Cytoskeleton regulators CAPZA2 and INF2 associate with CFTR to control its plasma membrane levels under EPAC1 activation

João D. Santos1, Francisco R. Pinto1, João F. Ferreira1, Margarida D. Amaral1, Manuela Zaccolo2, Carlos M. Farinha1*

1 University of Lisboa, Faculty of Sciences, BioISI-Biosystems and Integrative Sciences Institute, Campo Grande 1749-016 Lisboa, Portugal
2 Department of Physiology, Anatomy & Genetics, University of Oxford, Oxford OX1 3PT, UK

*corresponding author: Carlos M Farinha, Department of Chemistry and Biochemistry, Faculty of Sciences, University of Lisboa, Campo Grande, 1749-016 Lisboa, Portugal; Tel.: +351 217500864; e-mail: cmfarinha@fc.ul.pt
Abstract

Cystic Fibrosis (CF), the most common lethal autosomal recessive disorder among Caucasians, is caused by mutations in the gene encoding the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) protein, a cAMP-regulated chloride channel expressed at the apical surface of epithelial cells. Cyclic AMP regulates both CFTR channel gating through a protein kinase A (PKA)-dependent process and plasma membrane (PM) stability through activation of the exchange protein directly activated by cAMP1 (EPAC1). This cAMP effector, when activated promotes the NHERF1:CFTR interaction leading to an increase in CFTR at the PM by decreasing its endocytosis. Here, we used protein interaction profiling and bioinformatic analysis to identify proteins that interact with CFTR under EPAC1 activation as possible regulators of this CFTR PM anchoring.

We identified an enrichment in cytoskeleton related proteins among which we characterized CAPZA2 and INF2 as regulators of CFTR trafficking to the PM. We found that CAPZA2 promotes wt-CFTR trafficking under EPAC1 activation at the PM whereas reduction of INF2 levels leads to a similar trafficking promotion effect. These results suggest that CAPZA2 is a positive regulator and INF2 a negative one for the increase of CFTR at the PM after an increase of cAMP and concomitant EPAC1 activation. Identifying the specific interactions involving CFTR and elicited by EPAC1 activation provides novel insights into late CFTR trafficking, insertion and/or stabilization at the PM and highlights new potential therapeutic targets to tackle CF disease.

Abbreviations: APID, Agile protein interactome data server; CAPZA2, Capping actin protein of muscle Z-line alpha subunit 2; CFTR, Cystic fibrosis transmembrane conductance regulator; EPAC, Exchange protein directly activated by cAMP; ERM, Ezrin/radixin/moesin; INF2, inverted formin 2; PM, plasma membrane.

Keywords CFTR; EPAC1; cAMP; Actin cytoskeleton; protein trafficking
Introduction

Cystic Fibrosis (CF) is the most common lethal and life-limiting autosomic recessive disorder among Caucasian population, affecting about 1:2500-6000 new borns (1) and it is caused by absence or dysfunction of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein. This is a member of the ABC (ATP-binding cassette) transporter superfamily and functions as a cAMP-regulated chloride (Cl⁻) and bicarbonate (HCO₃⁻) channel in the apical membrane of epithelial cells to maintain ion and fluid homeostasis(2, 3). CFTR is composed by two membrane spanning domains (MSD1 and MSD2), forming the pore of the channel; two cytosolic nucleotide binding domains (NBD1 and NBD2), where ATP binds and is hydrolysed regulating channel gating and one CFTR-exclusive regulatory domain (RD) that contains multiple phosphorylation sites essential for channel activity (4).

The most common CF-causing mutation corresponds to a deletion of phenylalanine (Phe) at position 508, located in NBD1 (F508del), which occurs in ~85% of CF patients in at least one allele and leads to CFTR misfolding and endoplasmic reticulum (ER) retention. The mutant protein is prematurely degraded precluding its delivery to the cell surface (3).

Levels of CFTR at the PM have been shown to be determined by a balance between three main processes – 1) anterograde transport, 2) endocytosis and 3) recycling (5, 6) – all of which rely on CFTR interaction with several proteins among which PDZ domain-containing proteins (PDZ proteins) play an essential role. Indeed, NHERF1 anchors CFTR to the PM and to the actin cytoskeleton (5, 7). In the PDZ-dependent CFTR-NHERF1 complex, NHERF1 interacts with ezrin and this NHERF1-ezrin interaction locks CFTR in an immobile and actin-tethered complex preventing its endocytosis (8, 9). NHERF1 not only regulates wt-CFTR at the PM but also of F508del-CFTR by protecting it from lysosomal degradation(10). Recently, it was shown that activation of the exchange protein directly activated by cAMP 1 (EPAC1) promotes its interaction with NHERF1 and CFTR, leading to an increase in the amount of CFTR at the PM at steady-state (11).

EPAC proteins function as guanine nucleotide exchange factor (GEF) for both Rap1 and Rap2 (12) and are involved in the regulation of cell-cell and cell-matrix adhesion, cytoskeleton rearrangements and cell polarization (13). Upon activation by cAMP, EPAC1 – the major EPAC expressed in the lung (14) is targeted to the PM where it tethers to phosphatidic acid or to phosphorylated proteins in the ezrin/radixin/moesin (ERM) family inducing its downstream effectors such as Rap (15). Identification of NHERF1-dependent CFTR-EPAC1 interaction (11) highlighted a two-level regulation of the channel by cAMP – low concentrations of cAMP activate PKA to regulate CFTR function whereas high cAMP levels promote EPAC1-dependent increase of its PM levels (11).
Recently, it was also reported that EPAC1 plays an important role in Cl− secretion not only through CFTR but also through TMEM16A (or anoctamin 1), a Ca2+-activated Cl− channel (16), since activation of EPAC1 by cAMP, activates Rap2 leading to an increase in Ca2+ (17). Thus, EPAC1 appears to be a hub in the cAMP/Ca2+ crosstalk. Despite these recent advances the mechanism and the macromolecular complexes elicited by EPAC1 activation at the PM are still largely uncharacterized.

We used a proteomic interaction profiling approach coupled to global bioinformatic analysis to identify specific CFTR interactions elicited by EPAC1 activation. We identified several novel interactors and characterized two of them involved in its PM anchoring, CAPZA2 and INF2. Our data show that while CAPZA2 downregulation, leads to an effect by EPAC1 on wt-CFTR by enhancing PM levels, in contrast, INF2 downregulation, leads to the opposite effect i.e., reduced levels of wt-CFTR at the PM. We report here for the first time the acting cytoskeleton dynamics regulators CAPZA2 and INF2 as modulators of CFTR anchoring at the PM.

Considering that the therapeutic opportunities of restoring CFTR PM stability are still poorly explored, characterization of the molecular mechanism of CFTR anchoring as described here may contribute to identify novel targets that can be used in combination with other therapeutic strategies.
Experimental (Materials and methods)

Cell culture and compound treatment

Parental CFBE41o- cells and CFBE stably transduced with wt-CFTR were grown in EMEM (Lonza, #BE12-611F) supplemented with 10% (v/v) FBS (GIBCO® Life Technologies, #10270-106) and 5 µg/ml puromycin (Sigma, #P8833). CFBE41o- cells expressing double tagged mCherry-FLAG-wt-CFTR, under a doxycycline inducible promoter were grown in DMEM with 4.5 g/L glucose and L-Glutamine supplemented with 10% (v/v) FBS, 2 µg/ml puromycin and 10µg/ml blasticidin (InvivoGen, #ant-bl). CFTR expression was induced with doxycycline 1 µg/ml (Sigma #9891) for 24h. All cell lines were grown at 37ºC in 5% CO2.

When applicable, cells were treated 2h with 1 µM 8-pCPT-2′-O-Me-cAMP-AM (007-AM) (BioLog, #c041-05), 10 µM forskolin or vehicle control (DMSO) in the appropriate serum-free media. Treatment with corrector VX-661 5 µM was initiated 24h after transfection and extracts were collected at 48h incubation. All the incubation times and concentrations are the standard, established ones and are in agreement with previous usage (11, 18). When compound incubation was combined with transfections, compounds were added in the final periods (2h or 24h) prior to cell harvesting.

CFTR immunoprecipitation

CFTR was immunoprecipitated (IP) from stable CFBE41o- cells expressing wt-CFTR previously treated with the in vivo cross link agent dithiobis(succinimidylpropinate) (DSP) (Thermo Scientific, #22585). Parental CFBE cells (not expressing CFTR) were used to define the background. Immunoprecipitation was performed using the anti-CFTR 596 monoclonal antibody coupled to Protein G agarose beads coupled with the 596 as previously described (Canato et al 2018; Santos et al 2019). Cell lysis was performed for 30 min with Triton lysis buffer – 25 mM Tris-HCl pH7.7, 150 mM NaCl, 1% (v/v) Triton-X 100, supplemented with protease inhibitors 2mg/ml – Complete Protease Inhibitor Cocktail (Roche, #11697498001). Pre-cleared lysates were incubated with the antibody-beads complex overnight at 4ºC. After washing twice with wash buffer (Tris-HCl 100 mM pH 7.4; NaCl 300 mM) supplemented with 1% (v/v) triton-X100 and twice with wash buffer without Triton, proteins were eluted in Laemmli buffer supplemented with 100mM DTT. Eluted proteins were loaded into a SDS-PAGE gel and run until reaching 1 cm into the separating gel. Silver nitrate staining was performed and the upper 1cm gel segment was preserved in PBS and used for MS analysis. All the analysis was performed in triplicate.

