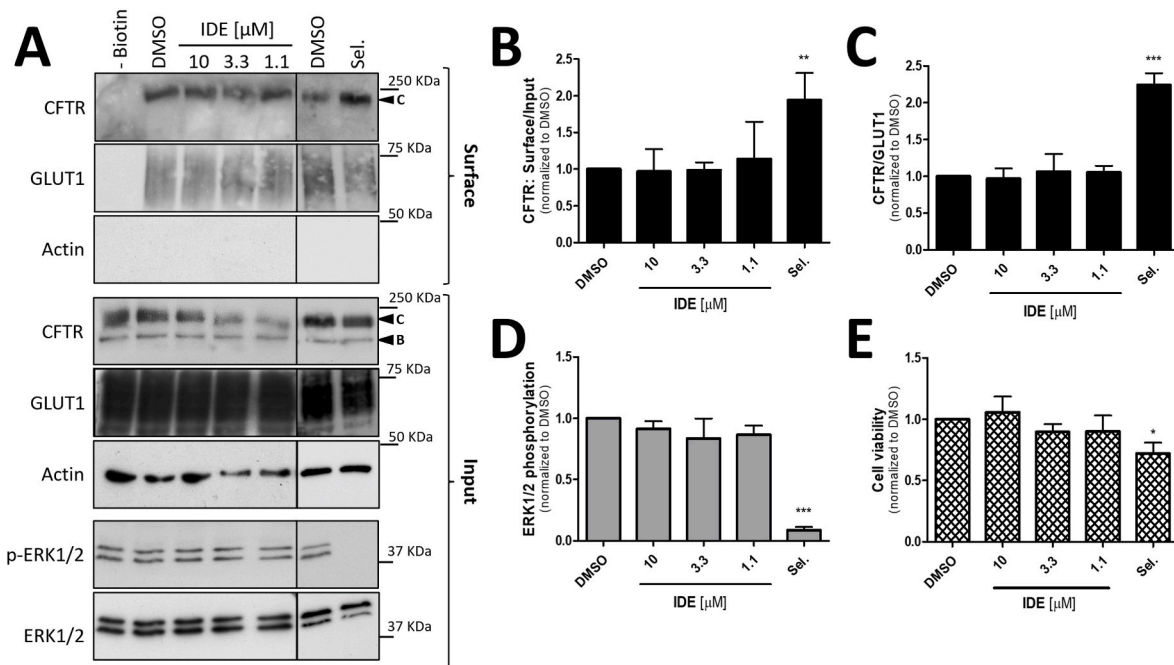


Fig. 5. IDE has no effect on CFTR plasma membrane abundance, MAPK pathway activity, or cell viability in 16HBE cells. 16HBE cells were treated for 4 h with vehicle (DMSO), increasing concentrations of idebenone (IDE, 1.1–10 μ M), or 10 μ M of the MEK inhibitor selumetinib (Sel.), as indicated. (A) Cell surface (biotinylated) and total (input) protein fractions were analysed by immunoblotting to assess CFTR abundance at the plasma membrane (Surface; upper panels) and in total lysates (Input; lower panels). “-Biotin”, correspond to vehicle-treated cells exposed to no biotin reagent during surface labelling. Actin was used as a control for intracellular protein contamination. Selumetinib samples were analysed in separate but equivalent experiments also using DMSO (vehicle) as control. Surface CFTR signal was quantified relatively to either total CFTR in the corresponding input samples (B) or to GLUT1 signals in the biotinylated fraction (C) to assess response specificity. (D) MAPK pathway activity was evaluated by densitometric analysis of Western blots for phosphorylated ERK1/2 (p-ERK1/2) relative to total ERK1/2 levels (representative immunoblots shown in (A), input fraction). (E) 16HBE cell viability measured 20 h after the above indicated treatments, using the MTT assay. Graph bars represent means $\pm$ SD of $n = 4$ independent experiments (biotinylation and p-ERK1/2) or $n = 3$ (MTT). $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA followed by Dunnett's post-test.

total and cell surface fractions following 110#3 exposure (Fig. 7B).

