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**Pharmacological chaperone-rescued cystic fibrosis CFTR-F508del mutant overcomes PRAF2-gated access to endoplasmic reticulum exit sites**

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## **Abstract**

The endoplasmic reticulum exit of some polytopic plasma membrane proteins (PMPs) is controlled by arginin-based retention motifs. PRAF2, a gatekeeper which recognizes these motifs, was shown to retain the GABA<sub>B</sub>-receptor GB1 subunit in the ER. We report that PRAF2 can interact on a stoichiometric basis with both wild type and mutant F508del Cystic Fibrosis (CF) Transmembrane Conductance Regulator (CFTR), preventing the access of newly synthesized cargo to ER exit sites. Because of its lower abundance, compared to wild type CFTR, CFTR-F508del recruitment into COPII vesicles is suppressed by the ER-resident PRAF2. We also demonstrate that some pharmacological chaperones that efficiently rescue CFTR-F508del loss of function in CF patients, target CFTR-F508del retention by PRAF2 operating with various mechanisms. Our findings open new therapeutic perspectives for diseases caused by the impaired cell surface trafficking of mutant PMPs, which contain RXR-based retention motifs that might be recognized by PRAF2.

## **Keywords**

CFTR, gatekeeper, corrector, Sec 31, Sec 24, protein export, ERES, arginin-based retention motif, BRET

## Introduction

Control-quality checkpoints regulate the delivery of neo-synthesized polytopic plasma membrane proteins (PMPs), such as receptors, ion channels or transporters, from the Endoplasmic Reticulum (ER) to the plasma membrane [1-3]. Cargo PMPs are loaded in coatomer COPII transport vesicles at ER exit sites (ERES) [4]. Cargo capture requires the recognition of export signals, such as di-acidic DXE/D motifs [5], by the COPII vesicle adaptor protein Sec24 [6]. Retention mechanisms in the ER, on the other hand, control cargo access to COPII vesicles. Arginine-based (RXR) retention motifs, which supposedly mediate cargo interaction with putative ER resident gatekeepers, were identified in inwardly rectifying potassium channels [7] and in the GB1 subunit of the GABA<sub>B</sub> receptor [8]. We previously found that the Prenylated Rab Acceptor Family member 2 (PRAF2), an ubiquitous membrane-associated protein [9] particularly abundant in the ER [10], was the gatekeeper that retains the GB1 GABA<sub>B</sub> receptor subunit in the ER via the recognition of a specific RXR-di-leucine (LL) motif [11]. PRAF2 tightly controls cell-surface GABA<sub>B</sub> density and function in neurons, but its precise point of impact within the secretory pathway remained unknown.

Mutations, which cause abnormal folding and/or trafficking defects, reduce PMP cell surface abundance and may lead to human diseases. The cystic fibrosis (CF) transmembrane conductance regulator (CFTR) is a prototypical PMP with multiple disease-associated mutations. This cAMP-regulated chloride (Cl<sup>-</sup>) channel, member of the ATP-binding cassette (ABC) transporter superfamily, is composed of 2 membrane-spanning domains (MSD1 and MSD2), each containing six transmembrane segments, which form the pore of the channel, two cytoplasmic nucleotide-binding domains (NBD1 and NBD2) and a regulatory domain [12]. More than 2000 single or multiple non-synonymous mutations throughout the sequence of the *CFTR* coding region have been identified in patients with variable forms of CF (<https://cftr2.org>). The deletion of the Phe-508 residue in the NBD1 domain (p.Phe508Del, CFTR-F508del thereafter) is found in more than 80% of the patients. This deletion causes a folding defect that compromises both NBD1 stability and channel assembly [13, 14]. It also induces ER retention and enhances degradation by the ER quality-control system [15], so

that only minimal residual levels of mature fully-glycosylated CFTR-F508del reach the cell surface [13]. Sec24-dependent accumulation of CFTR in COPII vesicles involves a DXD/E (DAD) motif located at position 565-567 in its NBD1 domain [16]. Conversely, partially characterized RXR-driven retention mechanisms prevent CFTR leaving the ER, the mutagenesis of these motifs partly rescuing CFTR-F508del transport to the cell surface [17-20]. Pharmacological chaperones (or correctors), which increase cell-surface release of CFTR-F508del by improving its folding, processing and trafficking were developed to treat CF patients [21]. The combination of two correctors with a potentiator that augment channel gating led to an unprecedented clinical improvement [22] even if a full recovery of channel function is not obtained [23]. Despite the recent identification of corrector binding sites [24, 25], the molecular mechanisms of their effect have not been completely elucidated.

Here we have uncovered the involvement of PRAF2 in the RXR-dependent retention of CFTR in the ER. PRAF2 binds to and retains both wild type CFTR (CFTR-WT) and CFTR-F508del upstream of ERES. Reducing the concentration of endogenous PRAF2 or mutation of the PRAF2-interaction motif both enhanced the maturation of CFTR-F508del. Moreover, second generation pharmacological chaperones used to treat CF patients targeted PRAF-2 dependent CFTR-F508del retention.

Our study unravels unknown mechanistic aspects of cargo-PMP transport via the ERES and provides key information on how some pharmacological chaperones used in the treatment of CF patients operate.

## METHODS

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Stefano Marullo ([stefano.marullo@inserm.fr](mailto:stefano.marullo@inserm.fr))

### Materials availability

This study did not generate new unique reagents.

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human Embryonic Kidney 293 (HEK-293) cell (ATCC®) were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% FBS (Sigma) and 1% Penicillin-Streptomycin (Gibco) at 37°C in 5% CO<sub>2</sub> humidified chamber. The human bronchial epithelial CFBE41o- parental cell line, which express low amounts of endogenous CFTR-F508del protein [26] was cultured in minimum essential medium (MEM; Gibco) with 10% FBS (Sigma) and 1% Penicillin-Streptomycin (Gibco) at 37°C in 5% CO<sub>2</sub> humidified chamber.

CFBE41o-WT-CFTR (HS-YFP) and CFBE41o-F508del-CFTR (HS-YFP) cell lines were both derived CFBE41o- cells. They respectively express exogenous CFTR-WT and CFTR-F508del in addition to an YFP-tagged halide sensor [27]. They were cultured in minimum essential medium (MEM 31095029; Gibco) with 10% FBS (F7524; Sigma) and 1% Penicillin-Streptomycin (15140-122; Gibco) supplemented with 1 or 2 µg/ml puromycin (Gibco), for CFBE41o-WT-CFTR (HS-YFP) and CFBE41o-F508del-CFTR (HS-YFP), respectively) at 37°C in 5% CO<sub>2</sub> humidified chamber. Culture of CFBE41o-F508del-CFTR (HS-YFP) cells also required addition of 600 µg/ml G418 (Gibco).

## METHOD DETAILS

### Site-Directed mutagenesis, DNA constructs and siRNAs

The cDNA for CFTR, sub-cloned in pTracer, was described previously [28]. The Rluc-CFTR used in BRET experiments was generated by subcloning in frame a humanized form of Renilla Luciferase (Biosignal; PerkinElmer Life Sciences) upstream of the CFTR cDNA.

QuickChange Site-Directed Mutagenesis kit (Agilent) was used to perform mutagenesis following manufacturer's protocol and all mutated plasmids sequences were verified by DNA sequencing. PRAF2-YFP and PRAF2-V5 were described previously [11]. GB1-Rluc was described previously [29].

The cDNA region coding for the NBD1 of CFTR-F508del was subcloned in the pCMV-tag2B (Agilent). The final construct contains a sequence coding for the Flag epitope followed by the 27 N-terminal amino-acid residues of the ER resident P450 oxidase 2C1 (Addgene, plasmid#79055), which target the NBD1 to the cytoplasmic face of the ER [30].

