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RESEARCH ARTICLE

Correctors of mutant CFTR enhance subcortical cAMP–PKA signaling through modulating ezrin phosphorylation and cytoskeleton organization

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ABSTRACT

The most common mutation of the cystic fibrosis transmembrane regulator (*CFTR*) gene, F508del, produces a misfolded protein resulting in its defective trafficking to the cell surface and an impaired chloride secretion. Pharmacological treatments partially rescue F508del CFTR activity either directly by interacting with the mutant protein and/or indirectly by altering the cellular protein homeostasis. Here, we show that the phosphorylation of ezrin together with its binding to phosphatidylinositol-4,5-bisphosphate (PIP$_2$) tethers the F508del CFTR to the actin cytoskeleton, stabilizing it on the apical membrane and rescuing the sub-membrane compartmentalization of cAMP and activated PKA. Both the small molecules trimethylangelicin (TMA) and VX-809, which act as 'correctors' for F508del CFTR by rescuing F508del-CFTR-dependent chloride secretion, also restore the apical expression of phosphorylated ezrin and actin organization and increase cAMP and activated PKA submembrane compartmentalization in both primary and secondary cystic fibrosis airway cells. Latrunculin B treatment or expression of the inactive ezrin mutant T567A reverse the TMA and VX-809-induced effects highlighting the role of corrector-dependent ezrin activation and actin re-organization in creating the conditions to generate a sub-cortical cAMP pool of adequate amplitude to activate the F508del-CFTR-dependent chloride secretion.

KEY WORDS: Cystic fibrosis, Correctors, cAMP, PKA, Airway cells, FRET

INTRODUCTION

The most prevalent mutation of the *CFTR* gene associated with cystic fibrosis is the deletion of phenylalanine at residue 508 (F508del CFTR) in the nucleotide-binding domain 1 (NBD1). The mutation causes an improperly folded CFTR protein that in large part is unable to traffic to the cell surface due to premature degradation. The F508del CFTR that can reach the membrane displays reduced stability (Lukacs et al., 1993) as peripheral quality control removes the unfolded CFTR from the plasma membrane (Okiyoneda et al., 2010). In the airway cells, the lack of a functional CFTR protein on the cell surface causes impaired mucociliary clearance and consequent opportunistic bacterial infections and progressive deterioration of lung function (Clunes and Boucher, 2011). The complexity of the defects of F508del CFTR trafficking and activation can be overcome by small-molecule modulators that can rescue the biosynthetic defect of F508del CFTR (termed 'correctors') and those that increase its regulated function once rescued to the cell surface (termed 'potentiators') (for reviews, see Rowe and Verkman, 2013; Zhang et al., 2012). However, the levels of plasma-membrane-recovered F508del CFTR obtained by the correctors discovered until now, as well as the knowledge of their mechanisms of action, are still limited. The small-molecule correctors discovered to date might either directly interact with the misfolded F508del CFTR protein favoring its folding and/or with one or more F508del-CFTR-interacting proteins that promote the processing and delivery of the protein on the apical membrane (Cai et al., 2011).

It has been shown that CFTR is spatially coupled with various interacting proteins through a physical interaction with both the N-terminal and C-terminal tails, and these interactions play an important role in regulating CFTR-mediated transepithelial ion transport (Li and Naren, 2010). In particular, the C-terminal postsynaptic density 95, disclarge, zona occludens (PDZ)-binding motif of CFTR interacts with several PDZ-domain-containing proteins that assist CFTR delivery and recycling to the cell surface and finely regulate its activity (Guggino and Stanton, 2006; Swiatecka-Urban et al., 2002).

In airway epithelial cells, CFTR regulation has been demonstrated to depend on the organization of a multi-protein complex involving F-actin, the scaffolding protein NHERF1 (also known as SLC9A3R1) and ezrin. This protein complex, besides stabilizing CFTR in highly restricted domains at the plasma membrane (Haggie et al., 2006; Jin et al., 2007), plays an important role in the control of CFTR function as ezrin, an A-kinase anchoring protein, tethers PKA in the proximity of CFTR, allowing cAMP-dependent control of chloride efflux (Moyer et al., 2000; Short et al., 1998; Sun et al., 2000).

In the cytoplasm, most ezrin is maintained in an inactive conformation through intramolecular interactions between its N-terminal FERM and the C-terminal domains (Bretscher et al., 2002). The phosphorylation of a conserved threonine residue in the actin-binding domain (T567) and the interaction with phosphatidylinositol 4,5-bisphosphate (PIP$_2$), through its FERM domain (Bretscher et al., 2002; Yonemura et al., 2002), are both required for the activation and recruitment of ezrin to the plasma membrane, whereas a reduction in PIP$_2$ levels mediated by activation of phospholipase C promotes the release and dephosphorylation of ezrin (Hao et al., 2009).

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We have previously reported that the cellular localization of both NHERF1 and phosphorylated ezrin (phospho-ezrin) differs between airway cells expressing wild-type (wt) CFTR (16HBE14o-, denoted as HBE) and F508del CFTR (CFBE41o-, denoted as CFBE), such that both proteins are mainly localized in the apical region in HBE cell monolayers, whereas they are diffusively expressed in the cytosol of CFBE cell monolayers (Favia et al., 2010). Moreover, the actin cytoskeleton is disorganized in CFBE cells compared to HBE cells. NHERF1 overexpression in CFBE cells induces the formation of the multi-protein complex NHERF1–ezrin–RhoA–ROCK–actin that, in turn, increases the F-actin organization and rescues the functional expression of F508del CFTR on the apical membrane of polarized cell monolayers (Favia et al., 2010). These results, together with the findings demonstrating that the increased cell surface mobility of the rescued F508del CFTR observed at 37°C involves a reduced level of PDZ interaction in comparison to wt CFTR (Valentine et al., 2012), indicate that the inclusion of F508del CFTR in a multi-protein complex and its interaction with actin cytoskeleton might be important in modulating its functional expression on the plasma membrane.

Here, we investigated the role of ezrin activation in rescuing both the functional expression of F508del CFTR and the compartmentalization of cAMP and activated PKA on the apical region of both primary and secondary airway cystic fibrosis cells. Moreover, we asked whether small-molecule correctors might mediate the activation of ezrin and the inclusion of F508del CFTR in multiprotein complex to achieve their rescue of F508del CFTR functional expression. In this regard, it has been recently demonstrated that the known small-molecule corrector VX-809 (Van Goor et al., 2011) enhances the stability and function on the cell surface of F508del CFTR (Eckford et al., 2014) and improves its interaction with NHERF1 (Arora et al., 2014; Loureiro et al., 2015). Indeed, we find here that both VX-809 (Van Goor et al., 2011) and 4,6,4′-trimethylangelicin (TMA), a recently reported corrector and potentiator of F508del CFTR (Favia et al., 2014; Tamanini et al., 2011), involve both ezrin activation and actin cytoskeleton re-organization in their rescuing effect. Moreover, both correctors restore the sub-cortical compartmentalization of cAMP and activated PKA resulting in the rescue of the cAMP–PKA-dependent chloride secretion in both primary and secondary cystic fibrosis airway cells.

RESULTS
Phosphoinositide binding and phosphorylation of ezrin are required for the rescue of F508del CFTR

To analyze the role of ezrin activation in regulating both the rescue of functional expression of F508del CFTR and the organization and translocation to the membrane region of the F508del-CFTR–NHERF1–ezrin–actin multiprotein complex, we expressed either the 'active' mutant of ezrin, T567D, in which the C-terminal T567 residue is mutated to aspartic acid to mimic the phosphorylated form of ezrin, or the double mutant PIP$_2^-$ T567D, in which the capacity of the ezrin active mutant to bind to PIP$_2$ is abolished, in CFBE cell monolayers. Both ezrin mutants constructs, T567D and PIP$_2^-$ T567D ezrin, had similar expression levels that were ~40% greater than the level of endogenous ezrin in non-transfected cells (see Materials and Methods). Confocal immunofluorescence analysis shows that (1) whereas GFP-tagged T567D ezrin was exclusively localized at the apical membrane, the GFP-tagged PIP$_2^-$ T567D construct was distributed in the cytoplasm, and (2) F508del CFTR expression (red) was rescued to the apical membrane only in CFBE cell monolayers transfected with T567D ezrin (CFBE/T567D)

(Fig. 1A). These findings were confirmed by the surface biotinylation assay performed in polarized CFBE cell monolayers showing that T567D but not PIP$_2^-$ T567D ezrin expression resulted in the appearance of the mature F508del CFTR (known as band C) (Fig. 1B). Moreover, analysis of F508del-CFTR-mediated chloride transport shows that only T567D ezrin expression significantly rescued apical F508del-CFTR-dependent chloride secretion in polarized cystic fibrosis cell monolayers (Fig. 1C). Taken together, these data demonstrate that both activation of ezrin and its interaction with PIP$_2$ are needed for F508del CFTR to be functionally expressed on the apical membrane of CFBE cell monolayers.

We have previously demonstrated that the rescue of CFTR functional expression is promoted by a change in actin cytoskeleton organization mediated by the formation of the NHERF1–ezrin–actin complex (Favia et al., 2010). Here, co-immunoprecipitation experiments in CFBE cells transfected with T567D or PIP$_2^-$ T567D ezrin, demonstrated that the interaction between NHERF1 and CFTR, as well as between ezrin and actin, was significantly higher in CFBE/T567D ezrin cells than in control CFBE or in CFBE/PIP$_2^-$ T567D ezrin cells (Fig. 1D,E). In addition, by performing a proximity ligation assay (PLA) analysis, which can detect endogenous protein–protein interactions that occur within 40 nm (Soderberg et al., 2006), we observed that F508del CFTR and NHERF1 colocalize in the sub-plasma-membrane region in CFBE cells when transfected with GFP-tagged T567D ezrin (Fig. 1F). Moreover, we observed that T567D ezrin expression significantly increased the F-actin assembly in CFBE cells, whereas PIP$_2^-$ T567D ezrin was ineffective (Fig. 1G).