4. Discussion

As mentioned previously, we have shown that MAPK signaling mediates the internalization of wt-CFTR from the PM in CFBE cells, a process facilitated by the interaction of SHC-1, via its PTB domain, with SYK-phosphorylated Y512 of the channel [10]. Here we show, using the MAPK inhibitor selumetinib, that this pathway regulates CFTR surface abundance not only in the CFBE airway cell line stably expressing wt-CFTR, but also in epithelial models of bronchial and intestinal origin expressing endogenous wild-type CFTR.

Following the identification of IDE and compound 110#3 as inhibitors of SHC-1 that disrupt the interaction between the PTB domain of SHC-1 and the phosphorylated insulin receptor (p-IR) [14,24], we investigated whether both compounds could also be used to pharmacologically disrupt the PTB-mediated pY512-CFTR/SHC-1 interaction and block CFTR internalization, mimicking the effect observed with SHC-1 depletion or selumetinib treatment.

Our experimental data led us to conclude that although IDE, and to a lesser extent 110#3, were capable of increasing CFTR abundance at the cell surface and enhancing CFTR-mediated ion transport in CFBE cells, these effects were absent in both the bronchial 16HBE and intestinal Caco-2 cell models. Moreover, further analysis of other common cell surface proteins in CFBE cells revealed that, in addition to CFTR, treatment with the SHC-1 inhibitor compounds, particularly IDE, also elevated the abundance of GLUT1 and E-cadherin. Because these proteins are not directly linked to CFTR or insulin receptor membrane residence [21,22,35], our data indicated a non-specific effect on PM proteins in CFBE cells. Therefore, while methodological factors such as differences in biotinylation efficiency cannot be entirely excluded, the

observation that selumetinib treatment caused a clear and selective increase in surface CFTR abundance across all three cellular models supports the robustness of the assay and argues against a generalized methodological artifact. Together, these findings indicate that CFBE cells display a heightened sensitivity to IDE-induced perturbation of membrane-associated proteins. Thus, the effects observed in CFBE cells are not indicative of selective CFTR modulation, but rather reflect cell line-specific properties, which may involve both direct interference with endocytosis and broader alterations in membrane trafficking.

Additionally, we observed that, in contrast to selumetinib, neither IDE nor 110#3 affected MAPK pathway activity in any of the three cell models tested, as would be expected from the known roles of SHC-1. This was evident not only from the lack of changes in intracellular levels of phosphorylated ERK kinases but also from the absence of reduced cell viability following treatment with either SHC-1 inhibitor, while a small but significant viability reduction was observed under equivalent experimental conditions with selumetinib. Although, CFBE cell viability was decreased when treated with 10 μ M IDE, this did not correlate with any detectable effect on MAPK activity. These findings further support the hypothesis that the effects of IDE on membrane protein abundance and cell viability in CFBE cells are likely mediated by mechanisms independent of SHC-1 inhibition or MAPK pathway modulation. Instead, they seem to reflect a CFBE cell line-specific response to the drug that results in a non-specific increase in the abundance of membrane-associated proteins at the cell surface, suggesting some kind of interference with the endocytic machinery.

In this respect, it should be noted that IDE is a synthetic quinone developed as an analogue of ubiquinone (coenzyme Q10), initially designed to enhance metabolic stimulation in neuronal cells for the treatment of neurological diseases [36]. This class of quinones is known to inhibit clathrin-mediated endocytosis by obstructing the activity of

