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HGF Stimulation of Rac1 Signaling Enhances Pharmacological Correction of the Most Prevalent Cystic Fibrosis Mutant F508del-CFTR

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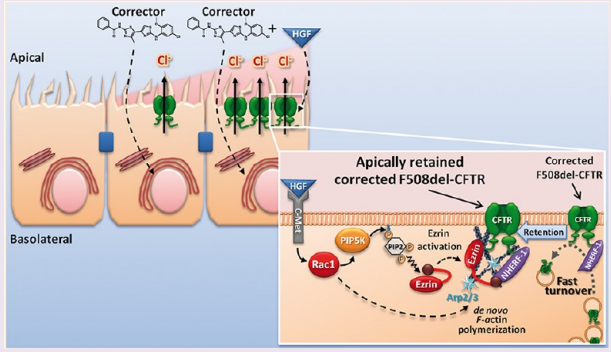
Supporting Information

ABSTRACT: Cystic fibrosis (CF), a major life-limiting genetic disease leading to severe respiratory symptoms, is caused by mutations in CF transmembrane conductance regulator (CFTR), a chloride (Cl^-) channel expressed at the apical membrane of epithelial cells. Absence of functional CFTR from the surface of respiratory cells reduces mucociliary clearance, promoting airways obstruction, chronic infection, and ultimately lung failure. The most frequent mutation, F508del, causes the channel to misfold, triggering its premature degradation and preventing it from reaching the cell surface. Recently, novel small-molecule correctors rescuing plasma membrane localization of F508del-CFTR underwent clinical trials but with limited success. Plausibly, this may be due to the mutant intrinsic plasma membrane (PM) instability. Herein, we show that restoration of F508del-CFTR PM localization by correctors can be dramatically improved through a novel pathway involving stimulation of signaling by the endogenous small GTPase Rac1 *via* hepatocyte growth factor (HGF). We first show that CFTR anchors to apical actin cytoskeleton (*via* Ezrin) upon activation of Rac1 signaling through PIP5K and Arp2/3. We then found that such anchoring retains pharmacologically rescued F508del-CFTR at the cell surface, boosting functional restoration by correctors up to 30% of wild-type channel levels in human airway epithelial cells. Our findings reveal that surface anchoring and retention is a major target pathway for CF pharmacotherapy, namely, to achieve maximal restoration of F508del-CFTR in patients in combination with correctors. Moreover, this approach may also translate to other disorders caused by trafficking-deficient surface proteins.



Cystic fibrosis (CF), the most common, life-limiting autosomal genetic disease in Caucasians, is caused by impaired functional expression of CFTR (CF transmembrane conductance regulator), a chloride (Cl^-) channel expressed at the plasma membrane (PM) of epithelial cells.[1] The most severe CF symptoms manifest in the lungs where hindered CFTR function impairs ionic homeostasis and airway hydration, which results in increased mucus viscosity and compromised mucociliary clearance, promotes chronic infection and inflammation, and ultimately leads to respiratory failure. CF-causing mutations can interfere with the biogenesis, transport, and activity of CFTR but also with its stability at the cell surface.[2] About 90% of CF patients have a deletion of Phe508 (F508del) in at least one of their CFTR genes.[2,3] Hence, most CF translational research efforts have aimed to functionally rescue F508del-CFTR through the correction of the folding defect induced by this mutation, which results in substantial endoplasmic reticulum (ER) retention and premature degradation, preventing the mutant protein from reaching the cell surface.[1,3] Pharmacological agents (termed correctors), recently identified by high-throughput screenings of small molecules, were shown to rescue of the trafficking defect associated with F508del-CFTR.[3,4] However, results from ongoing clinical trials for the most promising correctors have yielded limited success in restoring F508del-CFTR function in patients.[4]

Recent studies revealed that despite exhibiting partial channel function,[1] the F508del-CFTR molecules that reach the cell surface present a highly decreased PM half-life due to both