Finally, as it is known that the low-temperature-rescued F508del CFTR (rF508del CFTR) is unstable on the cell surface and is rapidly internalized and degraded at 37°C (Cholon et al., 2010; Sharma et al., 2004), we monitored the effect of T567D ezrin expression on the cell surface stability of rF508del CFTR by following the surface pool of rF508del CFTR using the surface biotinylation assay previously described (Favia et al., 2014; Jurkuvenaite et al., 2010). Fig. 1H shows that T567D expression stabilized the glycosylated band C of rF508del CFTR for up to 8 h at 37°C compared to non-transfected CFBE cell monolayers, suggesting that the expression of activated ezrin might partially protect the thermally destabilized rF508del CFTR from the peripheral degradation pathways.

Taken together, these data demonstrate that, through its interaction with PIP$_2$, ezrin is recruited to the membrane region and phosphorylated; this results in its activation, which then unmasks its actin-binding sites with the consequent formation of the multiprotein complex that stabilizes F508del CFTR on the cell surface.

Ezrin activation rescues subcortical compartmentalization of both cAMP and PKA in CFBE cells

We have previously reported that the lack of an organized subcortical cytoskeletal organization observed in CFBE cells compared to HBE cells, causes a cytosolic accumulation and a concomitant reduced compartmentalization of cAMP in the membrane region, resulting in reduced subcortical levels of PKA activity. Importantly, the stable expression of wt CFTR in CFBE cells, besides restoring the cortical actin cytoskeletal organization and rescuing the functional expression of F508del CFTR, also re-establishes the membrane compartmentalization of cAMP to a level similar to that found in HBE cells (Monterisi et al., 2012). In line with these data, CFTR knockdown in HBE cells induced a reverse in the cAMP distribution together with a loss in the cytoskeletal
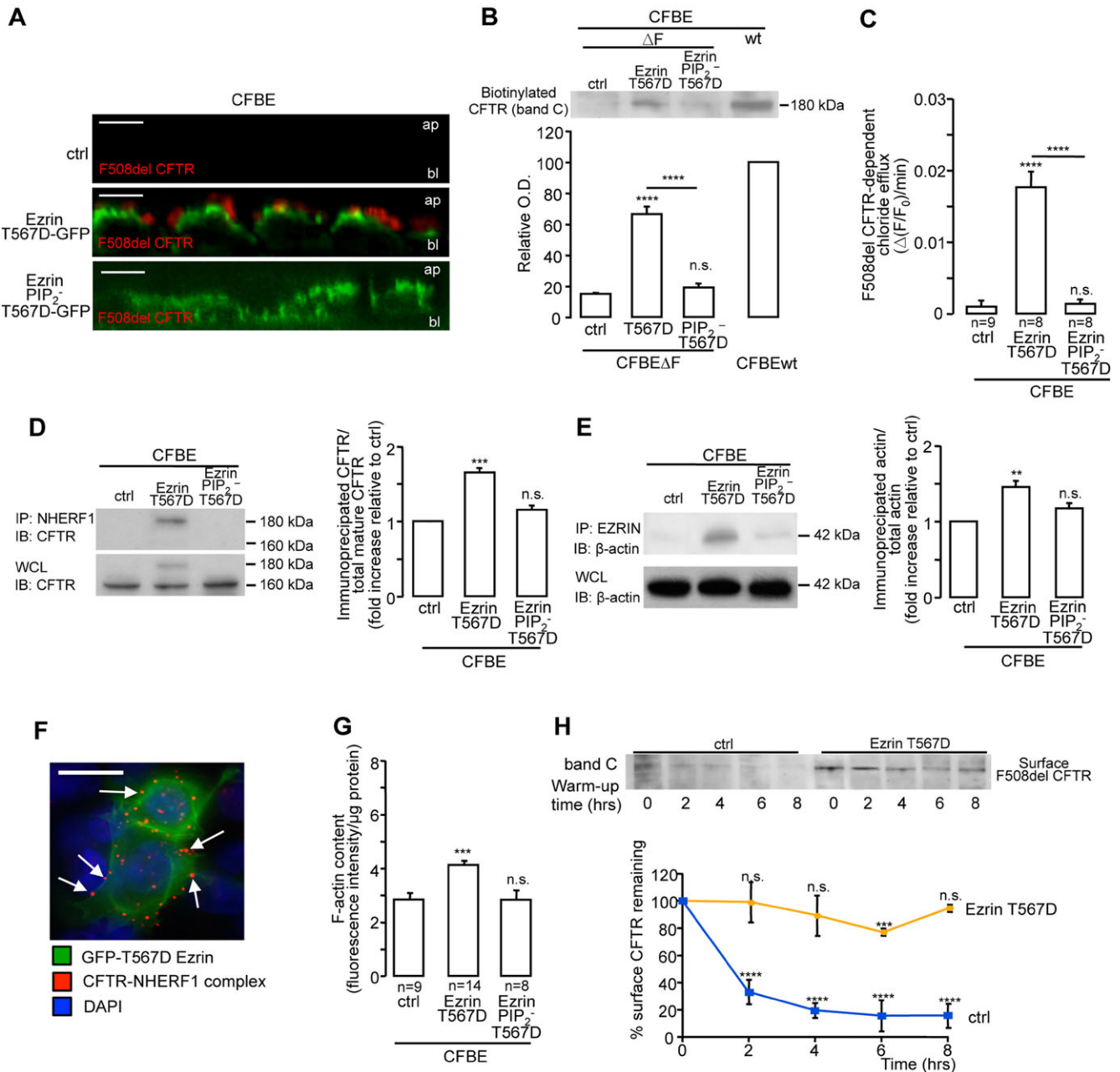
Fig. 1. Ezrin activation promotes apical F508del CFTR functional expression in CFBE monolayers through the formation of the multiprotein complex F508del-CFTR–NHERF1–ezrin–actin. CFBE cells were transfected with GFP-tagged ezrin mutants T567D ezrin or PIP$_2^-$ T567D ezrin or treated with the transfection vehicle (ctrl). (A) Representative confocal immunofluorescence images of polarized CFBE monolayers. Unpermeabilized cells were immunolabeled with a primary mouse monoclonal antibody raised against the first extracellular loop of CFTR. Confocal scans are shown in the vertical cross section (xz) plane. ap, apical membrane; bl, basolateral region. Scale bars: 10 µm. (B) Cell surface membrane proteins were biotinylated and analyzed by western blotting using anti-CFTR antibody. As a positive control, biotinylated CFTR from CFBE cells expressing wt CFTR is shown (wt). The histogram summarizes the amount of biotinylated CFTR relative to mature CFTR (n=4) as assessed by densitometry (O.D.). (C) CFTR-dependent chloride efflux was analyzed by spectrofluorimetric measurements in polarized CFBE cell monolayers transfected or not with ezrin mutants. (D,E) The interaction between NHERF1 and CFTR, and between ezrin and actin was measured in co-immunoprecipitation experiments as described in Materials and Methods. Representative blots of the immunoprecipitates (IP) and the respective whole-cell lysates (WCL) and histograms summarizing relative densitometry values are shown for immunoprecipitates with polyclonal NHERF1 antibody (D) or with monoclonal ezrin antibody (E) and immunoblotted (IB) respectively with monoclonal CFTR antibody (D) or with monoclonal β-actin antibody (E) (n=3). (F) Representative fluorescence microscopy image showing protein interaction between CFTR and NHERF1 (red signal) in CFBE cells transfected with GFP-tagged T567D ezrin. Fixed CFBE/T567D ezrin cells were permeabilized and labeled primary antibodies against CFTR (rabbit polyclonal) and NHERF1 (mouse monoclonal). Protein interaction (red fluorescent signals) were revealed by the PLA anti-rabbit minus probe and PLA anti-mouse plus probes (see Materials and Methods). Nuclei were stained with DAPI (blue). The arrows indicate the red spots representative of the interactions between the proteins of interest. Scale bar: 10 µm. (G) The histogram summarizes the F-actin content in CFBE cells transfected or not with ezrin mutants. (H) Stabilization of rF508del CFTR on cell surface was analyzed by biotinylation assay. Polarized CFBE cells were cultured at 27°C for 48 h, transfected (orange) or not (blue) with ezrin T567D for the last 24 h and then transferred to 37°C up to 8 h. A representative blot is shown (top). Quantification of cell surface CFTR is plotted as the percentage change in density of rF508del CFTR band C at different time points (bottom; n=4). Data represent mean±s.e.m. **P<0.01; ***P<0.001; ****P<0.0001; n.s., not significant (Student's t-test).

organization (Monterisi et al., 2012). Therefore, on the basis of those previous findings and the data of Fig. 1 demonstrating that phosphorylated ezrin together with its binding to PIP$_2$ stabilizes F508del CFTR on the plasma membrane and rescues its PKA-dependent chloride secretion, we hypothesize that this activated ezrin could directly affect the cAMP and PKA compartmentalization.