SMARTpool ON-TARGETplus PRAF2 siRNA (Dharmacon; L-019671) was used to silence PRAF2 expression. As controls siGENOME Non-Targeting siRNA pool (Dharmacon; L-080157) was used.

### **Antibodies, Reagents and Drugs**

The following monoclonal antibodies (mAb) or polyclonal antibodies were used for immunofluorescence or immunoblotting experiments: Anti-CFTR660 mouse ascites fluid (obtained from CF Foundation), Anti-CFTR (24-1) mouse monoclonal antibody (MAB25031; Novus Biologicals), Anti-JM4/PRAF2 rabbit polyclonal antibody (ab53113; Abcam), Anti-V5 mouse monoclonal antibody (R96025; Invitrogen), Anti-Calnexin goat polyclonal antibody (ab192439; Abcam), Anti-Sec31 mouse monoclonal antibody (sc-376587; Santa Cruz), Anti-Vinculin mouse monoclonal antibody (V9131; Sigma), Anti-Na<sup>+</sup>K<sup>+</sup>-ATPase  $\alpha$ 1 mouse monoclonal antibody (sc-514614; Santa Cruz), Anti HSP 70/HSC 70 mouse monoclonal antibody (sc-32239; Santa Cruz), Anti-GAPDH rabbit monoclonal antibody (mAB #2118; Cell Signalling) and Anti- $\beta$ -Tubulin rabbit monoclonal antibody (mAB #2128; Cell Signalling).

The following secondary antibodies were used in western blot experiments: IR Dye® 680 RD Donkey anti-Rabbit antibody (926-68073; Li-cor), IR Dye® 800 CW Donkey anti-Rabbit (926-32213; Li-cor), IR Dye® 800 CW Donkey anti-Mouse (925-32212; Li-cor), IR Dye® 680 RD Donkey anti-Mouse (925-68072; Li-cor).



The following secondary antibodies were used for immunofluorescence experiments: Donkey anti-Rabbit IgG (H+L) Alexa Fluor™ Plus 647 (A32795; Invitrogen), Donkey anti-Mouse IgG (H+L) Alexa Fluor™ 568 (A10037; Invitrogen) and Donkey anti-Goat IgG (H+L) Alexa Fluor Plus 488 (A32814; Invitrogen). The following secondary antibodies were used for STED experiments: Goat anti-mouse IgG Abberior STAR580 (ST580-1001) and Goat anti-rabbit IgG Abberior STAR635P (ST635P-1002). The mounting media was Prolong™ Glass Antifade (P36982; ThermoFisher).

The following reagents were used: Dulbecco's phosphate buffered saline (14190-094; Gibco), Hanks's balanced salt solution (14025-050; Gibco), 0.05% trypsin-EDTA (25300-054; Gibco), coelenterazine h (R3078b; Interchim), cOmplete EDTA free protease tablets (05056489001; Roche), Nonidet P-40 (11754599001; Roche), Pierce™ premium-grade-sulfo-NHS-SS-Biotin (PG82077; ThermoFisher), Strepta-vidin agarose (69203; EMD Millipore), 3-isobutyl-1-methyl-xanthine (IBMX) (I5879; Sigma), 8-(4-chlorophenylthio)-adenosine-3',5'-cyclic monophosphate (cpt-cAMP) (C3912; Sigma), Protein A/G Plus-Agarose (sc-2003; Santa Cruz), Pierce™ BCA protein assay (23225; ThermoFisher), Hoechst (33342; ThermoFisher), Paraformaldehyde 4% in PBS (sc-281692; Chem Cruz), Saponin (84510; Sigma) DMSO (D2650. Sigma), BSA (80763; Roth), Tween 20 (P2287; Sigma), triton X-100 (X100; Sigma) and glycerol (453751; CARLO ERBA). The following were used for microscopy: microscope slides (J1800AMNZ; ThermoFisher) and microscope cover glasses (0107052; Marienfeld).

The following CF correctors or potentiators were used: VX-809 (936727-05-8; AdooQ® Bioscience), VX-770 (S1144; Selleckchem), VX-661 (A12519; AdooQ® Bioscience) and VX-445 (S8851; Selleckchem).

## **Transfections**

CFTR and PRAF2 plasmids were transiently transfected in HEK-293 cells using GeneJuice (Merck Millipore) transfection reagent following the manufacturer's protocol. CFBE cells were

transfected with CFTR and PRAF2 plasmids and siRNA (50 nM) using Lipofectamine 3000 (ThermoFisher) following the manufacturer's protocol.

### **Drug treatment**

CFTR-F508del rescue assays with correctors were performed by treating the cells with VX-809 (3 $\mu$ M), VX-661 (18 $\mu$ M), VX-445 (2 $\mu$ M) for 24 hours before the assays DMSO was used as vehicle control. The potentiator VX-770 (10 $\mu$ M) was added in some experiments to various combinations of correctors as indicated in the figures.

### **Immunoprecipitation and Immunoblotting**

For immunoprecipitation experiments, cells were lysed in a lysis buffer containing 50mM HEPES (pH 7.4; adjusted with NaOH), 250mM NaCl, 2mM EDTA, 0.5% (v/v) Nonidet P-40, 10% (v/v) glycerol and cOmplete protease inhibitor mix. Lysates were cleared by centrifugation at 16200xg at 4°C for 15 minutes. Cell lysates were incubated with anti-V5 antibody (ThermoFisher, R960-25) or anti-PRAF2 antibody (Abcam, ab53113) overnight at 4°C. Immune complexes were collected the following day by incubating with the protein A or G beads for 3 hours at 4°C. The beads were subsequently washed three times and eluted in 2X sample loading buffer at 37°C for 15 minutes. The eluted samples were further analyzed by western blot.

For immunoblotting the cells were lysed in RIPA buffer (25mM Tris-HCl (pH 7.4), 150mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% (w/v) sodium deoxycholate and cOmplete protease inhibitor mix). Lysates were cleared by centrifugation at 16200xg at 4°C for 15 minutes.

The total amount of protein was measured by Pierce<sup>TM</sup> BCA protein assay and 25-50 $\mu$ g protein was separated by SDS-polyacrylamide gel electrophoresis. The samples were divided equally between a 7% gel to separate the CFTR fraction ( $\approx$ 250kDa - 100kDa) and 12% gel to separate the PRAF2 fraction ( $\approx$ 100kDa - 10kDa). The separated proteins were transferred to nitrocellulose membrane. The membranes were blocked in 5% skimmed milk in Tris buffered Saline containing 0.1% Tween-20 (TBST) for 1 hour at room temperature

followed by incubation with the respective primary antibodies (dilution used 1:1000) at 4°C overnight. The next day the membranes were incubated with secondary antibodies from Li-cor for 1 hour at room temperature and labeled material visualized/analyzed in the Odyssey CLx Imager.

Quantification of specific proteins after IP, co-IP and/or western blot experiments was performed using the Image J software. Briefly, images recorded with the imager were opened in Image J and analyzed lane-by-lane using the gel analysis plugins; the obtained pixel areas in each column were measured with the "wand" tool. WB densitometric values were normalized to the loading control. For co-IP experiments, the densitometric values were first normalized to the V5 signal, then the % of Co-IP material was calculated by dividing the the normalized value of the CO-IP signal by that of the normalized input, taking in account the % of loaded material.