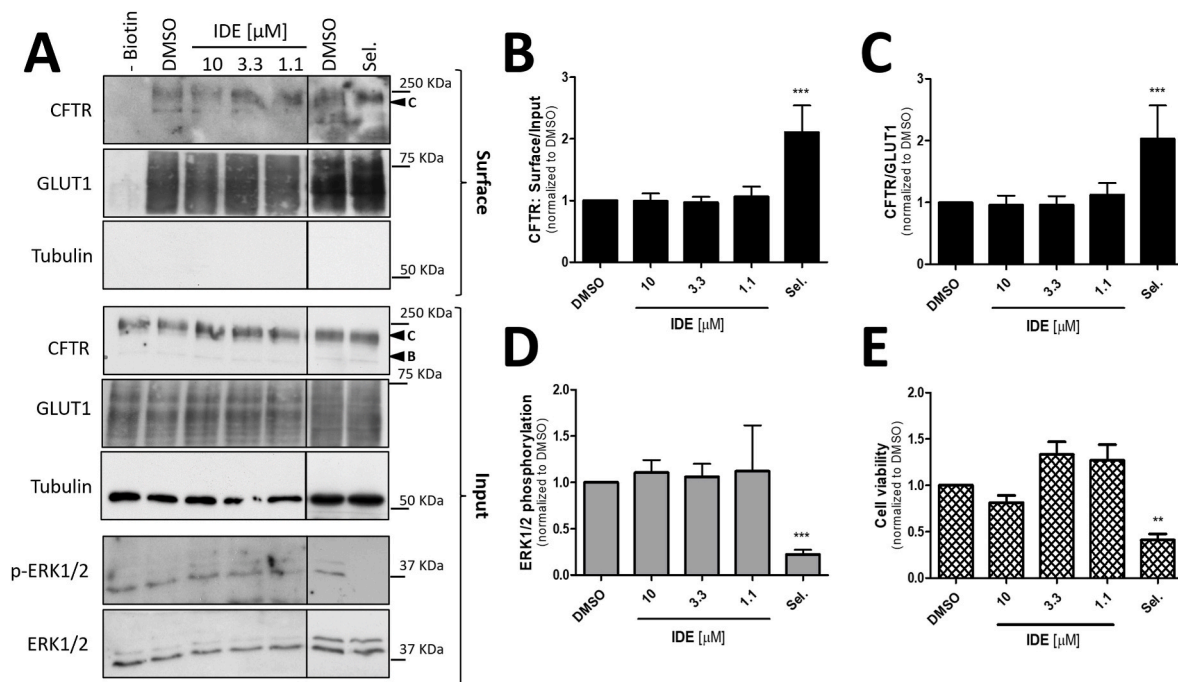


Fig. 6. IDE produces no significant changes on CFTR plasma membrane abundance, MAPK pathway activity, or cell viability in Caco-2 cells. Caco-2 cells were treated for 4 h with vehicle (DMSO), increasing concentrations of idebenone (IDE, 1.1–10 μ M), or 10 μ M of the MEK inhibitor selumetinib (Sel.), as indicated. (A) Cell surface (biotinylated) and total (input) protein fractions were analysed by immunoblotting to assess CFTR abundance at the plasma membrane (Surface; upper panels) and in total lysates (Input; lower panels). “-Biotin”, correspond to vehicle-treated cells exposed to no biotin reagent during surface labelling. Tubulin was used as a control for intracellular protein contamination. Surface CFTR signal was quantified relatively to either total CFTR in the corresponding input samples (B) or to GLUT1 signals in the biotinylated fraction (C). (D) MAPK pathway activity was evaluated by densitometric analysis of Western blots for phosphorylated ERK1/2 (p-ERK1/2) relative to total ERK1/2 levels (representative immunoblots shown in (a), input fraction). (E) Cell viability was measured 20 h after the above indicated treatments, using the MTT assay. Graph bars represent means $\pm$ SD of $n = 3$ independent experiments. $p < 0.05$, $**p < 0.01$, $***p < 0.001$ by one-way ANOVA followed by Dunnett's post-test. IDE-treated samples within each experiment derive from the same blotting membrane. Selumetinib experiments were performed in parallel under equivalent conditions, also using DMSO (vehicle) as control, but the lysates were analysed on different membranes. Exposure levels were selected to allow comparable visualization across membranes, and acquisition and processing settings were kept identical for all lanes within each membrane.

dynamin GTPase, a key regulator of cellular functions. The extent of this inhibition, however, varies depending on the compound, cell type, and cellular context [37,38].

Based on our results, neither IDE nor 110#3 appear to be highly effective inhibitors of endocytosis, as they did not alter the overall levels of PM proteins in 16HBE or Caco-2 cells. However, CFBE-wt-CFTR cells are a clonal line derived from a cystic fibrosis patient-derived cell line (CFBE41o-) that has been engineered to stably express high levels of wt-CFTR protein [25]. Thus, the clonal selection process may have altered membrane trafficking regulation, rendering this cell line more susceptible to quinone-induced inhibition of endocytosis. This could account for the general increase in membrane protein abundance observed following IDE treatment, which was less pronounced with the more SHC-1-specific compound 110#3.