We then analyzed the role of ezrin phosphorylation and its interaction with PIP$_2$ on cAMP compartmentalization in living CFBE cells utilizing a membrane-targeted version (mpH30) (Terrin et al., 2006) and a cytosolic version (H30) (Ponsioen et al., 2004) of a FRET-based reporter for cAMP. As shown in Fig. 2A,B, T567D ezrin expression in CFBE cells resulted in a large increase of cAMP at the sub-plasma-membrane region in

response to 1 μM forskolin (FSK) stimulation, whereas PIP$_2^-$ T567D expression had no effect. By contrast, when the cAMP responses to FSK were measured in cells expressing the cytosolic H30 reporter, we detected a significantly lower cAMP response in the bulk cytosol of CFBE cells expressing T567D ezrin than in control CFBE or CFBE cells transfected with PIP$_2^-$ T567D (Fig. 2C,D).

Importantly, we found that the increase of the total cAMP in response to 1 μM FSK stimulation was not significantly different in the CFBE cells transfected with T567D ezrin from that measured in control CFBE cells [25.7±1.57 (mean±s.e.m.; $n=5$) and 28.7± 3.69 nM cAMP ($n=6$) in CFBE cells transfected with T567D ezrin and in control CFBE cells, respectively; not significant as determined by Student's t-test]. These results confirm that T567D
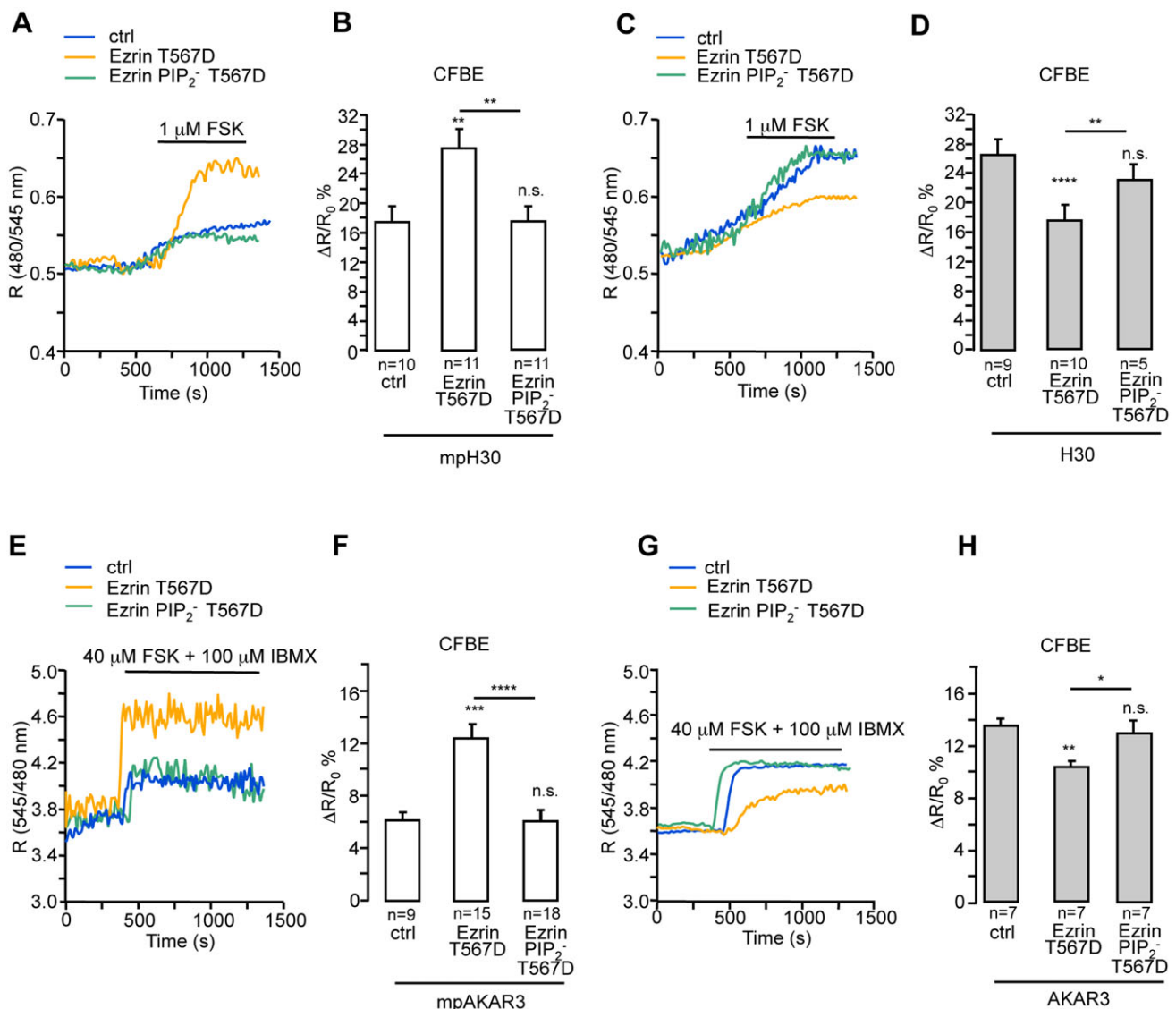

Fig. 2. Activated ezrin rescues cAMP levels and PKA activity in the sub-plasma-membrane region of CFBE cells. cAMP levels and PKA activity in both the sub-plasma-membrane and cytosolic region of CFBE cells transfected or not with ezrin mutants were analyzed by FRET using H30 and AKAR3 reporters and their membrane-targeted version, mpH30 and mpAKAR3, respectively. (A,C) Representative kinetics of cAMP changes on application of the stimulus as indicated. R is the ratio of the 480 nm to the 545 nm value calculated at each acquisition time point. (B,D) Summary of FRET changes measured in CFBE cells transfected or not with ezrin mutants and expressing the mpH30 and H30 sensors upon stimulation with FSK. (E,G) Representative kinetics of PKA-mediated phosphorylation. R is the ratio of the 480 nm to the 545 nm value calculated at each acquisition time point. (F,H) Summary of FRET changes recorded in CFBE cells transfected or not with ezrin mutants and expressing the mpAKAR3 and AKAR3 probes upon stimulation with FSK and IBMX. Data represent mean± s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; n.s., not significant (Student's t-test).

ezrin expression does not change the total cAMP levels but only re-compartmentalizes cAMP to the sub-membrane region.

In a parallel series of experiments, we analyzed the effect of ezrin activation in CFBE cells expressing T567D or PIP$_2^-$ T567D mutants together with the genetically encoded reporter for the plasma-membrane-targeted A-kinase protein activity, mpAKAR3, which detects PKA-mediated phosphorylation following stimulation with FSK (40 µM) and IBMX (100 µM) in the membrane region. In analogy to what was observed for cAMP levels, there was a significantly higher PKA-mediated phosphorylation activity at the plasma membrane region of CFBE cells transfected with T567D ezrin compared to either control CFBE cells or CFBE cells expressing PIP$_2^-$ T567D ezrin (Fig. 2E,F). To assess whether this increase in subcortical PKA activity was associated with a decrease in cytosolic PKA activity, we measured the PKA activity in the cytoplasmic region using AKAR3, an untargeted cytosolic version of the PKA activity FRET reporter and, indeed, we found a significantly lower level of PKA-mediated phosphorylation in the cytosol of CFBE T567D compared to either control CFBE cells or CFBE cells expressing PIP$_2^-$ T567D ezrin (Fig. 2G,H). These data indicate that membrane-bound and activated ezrin favors subcortical cAMP compartmentalization and the subsequent local activation of PKA. As would be predicted, in HBE cells transfected with a phosphorylation-dead ezrin mutant, T567A ezrin, a substantial reduction of both cAMP and PKA compartmentalization at plasma membrane region was observed (data not shown). Taken together, these data suggest that both ezrin activation and its interaction with membrane PIP$_2$ are crucial in controlling the apical compartmentalized functional 'signalsome' that allows cAMP- and PKA-mediated activation of F508del CFTR.

TMA and VX-809 correctors rescue subcortical cAMP compartmentalization in cells expressing F508del and not in cells expressing the G551D mutation of CFTR

We recently reported that preincubation with the 'corrector' molecules TMA and VX-809 significantly rescues the F508del CFTR-dependent chloride efflux in response to FSK stimulation in polarized monolayers of CFBE cells overexpressing F508del CFTR (Favia et al., 2014). However, the cellular mechanisms by which

corrector molecules improve F508del CFTR folding and its trafficking to and stabilization on the apical membrane to rescue the cAMP- and PKA-dependent F508del-CFTR-driven chloride efflux are still largely unknown.

As shown in Fig. 3A–C, both the TMA (100 nM)- and VX-809 (5 µM)-dependent rescue of F508del CFTR-dependent chloride secretion was paralleled by a significant increase of both the cAMP levels and PKA-mediated phosphorylation activity at the plasma membrane region of CFBE cells which reached levels similar to those recorded in HBE cells expressing wt CFTR.

In addition in this case, as previously observed for T567D ezrin expression in CFBE cells, neither VX-809 nor TMA treatment changed the total cAMP level in response to 1 µM FSK [27.7±3.7 (n=6), 27.7±3.2 (n=9) and 29.50±3.5 (n=9) (mean±s.e.m. nM cAMP) in control CFBE, TMA-treated and VX-809-treated cells, respectively; not significant as determined by Student's t-test], whereas they rescued the cAMP localization in the membrane region.

To analyze whether the corrector-induced cAMP re-compartmentalization in the sub-apical region was limited to CFBE cells or other cells expressing F508del CFTR, we compared the TMA or VX-809 corrector effect in FRT cells expressing either F508del CFTR (F508del CFTR-FRT) or G551D CFTR (G551D CFTR-FRT), and in FRT cells not expressing CFTR at all (null CFTR-FRT) (Zegarra-Moran et al., 2002). As shown in the Fig. 4A,B, in the absence of the correctors, when we measured the cAMP response generated by 1 µM FSK using a membrane-targeted version of the FRET-based reporter H30 (mpH30), we found that the subcortical cAMP response was significantly higher in G551D CFTR-FRT cells than in either the F508del CFTR-FRT or null CFTR-FRT cells. By contrast, when we analyzed the cAMP changes in cells expressing the cytosolic reporter H30, we found that, after FSK stimulation, there was a lower cytosolic cAMP response in the G551D CFTR-FRT cells than in F508del CFTR-FRT or null CFTR-FRT cells.