### **Surface Biotinylation**

Surface biotinylation was performed on cells following plasmid transfection and drug treatment. The cells were washed thrice in PBS<sup>2+</sup> (PBS supplemented with 1 mM MgCl<sub>2</sub> and 0.1 mM CaCl<sub>2</sub>). The cells were incubated with sulfo-NHS-SS-Biotin in PBS<sup>2+</sup> (0.5 mg/ml) for 30 minutes at 4°C. The unbound biotin was subsequently quenched with 100 mM glycine in PBS<sup>2+</sup>. The cells were lysed in RIPA buffer and the biotinylated fraction was enriched by incubating with streptavidin-Agarose beads for 2 hours at 4°C. The beads were washed three times and eluted in 2X sample loading dye at 37°C for 15 minutes. The eluted samples were further analyzed by western blot.

### **Proximity labeling experiments**

Original TurboID plasmids were from Dr. Alice Ting laboratory (Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA) and were used to prepare the PRAF2-V5-TurboID and C1-TurboID-V5 constructs. HEK-293 cells, grown in T150 flasks at 60-70% confluency, were transfected with TurboID and control constructs

(30µg DNA and 150µL Lipofectamine 3000). The next day transfected cells were labeled using 500µM biotin for 10 min; Labeling was stopped by placing cells on ice and washing five times with ice-cold PBS. Cells were detached by pipetting and cell suspensions were centrifuged at 400xg for 3min. The supernatant was removed, and the pellet was lysed in 1.5 mL RIPA lysis buffer by gentle pipetting and incubating for 5min at 4°C. Lysates were clarified by centrifugation at 9600xg for 10 min at 4°C. 350 µL streptavidin-coated magnetic beads (Pierce), previously washed twice with RIPA buffer, were incubated with 3mg protein from clarified lysates overnight under rotation at 4°C. The beads were subsequently washed twice at 4°C with 1mL of RIPA lysis buffer, then with 1 mL of 1 M KCl, then with 1 mL of 0.1 M Na<sub>2</sub>CO<sub>3</sub>, once with 1 mL of 2 M urea in 10 mM Tris-HCl (pH 8.0), and twice with 1 mL RIPA lysis buffer. The beads were then resuspended in 1 mL fresh RIPA lysis buffer for further processing for LC-MS/MS analysis.

## **Mass Spectrometry**

### Protein digestion

S-Trap<sup>TM</sup> micro spin column (Protifi, Hutington, USA) digestion was performed on half (50µl) of the elution volumes according to manufacturer's instructions. Briefly, samples were reduced with 20mM TCEP and alkylated with 50mM CAA (chloracetamide) for 15min at room temperature. Aqueous phosphoric acid was then added to a final concentration of 2.5% following by the addition of S-Trap binding buffer (90% aqueous methanol, 100mM TEAB, pH7.1). Mixtures were then loaded on S-Trap columns. Four extra washing steps were performed for thorough SDS elimination. Samples were digested with 1µg of trypsin (Promega) at 47°C for 2h30. After elution, peptides were vacuum dried and resuspended in 35µl of 2% ACN, 0.1% formic acid in HPLC-grade water prior to MS analysis.

### NanoLC-MS/MS protein identification and quantification

The tryptic peptides (500ng) were injected on a nanoelute (Bruker Daltonics, Germany) HPLC (high-performance liquid chromatography) system coupled to a timsTOF Pro (Bruker Daltonics, Germany) mass spectrometer. HPLC separation (Solvent A: 0.1% formic acid in

water; Solvent B: 0.1% formic acid in acetonitrile) was carried out at 250nL/min using a packed emitter column (C18, 25 cm×75µm 1.6µm) (IonOpticks, Australia) using a 40min gradient elution (2 to 11% solvent B during 19min; 11 to 16% during 7min; 16% to 25% during 4min; 25% to 80% for 3min and finally 80% for 7min to wash the column). Mass-spectrometric data were acquired using the parallel accumulation serial fragmentation (PASEF) acquisition method. The measurements were carried out over the m/z range from 100 to 1700 Th. The range of ion-mobility values was from 0.8 to 1.3 V s/cm<sup>2</sup> (1/k<sub>0</sub>). The total cycle time was set to 1.17s and the number of PASEF MS/MS scans was set to 10.

#### Data Processing Following LC-MS/MS acquisition

The MS files were processed with the MaxQuant software 1.6.17.0 and searched with Andromeda search engine against the UniProtKB/Swiss-Prot *Homo Sapiens* database (release 01-02-2021, 20396 entries). To search parent mass and fragment ions, we set an initial mass deviation of 4.5 ppm and 20 ppm, respectively. The minimum peptide length was set to 7 amino-acid residues and strict specificity for trypsin cleavage was required, allowing up to two missed cleavage sites. Carbamidomethylation (Cys) was set as fixed modification, whereas oxidation (Met) and N-term acetylation were set as variable modifications. Match between runs was not allowed. LFQ minimum ratio count was set to 2. The false discovery rates (FDRs) at the protein and peptide level were set to 1%. Scores were calculated in MaxQuant as described previously [31]. The reverse and common contaminants hits were removed from MaxQuant output. Proteins were quantified according to the MaxQuant label-free algorithm using intensities [31, 32].

Three independent streptavidin immuno-precipitation replicates for PRAF2-V5-TurboID, C1-TurboID-V5 and untagged PRAF2 cells were analyzed with Perseus software (version 1.6.14.0) freely available at [www.perseus-framework.org](http://www.perseus-framework.org) [33]. The protein intensities were transformed in log<sub>2</sub>, and proteins identified in at least 3 replicates in at least one group were submitted to statistical test (volcano plot, FDR=0.05 and S<sub>0</sub>=0.5) after imputation of the missing value by a Gaussian distribution of random numbers with a standard deviation of

30% relative to the standard deviation of the measured values and 2.5 standard deviation downshift of the mean. Proteins never identified in the negative control (untagged PRAF2) were labelled as "discard".

Differentially abundant proteins enriched in PRAF2-V5-TurboID compared to C1-TurboID-V5 were subjected to bioinformatic analysis using EnrichR software freely available at <https://maayanlab.cloud/Enrichr/> for enrichment of GO terms using Reactome library. Ranking of enriched term was performed using adjusted p-value. Proteins network was built using String database [34] (<https://string-db.org/>) using highest confidence (0.900) for minimum interaction score. The top non-redundant networks enriched according to "local network cluster (STRING)" were selected and highlighted in color.

### **STED microscopy experiments**

Cells were cultured in 22 mm round cover slips and fixed with 4% paraformaldehyde 4% in PBS for 10 minutes at room temperature. The cells were permeabilized with PBS containing 0.05% saponin (PBS-saponin) for 15 minutes at room temperature. The cells were blocked in PBS-Saponin containing 3% BSA (PBS-Saponin-BSA) for 1 hour at room temperature. The cells were subsequently incubated with the primary antibodies in PBS-Saponin-BSA for 1 hour and 30 minutes at room temperature. The cells were subsequently washed three times with PBS-Saponin and were incubated with STED Abberior specific secondary antibodies. The cells were subsequently washed three times with PBS-saponin and mounted on glass slides using Prolong Glass Antifade mounting media.

### **BRET**

Saturation BRET experiments were performed as described [35]. Briefly HEK-293 cells were transfected with a constant amount of the donor plasmid and increasing amount of acceptor plasmid; the total amount of DNA is kept constant with empty vector DNA.

Cells were detached in PBS-EDTA and centrifuged at 750xg for 5 minutes. After resuspension in HBSS cells were plated in 96 well plate and coelenterazine h (5 $\mu$ M) added. The luminescence (filter 485 $\pm$ 10nm) and fluorescence (filter 530 $\pm$ 12.5nm) signals were measured simultaneously in a Mithras fluorescence-luminescence detector. The BRET ratio is the fluorescence signal over the luminescence signal and the specific BRET is obtained by subtracting the signal from cells expressing donor alone. The specific BRET is then multiplied by 1000 to be expressed as milli-Bret. Specific BRET values were then plotted as a function of  $[(YFP-YFP_0)/Rluc]$  where: YFP is the fluorescence measured after excitation at 480 nm; YFP<sub>0</sub> is the background fluorescence measured in cells not expressing the BRET-acceptor; Rluc is the luminescence measured after coelenterazine h addition. The data were analyzed in GraphPad Prism in a non-linear regression equation assuming a single binding site and the BRET<sub>max</sub> and BRET<sub>50</sub> were calculated.