Mass spectrometry: sample preparation, analysis and dataset filtering
Eluted proteins were reduced with 10 mM of DTT for 40 min at 56°C followed by alkylation with 55mM iodoacetamide for 30 min. The remaining iodoacetamide was quenched by a second incubation with 10mM DTT for 10 min in the dark. Then, the resulting sample was digested overnight with trypsin at 37°C (1:50 protein/trypsin ratio), dried and resuspended in 8 µL LCMS water 0.1% formic acid.

NanoLC-MS/MS analysis was performed on an ekspert™ NanoLC 425 cHiPLC® system coupled with a TripleTOF® 6600 with a NanoSpray® III source (Sciex). Peptides were separated through reversed-phase chromatography (RP-LC) in a trap-and-elute mode. Trapping was performed at 2 µl/min with 100% A (0.1% formic acid in water, Fisher Chemicals, Geel, Belgium), for 10 min, on a Nano cHiPLC Trap column (Sciex 200 µm x 0.5 mm, ChromXP C18-CL, 3 µm, 120 Å). Separation was performed at 300 nL/min, on a Nano cHiPLC column (Sciex 75 µm x 15 cm, ChromXP C18-CL, 3 µm, 120 Å). The gradient was as follows: 0-1 min, 5% B (0.1% formic acid in acetonitrile, Fisher Chemicals); 1-91 min, 5-30% B; 91-93 min, 30-80% B; 93-108 min, 80% B; 108-110 min, 80-5% B; 110-127 min, 5% B. Peptides were sprayed into the MS through an uncoated fused-silica PicoTip™ emitter (360 µm O.D., 20 µm I.D., 10 ± 1.0 µm tip I.D., New Objective). The source parameters were set as follows: 12 GS1, 0 GS2, 30 CUR, 2.5 keV ISVF and 100 °C IHT. An information dependent acquisition (IDA) method was set with a TOF-MS scan range of 400-2000 m/z for 250 msec. The 50 most intense precursors were selected for subsequent fragmentation and the MS/MS were acquired in high sensitivity mode (150-1800 m/z for 40 msec each). The selection criteria for parent ions included a charge state between +2 and +5 and counts above a minimum threshold of 125 counts per second. Ions were excluded from further MSMS analysis for 12 s. Fragmentation was performed using rolling collision energy with a collision energy spread of 5.

The processing and analysis of the obtained mass spectra list were done using ProteinPilot™ software, with the Paragon search engine (version 5.0, Sciex). The following search parameters were set: search against Homo sapiens from Uniprot/SwissProt database (release 2015_05); Iodoacetamide, as Cys alkylation; Trypsin, as digestion; TripleTOF 6600, as the Instrument; ID focus as biological modifications and Amino acid substitutions; search effort as thorough; and a FDR analysis. Only the proteins with Unused Protein Score above 0.47 (corresponding to 66% confidence) were considered. This lower confidence threshold was selected to allow the detection of proteins that compensate their lower scores in single replicates by their identification in multiple replicates. If a protein is detected with 66% confidence in three replicates, the chance that all identifications were false positives is below 4% (below 11% for two replicates). To account for this effect, a Confidence score was developed, based on the unused ProtScore and the number of replicates in which each protein was detected (compared to controls). The range varies between 0 - protein identified with low robustness and confidence, and 5 - protein identified with high robustness and confidence as shown in Table S1.
proteins identified for parental CFBE41o- cells and for CFBE cells expressing wt-CFTR incubated with non-conjugated beads were considered as non-specific and removed from the protein lists identified for CFBE cells expressing wt-CFTR incubated with Fsk, 007-AM and DMSO. In this analysis, a protein is only excluded (appearing in controls) or considered (appearing in samples under analysis) as a specific protein when present in at least two replicates. To determine the score of each protein, the lists for each condition (CFBE cells incubated with DMSO, Fsk or 007-AM) were compared with the lists for the 2 controls used (parental cells and wt-CFTR cells incubated with beads only) to evaluate the associated Unused ProtScore and the number of replicates where the protein was identified in both samples and controls. R programming was used to calculate the Confidence score for each protein\(^1\). Lists of proteins identified in each of the three conditions tested were compared to identify common and specific proteins for each condition.

**Networks and gene ontology**

The Database for Annotation, Visualization and Integrated Discovery (DAVID)\(^2\) (19, 20) was used to analyse the obtained data set and identify the Gene Ontology (GO) terms which were enriched in our data. The GO terms considered were the Biologic process (BP) and Cellular Component (CC) using version 6.8.

Data were also analysed with Agile Protein Interactomes DataServer (APID) in combination with R programming (21). APID human interactomes of quality level 0 (all reported interactions) or level 1 (interactions proven by 2 or more experimental evidences) were used to assess the distance (1 to 5 edges distance) of the identified proteins (proteins interacting with CFTR specifically after cell incubation with 007-AM) to proteins of interest (CFTR and EPAC1). APID level 0 was also used to generate protein networks. Finally, the shortest path in the network between two proteins of interest was determined using APID level 0 or APID level 1. Networks generated using APID (22) were accessed and visualized/analyzed using Cytoscape (23). To generate the wt-CFTR general network, we used the proteins detected in our assays with a Confidence score of 2 or higher (Table S1) for all sample-control combinations, using APID level 0 as the basis for the PPI.

**siRNA transfection**

siRNA transfection was performed as previously described (24). Transfection mixture using Lipofectamine 2000 (1 mg/ml, Invitrogen #11668019) was prepared containing 75nM of

---

1 R program, https://www.r-project.org/ (Last accessed March 09, 2019).
2 DAVID database, https://david.ncifcrf.gov/ (Last accessed March 08, 2019)
siRNA (CAPZA2 (Dharmacon #D-012213-17), INF2 (Dharmacon # D-014097-03), EGFP (Eurofins MWG operon #921154)) and 2.5 ng Lipofectamine to a final volume of 500 µL in OptiMEM, according to the manufacturer's instructions. After transfection, cells were grown in FBS-free media. After 24h the media was changed to EMEM supplemented with 10% FBS (v/v). 48h after transfection, cells were harvested.

**CFTR PM traffic assay and image analysis**

CFTR trafficking was assessed as described previously (25). For cell reverse transfection, 96 well plates were coated with customized siRNAs for solid-phase reverse transfection. In short, a transfection mix (final volume of 300 µl) containing 161.5 µl of lipofectamine 2000 (Invitrogen #11668019) and 138.5 µl of 0.4 M sucrose in OptiMEM solution was prepared. Then, 1.25 µl siRNA 20 µM (Table S2 - Supplementary Data) were added to the respective well into the 96 v-shaped well plates (siRNA plate). 1.75 µl of transfection mix were added to siRNA plate, followed by mixing and centrifugation for 1 min at 50g. 1.75 µl of 0.2% (w/v) gelatin solution were added to the siRNA plate and centrifuged at 50g. A total of 4.25 µl of the siRNA plate mix (siRNA plus transfection mix plus gelatine) was diluted 1:50 in 96 v-shaped well plate using double distilled water. Finally, 50 µl from diluted mix were lyophilized and stored in an anhydrous atmosphere before cell seeding.

After 24h, cells were seeded in the siRNA coated 96-well plate (7 x 10⁷ cells/well) using a Multidrop™ Combi Reagent Dispenser (Thermo Scientific #5840300). CFTR expression was induced after 24h of reverse transfection by incubating the cells with 1 µg/ml doxycycline in antibiotic-free medium. CFTR expression was induced for 24h. 72h after cell seeding and siRNA knockdown, cells were washed once with cold PBS+/+ using Tecan Hydrospeed™ and immunostained for the extracellular FLAG-tag (in non-permeabilized cells) for 1h at 4°C with 1:500 monoclonal anti-FLAG® M2 antibody (Sigma-Aldrich, #F1804). Then, cells were washed three times in ice with cold PBS+/+ and incubated 20 min with 3% (w/v) paraformaldehyde (PFA) at 4°C. Then, cells were then washed three times in ice with cold PBS+/+ and incubated with the anti-mouse Cy5 conjugated secondary antibody (1:500) (Invitrogen, #A10524) for 1h at 4°C. Cells were washed three times in cold PBS+/+ and incubated for 1h with Hoechst 33342 200 ng/ml (Sigma-Aldrich, #B2261) for nuclei staining. Finally, cells were washed three times in cold PBS+/+ and immersed in PBS+/+ until cell imaging. All solutions were prepared in PBS+/+ and primary and secondary antibodies were diluted in PBS+/+ supplemented with 1% (v/v) BSA.

For image acquisition, cell imaging was performed with inverted widefield fluorescence microscope Leica DMI6000 equipped with a DFC360FX camera (12-bit, 1344x1024 pixel resolution) (Leica) and a 10x objective. The exposure times at maximum light brightness for
Hoechst, mCherry and Cy5 were 70ms, 1300ms and 8s respectively. The Hoechst channel was used for contrast-based autofocus. The experiment was performed in triplicate and five images were collected per well.