Our results have several relevant implications. First, they indicate limitations when using CFBE cells as a model in CFTR membrane trafficking studies. Since a non-endocytosis-specific compound like IDE can disrupt trafficking in CFBE cells to the extent that it increases the surface abundance of multiple unrelated membrane proteins - not just CFTR, this suggests that these cells possess an atypically sensitive endocytic system. Importantly, this unspecific membrane protein accumulation was not observed in 16HBE or Caco-2 cells, indicating that the effect is specific to CFBE cells and not to IDE. As a result, caution is required when CFBE cells are used for assessing the impact of candidate compounds on CFTR PM stability, as observed changes might reflect generalized trafficking defects rather than CFTR-specific mechanisms. In contrast, 16HBE and Caco-2 cells, which respond to selumetinib but not IDE, may offer a more reliable system for studying targeted modulation of CFTR surface retention, particularly for evaluating inhibitors that

specifically disrupt the pY512-CFTR/SHC-1 interaction. Future efforts to identify such modulators should therefore prioritize cellular models with more selective and physiologically relevant trafficking responses. Within such models, quantitative surface biotinylation remains a robust approach to assess CFTR PM abundance, an approach fully supported by functional validation through iodide influx measurements in CFBE cells. Complementary trafficking analyses in 16HBE and Caco-2 cells could nonetheless further refine the mechanistic understanding of CFTR surface regulation in these physiologically relevant epithelial contexts.

Second, our observations suggest that the effectiveness of IDE and 110#3 in disrupting the interaction between the PTB domain of SHC-1 and its phosphorylated partners, may be context-dependent, relying on specific peptide motifs. Notably, the SHC-1 PTB domain typically recognizes canonical NPXY motifs, such as the phosphorylated Y960 residue within the LDNPEY sequence of the IR, which is known to be strongly inhibited by IDE [14,15,24]. However, in the case of CFTR, SHC-1 binds through its PTB domain to the pY512 residue within an atypical peptide motif - IFGVSY [10]. Thus, the unique pY512-CFTR/PTB-SHC-1 interaction may create a molecular context that affects the binding of IDE and 110#3, which could explain why these compounds failed to prevent CFTR internalization in 16HBE and Caco-2 cells.

Finally, the observed discrepancy underscores the complexity of SHC-1-mediated signalling and suggests that IDE and 110#3 may not be universally effective inhibitors of SHC-1-mediated interactions. The nature of the SHC-1 binding site and the specific phosphorylation sites involved, appear to play a crucial role in determining the sensitivity of the pathway to these compounds. Therefore, the differential efficacy of these inhibitors in various protein contexts emphasizes the need for a