### **YFP halide assay**

CFBE41o-F508del-CFTR (HS-YFP) were plated in 96-well black microplates and transfected with siRNA for 24 hours. After 24 hours of siRNA transfection drug treatment was performed for 24 hours for CFBE41o-F508del-CFTR (HS-YFP) cells. After 24 hours of PRAF2-V5 plasmid transfection drug treatment was done for another 24 hours. The CFBE41o-WT-CFTR (HS-YFP) cells expressing CFTR WT were used as a positive control. After 24 hours of drug treatment the YFP halide assay was performed. The cells were washed two times with PBS-Cl (137mM NaCl, 2.7mM KCl, 8.1mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5mM KH<sub>2</sub>PO<sub>4</sub>, 1.0mM CaCl<sub>2</sub>, 0.5mM MgCl<sub>2</sub>, pH 7.4). The cells were incubated with 100  $\mu$ l PBS-chloride containing 100  $\mu$ M 3-isobutyl-1-methyl-xanthine (IBMX), 100 $\mu$ M 8-(4-chlorophenylthio)-adenosine-3',5'-cyclic monophosphate (cpt-cAMP) with 10  $\mu$ M VX-770 (mentioned in figure legends) for 30 minutes. The plate was transferred to a CLARIOstar plate reader (BMG LABTECH, Germany). Cell fluorescence (excitation: 485nm; emission: 535nm) was continuously measured before and after injection of 200 $\mu$ l of PBS-Nal (137mM NaI, 2.7mM KCl, 8.1mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5mM KH<sub>2</sub>PO<sub>4</sub>, 1.0mM CaCl<sub>2</sub>, 0.5mM MgCl<sub>2</sub>, pH 7.4).

Fluorescence recordings were normalized to initial average values and signal decay was fitted to an exponential function to obtain maximal slope. The maximal slope is represented by conversion to rates of change in intracellular  $I^-$  concentration expressed in mM/s.

### **Quantifications and statistical analysis**

For quantification of % total band B immunoprecipitated (IP) with PRAF2-V5/PRAF2 the following steps were performed. Densitometric quantification of band B (ER-core glycosylated) (10% input) was performed and normalized to the internal loading control. Densitometric quantification of coimmunoprecipitated band B (ER-core glycosylated) was performed. The ratio of coimmunoprecipitated band B vs. normalized band B was determined. The ratio was then multiplied with 100 and expressed as a percentage.

For quantification of % band C and normalization of the data the following steps were performed. Densitometric quantification of band C (Golgi-fully glycosylated) and band B (ER-core glycosylated) was performed. Total protein was obtained as the sum of band C and band B. Total protein was normalized to the internal loading control. The ratio of band C vs total protein was determined and then normalized to CFTR-WT, which is considered as 1. The ratio was then multiplied with 100 and expressed as a percentage.

For quantification of Manders' colocalization coefficient M1 indicating colocalization between PRAF2 and Sec31, PRAF2, and Sec31 images were simultaneously acquired during imaging. The Manders' colocalization coefficient M1 was calculated using the Image J plugin JACoP. The n represents the number of cells analyzed.

For statistical analysis of BRET curves, the one-site specific binding model was compared using extra sum-of-squares F Test for BRET<sub>max</sub> and BRET<sub>50</sub>. The null hypothesis is that BRET<sub>max</sub> or BRET<sub>50</sub> were statistically the same between each data set. The null hypothesis was rejected if the p value was less than 0.05. The exact p value is reported for each experiment.



All data are expressed as mean  $\pm$  SEM or mean  $\pm$  SD. Data sets were compared with one-way ANOVA and multiple comparison test (Sidak's) or t-test and were performed in GraphPad Prism (Prism 9.0).

## Results

### ***PRAF2 interacts in the ER with both -WT and -F508del CFTR***

PRAF2 is abundant in human lung extracts [9] and expressed in primary respiratory cells from both control and CF patients (Supplementary Fig.1). Two principal forms of CFTR-WT are generally visible in immunoblot experiments in primary or transfected cells (Fig. 1a, input): a 145 kDa form (referred to as band B), which corresponds to the immature, core-glycosylated CFTR present in the ER, and a 170-190 kDa form (referred to as band C), corresponding to the fully-glycosylated protein found in the Golgi apparatus or at the plasma membrane [15]. Band C is poorly expressed or even undetectable in cells expressing the misfolded, ER-retained CFTR-F508del. Endogenous PRAF2 co-immunoprecipitated core-glycosylated CFTR-F508del in CFBE41o- cells, a CF human bronchial epithelial cell line derived from a CF patient homozygous for F508del, expressing exogenous CFTR-F508del (Supplementary Fig. 2). In extracts of HEK-293 cells expressing exogenous constructs, V5-epitope tagged PRAF2 (PRAF2-V5) co-immunoprecipitated the bands B of both CFTR-WT and CFTR-F508del (Fig. 1a,b). Importantly, the absence of band C co-immunoprecipitation with PRAF2-V5 in cells expressing CFTR-WT indicates that PRAF2 only interacts with the ER-resident core-glycosylated CFTR. The proximity of CFTR and PRAF2 in intact cells was further examined using Bioluminescence Resonance Energy Transfer (BRET) [36]. Saturation BRET experiments [35, 37] were conducted in HEK-293 cells expressing the BRET donor (*Renilla luciferase*, Rluc) fused to either wild-type or mutant CFTR (Rluc-CFTR-WT and Rluc-CFTR-F508del, respectively) and increasing concentrations of the BRET acceptor (yellow fluorescent protein, YFP) fused to PRAF2 (PRAF2-YFP) (Fig. 1c,d). Hyperbolic saturating curves were obtained in both cases, indicative of specific BRET [35], and thus of CFTR-PRAF2 proximity (<10 nm). BRET<sub>50</sub> (YFP/Rluc value calculated for half-maximal BRET) for CFTR-F508del and CFTR-WT was comparable (1.9±0.2 and 1.7±0.2, respectively) denoting a similar propensity for CFTR-WT and CFTR-F508del to be in proximity with PRAF2. In contrast, the maximal BRET (BRET<sub>max</sub>) measured with CFTR-

F508del was significantly higher than for CFTR-WT ( $197.5 \pm 4.2$  vs.  $110.5 \pm 5.2$  mBRET) (Fig. 1,d). This difference is consistent with the absence of band C co-immunoprecipitation with PRAF2 and confirms that PRAF2 and CFTR only interact in the ER. Indeed, whereas maximal energy can be transferred by the ER-retained Rluc-CFTR-F508del to the predominantly ER-resident PRAF2-YFP acceptor, the fraction of the Rluc-CFTR-WT donor present in the Golgi and at the plasma membrane cannot transfer any energy to it (Fig. 1,c). To examine the impact of PRAF2 interaction in the ER on subsequent CFTR maturation, HEK-293 cells, which contain low concentrations of endogenous PRAF2 [11], were transfected with appropriate concentrations of plasmids to express constant amounts of CFTR-WT and increasing amounts of PRAF2-V5. CFTR-WT was progressively retained in the ER with increasing PRAF2 expression, as shown by both the continued decrease of band C and the increase of band B in immunoblot experiments (Fig. 1e,f). CFTR-WT retention by exogenous PRAF2 was also observed in CFBE41o- cells (Supplementary Fig. 3). In these cells the CF-corrector VX-809 (generic name-Lumacaftor) promoted the partial maturation of CFTR-F508del, as shown by the rescue of about 30% of band C. Interestingly, PRAF2 overexpression inhibited the VX-809 effect, demonstrating that PRAF2 can retain both WT and mutant F508del CFTR in the ER.