For image analysis, automatic image analysis was performed using open source software tools namely cell image analysis software CellProfiler\(^3\) (26), R programing (21) and a pipeline to measure CFTR traffic efficiency developed previously (25). Using the pipeline, the background was subtracted to each image. Then, a quality control was applied for each image excluding all cells which do not significantly express CFTR, present abnormal morphology or contain a significant number of saturated pixels. Out-of-focus images, images with high background fluorescence and images with lower number of cells (less than 15 cells) were also removed with this pipeline. Finally, CFTR levels were measured in each cell using the fluorescence quantification to determine Total CFTR, given by the mCherry fluorescence intensity and PM CFTR, given by Cy5 fluorescence intensity. CFTR traffic efficiency was calculated using the median values for all cells in the image and calculated according to the ratio PM CFTR (Cy5 Integrated Fluorescence) / TOTAL CFTR (mCherry Integrated Fluorescence). Then, the deviation score was calculated according to the following formula:

\[
\text{Deviation Score} = \frac{\text{Traffic Efficiency}_{\text{test}} - \text{Traffic Efficiency}_{\text{control}}}{2 \times \text{SEM}_{\text{control}}}
\]

The Traffic Efficiency\(_{\text{test}}\) corresponds to the average of CFTR traffic efficiency for all images transfected with the same siRNAs or treated with the same compound which passed the QC. The Traffic Efficiency\(_{\text{control}}\) corresponds to the average of CFTR traffic efficiency for all the conditions in the plate or in case of compound incubation the DMSO - All Conditions or DMSO. The standard error of the mean of the control (SEM\(_{\text{control}}\)) corresponds to the SEM recorded for the control condition - All Conditions or DMSO. Deviation score identifies traffic enhancers (when it is positive) or inhibitors (when it is negative).

**Cell-surface biotinylation assay**

Cell surface biotinylation was performed as previously described (11, 27). Cells were grown in P60 plates until pre-confluence. After three washes with PBS\(^{14}\) cells were incubated at 4ºC with 3mL of Sulfo-NHS-SS-Biotin buffer (0.1mg biotin (Thermo Scientific, #21331)/1 mL PBS with 1mM MgCl\(_2\), 0.1mM CaCl\(_2\), pH 8.2) for 30 min and then washed three times with PBS\(^{14}\). Cells were lysed with 1.5mL of BL buffer (HEPES 25mM, Triton X-100 1% (v/v), glycerol 10% (v/v), supplemented with complete protease inhibitor cocktail, pH 8.2) and incubated for 15min at 4ºC. Cell membranes and debris were pelleted by centrifugation at

\(^3\)CellProfiler, [http://cellprofiler.org/](http://cellprofiler.org/) (Last accessed February 21, 2019).
14000g for 10min at 4°C following by supernatant incubation at 4°C overnight with streptavidin beads previously washed twice with PBS and once with BL buffer. The beads were pelleted by centrifugation 1min at maximum speed and next washed once with BL buffer and twice with PBS. Then, beads were incubated with 65µL SB suplemented with DTT 1M at 85°C for 5min. Finally, samples were loaded into a SDS-PAGE gel for Western blotting (WB) analysis.

**Western blotting**

Protein extracts were separated on 7% SDS polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk in PBS supplemented with 0.1% (v/v) Tween-20 (PBS-T) and probed overnight with primary antibody at 4°C (1:5000 mouse anti-CFTR 596 (Cystic Fibrosis Foundation, Cat No A4), 1:1000 rabbit polyclonal RAPGEF3 antibody - middle region (Aviva Systems Biology, ARP52140_P050), 1:1000 rabbit polyclonal anti-CAPZA2 (ProteinTech, 15948-1-AP), 1:1000 rabbit polyclonal anti-INF2 (ProteinTech, 20466-1AP). Calnexin, tubulin or GAPDH (1:3000 purified mouse anti-Calnexin (BD Transduction Laboratories™, 610523), 1:10000 mouse monoclonal anti-α-tubulin1 (Sigma, T5168), 1:1000 mouse monoclonal anti-GAPDH (6C5) (Abcam, ab8245)) were detected as loading control. Membranes were washed three times followed by incubation for 1h with the adequate secondary antibody at room temperature (1:3000 goat anti-mouse IgG (H+L) HRP conjugate (Bio-Rad, 170-6516), 1:3000 goat anti-rabbit IgG (H+L) HRP conjugate (Bio-Rad, 170-6515)). Finally, membranes were washed three times with PBS-T. Antibody dilutions were all made in blocking solution (PBS-T plus 5% milk). Chemiluminescent detection was performed using the Clarity™ Western ECL substrate (BioRad, #170-5061) and the Chemidoc™ XRS system (BioRad). The quantification of band intensity was performed using the Image Lab software (BioRad) and normalized to loading control as appropriate.

**Statistical analyses**

Data are presented as mean ± standard error of the mean (SEM). Two-tailed Student’s t-tests were performed to assess statistical significance. In these analyses, p<0.05 was considered as significant.
Results

Proteomic profiling of wt-CFTR under activation of EPAC1

In an attempt to identify the proteins involved in CFTR anchoring/stabilization at the PM under EPAC1 activation, CFTR was immunoprecipitated from CFBE wt-CFTR cells under low-stringency conditions so as to capture multiprotein complexes and thus maximize the number of interacting proteins obtained. As we were interested in identifying the differential interactors involved in CFTR stabilization by EPAC1, cells were incubated with: i) forskolin (Fsk); ii) the specific EPAC1 agonist 007-AM (8-pCPT-2′-O-Me-cAMP); or iii) DMSO (vehicle control). Non-specific interactions (“background”) were discarded using an equivalent approach performed in CFBE cells which do not express CFTR (parental CFBE cells) and in CFBE wt-CFTR cells incubated with non-conjugated beads. Isolated proteins were then identified using nanoLC-MS/MS.

We were able to pull-down a total of 1,599 proteins (Table S3A - Supplementary Data). For CFBE cells expressing wt-CFTR incubated with DMSO, Fsk or 007-AM, a total of 311, 263 and 399 proteins were identified respectively (Table 1, Table S3B - Supplementary Data). A total of 374 proteins were identified for CFBE parental cells and 251 proteins were identified for CFBE cells expressing wt-CFTR incubated with non-conjugated beads, i.e., without CFTR Ab (Table 1, Table S3B - Supplementary Data).

Removal of proteins identified in the controls resulted in a total of 207 different proteins (Table S4A - Supplementary Data) for the sum of the three conditions tested, that were then crossed to identify the specific proteins for each condition (Table 1, Figure S1). CFTR is one of the three proteins that is common to all the conditions tested. Interestingly, the higher number of specific wt-CFTR-interacting proteins (CIPs) was identified when EPAC1 is activated (CFBE cells incubated with 007-AM) – 101 proteins (Table S4B - Supplementary Data). In addition, 23 CFTR interacting proteins were identified specifically when adenylyl cyclase (AC) is activated (CFBE cells incubated with Fsk) and 62 CFTR interacting proteins were identified exclusively for the incubation with DMSO control condition (Figure S1; Table S4B - Supplementary Data).

Bioinformatic and comparative analysis of CFTR interacting proteins

We used available bioinformatics tools to analyse the lists of CIPs upon activation of either AC or EPAC1, i.e., in cells incubated with either Fsk or 007-AM, respectively.

DAVID 6.8 was used to identify the enriched GO terms among the 110 EPAC1-specific CIPs (Figure 1). For GO term – Biological Process (BP), cellular component biogenesis, cellular component assembly and cell-cell adhesion are the most represented terms. There is also an enrichment of categories associated to the cytoskeleton and to actin filaments. For GO
term – Cellular Compartment (CC), adherent junction, anchoring junction and extracellular vesicles are the most represented terms. At the CC level, the enrichment in categories of cytoskeleton and actin filaments-associated is even more evident since more than 2/3 of all categories represent cellular components which are strongly connected with cytoskeleton and actin filaments as shown in Figure 1B.

We then used APID to calculate the distance between the 110 proteins specifically identified upon EPAC1 activation to either CFTR or EPAC1. The proteins were analysed with APID level 0 (accounts for all known interactions) and APID level 1 (accounts for interactions proven by two or more experiments). Among these 110 proteins, 6 (based on both APID level 0 and APID level 1 – Figure S2A) had been previously reported as directly interacting with CFTR, namely: AHSA1, CSE1L, HSPA2, PPP2R1A, PSMD2 and NHERF1. Moreover, we found that more than 85% of the 110 proteins are within 2 to 3 edges of distance from CFTR (with the more stringent APID level 1). When measuring the distance of the 110 proteins to EPAC1, there was no protein detected in APID previously reported to interact directly with EPAC1 (Figure S2B). The fact that we pulled-down CFTR and not EPAC1 is a plausible explanation for this observation. However, more than 89% of these proteins are within 3 to 4 edges of distance from EPAC1.