On the basis of these data, we hypothesize that the lack of CFTR expression on the cell plasma membrane of F508del CFTR-FRT or null CFTR-FRT cells, results in a mislocalized cAMP membrane compartmentalization and a concomitant
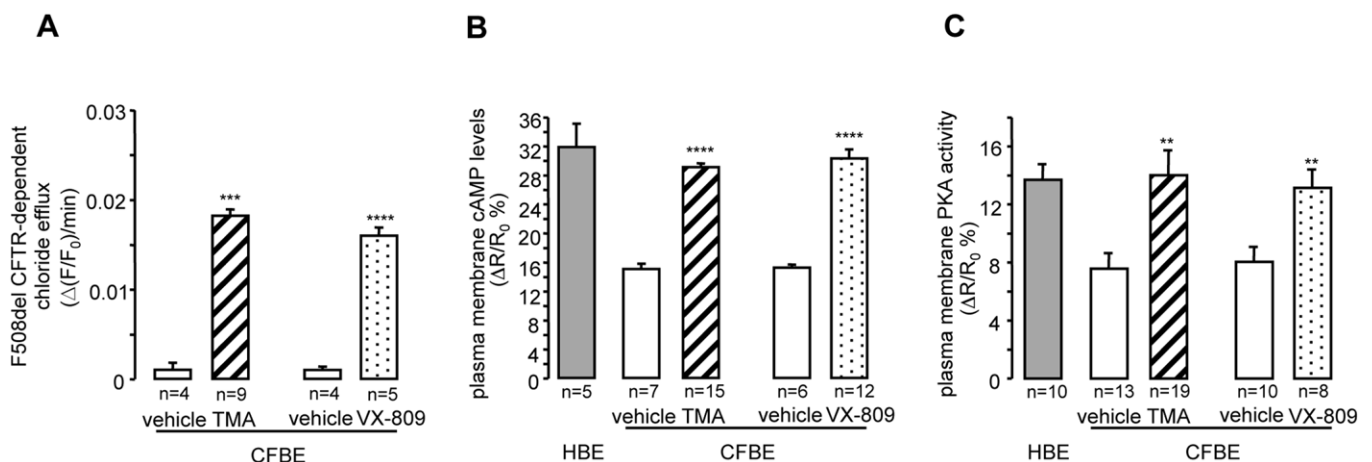

Fig. 3. TMA and VX-809 correctors rescue cAMP and PKA membrane compartmentalization in CFBE cells. CFBE cells were incubated with vehicle or with 100 nM TMA or 5 µM VX-809 for 24 h before functional and FRET experiments. HBE cells were considered as a positive control. (A) Summary of CFTR-dependent chloride effluxes [expressed as Δ(F/F$_0$)/min] and analyzed by spectrofluorimetric measurements in polarized CFBE monolayers treated with vehicle or correctors. (B,C) Summary of FRET cAMP changes (B) and PKA activity (C) measured in HBE and CFBE cells expressing the mpH30 sensor (B) and mpAKAR probe (C), pre-incubated with vehicle alone or with correctors. Data represent mean±s.e.m. **P<0.01; ***P<0.001; ****P<0.0001 (Student's t-test).
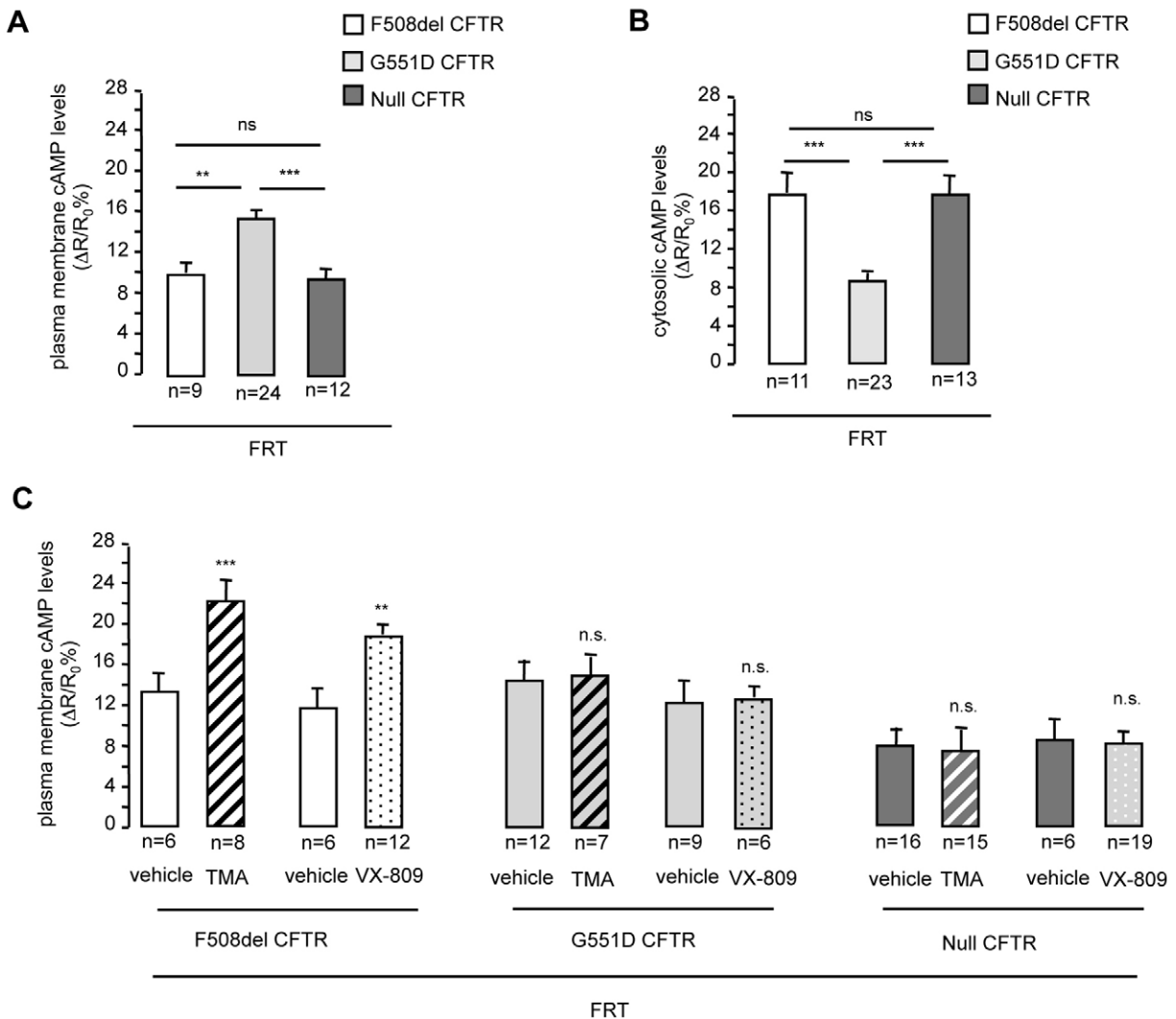
Fig. 4. FRET analysis of cAMP localization in FRT cells expressing the F508del CFTR or the defective conductance G551D CFTR mutant in basal conditions and in response to corrector treatment. cAMP levels in both the sub-plasma-membrane (A) and cytosolic regions (B) of FRT cells expressing either the F508del CFTR or the G551D CFTR transport defect mutants and in FRT cells not expressing CFTR (null CFTR-FRT) were analyzed by FRET using mpH30 and H30 reporter, respectively. FRET changes were recorded after 1 µM FSK stimulation. **$P<0.01$; ***$P<0.001$; n.s., not significant (comparisons among the three cell lines using one-way ANOVA followed by Bonferroni post hoc test). (C) Plasma membrane cAMP-mediated FRET signals were measured in FRT cells expressing either F508del CFTR or G551D CFTR mutants and in null CFTR-FRT cells transfected with the mpH30 sensor and pre-incubated with the indicated correctors (100 nM TMA or 5 µM VX-809) or their corresponding vehicles. FRET analysis was performed 48 h after transfection and 24 h after corrector treatment. FRET changes were recorded upon 1 µM FSK stimulation. Data represent mean±s.e.m. **$P<0.01$; ***$P<0.001$ (Student's t-test).

cAMP accumulation in the cytosol. By contrast, when CFTR is expressed on the plasma membrane, even if not functional as in G551D CFTR-FRT cells (Welsh and Smith, 1993), sub-membrane cAMP compartmentalization is re-established. As shown in Fig. 4C, both TMA and VX-809 correctors rescue the plasma membrane cAMP compartmentalization only in the F508del CFTR-FRT cells.

These data support the hypothesis that the corrector-induced rescue of F508del CFTR expression on the plasma membrane, by promoting its stabilization on the plasma membrane favors the formation of an intracellular milieu more similar to that found in cells expressing the wt CFTR. The correctors had no effect on cAMP compartmentalization in either the G551D CFTR-FRT cells, where CFTR was already expressed on the plasma membrane but not functional, nor in the null CFTR-FRT cells where CFTR was not expressed.