### **PRAF2 interaction involves an arginine-based and a di-leucine motif in the NBD1**

We previously established that PRAF2 interaction with the GB1 subunit of the GABA<sub>B</sub> receptor in the ER depends on adjacent RXR and LL motifs in the C-terminal region of the receptor [11]. Previous *in vitro* studies showed that ER retention of the CFTR-F508del could partially be rescued by substitutions within arginine-framed retention signals [17, 18]. We thus examined whether one of them might be involved in CFTR interaction with PRAF2. Among the four arginine-based motifs found in the CFTR sequence, <sup>553</sup>RXR<sup>555</sup>, located in the NBD1 was the most likely candidate, as its removal was shown to be sufficient to restore some cell surface trafficking of CFTR-F508del [20]. Consistently, a larger fraction of CFTR-F508del-R555K (the R555K substitution disrupting the RXR motif) achieved full

glycosylation, compared to CFTR-F508del (% of band C in Fig. 2 a,b). The additional substitution of the adjacent di-leucine motif within the NBD1 by alanine residues (R555K, LL435-436AA) further enhanced the proportion of band C. The partial rescue of CFTR-F508del-R555K, LL435-436AA glycosylation was also observed in CFBE41o- cells (Supplementary Fig. 4). Co-IP experiments were then conducted to examine whether this effect was associated with a decreased interaction with PRAF2. For both CFTR-WT and CFTR-F508del, the R555K, LL435-436AA substitutions significantly reduced the amount of band B co-immunoprecipitated with PRAF2 (Fig. 2c,d). Since both <sup>553</sup>RAR<sub>555</sub> and <sup>435</sup>LL<sub>436</sub> motifs are contained in the NBD1 domain, we generated a construct coding for a Flag-tagged isolated NBD1 domain (Flag-C1-NBD1). The expression of Flag-C1-NBD1 at the surface of the endoplasmic reticulum facing the cytosol was driven by the addition of the first 29 aminoacid residues of cytochrome P450 (C1). Flag-C1-NBD1 could be co-immunoprecipitated with PRAF2 (Fig. 2e). Moreover, the expression of increasing amounts of this construct progressively inhibited the BRET between Rluc-CFTR-F508del, and PRAF2-YFP (Fig. 2f). Together, these data demonstrate that the <sup>553</sup>RAR<sub>555</sub> retention signal and the proximal di-leucine motif in the NBD1 domain are involved in the interaction of WT or F508del CFTR with PRAF2.

### **PRAF2 controls CFTR cargo recruitment into COPII vesicles.**

To characterize more precisely the pathway(s) and the interacting proteins involved in the PRAF2-dependent ER retention of CFTR-WT and CFTR-F508del, we conducted an enzyme-catalyzed proximity labeling experiment using the TurboID approach. TurboID, a promiscuous mutant of *Escherichia coli* biotin ligase with high catalytic efficacy, allows rapid (10 min) and non-toxic biotin proximity labeling in yeast, fly and mammalian cells [38]. Proximity labeling experiments (see Methods) were carried out in triplicate in HEK-293 cells expressing one of the following constructs: i) the PRAF2-TurboID fusion protein (PRAF2-V5-TurboID); ii) the ERM (Endoplasmic Reticulum Membrane)-Turbo ID control (C1-TurboID-V5), with the ligase targeted to the ER membrane facing the cytosol; iii) PRAF2-V5 alone, to

determine background biotinylation (Fig. 3a, left panel). All constructs contained a V5 epitope for the comparative quantification of protein expression (Supplementary Fig. 5a, left panel). Streptavidin blot analysis of whole cell lysates were performed to compare protein biotinylation in cells expressing C1-TurboID-V5 or PRAF2-V5-TurboID (Supplementary Fig. 5a, middle panel). Although global biotinylation was comparable, some molecular species were enriched in either condition. The experiment in cells expressing PRAF2-V5 allowed to appreciate the low number of background labeled proteins. Mass spectrometry analysis identified about 1600 and 2000 proteins (Supplementary Fig. 5a, right panel and Supplementary Table 1) with PRAF2-V5-TurboID and C1-TurboID-V5, respectively, showing important overlap (Supplementary Fig. 5b and c), which was expected for proteins localized totally (C1-TurboID-V5) or partially (PRAF2-V5-TurboID) to the cytoplasmic face of the ER. These sets of proteins were analyzed using the STRING database [39] to identify enriched pathways and predicted protein networks. As expected, these two sets of data showed common enrichments, in pathways involved in protein synthesis, mRNA translation, membrane trafficking (Supplementary Fig. 5b and c). However, the comparison of PRAF2-V5-TurboID- and C1-TurboID-V5-data highlighted 183 proteins, which were significantly enriched in PRAF2 samples (Fig. 3b, Supplementary Table 2). Their classification, using the Gene Ontology (GO) repository "cellular component", showed an enrichment of proteins involved "vesicular trafficking", with the highest enrichment being for COPII-coated vesicles components (Fig. 3c). The STRING network revealed a preferential proximity of PRAF2 with the Sec machinery (in green) as well as SNARE proteins (in red) (Fig. 3b,d). Rab GDP dissociation inhibitor beta, previously associated to PRAF function [40], was also present among the 183 proteins enriched in PRAF2-V5-TurboID samples, compared to the C1-TurboID-V5 control, as well as PRAF3, likely due to the propensity of PRAFs to form hetero-oligomers [41].

Altogether, MS experiments identified a large network of PRAF2-proximity proteins operating at the ER level involved in COPII vesicle cargo loading, coating and budding at ER exit sites (ERES). Stimulation Emission Depletion (STED) microscopy experiments were then

conducted to examine, at high resolution, the proximity of PRAF2 with Sec31, one of the proteins labeled in the proximity assay used as a marker of COPII-coated vesicles. These experiments confirmed that, in the ER, PRAF2 was localized close to COPII vesicles, in proximity of the Sec31 but without superposition (Fig. 3e).

The export of CFTR from the ER was reported to involve a di-acidic (DAD) exit code, which controls the Sec24-dependent accumulation of cargo molecules into COPII vesicles [16]. This DAD motif is located at position 565-567 in the NBD1 domain, close to the RXR motif involved in PRAF2-mediated retention. Consistent with previous observations [16], disruption of this motif (by D567A substitution) inhibited the terminal glycosylation (amount of band C, Fig. 4a,b) as well as surface export (Fig. 4c) of CFTR-WT. It also blocked the export rescue observed for CFTR-F508del-R555K, LL435-436AA, indicating that PRAF-2 retention and Sec-24-recruitment in COPII vesicles operate sequentially in the same canonical ER-exiting pathway.

### **Effect of pharmacological chaperones on PRAF-2 mediated retention of CFTR F508del in the ER**

We investigated whether the pharmacological chaperones developed to treat CF patients, and which are known to increase cell-surface expression of CFTR-F508del, might interfere with PRAF2 retention in the ER.