To analyse the robustness and confidence for the identified CIPs, a scoring system (named Confidence score and detailed in Table S1) was developed. This Confidence score takes into consideration the Unused ProtScore and the number of replicates in which each protein was detected (comparing samples and controls). 5 proteins were identified with a high robustness and confidence (score – 5) for their interactions upon EPAC1 activation (Table S5 - Supplementary Data). CFTR is one of these proteins supporting the approach used as well as the reliability of the scoring methodology. Overall, for the proteins detected under EPAC1 activation, 27% were scored above or equal to 3.

Comparison with previously published CFTR interactomes revealed a very low overlap (around 2% with 20 and 13 proteins in common respectively) (28, 29) - suggesting that the 110 CIPs correspond to proteins that specifically interact with CFTR when EPAC1 is activated and thus do not belonging to the “core” CFTR interactome.

In order to select the hits for validation among the 110 interactors, the above-described data were integrated (Table S6 - Supplementary Data) and complemented with extensive literature-mining. We then selected for validation: i) 3 proteins with a Confidence score of 5; ii) 7 proteins with a score of 4; and iii) 3 proteins with a score of 3 (Table S6 - Supplementary Data). In addition, we also selected 5 proteins with a score of 2 (lower robustness and confidence) in order to evaluate the strength of the selection methodology used. Interestingly, among the 18 selected hits, 16 are within 2 edges of distance to CFTR and, from these 16, 15
proteins are within 3 edges of distance to EPAC1 showing the high proximity of the selected hits to CFTR and EPAC1 (Table S6 - Supplementary Data).

**Generation of protein networks**

To assess the possible connections between the identified CIPs, we generated a network using all proteins with a Confidence score of 2 or higher. For this, proteins identified in the controls were removed from the lists identified for each sample (Fsk, DMSO or 007-AM) resulting in a total of 260 proteins that were used for the network generation (Table S7 - Supplementary Data).

Using APID, we created a network exhibiting the association between the proteins identified based on previously reported interactions (Figure S3 - Supplementary Data). This large network shows that 201 proteins are connected. Moreover, the network shows that the proteins detected specifically after treatment with Fsk or DMSO (shown in green and blue respectively) as well as the proteins detected in more than one sample (shown in grey) are relevant in establishing the core of the network as they mediate the paths between the 110 proteins detected upon EPAC1 activation (shown in pink). CFTR, NHERF1 and EPAC1 (represented in yellow) appear as key components in this network, supporting the robustness (and relevance) of the results obtained. Among the 251 proteins (interaction score ≥2), there are only 51 proteins that do not participate in previously described interactions. The general network obtained also shows that the 18 top hits selected for validation (shown with red border) are homogenously distributed rather than concentrated in a restricted area. This list of 18 selected proteins comprises proteins which are: 1) in close proximity to CFTR, with just one edge of distance; 2) distant from CFTR, with two or more edges of distance; 3) proteins creating small clusters among themselves and 4) proteins not present in the general network obtained.

As the 18 hits selected for validation seem to be in close proximity among themselves, we built a minimum network needed to link them to CFTR and EPAC1 (Figure 2) with APID level 1 as the source of interactions. This confirms that they can be linked through a minimal network to which only 8 additional proteins (shown in blue) are needed to connect them to CFTR and EPAC1 (shown in yellow) (Figure 2).

**Impact of hit knockdown on CFTR trafficking**

We next assessed the impact upon CFTR trafficking of knocking down by siRNA the 18 hits selected for validation using a previously described assay (25).

We started by validating the assay for the previously reported increase of CFTR trafficking upon EPAC1 activation (as reported by us in (11)). For that, we used CFBF cells expressing mCherry-Flag-wt-CFTR under the control of a doxycycline-inducible promoter, that
were incubated with either the EPAC1 agonist 007-AM or DMSO (vehicle control). Immunostaining assay was performed and CFTR at the PM was monitored by the Flag (Cy5) signal. CFTR trafficking (given by the ratio of PM CFTR to total CFTR) and the deviation score was also calculated as described (25). Results show that EPAC1 activation leads to an increase in wt-CFTR at the PM corresponding to a statistically significant increase in traffic efficiency (Figure S4). This result confirms that this trafficking assay is robust to assess the impact of hit knockdown in combination with EPAC1 activation.

The impact of siRNA knockdown of the 18 selected hits on wt-CFTR traffic was then assessed under 007-AM treatment vs the vehicle control (DMSO). For this assay, cells were reverse transfected with 2 independent siRNA for each of the 18 selected genes and also with siRNAs to CFTR, COPB1, EPAC1 and EGFP (non-targeting siRNA) as controls. Twenty-four hours after siRNA transfection with, expression of mCherry-Flag-CFTR was induced by addition of doxycycline and 24h post-induction, immunofluorescence was performed. Total and PM for CFTR were assessed and the trafficking efficiency plotted (Figure 3). An effect on CFTR traffic efficiency was considered when the two siRNAs targeting the same gene resulted in an equivalent effect (both positive or both negative) and a deviation score significantly different from the control

Analysis of results (Figure 3C) shows that only the knockdown of INF2 (Inverted formin-2) by both siRNAs has a major positive impact in the traffic efficiency of wt-CFTR (deviation score >1) under EPAC1 activation by 007-AM, but not under DMSO as clearly observed in the representative images (Figure 3B-INF2_2).

With the opposite effect, CAPZA2 (Capping actin protein of muscle Z-line alpha subunit 2) was the only hit whose knockdown had a major negative impact on wt-CFTR traffic efficiency with both siRNAs showing a deviation score lower than -1 under EPAC1 treatment but not under DMSO. CAPZA2 representative images clearly show the negative impact of knocking down CAPZA2 on the levels of wt-CFTR at the PM after incubation with 007-AM (Figure 4A and B-CAPZA2_2). Thus, INF2 and CAPZA2 were selected to be further investigated regarding their impact on wt-CFTR PM levels/stability. The Confidence score of INF2 was 4, while that of CAPZA2 was 3.

Inverted formin-2 (INF2) is an unusual formin since it is the only one with the ability to accelerate both actin polymerization and depolymerization controlling filament dynamics and regulating cell polarity, mitochondrial fission, intracellular trafficking as well as cell and tissue morphogenesis (30, 31). Capping actin protein of muscle Z-line alpha subunit 2 (CAPZA2) is a component of the heterodimeric actin-capping protein which binds to the fast growing ends of actin filaments (barbed end) by blocking the exchange of subunits at these ends (32).

As to the Confidence score of the other selected proteins, it was 4 for CALD1 and MYO1D but their KD had no relevant impact in CFTR trafficking efficiency under 007-AM
(Figure 3). Both siRNAs used against CALD1 showed a trend to decrease CFTR trafficking efficiency in the presence of 007-AM, however, the deviation score was > -1. The representative images show that CALD1 knockdown does not influence wt-CFTR levels at the PM (Figures 3A-CALD1_2 and 3B-CALD1_2). Knockdown of MYO1D had a null impact on wt-CFTR trafficking, despite its high Confidence score (Figure 3-MYO1D_2). TPM4 score was 2 and thus it was selected to evaluate the strength of the selection strategy used. CFTR trafficking assay showed that knockdown of this gene has no impact in wt-CFTR trafficking to the PM (Figure 3-TPM4_2) similarly to siRNAs knocking-down all the other proteins scored with 2 (FLG2, PSMB6, RPS14 and RPS18) supporting the selection strategy adopted. Being this a screening assay, we cannot of course exclude the fact that lack of an effect may correspond to low knock-down efficiency by the siRNA(s) used.

Validation of INF2 and CAPZA2 as CFTR interactors under EPAC1 activation

As we aim to identify proteins involved in CFTR PM stabilization by EPAC1 activation, we then validated and characterized the role of the two hits identified, namely CAPZA2 and INF2. For that, we validated the interaction between CAPZA2 or INF2 proteins and CFTR and assessed the effect of knocking down CAPZA2 and INF2 at 3 levels: i) the amount of wt-CFTR at the PM; ii) amount of EPAC1 interacting with CFTR and iii) the cellular localization of CFTR and EPAC1 vs that of INF2 and CAPZA2. Interaction between EPAC1 and CAPZA2 or INF2 was also assessed to get additional information on its mechanism of action.

Firstly, we assessed the efficiency of KD of both siRNAs used against CAPZA2 or INF2 (Figure S5). Results show that both siCAPZA2 and siINF2 decrease the protein levels of CAPZA2 or INF2 by more than 50% or 40-60%, respectively as compared to siRNA against EGFP as a negative control (Figure S5B and C, respectively).

We then assessed the interaction between CAPZA2 and CFTR by co-IP to validate the MS results. For that, CFTR was immunoprecipitated from CFBE wt-CFTR cells incubated with 007-AM or DMSO. Lysates incubated with beads only were used as a control. WB analysis shows that CAPZA2 is detected after CFTR IP when EPAC1 is activated, but not under DMSO (Figure 4A). The quantification of these data shows a significant increase in the amount of CAPZA2 in the CFTR co-IP when cells were incubated with 007-AM vs DMSO (Figure 4B), confirming that CFTR:CAPZA2 interaction only occurs under EPAC1 activation.