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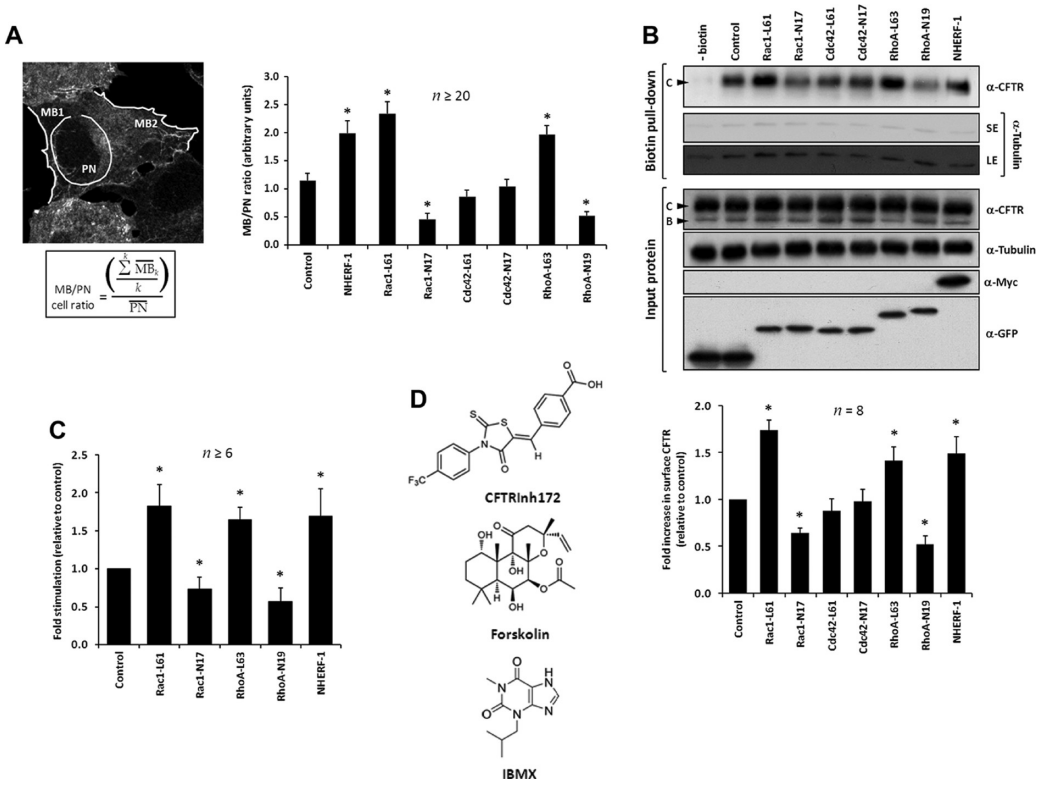

Figure 1. Expression of active Rac1 or RhoA mutants increases CFTR levels at the cell surface. Wt-BHK cells transfected as indicated were analyzed by (A) semi-quantitative confocal immunofluorescence (qIF, see also Supplementary Figure S1), where the ratio between the mean pixel intensity from CFTR signals at the plasma membrane (MB) and at the perinuclear region (PN) was determined (MB/PN; see left insert); (B) biotinylation of cell surface proteins (quantitated in the lower graph). Shown are representative WBs of CFTR protein in either the pulled-down fraction (biotin pull-down) or the correspondent whole cell lysates (input). Membranes were further stained for α-tubulin both as loading control and to assess contamination of pull-downs with intracellular proteins from permeable cells. Note that only residual contamination was detectable (SE, similar exposure), similar to the background levels in "- biotin" control (longer exposure, LE). The input WBs were also stained to document transfected protein levels; (C) fold change, relative to control cells, in forskolin (10 μM) plus IBMX (50 μM)-stimulated, CFTRInh172 (20 μM)-blocked iodide efflux (details in Supporting Information). (D) Chemical structures of CFTRInh172, forskolin, and IBMX. All panels show means ± SEM. Asterisk indicates $p < 0.05$ relative to control cells.

accelerated endocytosis[5] and fast turnover.[6] Given this accelerated endocytic rate of F508del-CFTR, part of the incomplete effectiveness of these compounds may derive from an inability to sustain sufficient surface CFTR levels at patient-tolerated doses. Accordingly, molecular strategies achieving the PM retention of F508del-CFTR could be relevant as to enhance the efficacy of small-molecule correctors.