The reference corrector VX-809 [21] interacts with both the membrane spanning domain 1 (MSD1) [42] and NBD1 [43] to promote intra- and inter-domain folding and assembly [24, 44]. In HEK-293 cells expressing CFTR-F508del, the siRNA-mediated inhibition of PRAF2 (Fig. 5a-c) did not significantly enhance the amount of CFTR-F508del band C, comparatively to the effect promoted by the incubation with VX-809. However, combining both conditions (siRNA-mediated PRAF2 inhibition and VX-809 incubation) resulted in a significantly larger rescue than pharmacological correction alone (Fig. 5a,b). These data indicate that the reported chaperone effects of VX-809 [21], which enhance the flow of CFTR-F508del directed to the cell surface, are likely amplified by simultaneous PRAF2 depletion. To

quantify the functional significance of the increased surface expression of CFTR-F508del upon VX-809 and siRNA-PRAF2 treatment, we used an YFP-based halide sensitive assay [45] in CFBE41o- cells (Fig. 5d,e). The anion channel function of WT or mutant CFTR was determined from the time course of YFP fluorescence quenching in response to the extracellular addition of iodide, which is transported into the cells via the investigated channel. Using this assay, we observed that PRAF2 depletion enhanced the level of CFTR-F508del functional rescue caused by VX-809 alone and that this effect was inhibited by PRAF2 overexpression.

We next examined the possible interplay of new generation pharmacological chaperones developed to treat CF patients [22], with PRAF2-mediated retention of CFTR-F508del. In association with the potentiator VX-770 (generic name-Ivacaftor), the corrector VX-661 (generic name-Tezacaftor), a derivative of VX-809 with similar effects, enhanced the ion transport of CFTR-F508del in function-rescue assays; this effect was augmented by cell pre-incubation with PRAF2 siRNA (Fig. 6a,c). In association with VX-770, the function rescue induced by VX-445, a corrector which suppress the F508del-NBD1 thermal instability *in vitro* [46], was comparatively much larger. However, PRAF2 siRNA treatment did not provide any additional effect in this case (Fig. 6b,c), indicating a different mechanism of action, compared to VX-809 and VX-661. A BRET saturation assay was conducted with Rluc-CFTR-F508del and PRAF2-YFP in the absence or presence of the three correctors (Fig. 7a). Pre-incubation with VX-661 or VX-809 did not significantly affect  $BRET_{max}$  or  $BRET_{50}$  values, whereas both values were markedly modified by pre-incubation with VX-445. The decrease in  $BRET_{max}$  (similar to that observed for CFTR-WT in Fig. 1d) reflected a higher Rluc-CFTR-F508del exit from the ER, whereas the higher  $BRET_{50}$  indicated an inhibiting effect of VX-445 on CFTR-F508del - PRAF2 interaction. Interestingly, pre-incubation with VX-445 also inhibited the ER retention of CFTR-WT by overexpressed PRAF2 (Fig. 7b,c) and the amount of both CFTR-WT and CFTR-F508del co-immuno-precipitating with PRAF2 from cell extracts (Fig. 7d,e). The effect of VX-445 was specific for CFTR, since the drug had no effect in saturation BRET experiments carried with the GB1 subunit of the GABA<sub>B</sub> receptor, which also interacts with

PRAF2. (Supplementary Fig. 6). Together, the data above suggest that VX-445 behaves as a specific negative modulator of CFTR-PRAF2 interaction.



## Discussion

In the present study we have identified the mechanism by which the ER gatekeeper PRAF2 controls CFTR targeting to the cell surface, upstream of its recruitment into ERES. In the ER, PRAF2 appears enriched close to ERES and the ERES-associated molecular machinery involved in cargo recruitment within COPII vesicles. Membrane associated cargo harboring an appropriate RXR / LL motif, such as CFTR (the present work) or GB1 [11], interacts with PRAF2 and is retained on a stoichiometric basis, before it can interact with ERES cargo receptors such as Sec24. Early studies showed that a substantial fraction of CFTR-WT precursors leave the folding pathway, which are rapidly poly-ubiquitinated and targeted to the ER-associated proteasome for degradation [47-49]. Nevertheless, in normal conditions, a sufficient flow of functional channel can largely overcome the subsequent concentration-dependent retention by PRAF2 and reach the ERES before being targeted to the cell surface. The deletion of the Phe residue at position 508 in the NBD1 domain in CFTR-F508del further impairs its folding capacity, as shown by its persistent association with the quality control chaperone calnexin [49, 50] and leads to the degradation of nearly all neo-synthesized mutant channels. Under these conditions endogenous PRAF2 is sufficient to retain most CFTR-F508del upstream of the ERES.

The pharmacological chaperones, which have recently revolutionized the clinical status of CF patients [22], interfere with PRAF2 retention. By improving CFTR-F508del folding, correctors may enhance the flow of mutant channel to the ERES, overriding PRAF2 retention. Some sufficiently folded mutant channels can then reach the cell surface to partially restore Cl-transport [51], which is further enhanced by potentiators. Consistent with this proposed flow-enhancing mechanism, the effect of VX-809 and VX-661 is amplified when the concentration of PRAF2 is decreased. Our data indicate that VX-445 behaves, in addition, as a specific negative modulator of CFTR-PRAF2 interaction. It has recently been proposed that VX-445, directly binds to the CFTR-F508del NBD1 domain reducing its thermo-instability [46]. Since NBD1 also contains the RXR-LL retention motif, which drives the interaction with PRAF2, it can be hypothesized that VX-445 binding in the proximity of this motif (Baatallah et al, 2021)

might be masking it or reducing its affinity for PRAF2 (Supplementary Fig. 7). This mechanism is consistent with the minimal additive functional effect of the simultaneous inhibition of PRAF2, and the reduction in the amount of band B co-immunoprecipitated with PRAF2 in the presence of VX-445. Finally, it has been recently proposed that VX-445 also has a potentiator effect on CFTR-F508del [52]. Overall, the different points of impact of CF correctors, postulated in previous studies [53, 54], likely determine the superior effect of their combination observed in CF patients.

Since PRAF2 appears as a constitutive ubiquitous distal component of the ER quality control system, it might also regulate other types of PMPs, which express appropriate RXR-LL retention motifs, such as the V2 vasopressin receptor, mutated in the nephrogenic diabetes insipidus, the calcium sensing CaSR receptors, mutated in familial hypocalciuric hypercalcemia, or the gonadotrophin GnRH receptor, mutated in hypogonadotropic hypogonadism. Similarly to what we have reported for CFTR-F508del, PRAF2 might contribute to the trafficking defect of these mutants, which might be corrected by appropriate pharmacological chaperones. Finally, it has been known for some time that there is a substantial variability in disease expression among CF patients carrying the same mutation [55]. Based on our findings, PRAF2 concentration and/or polymorphism as well as the intracellular competition of other PMPs for PRAF2 interaction might contribute to this diversity by modulating CFTR proteostasis.

In conclusion, the gatekeeper PRAF2 is a previously unappreciated distal component of the ER quality control system, which appears to regulate wild type and mutated PMP trafficking recruitment into ERES. In the context of CF treatment, the new corrector VX-445 enhances CFTR F508del trafficking to the cell surface functioning in part as an inhibitor of its interaction with PRAF2. These findings open new therapeutic perspectives for rare diseases caused by the impaired cell surface trafficking of misfolded PMPs.