Next, we assessed the CFTR:INF2 interaction, also by co-IP. For technical reasons (antibody quality), we used the reverse approach, i.e, detecting CFTR in INF2 co-IP. Results show that CFTR can be detected in the INF2 co-IP in cells treated with either 007-AM or DMSO, but a significant increase in the amount of INF2 was observed after EPAC1 activation vs DMSO (Figure 4C and D). Moreover, our data suggest that INF2 interacts mostly with the
mature form of CFTR, as evidenced by a clear increase in Band C levels in Figure 4C in 007-AM- vs DMSO-treated cells. In conclusion, this result confirms INF2 as a stronger interactor of CFTR under EPAC1 activation.

**CFTR levels at the PM after modulation of CAPZA2 and INF2**

Results from the trafficking assay (Fig.3) indicate that INF2 and CAPZA2 influence CFTR PM traffic with opposite effects. To confirm such observations, we assessed the impact of knocking down INF2 and CAPZA2 on CFTR PM levels using cell surface biotinylation in CFBE wt-CFTR cells incubated with 007-AM or DMSO.

Results show a statistically significant increase in the levels of PM CFTR after INF2 KD (Figure 5B) when compared to siRNA EGFP (control). Thus, our results indicate that INF2 knockdown further potentiates the increase in levels of PM CFTR (Figure 5B) previously described for EPAC1 activation (11).

On the other hand, for CAPZA2 knockdown, results show that it leads to a statistically significant decrease in CFTR PM levels (Figure 5B) when compared to the control siRNA EGFP.

So, cell surface biotinylation results further support the previous observations that INF2 and CAPZA2 influence the amount of CFTR at the PM, with opposite effects.

**Interaction between CAPZA2, INF2 and EPAC1**

Since both INF2 and CAPZA2 influence CFTR PM levels upon EPAC1 activation, we then evaluate whether these two proteins interact with EPAC1. Co-IP of EPAC1 was performed in CFBE wt-CFTR cells with a specific antibody and using DMSO or beads only as controls. Presence of INF2 and CAPZA2 was assessed by WB.

We were able to detect CAPZA2 after EPAC1 co-IP mainly after 007-AM treatment (Figure 6A). Results show that the EPAC1:CAPZA2 interaction is increased by 2-fold upon EPAC1 is activated (Figure 6B), thus, indicating that CAPZA2 interacts with EPAC1 as well as with CFTR. For INF2, we were able to detect it after treatment with either 007-AM or DMSO, and observed a slight, albeit not significant, increase when cells are incubated with 007-AM (Figure 6A).

In summary, our results show a strong EPAC1:CAPZA2 interaction promoted by 007-AM and also an interaction between INF2:EPAC1 (but in this case independent of EPAC1 activation). Thus, next we assessed the impact of CAPZA2 or INF2 knockdown on the EPAC1:CFTR reported interaction. For that, we used CFTR co-IP followed by detection of EPAC1 in wt-CFTR expressing CFBE cells transfected with siRNAs against INF2 or CAPZA2 and incubated with 007-AM or DMSO. Results show that knockdown of either CAPZA2 or
INF2 does not influence significantly the CFTR:EPAC1 interaction (Figure 6C and D). Thus, our results indicate that although both INF2 and CAPZA2 interact with CFTR and EPAC1, their KD does not compromise the CFTR:EPAC1 interaction.

**INF2 knockdown increases the amount of rescued F508del-CFTR**

Lastly, we assessed whether the knockdown of INF2 is able to increase the rescue of F508del-CFTR observed under EPAC1 activation and treatment with the corrector VX-661. To this end, CFBE-F508del cells were incubated with 5µM VX-661 to promote the rescue of the mutant protein combined with 1µM 007-AM to activate EPAC1 (Figure 7A) and the knockdown of either GFP (as negative control) or INF2. Levels of mature CFTR (band C) were assessed by WB. Results show that, under treatment of both drugs, knockdown of INF2 leads to a 6-fold increase in the amount of band C (Figure 7B) when compared to the siEGFP control. These findings indicate that increasing the amount of EPAC1-stabilized PM F508del-CFTR through knockdown of INF2 may be the basis of an improved combinatorial approach towards mutant CFTR rescue.
Discussion

The net flow of CFTR activity depends on both the number of these channels at the PM as well as the function of each individual channel. Understanding the mechanisms through which CFTR PM levels are regulated is likely to identify targets amenable to modulation to design potential innovative therapeutic strategies. This is of particular interest because although F508del-CFTR can be rescued to the PM by different mechanisms, the half-life of the rescued mutant protein is significantly reduced due to the high endocytosis rate and/or a reduction in recycling back to the PM (2, 33) and thus its stabilization at the PM can be targeted in a combinatorial therapeutical approach (34).

Here, we aimed at better understanding the mechanism through which EPAC1 activation leads to CFTR stabilization at the PM by identifying the molecular complexes elicited during this process. Using co-IP of CFTR followed by mass spectrometry, we performed a comparative interactomics profiling of wt-CFTR under activation of adenylyl cyclase (by Fsk) or EPAC1 (by 007-AM). From a total of 1,599 CFTR-interacting proteins (CIPs) identified, 110 were exclusively identified after EPAC1 activation (Figure S1). GO terms enrichment analysis (Figure 1) in this set of proteins identified a high representation of actin cytoskeleton associated categories. Whereas it is known that CFTR stability and function is correlated with the actin cytoskeleton (35) and that EPAC1 activation is also involved in actin cytoskeleton organization (36), these results suggest that the previously described increase in CFTR:NHERF1 interaction (11) under EPAC1 activation is related to the formation of macromolecular complexes that stabilize the anchoring of CFTR to the actin cytoskeleton.

From the 110 EPAC1-specific CIPs, we selected 18 for validation based on a “Confidence score” (based on confidence levels and robustness of the MS data) and on the distance to CFTR and EPAC1 supported by extensive literature-mining (Table S6). This set of 18 proteins includes mostly proteins related to the actin cytoskeleton, such as: actin related protein 3 homolog B (ACTR3B), which has 89% identity with the Arp2/3 complex member Arp3 (37); caldesmon (CALD1), an actin-binding protein that influences contractibility by interfering with focal adhesion and stress fiber assembly (38); catenin alpha 1 (CTNNA1), a member of catenin family of proteins which affects actin dynamics by limiting Arp2/3-based polymerization (39); myosin-1D (MYO1D), a monomeric actin-based motor protein that plays a role in membrane trafficking and in the control of membrane tension (40), previously shown to regulate CFTR trafficking (41); vinculin (VCL), an actin-binding protein involved in the mechanical coupling among the actin cytoskeleton and the extracellular matrix (42); zyxin (ZYX), an adhesion protein that regulates actin assembly (43); as well as the inverted formin INF2 and the capping protein CAPZA2.

To evaluate the relative proximity between CFTR, EPAC1 and the 18 selected CIPs, we
refined the analysis to create a minimal network needed to link those 18 CIPs (Figure 2). Only 8 additional proteins (XPO1, RAP1A, EZR, CDK2, PPP1CB, PSMC4, ACTB and PAN2) were needed to connect the selection. Interestingly, EZR, a well known CFTR and NHERF1 interactor (11, 44), was one of these added proteins. Among these added proteins, ACTB and XPO1 were also previously described as CFTR interactors (45, 46). RAP1A was also added to build the minimal network – despite not being found as a direct CIP, it is regulated by EPAC as cAMP induces the GEF activity of EPAC towards RAP1A (47).

We then assessed the impact of the KD of the selected proteins on CFTR PM traffic by an immunofluorescence assay. A major impact (statistically significant) was obtained under knockdown of INF2 and CAPZA2 (Figure 3) under 007-AM, while the remaining siRNAs showed only minor impact on CFTR traffic efficiency. While wt-CFTR traffic was significantly increased for both INF2 siRNAs, both CAPZA2 siRNAs showed a negative impact under EPAC1 activation. These results suggest that INF2 impairs and CAPZA2 positively influences CFTR PM traffic and/or stability under EPAC1 activation.

These two CIPs (INF2 and CAPZA2) were both classified with high “Confidence score” (3 or higher) and, according to protein interaction databases, located at 2- and 3-edge distance from CFTR and EPAC1. To our knowledge, this is the first time that these two proteins are identified as CFTR interactors and that their silencing has an effect in CFTR trafficking. We thus proceeded with additional validation/characterization of these 2 hits.

CAPZ proteins are highly conserved proteins from yeast to humans and are composed of two unrelated subunits - α and β (48). CAPZ proteins plays a key role in Arp2/3 mediated actin polymerization, influencing several cellular processes such as cell migration and cell invasion (48). They control actin dynamics by blocking the addition of actin monomers to the filament end thus effectively terminating its elongation (48, 49). CAPZA2 protein (CAPZ subunit α2) has been associated with cancer aggressiveness (32) and is regulated by different scaffold proteins, such as TNKS1BP1, whose depletion causes a decrease in the association between CAPZA2 and actin filaments.