TMA- and VX-809-dependent rescue of F508del CFTR functional expression and subcortical cAMP compartmentalization involves ezrin activation and actin cytoskeletal re-organization in both secondary and primary airway cystic fibrosis cells

We previously observed that both the cytoskeleton organization and the intracellular distribution of cAMP and PKA are dependent on the formation of the multiprotein CFTR–NHERF1–ezrin–actin complex, which is driven by the presence of wt CFTR on the apical membrane (Monterisi et al., 2012). In line with these observations, we found that both TMA and VX-809 rescued the organization of the actin network and the expression of phospho-ezrin at the apical region of polarized CFBE cell monolayers and significantly increased the F-actin content (Fig. 5A,B). In addition, we found that both TMA and VX-809 increase the interaction between F508del CFTR and ezrin as well as between NHERF1 and ezrin (Fig. 5C).
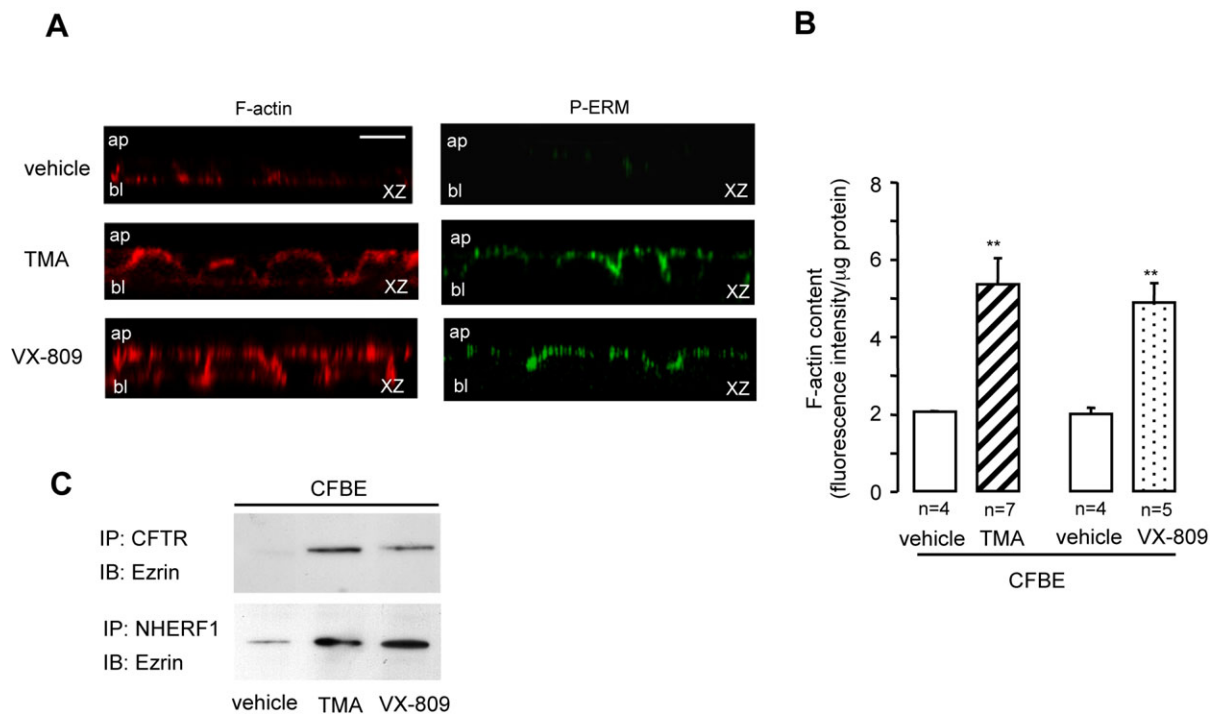
Fig. 5. TMA and VX-809 correctors induce the apical localization of F-actin and phospho-ezrin, enhance the F-actin content and favor the interaction between CFTR and ezrin and between NHERF1 and ezrin in CFBE cells. (A) Confocal immunofluorescence microscopy images of polarized cell monolayers incubated with phalloidin–TRITC to visualize F-actin and with a polyclonal antibody against phospho-ERM (P-ERM). All images show the cell's vertical cross section (xz) planes. ap, apical region; bl, basolateral region. Scale bar: 10 µm. (B) Summary of F-actin content measurements. Data represent mean±s.e.m. **$P<0.01$ (Student's t-test). (C) The interactions between CFTR and ezrin, and between NHERF1 and ezrin were analyzed in co-immunoprecipitation (IP) experiments as described in the Materials and Methods.

As shown in Fig. 6, either the pre-treatment of CFBE cells with Latrunculin B (Fig. 6A,B) or the transfection with the phospho-dead ezrin mutant, T567A (Fig. 6C,D) inhibited the corrector-induced rescue of both the F508del CFTR-dependent chloride efflux and the subcortical cAMP re-compartmentalization. By contrast, transfection of CFBE cells with T567D ezrin did not significantly alter the rescuing effect of the corrector (Fig. 6E,F). Taken together, these data indicate that the corrector-induced rescue of F508del CFTR-dependent chloride secretion and sub-plasma-membrane cAMP compartmentalization involves both the cytoskeleton organization and ezrin activation.

As primary airway epithelial cells provide the closest 'in vitro' representation of the airway epithelium by providing a microenvironment or architecture closer to 'in vivo' situations, we analyzed the corrector effects on cAMP compartmentalization in primary human bronchial epithelial cells. As primary cells are more resistant to transfection, the FRET H30 and mpH30 probes were cloned into adenoviral vectors. In these experiments, we averaged together the results obtained from two different F508del CFTR homozygous individuals (CF-BE) and two non-cystic fibrosis donors (wt-BE). As shown in Fig. 7A, the wt-BE cells expressing wt CFTR displayed higher cAMP levels in the sub-plasma-membrane region, whereas, in the CF-BE cells, cAMP was more compartmentalized in the cytosol at the expense of the sub-plasma-membrane region. Similar results were obtained when the primary wt-BE and CF-BE cells were grown as polarized monolayers and cultured in air–liquid interface (ALI) conditions on permeable filters mimicking the in vivo situation (Fig. 7B). Taken together, these data demonstrate that the difference in cAMP compartmentalization between cystic fibrosis and non-

cystic fibrosis immortalized airway cells is conserved in primary cells.

We have previously reported that both TMA (200 nM) and VX-809 (5 µM) rescue the functional expression of F508del CFTR on the apical membrane of polarized primary CF-BE cell monolayers (Favia et al., 2014). In Fig. 8A, we show that in the primary CF-BE cells both correctors also induced the apical rescue of actin organization and phospho-ezrin expression. In addition, we found that cAMP membrane compartmentalization was rescued by either TMA or VX-809, whereas Latrunculin B pretreatment significantly prevented this rescue (Fig. 8B). Importantly, the rescue of F508del-CFTR-dependent chloride secretion induced by TMA or VX-809 in CF-BE cells was not significantly changed by the transfection of CF-BE cells with T567D ezrin but was completely impeded by the T567A ezrin transfection (Fig. 8C).

Taken together, these results highlight the importance of corrector-dependent ezrin activation and actin re-organization in creating the conditions to generate a subcortical cAMP pool of adequate amplitude to activate the PKA-driven, F508del CFTR dependent chloride secretion.