## **Statements and Decarations**

**Ethics approval and consent to participate: Not Applicable**

**Consent for publication: Not Applicable**

**Availability of data and material**

The datasets generated during and/or analysed during the current study are available as supplemental tables and figures

**Competing interests**

The authors have no relevant financial or non-financial interests to disclose

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**Authors' contributions**

Stefano Marullo, Kusumika Saha and Alexandre Hinzpeter contributed to the study conception and design. Material preparation, data collection and/or analysis were performed by Kusumika Saha, Benoit Chevalier, Stephane Doly, Nesrine Baatallah, Thomas Guilbert, Iwona Pranke, Mark GH Scott, Hervé Enslén, Chiara Guerrera, Cérina Chuon, Aleksander Edelman, Isabelle Sermet-Gaudelus, Alexandre Hinzpeter and Stefano Marullo. The first draft of the manuscript was written by Stefano Marullo and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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## Figure Legends

### Fig. 1 - PRAF2 interacts with both CFTR WT and CFTR 508del

**a)** Whole cell lysate of HEK-293 cells expressing exogenous CFTR (WT or F508del) and V5-epitope tagged PRAF2 (PRAF2-V5) were immunoprecipitated with a monoclonal anti-V5 antibody. Immunoprecipitated and input material were separated by SDS-PAGE, blotted, and membranes probed with anti-CFTR, anti-tubulin and anti-V5 antibodies. The ER core-glycosylated CFTR, referred as band B, and the Golgi- glycosylated CFTR, referred as band C, are indicated by arrows in the input. Specific, immuno-precipitated band B is indicated by arrowheads. IP: Immunoprecipitated fraction. IB: Immunoblot. **b)** Densitometric quantification of the percent of band B (WT or F508del) immunoprecipitated with PRAF2 (see Methods) expressed as the percentage of total band B. Mean $\pm$ SEM; n=5; p= 0.71, unpaired t test; ns=not significant. **c)** Schematic representation of the non-radiative energy transfer occurring between the BRET donor (Rluc-CFTR or Rluc-CFTR-F508del) and the BRET acceptor (PRAF2-YFP) occurring in the ER (cytoplasmic side). **d)** BRET saturation experiments (see Methods) were conducted with constant, equivalent amounts of Rluc-CFTR or Rluc-CFTR-F508del and increasing concentrations of the PRAF2-YFP BRET acceptor. Mean values $\pm$ SD are shown; data were compiled from 4 independent experiments, each performed in triplicate. BRET<sub>max</sub> values: 197.5 $\pm$ 4.2 (95% CI:189.2-205.8; R<sup>2</sup>=0.95, 76 dof) for CFTR-F508del and 110.5 $\pm$ 5.2 (100.0-121.1; R<sup>2</sup>=0.89, 52 dof) for CFTR-WT, p<0.0001 CFTR-F508del vs CFTR-WT; BRET<sub>50</sub> values: 1.89 $\pm$ 0.2 and 1.72 $\pm$ 0.25 for CFTR-F508del and CFTR-WT, respectively, p=0.1775; not significant (see Methods for statistics). **e)** HEK-293 cells were transfected with a constant amount of CFTR-WT plasmid and increasing amounts of PRAF2-V5 plasmid. Immunoblots were probed with anti-CFTR, anti-tubulin and anti-V5 antibodies. **f)** Densitometric quantification of band C (black) and band B (blue) relative to control conditions in the absence of PRAF2-V5. Mean $\pm$ SEM; n=4 (including the experiment in **e**). \*\*\*\*p<0.0001, one-way Anova followed by Sidak's multiple comparison test.

**Fig. 2 - CFTR interacts with PRAF2 via <sup>553</sup>RXR<sub>555</sub> and <sup>435</sup>LL<sub>436</sub> motifs of the NBD1 domain.**

**a)** HEK-293 cells were transiently transfected or not (UT) with plasmids coding for CFTR-WT or CFTR-F508del or the indicated mutants. Whole cell lysates were processed for immunoblots and probed with anti-CFTR and anti-Na<sup>+</sup>K<sup>+</sup>-ATPase (loading control) antibodies. Arrows indicate band B and band C. IB: Immunoblot. **b)** Densitometric quantification (as in Fig.1b) of band C over total protein normalized to CFTR-WT; “+”: presence of the indicated mutations. Histograms correspond to the mean±SEM, n=4; significance was calculated by Anova with Sidak’s multiple comparison test; \*\*\*\*p<0.0001, \*p=0.0106. **c)** HEK-293 cells were transiently transfected with plasmids coding for CFTR-WT or CFTR-F508del or their mutated forms on RXR and LL motifs, and the plasmid for PRAF2-V5. Whole cell lysates were immunoprecipitated with the anti-V5 antibody and processed as in Fig. 1a. Arrows indicate band B and band C. **d)** Densitometric quantification of the percent of band B co-immunoprecipitated with PRAF2 expressed as a percentage of total band B. Histograms correspond to the mean±SEM, n=4; \*\*\*p<0.001, unpaired t-test. **e)** HEK-293 cells were transfected with a plasmid coding for the Flag epitope-tagged NBD1 domain of CFTR-F508del, which contains the F508del mutation, and both <sup>553</sup>RAR<sub>555</sub> and <sup>435</sup>LL<sub>436</sub> motifs, involved in the interaction with PRAF2, in the presence or absence of a plasmid coding for PRAF2-V5. The ER-localization signal of P450 oxidase 2C1 was placed immediately after the Flag epitope to drive the localization of NBD1 to the cytoplasmic side of the ER. After immunoprecipitation with the anti-V5 antibody, co-immunoprecipitated material was separated by PAGE, blotted and probed with the indicated antibodies. Input is shown in the right part of the panel. **f)** Saturation BRET experiments (described in Fig. 1c,d) were conducted in the absence or presence of 2 concentrations of the Flag-NBD1 plasmid; the Immunoblot at the top shows the expressed Flag-NBD1. Data were compiled from 3 independent experiments, each experiment performed in triplicate. Error bars correspond to the mean±SEM; calculated BRET<sub>max</sub> values were: 207.8±2.7 (95% CI: 202.4-213.2; R<sup>2</sup>=0.84, 52 dof) in the absence of NBD1; 181.5±10.1 (95% CI: 161.4-201.6; R<sup>2</sup>=0.98, 52 dof) for

300ng of NBD1 plasmid and  $168.0 \pm 13.32$  (95% CI: 141.3-194.7;  $R^2=0.80$ , 52 dof) for 600ng of NBD1 plasmid;  $p=0.0048$ , ANOVA with Extra sum-of-squares F Test; calculated  $BRET_{50}$  values were:  $1.0 \pm 0.06$  (95% CI: 0.9-1.2;  $R^2=0.98$ , 52 dof) in the absence of NBD1;  $3.7 \pm 0.7$  (95% CI: 2.4-5.1;  $R^2=0.98$ , 52 dof) for 300ng of NBD1 plasmid and  $7.8 \pm 1.7$  (95% CI: 4.4-11.3;  $R^2=0.80$ , 52 dof) for 600ng of NBD1 plasmid;  $p<0.0001$  ANOVA with Extra sum-of-squares F Test.

### **Fig. 3 - Proximity labeling experiments using TurboID**

**a)** Schematic representation of the constructs used for the mass spectrometry assay. PRAF2-V5-TurboID and C1-TurboID-V5, expressed at the ER surface, are expected to label protein partners at the ER membrane and in the cytoplasm. **b)** Volcano plot showing the proteins which are significantly enriched in samples expressing PRAF2-V5-TurboID, compared to the C1-TurboID-V5 control (183 proteins, right side of the plot). Proteins known to be involved in COPII vesicle formation (green) and SNARE complexes (red) are indicated. **c)** Lollipop chart with the best (higher fold enrichment) Gene Ontology (GO) terms for Cellular Component obtained with proteins enriched in PRAF2-V5-TurboID. Each line corresponds to a “cellular component”, sorted as a function of the fold enrichment. The false discovery rate (FDR) value obtained for each term is indicated by the color code. The diameter of the circles represents the number of proteins found enriched for each corresponding term. **d)** The search in the STRING database was conducted by setting high confidence (0.900). Connecting lines indicate physical interactions, and disconnected nodes in the network are hidden. Proteins implicated in COPII vesicle formation (green) and SNARE complexes (red) are indicated. **e)** STED microscopy was used to investigate at higher resolution the proximity of PRAF2-containing areas with ERES. Permeabilized CFBE41o-parental cells probed with anti-Sec31 and anti-PRAF2 antibodies were then labeled with appropriate secondary antibodies carrying STED adapted fluorochromes (see Methods). Images were acquired with a STED confocal microscope at 93x glycerol immersion magnification. Manders’ colocalization coefficient M1 between PRAF2 and Sec31

was  $0.089 \pm 0.004$  (mean  $\pm$  SEM, n=9). The enlarged area (right panel) corresponds to the blue square. Scale bars: 10  $\mu$ m.