Our results show that CAPZA2 interacts specifically with wt-CFTR and its KD decreases CFTR PM traffic under EPAC1 activation. We validated these observations using CFTR co-IP followed by detection of CAPZA2, confirming that the interaction is detected only upon EPAC1 activation (Figure 4A). Also, by cell surface biotinylation we showed that CAPZA2 knockdown in combination with the activation of EPAC1 pathway leads to a statistically significant decrease in wt-CFTR at the PM compared to the non-targeting siEGFP (Figure 5). This association suggests a role for CAPZA2 in regulating wt-CFTR at the cell surface. It was reported that CFTR anchoring to the actin filaments is associated to the activation of the small GTPase Rac1 through PIP5K and Arp2/3 such that Arp3 knockdown drastically impairs CFTR PM retention (50). Arp2/3 complex acts as an actin nucleator playing a key role in actin

19
dynamics (51). In the presence of capping proteins, branching by Arp2/3 complex is favoured, whereas capping protein depletion leads to a formation of actin bundles, thus affecting cell shape (51, 52).

The wt-CFTR:CAPZA2 association under EPAC1 activation is observed simultaneously with an increased interaction between EPAC1 and CAPZA2 (Figure 6A). Interestingly, CAPZA2 knockdown does not influence the EPAC1:CFTR interaction (Figure 6C). It is known that under EPAC1 activation, CFTR interacts with NHERF1 which then interacts with EPAC1 and ezrin. NHERF1:ezrin interaction locks CFTR in an immobile, stable and actin-tethered complex preventing its endocytosis (8, 9, 11). Thus, the increased EPAC1:CAPZA2 interaction verified under EPAC1 activation (Figure 6A) indicates that CAPZA2 further potentiate the CFTR anchoring at the PM. However, the fact that CAPZA2 KD does not influence CFTR:EPAC1 interaction (Figure 6C) may indicate that the presence of CAPZA2 plays an additive effect on CFTR anchoring at the PM and it is not a key element for the maintenance of the complex. We thus suggest that CAPZA2 promotes the anchoring of wt-CFTR at the PM by stabilizing actin cytoskeleton which consequently leads to a stabilization of the CFTR-NHERF1-Ezrin-EPAC1 complex (Figure 8, Stage I).

INF2 is unique among the formins due to its ability to accelerate depolymerization, in addition to the nucleation and elongation activity characteristic of most formins (31, 53).

Our results show that CFTR interacts with INF2, an association that is increased upon EPAC1 activation and that involves mainly the mature form of CFTR (Band C) (Figure 4C). Such association suggests that INF2 may participate in late stages of CFTR traffic. This effect was confirmed using cell surface biotinylation with INF2 KD (in the presence of 007-AM) promoting a statistically significant increase in wt-CFTR at the PM as compared to the control (Figure 5).

Biochemically, the FH2 domain of INF2 can accelerate significantly the actin nucleation regulating elongation by remaining processively bound to the fast-growing barbed end of the filaments. The FH1 domain binds the actin monomer-binding protein profilin, accelerating the elongation rate of the FH2-bound barbed end (31, 54). INF2 depolymerization activity is dependent on its unique C-terminus which contains an actin-binding WASP homology 2 (WH2) motif that sequester actin monomers in 1:1 complex. INF2 C-terminus is also required for fast filament severing (53). INF2 severing ability is directly associated with the increased rate of actin filament depolymerization since it increases the number of depolymerizable ends (53). This occurs as the FH2 domain of INF2 inhibits the barbed end capping by capping proteins (53).

This triphasic function suggests that the role of INF2 expression may depend on the context/environment, as increased INF2 overexpression has been associated with cell death (55) but also with cell migration, invasion and proliferation (56).
To further understand the mechanism through which INF2 impacts on CFTR PM stability under EPAC1 activation, we determined that EPAC1 and INF2 also interact (Figure 6A) but that, as described above for CAPZA2, INF2 knockdown does not affect the EPAC1:CFTR interaction (Figure 6C). These results suggest that INF2 is involved in CFTR anchoring at the PM but it may not be a key element in CFTR-EPAC1 complex maintenance. Thus, we suggest that INF2 hampers the anchoring of wt-CFTR at the PM by improving actin cytoskeleton dynamics which interfere with stabilization of the CFTR-EPAC1 complex (Figure 8, Stage II).

Our results, together with reports showing that INF2 is localized mainly in the cytoplasm (57, 58), suggest that the impact of INF2 in CFTR regulation may be associated with depolymerization events at the barbed ends of the filaments or with INF2-mediated severing near those ends. In both cases, INF2 activity likely affects actin cytoskeleton dynamics in a negative way which may explain the positive impact of INF2 KD observed in wt-CFTR traffic under EPAC1 activation – an effect that can also promote an increase in the rescue of F508del-CFTR (Figure 7).

We propose that CAPZA2 stabilizes the barbed end of actin filaments promoting wt-CFTR anchoring at the PM. On the other hand, our results suggest that INF2 negatively regulates CFTR anchoring at the PM through polymerization events at the barbed ends of the filaments or with INF2-mediated severing near to the barbed ends. Thus, considering our results and the fact that CAPZA2 and INF2 were simultaneously identified as CFTR interactors under EPAC1 activation, we may hypothesize a combined effect for the two proteins (Figure 8, Stage III). So, altogether our data suggest that while CAPZA2 stabilizes the barbed ends of actin filaments, INF2 binds laterally near to the barbed ends of the actin filaments promoting its severing. With an opposite effect, INF2 and CAPZA2 together promote a balanced effect on the regulation of actin cytoskeleton dynamics and thus of CFTR PM stability under EPAC1 activation (Figure 8, Stage III).

Despite the combined model proposed for CAPZA2/INF2 regulation of CFTR anchoring at the PM, a competitive model cannot be excluded. Interestingly, several studies report a competition between formins and capping proteins for the actin filaments barbed ends (59, 60). In fission yeast, cytokinesis formin Cdc12p competes with capping protein for the actin barbed end through a profilin mediated process (60). In human podocytes, INF2 interacts with profilin 2 and with the F-actin capping protein CAPZA1 playing a critical role in the tight regulation of actin and microtubule dynamics (61). Such reports suggest that the opposite effects of CAPZA2 and INF2 in regulating CFTR PM levels under EPAC1 activation may also be explained by a competition or at least a contrasting role in binding actin filaments barbed ends in bronchial epithelial cells.

In general the results presented here confirm the role of the cytoskeleton-related interactions in regulating CFTR PM levels and stability and identify such processes as relevant
for the increase of PM CFTR stability after an increase of cAMP and concomitant activation of EPAC1.
Acknowledgments & Funding

Work supported by center grant UID/MULTI/04046/2019 to BioISI and grant PTDC/BIA-CEL/28408/2017 from FCT, Portugal. J.D.S was recipient of PD/BD/106084/2015 PhD fellowship from BioSys PhD programme (FCT, Portugal).

Author contributions

J.D.S. designed and performed the experiments, analyzed data and wrote the paper; F.R.P. guided the experiments design for bioinformatic analysis and provided advice; J.F.F. designed and performed the experiments and analyzed data; M.D.A and M.Z. provided advice and comments on paper; C.M.F guided the project, guided the experiments design and reviewed the manuscript.

Conflict of interest

There is no conflict of interest to disclose.
References

1. Collins FS. Cystic fibrosis: molecular biology and therapeutic implications. Science. 1992;256:774-9.

2. Farinha CM, King-Underwood J, Sousa M, Correia AR, Henriques BJ, Roxo-Rosa M, et al. Revertants, Low Temperature, and Correctors Reveal the Mechanism of F508del-CFTR Rescue by VX-809 and Suggest Multiple Agents for Full Correction. Chem Biol. 2013;20(7):943-55.

3. Riordan JR. CFTR function and prospects for therapy. Annu Rev Biochem. 2008;77:701-26.

4. Rommens JM, Iannuzzi MC, Kerem B, Drumm ML, Melmer G, Dean M, et al. Identification of the cystic fibrosis gene: chromosome walking and jumping. Science. 1989;245(4922):1059-65.

5. Farinha CM, Canato S. From the endoplasmic reticulum to the plasma membrane: mechanisms of CFTR folding and trafficking. Cell Mol Life Sci. 2017;74(1):39-55.

6. Okiyoneda T, Barriere H, Bagdany M, Rabeh WM, Du K, Hohfeld J, et al. Peripheral protein quality control removes unfolded CFTR from the plasma membrane. Science. 2010;329(5993):805-10.

7. Haggie PM, Kim JK, Lukacs GL, Verkman AS. Tracking of quantum dot-labeled CFTR shows near immobilization by C-terminal PDZ interactions. Mol Biol Cell. 2006;17(12):4937-45.

8. Farinha CM, Matos P, Amaral MD. Control of cystic fibrosis transmembrane conductance regulator membrane trafficking: not just from the endoplasmic reticulum to the Golgi. FEBS J. 2013;280(18):4396-406.

9. Guggino WB, Stanton BA. New insights into cystic fibrosis: molecular switches that regulate CFTR. Nat Rev Mol Cell Biol. 2006;7(6):426-36.

10. Kwon SH, Pollard H, Guggino WB. Knockdown of NHERF1 enhances degradation of temperature rescued DeltaF508 CFTR from the cell surface of human airway cells. Cell Physiol Biochem. 2007;20(6):763-72.

11. Lobo MJ, Amaral MD, Zaccolo M, Farinha CM. EPAC1 activation by cAMP stabilizes CFTR at the membrane by promoting its interaction with NHERF1. J Cell Sci. 2016;129(13):2599-612.