DISCUSSION

It is well established that the interaction of wt CFTR with the actin cytoskeleton, through protein complexes including NHERF1 and ezrin, results in an increase of its stability on the plasma membrane (Haggie et al., 2006, 2004; Li et al., 2005). In comparison to wt CFTR, the interactions between the rescued F508del CFTR and the PDZ-domain-containing protein are reduced (Valentine et al., 2012) and this could partially explain why the temperature-rescued F508del CFTR, once returned to 37°C, shows surface instability,
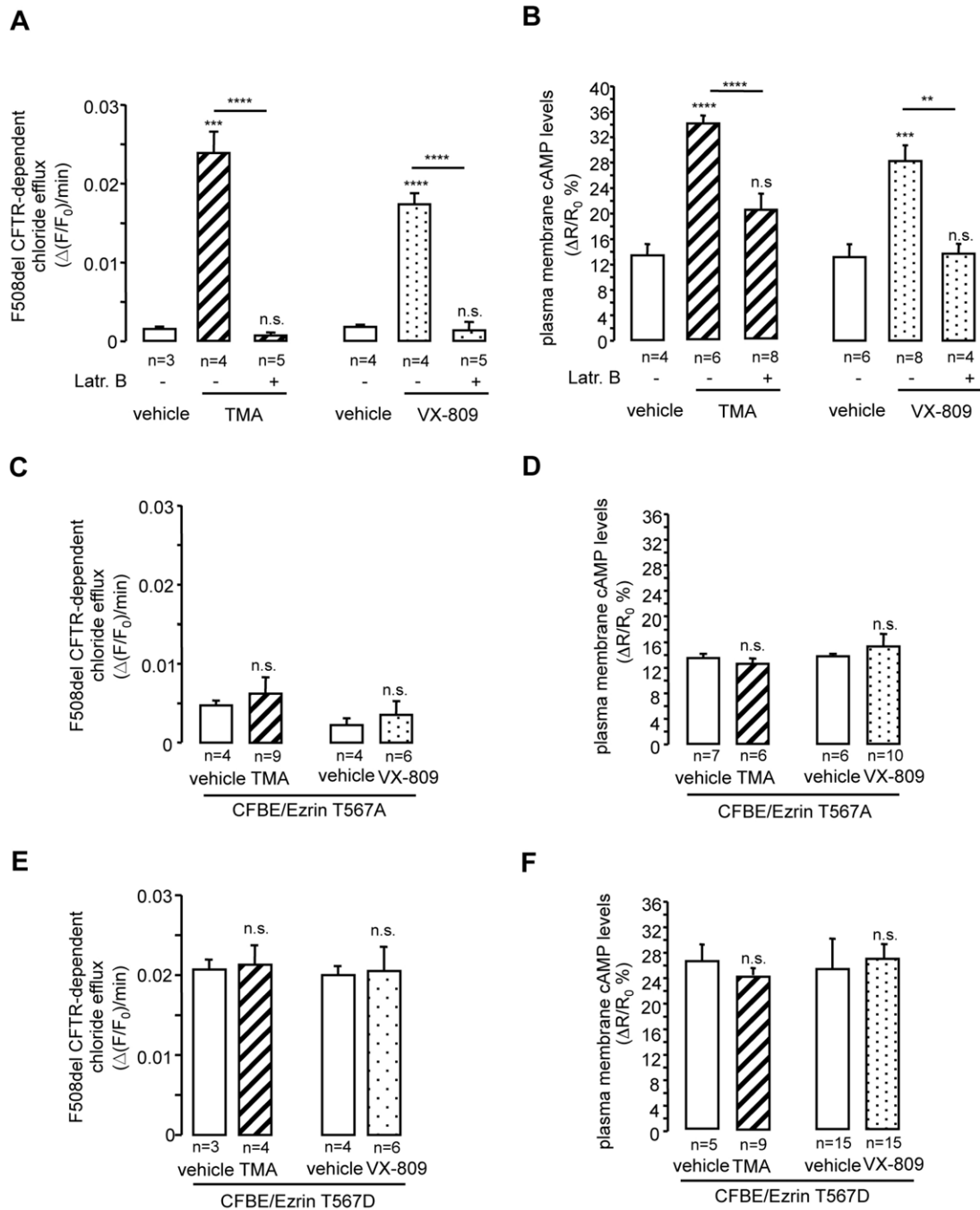
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Fig. 6. Both cytoskeleton disorganization and T567A ezrin expression reduce the corrector-induced rescue of cAMP plasma membrane redistribution and F508del CFTR activation in CFBE cells. CFBE cells were incubated with 100 nM TMA or 5 μM VX-809 for 24 h. (A) Summary of CFTR-dependent chloride efflux performed in polarized CFBE cell monolayers pre-incubated with vehicle or correctors and treated or not with 10 μM Latrunculin B for 30 min before the experiments. (B) Summary of FRET cAMP changes measured in CFBE cells expressing the mpH30 sensor, pre-incubated with corrector and treated or not with Latrunculin B. (C,D) CFBE cells transfected with non-phosphorylable ezrin, T567A (CFBE/ezrin T567A), were incubated with 100 nM TMA or 5 μM VX-809 for 24 h before functional and FRET experiments. (C) Summary of CFTR-dependent chloride effluxes analyzed by spectrofluorimetric measurements in polarized CFBE/ezrin T567A monolayers treated with correctors or their vehicle. (D) Summary of cAMP FRET changes measured in CFBE and CFBE/T567A cells expressing the mpH30 sensor upon stimulation with 1 μM FSK treated with correctors or their vehicle. (E,F) CFBE cells transfected with T567D ezrin (CFBE/ezrin T567D), were preincubated with 100 nM TMA or 5 μM VX-809 before functional and FRET experiments. (E) Summary of CFTR-dependent chloride effluxes analyzed by spectrofluorimetric measurements in polarized CFBE/ezrin T567D monolayers treated with the correctors or their vehicle. (F) Summary of cAMP FRET changes measured in CFBE/ezrin T567D cells expressing the mpH30 sensor and treated with the correctors or their vehicle. Data represent mean±s.e.m. **$P<0.01$; ***$P<0.001$; ****$P<0.0001$ (Student's t-test).

accelerated internalization and reduced recycling back to the cell surface (Cholon et al., 2010; Okiyoneda et al., 2010; Sharma et al., 2004). Based on these observations, we asked whether the

activation of ezrin and its binding to membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) are the key events necessary to rescue the functional expression of F508del CFTR on
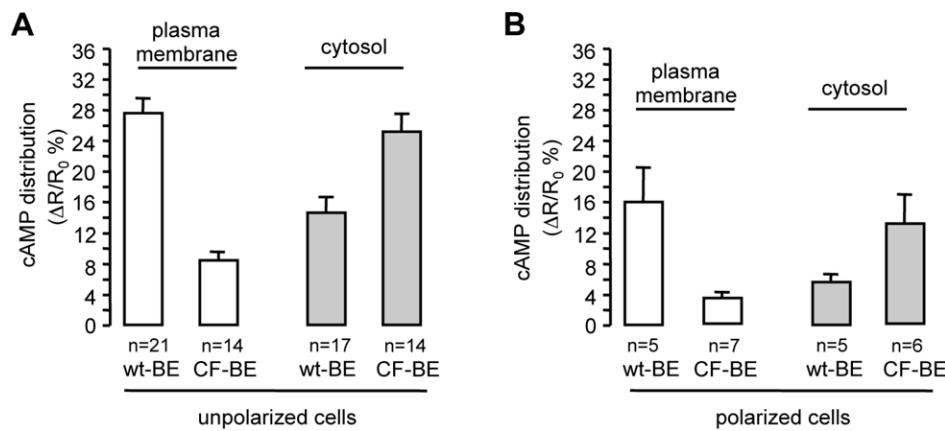
Fig. 7. Spatio-temporal distribution of cAMP in primary airway epithelial cells. wt-BE and CF-BE primary cells (obtained from two different donors and patients, respectively) grown on glass (A) or on permeable filters (B) were infected with adenoviral vectors encoding mpH30 (white bars) and H30 sensors (gray bars) (60 MOI) for 48 h. FRET changes were recorded upon 10 µM FSK stimulation. Data represent means±s.e.m.

the apical membrane of CFBE cells and, more importantly, if the small-molecule corrector-induced rescue of F508del CFTR to the plasma membrane involves ezrin activation and actin cytoskeleton reorganization.

Most of ezrin is maintained in an inactive conformation in the cytoplasm, through intramolecular interactions between its

N-terminal FERM and the C-terminal domains (Bretscher et al., 2002), and its activation and recruitment to the plasma membrane require both the phosphorylation of the threonine residue in the actin-binding domain (T567) (Bretscher et al., 2002; Yonemura et al., 2002) and the interaction of its FERM domain with PIP_2 present in compartmentalized pools at the apical surface of
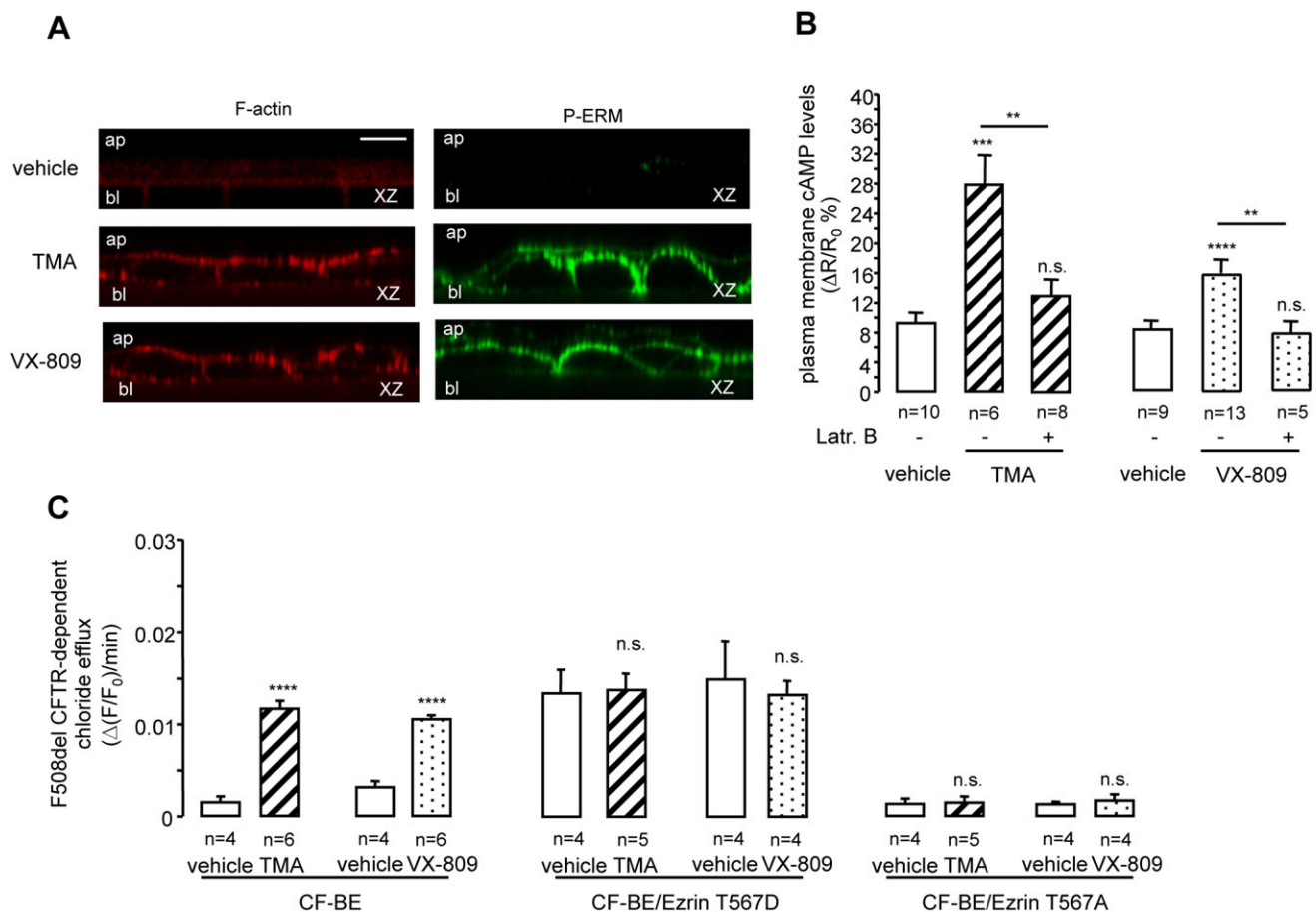