**Fig. 4 - PRAF-2 retention and Sec-24-recruitment in COPII vesicles occur sequentially**

**a)** HEK-293 cells were transiently transfected with the indicated plasmids, coding for CFTR-WT or CFTR-F508del, harboring or lacking the following individual mutations or their combinations: D567A, which corresponds to the mutation inhibiting the interaction with Sec24; K/AA which corresponds to the R555K-LL435-6AA double substitution that inhibits the interaction with PRAF2. Immunoblots (IB) were probed with anti-CFTR and anti-tubulin (loading control) antibodies. Arrows indicate bands B and C. UT: Untransfected. **b)** Quantification of the percentage of band C over total protein relative to the CFTR-WT normalized to the tubulin loading control. Mean  $\pm$  SEM; n=3; \*\*\*\*p<0.0001, one-way Anova followed by Sidak's multiple comparison test; \*\*\*p=0.006; ns=not significant. **c)** CFBE41o-parental cells were transfected with the indicated plasmids and a surface biotinylation assay was conducted as described in Methods. The biotinylated fraction, isolated with streptavidin beads, and 25% of the input were probed with anti-CFTR and anti-GAPDH antibodies (loading control). Arrows indicate bands B and C. IB: Immunoblot.

**Fig. 5 - Inhibiting PRAF2-mediated CFTR F508del retention can rescue transporter function**

**a)** CFBE41o-parental cells, treated with indicated siRNAs, were transfected with CFTR-WT or CFTR-F508del plasmids, then incubated with vehicle (DMSO) or VX-809 (3  $\mu$ M) for 24 hours. Whole cell lysates were separated by PAGE, blotted and probed with either anti-CFTR, anti-Na<sup>+</sup>K<sup>+</sup>-ATPase (loading control) or anti-PRAF2 antibodies. Arrows indicate band B and band C. IB: Immunoblot. **b)** Densitometric quantification of the percentage of band C over total protein and normalization relative to the CFTR-WT. Mean  $\pm$  SEM; n=4; \*\*\*\*p<0.0001 one-way Anova with Sidak's multiple comparison test; \*\*p<0.01; \*p<0.05; ns=not significant. **c)** Densitometric quantification of siRNA-mediated PRAF2 knockdown. Values were

normalized over the PRAF2 values in cells treated with control siRNA in DMSO. Mean $\pm$ SEM; n=4; \*\*\*\*p<0.0001 one-way Anova with Sidak's multiple comparison test. **d**) CFBE41o-F508del-CFTR stable cells expressing the halide sensor (HS-YFP) were treated with the indicated siRNAs and chemicals as in **(a)**, in the presence of the potentiator VX-770 to enhance channel opening probability [56]. In one control HS-YFP cells were transfected with a plasmid coding for PRAF2-V5. The transporter function was assayed following addition of iodide (see Methods). Representative traces of are shown. The curve was fitted to an exponential function to obtain the maximal slope. Histograms represent transport rates in mM/s. The stable cell line CFBE41o-WT-CFTR was used as positive control. Mean $\pm$ SEM; n=3; \*\*\*\*p<0.0001 one-way Anova with Tukey's multiple comparison test between the indicated groups; \*\*p=0.041; \*p<0.05; ns=not significant. **e**) Representative western blot showing knockdown of endogenous PRAF2 or PRAF2-V5 overexpression in **(d)**. Whole cell lysates were separated by PAGE, blotted and probed with anti-Tubulin (loading control) and anti-PRAF2 antibodies; IB: Immunoblot.

### **Fig. 6 – Functional interplay of new generation correctors, with PRAF2 retention**

**a**) CFBE41o-CFTR-F508del cells (see Fig. 5), were treated with indicated siRNAs, VX-661 or DMSO, VX-770 being present in all conditions; CFBE41o-CFTR-WT cells were used as positive control. Representative traces of the YFP halide assay are shown in the left panel. Note that the traces for WT (blue) Ctrl-siRNA (red) and PRAF2-siRNA (green) are same in Fig 5d, 6a and 6b to better compare the effects of the drugs. Histograms represent the transport rate. Mean $\pm$ SEM; n=5; \*\*\*\*p<0.0001, one-way Anova, with Tukey's multiple comparison test between indicated groups; \*\*\*p=0.0003; \*\*p=0.0014; \*p<0.05; ns=not significant. **b**) Same experiment as in **(a)** with the VX-445 corrector (n=6). \*\*\*\*p<0.0001 one-way Anova with Tukey's multiple comparison test between the indicated groups; ns=not significant. **c**) Representative western blot illustrating knockdown of endogenous PRAF2 in **(a-b)**. Whole cell lysates were separated by PAGE, blotted and probed with anti-Tubulin (loading control) and anti-PRAF2 antibodies; IB: Immunoblot.

**Fig. 7 – VX-445 is a negative modulator of PRAF2-CFTR F508del interaction**

**a)** BRET saturation experiments performed after 24h treatment with the indicated compounds. Mean±SD; data were compiled from 3 independent experiments, each performed in triplicate. BRET<sub>max</sub> values were: 196.2±3.3 (95%CI: 189.5-203.0; R<sup>2</sup>=0.96, 34 dof) for DMSO control; 195.6±5.2 (95%CI: 184.9-206.3; R<sup>2</sup>=0.95, 34 dof) for VX-809; 202.3±3.3 (95%CI: 195.7-209.0; R<sup>2</sup>=0.97, 52 dof) for VX-661 and 163.9±5.4 (95%CI: 152.9-174.9; R<sup>2</sup>=0.94, 52 dof); p<0.0001 versus control for VX-445. BRET<sub>50</sub> values were: 0.99±0.1 (95%CI: 0.6-1.3; R<sup>2</sup>=0.96, 34 dof) for control; 1.5±0.2 (95%CI: 1.0-2.0; R<sup>2</sup>=0.95, 34 dof) for VX-809; 1.9±0.2 (95%CI: 1.4-2.4; R<sup>2</sup>=0.97, 52 dof) for VX-661 and 7.2±0.8 (95%CI: 5.6-8.8; R<sup>2</sup>=0.94, 52 dof) p<0.0001 versus control for VX-445. **b)** HEK-293 cells were transfected with constant amount of CFTR-WT plasmid and increasing amounts of PRAF2-V5 plasmid. Next day cells were treated with DMSO or VX-445 and 24 hours later total lysates were processed for immunoblots, probed with anti-CFTR, anti-tubulin and anti-V5 antibodies. Arrows indicate bands B and C. UT: untransfected. **c)** After densitometric quantification of bands C and B, C/B ratios was calculated, relative to basal conditions (absence of PRAF2-V5) for DMSO (black dots) and VX-445 (red dots). Mean±SEM n=3. **d,e)** HEK-293 cells expressing exogenous CFTR-WT (**d**) or CFTR-F508del (**e**) and V5-epitope tagged PRAF2 (PRAF2-V5) were treated with VX-445 or control DMSO. Co-IP experiments were conducted as in Fig. 1a. The % of co-immunoprecipitated band B is shown in the histograms. Mean±SEM; n=3 (**d**) or 4 (**e**); \*\*\*\*p<0.0001, \*\*\*p=0.0002, unpaired t test between DMSO- and VX-445-treated, respectively.