12. de Rooij J, Rehmann H, van Triest M, Cool RH, Wittinghofer A, Bos JL. Mechanism of regulation of the Epac family of cAMP-dependent RapGEFs. J Biol Chem. 2000;275(27):20829-36.

13. Monterisi S, Favia M, Guerra L, Cardone RA, Marzulli D, Reshkin SJ, et al. CFTR regulation in human airway epithelial cells requires integrity of the actin cytoskeleton and compartmentalized cAMP and PKA activity. J Cell Sci. 2012;125(Pt 5):1106-17.

14. Ulucan C, Wang X, Baljinnyam E, Bai Y, Okumura S, Sato M, et al. Developmental changes in gene expression of Epac and its upregulation in myocardial hypertrophy. Am J Physiol Heart Circ Physiol. 2007;293(3):H1662-72.
15. Schmidt M, Dekker FJ, Maarsingh H. Exchange protein directly activated by cAMP (epac): a multidomain cAMP mediator in the regulation of diverse biological functions. Pharmacol Rev. 2013;65(2):670-709.

16. Lerias JR, Pinto MC, Botelho HM, Awatade NT, Quaresma MC, Silva IAL, et al. A novel microscopy-based assay identifies extended synaptotagmin-1 (ESYT1) as a positive regulator of anoctamin 1 traffic. Biochim Biophys Acta Mol Cell Res. 2018;1865(2):421-31.

17. Hoque KM, Woodward OM, van Rossum DB, Zachos NC, Chen L, Leung GP, et al. Epac1 mediates protein kinase A-independent mechanism of forskolin-activated intestinal chloride secretion. J Gen Physiol. 2010;135(1):43-58.

18. Awatade NT, Ramalho S, Silva IAL, Felicio V, Botelho HM, de Poel E, et al. R560S: A class II CFTR mutation that is not rescued by current modulators. J Cyst Fibros. 2019;18(2):182-9.

19. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009;4(1):44-57.

20. Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 2009;37(1):1-13.

21. Team RC. R: A language and environment for statistical computing Vienna, Austria. : R Foundation for Statistical Computing; 2018 [Available online at https://www.R-project.org/].

22. Alonso-Lopez D, Gutierrez MA, Lopes KP, Prieto C, Santamaria R, De Las Rivas J. APID interactomes: providing proteome-based interactomes with controlled quality for multiple species and derived networks. Nucleic Acids Res. 2016;44(W1):W529-35.

23. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 2003;13(11):2498-504.

24. Canato S, Santos JD, Carvalho AS, Aloria K, Amaral MD, Matthiesen R, et al. Proteomic interaction profiling reveals KIFC1 as a factor involved in early targeting of F508del-CFTR to degradation. Cell Mol Life Sci. 2018;75(24):4495-509.

25. Botelho HM, Uliyakina I, Awatade NT, Proenca MC, Tischer C, Sirianant L, et al. Protein traffic disorders: an effective high-throughput fluorescence microscopy pipeline for drug discovery. Sci Rep. 2015;5:9038.

26. Carpenter AE, Jones TR, Lamprecht MR, Clarke C, Kang IH, Friman O, et al. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. Genome Biol. 2006;7(10):R100.

27. Luz S, Cihil KM, Brautigan DL, Amaral MD, Farinha CM, Swiatecka-Urban A. LMTK2-mediated Phosphorylation Regulates CFTR Endocytosis in Human Airway Epithelial Cells. J Biol Chem. 2014;289(21):15080-93.

28. Hutt DM, Loguercio S, Campos AR, Balch WE. A Proteomic Variant Approach (ProVarA) for Personalized Medicine of Inherited and Somatic Disease. J Mol Biol. 2018;430(18 Pt A):2951-73.

25
29. Pankow S, Bamberger C, Calzolari D, Martinez-Bartolome S, Lavallee-Adam M, Balch WE, et al. F508 CFTR interactome remodelling promotes rescue of cystic fibrosis. Nature. 2015;528(7583):510-6.

30. Lamm KYB, Johnson ML, Baker Phillips J, Muntifering MB, James JM, Jones HN, et al. Inverted formin 2 regulates intracellular trafficking, placentation, and pregnancy outcome. Elife. 2018;7.

31. Ramabhadran V, Hatch AL, Higgs HN. Actin monomers activate inverted formin 2 by competing with its autoinhibitory interaction. J Biol Chem. 2013;288(37):26847-55.

32. Barron-Casella EA, Torres MA, Scherer SW, Heng HH, Tsui LC, Casella JF. Sequence analysis and chromosomal localization of human Cap Z. Conserved residues within the actin-binding domain may link Cap Z to gelsolin/severin and profilin protein families. J Biol Chem. 1995;270(37):21472-9.

33. Swiatecka-Urban A, Brown A, Moreau-Marquis S, Renuka J, Coutemarsh B, Barnaby R, et al. The short apical membrane half-life of rescued {Delta}F508-cystic fibrosis transmembrane conductance regulator (CFTR) results from accelerated endocytosis of {Delta}F508-CFTR in polarized human airway epithelial cells. J Biol Chem. 2005;280(44):36762-72.

34. Farinha CM, Matos P. Repairing the basic defect in cystic fibrosis - one approach is not enough. FEBS J. 2016;283(2):246-64.

35. Castellani S, Favia M, Guerra L, Carbone A, Abbattiscianni AC, Di Gioia S, et al. Emerging relationship between CFTR, actin and tight junction organization in cystic fibrosis airway epithelium. Histol Histopathol. 2017;32(5):445-59.

36. Kato Y, Yokoyama U, Yanai C, Ishige R, Kurotaki D, Umemura M, et al. Epa1 Deficiency Attenuated Vascular Smooth Muscle Cell Migration and Neointimal Formation. Arterioscler Thromb Vasc Biol. 2015;35(12):2617-25.

37. Shindo-Okada N, Shimizu K. Isolation of a novel actin-related gene expressed in low-metastatic PC-14 human lung adenocarcinoma. Biochem Biophys Res Commun. 2001;280(1):61-7.

38. Foster DB, Huang R, Hatch V, Craig R, Graceffa P, Lehman W, et al. Modes of caldesmon binding to actin: sites of caldesmon contact and modulation of interactions by phosphorylation. J Biol Chem. 2004;279(51):53873-94.

39. Escobar DJ, Desai R, Ishiyama N, Folmsbee SS, Novak MN, Flozak AS, et al. alpha-Catenin phosphorylation promotes intercellular adhesion through a dual-kinase mechanism. J Cell Sci. 2015;128(6):1150-65.

40. Benesh AE, Fleming JT, Chiang C, Carter BD, Tyska MJ. Expression and localization of myosin-1d in the developing nervous system. Brain Res. 2012;1440:9-22.

41. Hegan PS, Kravtsov DV, Caputo C, Egan ME, Ameen NA, Mooseker MS. Restoration of cytoskeletal and membrane tethering defects but not defects in membrane trafficking in the intestinal brush border of mice lacking both myosin Ia and myosin VI. Cytoskeleton (Hoboken). 2015;72(9):455-76.

42. Le Clainche C, Dwivedi SP, Didry D, Carlier MF. Vinculin is a dually regulated actin filament barbed-end-capping and side-binding protein. J Biol Chem. 2010;285(30):23420-32.
43. Oldenburg J, van der Krogt G, Twiss F, Bongaarts A, Habani Y, Slotman JA, et al. 
VASP, zyxin and TES are tension-dependent members of Focal Adherens Junctions independent of the alpha-catenin-vinculin module. Sci Rep. 2015;5:17225.

44. Li J, Dai Z, Jana D, Callaway DJ, Bu Z. Ezrin controls the macromolecular complexes formed between an adapter protein Na+/H+ exchanger regulatory factor and the cystic fibrosis transmembrane conductance regulator. J Biol Chem. 2005;280(45):37634-43.

45. Colas J, Faure G, Saussereau E, Trudel S, Rabeh WM, Bitam S, et al. Disruption of cytokeratin-8 interaction with F508del-CFTR corrects its functional defect. Hum Mol Genet. 2012;21(3):623-34.

46. Wang X, Venable J, LaPointe P, Hutt DM, Koulov AV, Coppinger J, et al. Hsp90 cochaperone Aha1 downregulation rescues misfolding of CFTR in cystic fibrosis. Cell. 2006;127(4):803-15.

47. de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, et al. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature. 1998;396(6710):474-7.

48. Jo YJ, Jang WI, Nangoong S, Kim NH. Actin-capping proteins play essential roles in the asymmetric division of maturing mouse oocytes. J Cell Sci. 2015;128(1):160-70.

49. Caldwell JE, Heiss SG, Mermall V, Cooper JA. Effects of CapZ, an actin capping protein of muscle, on the polymerization of actin. Biochemistry. 1989;28(21):8506-14.

50. Moniz S, Sousa M, Moraes BJ, Mendes AI, Palma M, Barreto C, et al. HGF stimulation of Rac1 signaling enhances pharmacological correction of the most prevalent cystic fibrosis mutant F508del-CFTR. ACS Chem Biol. 2013;8(2):432-42.