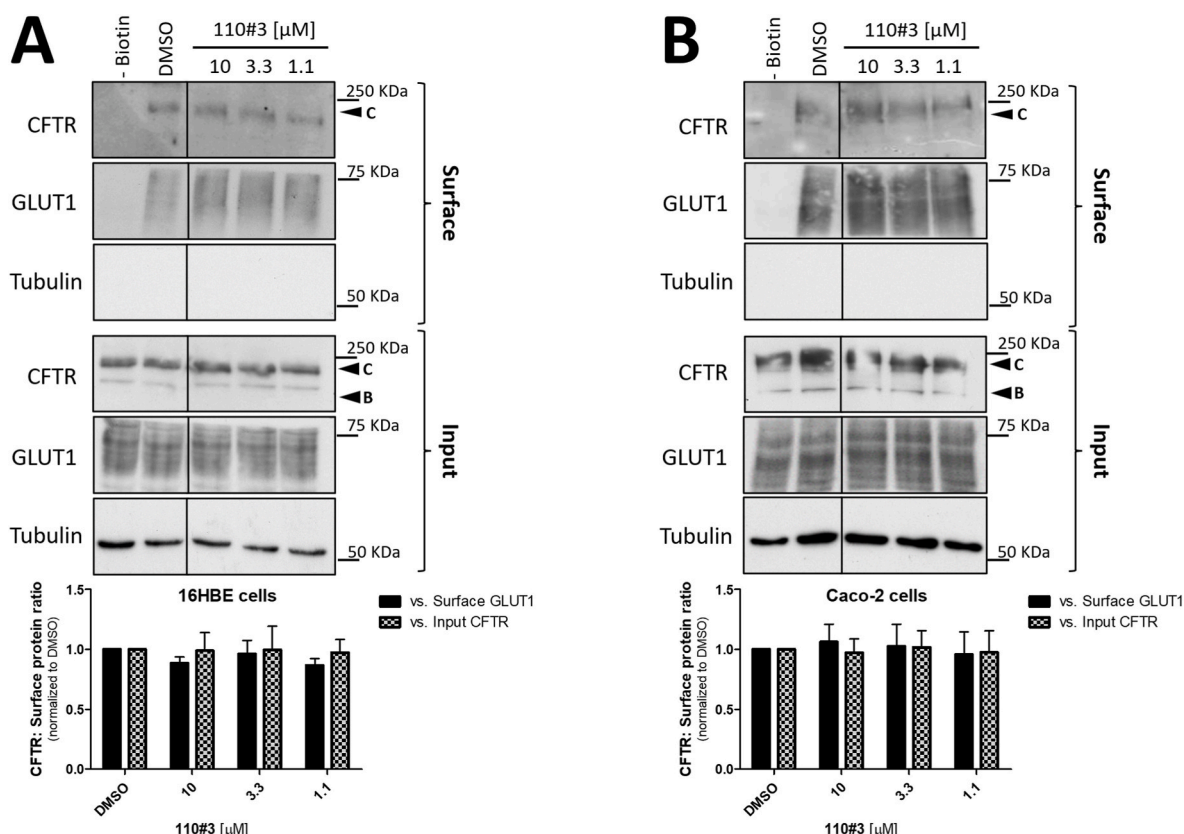


Fig. 7. Compound 110#3 does not alter CFTR plasma membrane abundance in either 16HBE or Caco-2 cells. (A) 16HBE and (B) Caco-2 cells were treated for 4 h with vehicle (DMSO) or in-creasing concentrations (1.1–10 μ M) of compound 110#3. Biotinylated surface proteins and total cell lysates were analysed by immunoblotting to assess CFTR and GLUT1 abundance at the plasma membrane (Surface) and in whole-cell extracts (Input). “-Biotin”, correspond to vehicle-treated cells exposed to no biotin reagent during surface labelling. Tubulin was used as a control for intracellular protein contamination. Surface CFTR levels were quantified relatively to either total CFTR in the input samples or to GLUT1 in the surface fraction (bottom graphs). Graph bars represent mean $\pm$ SD of $n = 4$ independent experiments for 16HBE or $n = 3$ for Caco-2 cells. No significant differences were detected by one-way ANOVA followed by Dunnett's post-test. All 110#3-treated samples derive from the same membrane, and densitometric quantification of lanes was performed on the full membranes and processed using identical settings.

more nuanced understanding of their binding mechanisms, which would allow to improve studying or targeting SHC-1-related signalling pathways.

In conclusion, we were unable to position IDE or 110#3 as potential therapeutic compounds for inhibiting CFTR internalization, which could be of interest for the treatment of COPD and some types of CF patients.

Our findings rather indicate that the effects of IDE or 110#3 are specific to the SHC-1/p-IR interaction observed in hepatocytes; however, this insight may open up new research avenues. First, being able to specifically target the interaction SHC-1/p-IR, suggests that other compounds may exist capable of selectively targeting the SHC-1/p-CFTR interaction, thus offering a strategy to specifically enhance CFTR abundance at the PM while minimizing off-target effects. Moreover, continued research on the role of adaptor proteins such as SHC-1 could yield valuable insights for the development of more precise and effective therapies for other conditions, including colon cancer, where both CFTR function and MAPK signalling are critically dysregulated [39,40].

Data statement

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

ChatGPT was used to enhance writing clarity and improve the

English language in certain sections of the manuscript. The authors reviewed and edited all content as necessary and take full responsibility for the final version of the manuscript.

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CRediT authorship contribution statement

Patrícia Barros: Formal analysis, Investigation, Writing – review & editing. **Mariana F.L. Pereira:** Formal analysis, Investigation, Writing – review & editing. **Alexey Tomilov:** Conceptualization, Writing – review & editing. **Gino Cortopassi:** Conceptualization, Writing – review & editing. **Peter Jordan:** Conceptualization, Funding acquisition, Writing – original draft. **Paulo Matos:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Writing – original draft.

Declaration of competing interest

The authors declare no competing interests.

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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrc.2026.153757>.

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