Fig. 8. Cytoskeleton organization and ezrin activation are required for corrector-induced rescue of plasma membrane cAMP redistribution and F508del CFTR activation in CF-BE primary cells. CF-BE primary cells were incubated with 100 nM TMA or 5 µM VX-809 for 24 h. (A) Confocal immunofluorescence microscopy images of polarized primary cell monolayers grown at air–liquid interface incubated with phalloidin–TRITC to visualize F-actin or with a polyclonal antibody anti-phospho-ERM (P-ERM). All images show the cell's vertical cross section (xz) plane. ap, apical region; bl, basolateral region. Scale bar: 10 µm. (B) Summary of FRET cAMP changes measured in CF-BE cells infected with the adenoviral vector encoding the mpH30 sensor, pre-incubated with correctors or their vehicle and treated or not with 10 µM Latrunculin B for 30 min. FRET analysis was performed 48 h after infection and 24 h after corrector treatment. (C) Summary of CFTR-dependent chloride effluxes analyzed by spectrofluorimetric measurements in polarized CF-BE, CF-BE/ezrin T567D and CF-BE/ezrin T567A monolayers treated with vehicles or correctors. Data represent mean±s.e.m. **$P<0.01$; ***$P<0.001$; ****$P<0.0001$; n.s., not significant (Student's t-test).

polarized epithelia (Yonemura et al., 2002). The binding of ezrin with PIP_2 is not only a prerequisite for its phosphorylation (Auvinen et al., 2007; Fievet et al., 2004; Hao et al., 2009), but renders its F-actin-binding site more accessible (Bosk et al., 2011; Janke et al., 2008).

In agreement with these findings, we show here that the phosphorylation of ezrin and its interaction with PIP_2 increases F-actin polymerization and tethers F508del CFTR to the actin cytoskeleton by enhancing its interaction with both NHERF1 and actin. Moreover, expression of the ezrin T567D mutant increases stability of the F508del CFTR at the apical cell surface and significantly rescues the apical PKA-driven F508del CFTR-dependent chloride efflux.

We have previously reported that, in CFBE cells, cAMP and activated PKA are more compartmentalized in the cytosol at the expense of the sub-plasma-membrane region, whereas HBE cells displayed higher levels of cAMP and activated PKA in the sub-plasma-membrane region compartment (Monterisi et al., 2012). Both an organized subcortical cytoskeleton and the CFTR expression on the plasma membrane are responsible for cAMP compartmentalization in the plasma membrane region as either silencing wt CFTR expression or disaggregating the actin cytoskeleton increased cAMP and activated PKA compartmentalization in the bulk cytoplasm in HBE cells (Monterisi et al., 2012). Here, by monitoring the intracellular levels of cAMP and PKA activity in response to FSK stimulation in living CFBE cells, we found that ezrin activation and its interaction with PIP_2 promotes the increase of cAMP compartmentalization in the subcortical region in such a way as to allow the activation of local PKA with the consequent rescue of apical F508del-CFTR-dependent chloride efflux.

Based on these findings, it was important to understand whether small-molecule correctors not only overcome the processing defect of F508del CFTR, but might also influence its functional expression on the plasma membrane by promoting the formation of a multiprotein complex that anchors it to the cortical cytoskeleton and restores a sufficiently high concentration of cAMP and activated PKA in the sub-plasma membrane region. In this regard, it has been recently demonstrated that one of the most promising correctors, VX-809 (Van Goor et al., 2011), stabilized F508del CFTR at the plasma membrane, improving its interaction with NHERF1 (Arora et al., 2014; Loureiro et al., 2015).

Here, we extended these studies and we found that both VX-809 and TMA, a molecule that both corrects and potentiates the F508del-CFTR-dependent chloride secretion (Favia et al., 2014; Tamanini et al., 2011), also restore the apical expression of phosphorylated ezrin and actin organization in polarized CFBE cell monolayers. Furthermore, in line with previous findings demonstrating that pharmacological molecules (correctors and potentiators) might increase the susceptibility of the R-domain of F508del CFTR to be phosphorylated (Pyle et al., 2011), we found that both correctors increase both the cAMP and the activated PKA compartmentalization in the sub-plasma-membrane region, allowing the consequent rescue of F508del-CFTR-dependent chloride efflux (Fig. 3). Both Latrunculin B treatment and transfection of CFBE cells with the phospho-dead ezrin mutant T567A reversed this corrector-induced rescue of the F508del-CFTR-mediated chloride efflux and the sub-cortical cAMP compartmentalization.

Importantly, the findings that both VX-809 and TMA, in analogy to what was observed in CFBE cells, were able to rescue the cAMP compartmentalization in the membrane region of FRT cells expressing F508del CFTR but not in FRT cells expressing

G551D CFTR (Fig. 4), confirm the hypothesis that the corrector-induced increase of cAMP compartmentalization in the membrane region is strictly correlated to the corrector-induced rescue of F508del CFTR expression to the plasma membrane.

As the effects of pharmacological correctors are significantly influenced by the cell background (Pedemonte et al., 2010), we analyzed the involvement of ezrin activation and actin reorganization in the rescuing effect of the two correctors in primary airway epithelial cells that provide the closest in vitro representation of the airway epithelium (Neuberger et al., 2011; Randell et al., 2011). As observed for the secondary airway cells (Monterisi et al., 2012), we found that the primary airway wt-BE cells expressing wt CFTR exhibited a higher cAMP compartmentalization in the sub-plasma-membrane region whereas the CF-BE cell monolayers expressing F508del CFTR display a defective accumulation of cAMP in the subcortical compartment in favor of its accumulation in the cytosolic compartment. Importantly, here we found that both TMA and VX-809, in addition to rescuing F508del CFTR chloride secretion, restored both the apical expression of phosphorylated ezrin and actin organization and increased cAMP compartmentalization in the sub-plasma-membrane region.

The findings that either T567A expression or Latrunculin pretreatment in both secondary (Fig. 6) and primary (Fig. 8) cells abrogated the corrector-induced rescue of both the F508del-CFTR-dependent chloride efflux and the subcortical cAMP compartmentalization underline the role of both ezrin activation and actin re-organization in the rescue of F508del CFTR induced by both correctors.

Taken together, these data suggest that both correctors modulate the balance between the macromolecular complexes regulating the cAMP signaling machinery in the vicinity of CFTR that either attenuate or enhance its activation (Blanchard et al., 2014; Li and Naren, 2005) and, in this way, restore the cAMP and PKA compartmentalization in the membrane region and improve the cellular environment that has been perturbed by the lack of a functional CFTR on the cell membrane.

Finally, the recent findings that chronic application of the potentiator VX-770 in combination with VX-809 resulted in a reduction of the correction efficacy of VX-809 due to destabilization of F508del CFTR and a consequent increase in its turnover rate (Cholon et al., 2014; Veit et al., 2014), highlight the need to identify new potential targets for therapeutically improving the formation of the multiprotein complex in order to favor the cAMP and PKA compartmentalization in the vicinity of F508del CFTR.

MATERIALS AND METHODS
Reagents and antibodies

The primary antibodies used for immunocytochemical analysis, immunoprecipitation and western blots were as follows (dilutions are given in the relevant sections): monoclonal anti-CFTR (CF3, Abcam), monoclonal anti-human CFTR against the C terminus (catalog no. MAB25031, R&D Systems), polyclonal anti-human CFTR (H-182, Santa Cruz Biotechnology, DBA, Italy), polyclonal anti-human phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) (catalog no. 3141, Cell Signaling Technology), monoclonal anti-β-actin (catalog no. A5441, Sigma-Aldrich), polyclonal anti-ezrin (catalog no. A00457, GenScript), polyclonal anti-human NHERF1 (catalog no. PA1-090, Thermo Fisher Scientific Inc.), monoclonal anti-human NHERF1 (catalog no. 611161, Becton Dickinson), monoclonal anti-ezrin (catalog no. 610603, Becton Dickinson). The appropriate secondary antibodies were purchased from Sigma-Aldrich or Invitrogen. Forskolin (FSK), $CFTR_{inh-172}$ and

Hygromycin B were purchased from Calbiochem. Sulfo-NHS-biotin, 3-isobutyl-1-methylxanthine (IBMX), phalloidin-tetramethylrhodamine B isothiocyanate (TRITC) and N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) were purchased from Sigma-Aldrich. EZ-LinkSulfo-NHS-SS-Biotin and streptavidin–agarose resin were purchased from Pierce Chemicals. Lipofectamine 3000 reagent was purchased from Invitrogen and JetPei from Polyplus transfection (Illkirch, France). Zeocin was purchased from Santa Cruz Biotechnology. The drug TMA was synthesized at the Department of Pharmaceutical Sciences, University of Padova, Italy and was dissolved in 3% DMSO in methanol (vehicle) at a concentration of 2.5 mM (stock solution), whereas VX-809 was purchased from Selleck Chemicals and was dissolved in DMSO at concentrations of 20 mM. FRET reporters, subcloned into adenoviral vectors, were generated by Vectors Biolabs (Philadelphia, Pennsylvania).