51. McMillen LM, Vavylonis D. Model of turnover kinetics in the lamellipodium: implications of slow- and fast-diffusing capping protein and Arp2/3 complex. Phys Biol. 2016;13(6):066009.

52. Durre K, Keber FC, Bleicher P, Brauns F, Cyron CJ, Faix J, et al. Capping protein-controlled actin polymerization shapes lipid membranes. Nat Commun. 2018;9(1):1630.

53. Chhabra ES, Higgs HN. INF2 Is a WASP homology 2 motif-containing formin that severs actin filaments and accelerates both polymerization and depolymerization. J Biol Chem. 2006;281(36):26754-67.

54. Paul AS, Pollard TD. Review of the mechanism of processive actin filament elongation by formins. Cell Motil Cytoskeleton. 2009;66(8):606-17.

55. Chen Z, Wang C, Yu N, Si L, Zhu L, Zeng A, et al. INF2 regulates oxidative stress-induced apoptosis in epidermal HaCaT cells by modulating the HIF1 signaling pathway. Biomed Pharmacother. 2019;111:151-61.

56. Heuser VD, Mansuri N, Mogg J, Kurki S, Repo H, Kronqvist P, et al. Formin Proteins FHOD1 and INF2 in Triple-Negative Breast Cancer: Association With Basal Markers and Functional Activities. Breast Cancer (Auckl). 2018;12:1178223418792247.

57. Chhabra ES, Ramabhadrnan V, Gerber SA, Higgs HN. INF2 is an endoplasmic reticulum-associated formin protein. J Cell Sci. 2009;122(Pt 9):1430-40.
58. Shao X, Kawauchi K, Shivashankar GV, Bershadsky AD. Novel localization of formin mDia2: importin beta-mediated delivery to and retention at the cytoplasmic side of the nuclear envelope. Biol Open. 2015;4(11):1569-75.

59. Harris ES, Li F, Higgs HN. The mouse formin, FRLalpha, slows actin filament barbed end elongation, competes with capping protein, accelerates polymerization from monomers, and severs filaments. J Biol Chem. 2004;279(19):20076-87.

60. Kovar DR, Wu JQ, Pollard TD. Profilin-mediated competition between capping protein and formin Cdc12p during cytokinesis in fission yeast. Mol Biol Cell. 2005;16(5):2313-24.

61. Rollason R, Wherlock M, Heath JA, Heesom KJ, Saleem MA, Welsh GI. Disease causing mutations in inverted formin 2 regulate its binding to G-actin, F-actin capping protein (CapZ alpha-1) and profilin 2. Biosci Rep. 2016;36(1):e00302.
Table 1. Summary of the number of proteins identified in each condition

|                         | Non-treated | DMSO  | Fsk   | 007-AM |
|-------------------------|-------------|-------|-------|--------|
| wt-CFTR CFBE (Ab)       |             | 311   | 263   | 399    |
| wt-CFTR CFBE (no Ab)    | 251         | -     | -     | -      |
| CFBE parental cells     | 374         | -     | -     | -      |
| “Condition Specific” CIPs* | -         | 62    | 23    | 101    |

*CIPs – CFTR-interacting proteins.
Legends to Figures

Fig. 1 Analysis of GO terms for the specific CFTR interacting proteins under EPAC1 activation using DAVID. From the total of 1599 proteins, 110 specific CFTR interacting proteins under EPAC1 activation were used to find the GO Terms: A) Biological process and B) Cellular component.

Fig. 2 Minimal network needed to connect the 18 selected hits to CFTR, NHERF1 and EPAC1. RAP1A, XPO1, EZR, CDK2, PPP1CB, PSMC4, ACTB and PAN2 (represented in blue) are the 8 proteins needed to link the 18 selected hits for validation (represented in red) to CFTR, EPAC1 and NHERF1 (represented in yellow). R programming was used to calculate the shortest path between all the 18 proteins to CFTR, EPAC1 and NHERF1. Cytoscape was used to visualize the network using APID1 PPIs as the background.

Fig. 3 CFTR trafficking efficiency under knockdown of the 18 selected hits. CFBE cell lines expressing mCherry-Flag-wt-CFTR under the control of a doxycycline-inducible promoter were transfected with siRNAs targeting the 18 selected hits. COPB1, EPAC1 and EGFP (non-targeting siRNA) siRNA were used as controls. A) Cells were incubated with DMSO (vehicle control) for 2h (representative images). B) Cells were incubated with 007-AM (1µM) for 2h. C) CFTR trafficking was given by the ratio of PM CFTR (Cy5)/TOTAL CFTR (mCherry). The deviation score was calculated and plotted. Data are shown as mean ± SEM, n=3. * p<0.05. Statistical analysis was performed using two-tailed unpaired Student’s t-test.

Fig. 4 Interaction between CFTR and CAPZA2 or INF2. Co-IP was performed using CFBE cells expressing wt-CFTR incubated with 007-AM (2h, 1 µM) or DMSO (vehicle control). Cell lysates incubated with non-conjugated beads were used as a control. A) CAPZA2 was detected by WB after IP of CFTR (CFTR 596 antibody cross-linked to rProtein G agarose beads). GAPDH was used as the loading control. B) Quantification of CFTR:CAPZA2 interaction was performed by calculating the CAPZA2 detected normalized to the amount of IP CFTR. C) CFTR was detected by WB after IP of INF2 (Rabbit polyclonal anti-INF2 antibody cross-linked to rProtein G agarose beads). Calnexin was used as the loading control. D) Quantification of INF2:CFTR interaction was performed by calculating the CFTR detected normalized to the amount of INF2 immunoprecipitated. 20% of the total lysate was analysed as WCL. Data are
shown as the mean ± SEM, n=3. * p<0.05. Statistical analysis was performed using two-tailed unpaired Student’s t-test.

**Fig. 5 Impact of INF2 and CAPZA2 knockdown in the levels of wt-CFTR at the cell surface under EPAC1 activation.** Cell surface biotinylation assay was performed using CFBCE cells expressing wt-CFTR transfected with a siRNA against INF2, CAPZA2 or EGFP (non-targeting siRNA) and treated with 007-AM (2h, 1 µM) or DMSO (vehicle control). Cells were incubated with biotin and then cell lysates were incubated overnight with streptavidin beads. Cells not incubated with biotin solution were used as a control. A) CFTR was detected by WB after streptavidin pulled-down. 20% of the total lysate was analysed as WCL. αTubulin was used as the loading control and Ezrin was used as an intracellular protein control. B) Quantification of CFTR detected after the pull-down was performed and the change (Δ) normalized to DMSO was plotted. Data are shown as the mean ± SEM, n=3. * p<0.05. Statistical analysis was performed using two-tailed unpaired Student’s t-test.

**Fig. 6 Impact of EPAC1 in CFTR and INF2 or CAPZA2 interaction.** Interaction between EPAC1 and CAPZA2 or INF2 were analysed by Co-IP using CFBCE cells expressing wt-CFTR incubated with 007-AM (2h, 1 µM) or DMSO (vehicle control). Cell lysates incubated with non-conjugated beads were used as a control. A) CAPZA2 and INF2 were detected by WB after EPAC1 IP. GAPDH was used as the loading control. B) Quantification of EPAC1:CAPZA2 or EPAC1:INF2 interaction was performed by calculating the CAPZA2 or INF2 detected normalized to the amount of EPAC1 immunoprecipitated. The impact of INF2 or CAPZA2 knockdown in CFTR:EPAC1 interaction was analysed by Co-IP using CFBCE cells expressing wt-CFTR transfected with an siRNA against INF2 or CAPZA2 and incubated with 007-AM (2h, 1 µM). Cell lysates from CFBCE cells incubated with non-conjugated beads were used as a control. C) EPAC1 was detected by WB after CFTR immunoprecipitated. GAPDH was used as the loading control. D) Quantification of CFTR:EPAC1 interaction was performed by calculating the EPAC1 detected normalized to the amount of CFTR immunoprecipitated. 20% of the total lysate was analysed as WCL. The results are normalized to DMSO and using the mean ± SEM, n=3. * p<0.05. Statistical analysis was performed using two-tailed unpaired Student’s t-test.

**Fig. 7 Impact on INF2 knockdown in the rescue of F508del-CFTR.** CFBCE cells overexpressing F508del-CFTR were transfected with siRNA against INF2 or EGFP (as control).
24h post transfection, treatment with VX-661 was initiated for 24h. 2h prior to extraction, cells were treated with 007-AM. A) CFTR, INF2 and calnexin (loading control) were detected by WB. B) Quantification of the amount of mature CFTR (band C) normalizaed to the loading control and to siRNA EGFP, using the mean ± SEM, n=4. * p<0.05. Statistical analysis was performed using two-tailed unpaired Student’s t-test.

Fig. 8 Role for CAPZA2 and INF2 in the regulation of CFTR anchoring at the PM. Stage I) CFTR-EPAC1 complex is inserted at the PM through an actin filament stabilized by CAPZA2; Stage II) INF2 binds laterally to the actin filament promoting it severing; Stage III) Actin filament severing increases the number of depolymerizable pointed ends which hampers the CFTR anchoring at the PM. The combination of the 3 stages contribute to the maintenance of cellular homeostasis.