Cell culture
Experiments were performed with human immortalized bronchial epithelial cell lines: the CFBE41o- cells homozygous for the F508del allele (F508del/F508del), CFBE wt CFTR cells (CFBE41o-, with stable expression of wild-type CFTR) and 16HBE14o-, endogenously expressing wt CFTR. Cells were a generous gift from Dieter Gruenert (University of California, San Francisco, CA). The cells were grown in Eagle's minimal essential medium (MEM; EuroClone) supplemented with 10% fetal bovine serum (Lonza), L-glutamine, and penicillin-streptomycin at 37°C under 5% CO$_2$. CFBE wt CFTR cells were maintained in presence of Hygromycin-B-positive selection. They were routinely grown in plastic flasks coated with an extracellular matrix. For chloride efflux experiments, confocal immunofluorescence analysis, biotinylation assay and cell surface stability studies, cells were seeded on 0.4-μm pore size PET filter inserts (Falcon; BD Biosciences Discovery Labware) coated with the same extracellular matrix. Primary human bronchial airway epithelial cells, derived either from F508del CFTR homozygous donors, denoted CF-BE, and from non-cystic fibrosis donors, were provided by 'Italian Cystic Fibrosis Foundation Primary Culture Core Facility'. The collection of bronchial epithelial cell and their use to investigate the mechanisms of transepithelial ion transport in cystic fibrosis were specifically approved by the Ethics Committee of the Istituto Giannina Gaslini (Genova Italy) following the guidelines of the Italian Ministry of Health (no. IGG:192). All primary cells were maintained at an air–liquid interface (ALI) according to the manufacturer's instruction. Primary cell differentiation was verified by immunostaining with a monoclonal anti-acetylated tubulin antibody (Sigma-Aldrich) for ciliated cells (Scudieri et al., 2012).

Fischer rat thyroid (FRT) epithelial cells, non-expressing CFTR either stably expressing human F508del CFTR or G551D CFTR and carrying the resistance gene for zeocin were a generous gift from Luis Galietta (Gaslini Institute, Genoa, Italy). Null CFTR-FRT, F508del CFTR-FRT and G551D CFTR-FRT cells were grown in Coon's modified Ham's F-12 medium plus 10% FBS, L-glutamine, and penicillin-streptomycin at 37°C under 5% CO$_2$. The stably transfected clones were maintained in presence of zeocin-positive selection (600 μg ml^{-1}). All cell lines were recently authenticated and tested for contamination.

Transfection of cells
At 90–95% confluence, cells were transiently transfected with human ezrin cDNA mutant constructs (Babich and Di Sole, 2015; Di Sole et al., 2009) by using Lipofectamine 3000 reagent according to the manufacturer's protocol and the experiments were conducted 48 h later. All of these clones behave as dominant mutants over the endogenous protein. Levels of construct expression were analyzed both by immunoblotting and immunostaining for GFP and the transfection efficiency was ∼60%. Western blotting analysis showed that exogenously expressed ezrin protein mutants had ∼25% higher expression levels than endogenous ezrin in cultured cells (data not shown). Considering a transfection efficiency of ∼60%, the actual expression level of GFP–ezrin in positively transfected cells was ∼40% higher than that of endogenous protein. For FRET experiments, immortalized cells were transiently transfected with plasmid constructs encoding for the FRET sensors, using JetPei transfection reagent according to the manufacturer's protocol. FRET imaging was conducted 24 h later.

Measurements of cAMP levels and PKA activity by FRET
FRET measurements were performed as described previously (Di Benedetto et al., 2008; Monterisi et al., 2012). A total of 5×10^4 primary cells seeded on glass or on permeable inserts were infected with adenoviral FRET probes at 60 multiplicity of infection (MOI) 48 h before the experiment.

Measurements of cAMP concentration
Changes of cAMP concentrations induced by 1 μM FSK stimulation in cells transfected with ezrin mutants or pretreated with the correctors were monitored by a bioluminescent assay (cAMP-Glo Assay-Promega) according to the manufacturer's instructions.

F-actin content in adherent cells
The actin polymerization assay was performed as previously described (Favia et al., 2010).

Immunofluorescence and confocal analysis
At 48 h after transfection, polarized cell monolayers were fixed in 3.7% paraformaldehyde and permeabilized in 0.1% Triton X-100 in PBS. The permeabilization was omitted for cell monolayers stained for anti-CFTR (CF3) mouse monoclonal (1:500). Goat anti-mouse-IgG conjugated to Alexa Fluor 568 (1:1000) was used as secondary antibody. To analyze the distribution of phosphorylated ERM, monolayers were incubated with polyclonal anti-human phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) (Cell Signaling, catalog no. 3141; dilution 1:100; this antibody recognizes all the phosphorylated ERM proteins.) according to the manufacturer's protocol. Goat anti-rabbit-IgG conjugated to Alexa Fluor 488 (dilution 1:1000) was used as secondary antibody for phospho-ezrin. Monolayers were stained with 20 nM phalloidin–TRITC to visualize F-actin. Monolayers were then mounted onto slides with Prolong Gold antifade reagent (Invitrogen) and examined with a Leica TCS SP5 II microscope equipped with a laser-scanning confocal unit containing a He-Ne argon laser (Leica). Specimens were viewed through a Planapo 63×/1.25 oil immersion objective and images were acquired with the LAS-AF Version 2.2.1 software.

Fluorescence measurements of apical chloride efflux
Chloride efflux was performed as previously described (Guerra et al., 2005). Confluent cell monolayers grown on permeable filters and at 48 h after transfection were loaded overnight in culture medium containing 5 mM MQAE at 37°C in a CO$_2$ incubator. These were then inserted into a perfusion chamber that allowed independent perfusion of apical and basolateral cell surfaces. Fluorescence was recorded with a Cary Eclipse spectrofluorometer. The apical CFTR-dependent chloride secretion was measured as the difference in the rate of change of FSK- plus IBMX-stimulated fluorescence in the absence or presence of apical treatment with CFTR$_{Inh}$-172 [$\Delta(F/F_0)$/min].

Co-immunoprecipitation and western blotting
At 48 h after transfection or 24 h after corrector incubation cells were lysed in co-immunoprecipitation buffer [50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 5 mM MgCl$_2$, 10% (v/v) glycerol, 0.5% (w/v) sodium deoxycholate and 1% Triton X-100, plus a protease inhibitor cocktail (Sigma-Aldrich)] and centrifuged at 10,000 g for 5 min at 4°C. An aliquot of 350 μg of protein was incubated with anti-ezrin polyclonal antibody (2 μg), anti-NHERF1 polyclonal antibody (2 μg) or anti-CFTR polyclonal antibody (2 μg) with rotation overnight at 4°C, followed by addition of 50 μl of PureProteome Protein A Magnetic Beads (Millipore) for an additional 2 h. Then, immunocomplexes were washed with PBS containing 0.1% Tween 20 and then eluted in Laemmli buffer, heated at 95°C for 5 min. Samples were run on 4–12% SDS-PAGE Criterion XT Precast gels (BioRad) and then transferred onto a polyvinylidene fluoride (PVDF) microporous membrane (Millipore). Proteins were probed by appropriate primary (CFTR, 1:500, β-actin, 1:5000 or ezrin, 1:250) and secondary antibodies. Following a final wash, proteins were detected using enhanced chemiluminescence (Cell Signaling). Densitometric quantification and image processing were carried out using Photoshop (Adobe Systems) and

the NIH Image software package version 1.61 (National Institutes of Health, Bethesda, MD).

Biotinylation assay and cell surface stability of Apical Membrane Proteins

Cells, transfected with the mutant ezrin cDNA constructs or the empty vector, were washed with PBS and incubated with 1 mg ml^{-1} sulfo-NHS-biotin (Sigma) in PBS for 30 min at 4°C. Free sulfo-NHS-biotin was removed by washing cells twice at 4°C with 0.1 M glycine in PBS and then with PBS. Cells were lysed in biotinylation lysis buffer (BLB: 0.4% deoxycholate, 1% NP-40, 50 mM EGTA, 10 mM Tris-Cl, pH 7.4, and Protease Inhibitor Cocktail), sonicated, and centrifuged, and the pellet was discarded. Volumes of supernatant, containing equal amounts of protein, were incubated overnight at 4°C with gentle mixing with streptavidin-agarose beads (50 µl of streptavidin/mg of biotin). Streptavidin-bound complexes were pelleted (16,000 g), and after two washes with BLB, biotinylated proteins were eluted in Laemmli buffer. The eluted proteins were subjected to 4-12% SDS-PAGE Criterion XT Precast gels and western blotting.

To analyze the effect of mutant ezrin constructs on rescued F508del CFTR cell surface stability, cell monolayers, cultured at 27°C for 48 h, were transfected, and 24 h after transfection were transferred at 37°C and incubated for 0 h up to 8 h. Then cell surface proteins were biotinylated and lysed in BLB buffer and processed as described above.

In situ proximity ligation assay

At 48 h after transfection, cells were fixed in 3.7% paraformaldehyde and permeabilized in 0.1% Triton X-100 in PBS. Then cells were incubated with rabbit CFTR polyclonal antibody (1:50) and mouse NHERF1 monoclonal antibody (1:50). The PLA was performed according to the manufacturer's instructions using anti-rabbit (plus) and anti-mouse (minus) Duolink In Situ PLA probes (Sigma). The images were taken using a fluorescence microscope (Nikon Eclipse E400), and the PLA signals that represent the site of the protein−protein interaction appear in red. Specimens were viewed through a 40×0.75 NA objective, and images were acquired by the NIS-Elements version 3.0 software.

Data analysis

The data for all of the experiments are presented as mean±s.e.m. for the number of samples indicated (n). Differences between control and test conditions were assessed statistically using unpaired data Student's t-test or ANOVA as appropriate. Differences were considered significant when $P<0.05$.

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