Current data indicate that up to 50% of surface CFTR exists in an immobile pool, tethered to filamentous (F-) actin.[7] Such tethering involves the interaction of CFTR C-terminal domain with the PDZ adaptor protein Na+/H+ exchanger regulatory factor isoform-1 (NHERF-1),[8] also important to target CFTR to exosomes and endosomes in polarized epithelial cells.[9] By interacting with Ezrin, an ERM (Ezrin/Radixin/Moesin) family protein, NHERF-1 anchors CFTR to the apical actin cytoskeleton, preventing its endocytosis.[7,10] While the integrity of the actin cytoskeleton was shown to be critical for CFTR recycling and PM retention,[11] the mechanisms regulating CFTR tethering and immobilization at cell surface still require elucidation.[12]

Small GTPases of the Rho family, found in all eukaryotic organisms, are key regulators of actin cytoskeleton dynamics.[13,14] The canonical members of this family, RhoA, Rac1, and Cdc42, have been implicated in the regulation of cell polarity and membrane trafficking through their modulation of F-actin remodeling.[14−16] Notably, the reciprocal regulation of Rho GTPases and ERM proteins controls the distribution and anchorage of macromolecular protein complexes essential to maintain cell polarity.[12,16,17] These data suggest Rho GTPases as good candidates to regulate F-actin-dependent tethering and surface retention of CFTR.

Herein, we reveal the Rho GTPase Rac1 as a key regulator of Ezrin-mediated anchoring of CFTR to F-actin in airway epithelial cells. Furthermore, we demonstrate that a physiological stimulus, HGF-induced activation of endogenous Rac1 signaling, is sufficient to coax the PM anchoring of F508del-CFTR and dramatically improves its apical retention and function upon pharmacological rescue in bronchial epithelial cells. Altogether, our data provide important new insights into the mechanisms regulating CFTR expression at the cell surface and reveal how CFTR PM anchoring and retention pathways can be pharmacologically manipulated to the benefit of CF patients. This approach may apply to other diseases resulting from deficient traffic of cell surface proteins.

RESULTS AND DISCUSSION

The purpose of the present work was to get new insights on the molecular pathways involved in the regulation of CFTR retention at the cell surface, namely, those responsible for its anchoring to the actin cytoskeleton, and to determine whether

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these pathways could be manipulated to enhance the restoration of F508del-CFTR function by chemical correctors.

Active Rac1 and RhoA Increase wt CFTR Cell Surface Levels. To address whether canonical small Rho GTPases participate in the regulation of CFTR retention at the PM, we started by overexpressing constitutively active (CA) or dominant negative (DN) mutants of RhoA, Rac1, and Cdc42 in the commonly used BHK cell model, stably expressing human wt-CFTR (wt-BHK).[18,19] NHERF-1 overexpression served as positive control as it was shown to increase CFTR PM levels.[20] The effects of each GTPase mutant on CFTR intracellular distribution were monitored by confocal immunofluorescence (IF) microscopy. A semi-quantitative approach (qIF), based on the ratio between membrane-associated (MB) and perinuclear (PN) CFTR signals, was used to distinguish selective PM enrichments from overall increased CFTR levels (MB/PN ratio, Figure 1A and see also Supplementary Figure S1). This showed that CA variants of both Rac1 (Rac1-L61) and RhoA (RhoA-L63) promoted a ∼2-fold increase in CFTR staining at the PM region, similar to that induced by NHERF-1. Conversely, the DN mutants of both GTPases (Rac1-N17 and RhoA-N19) produced a proportional decrease in CFTR PM signals. No significant ratio changes were observed with either DN or CA forms of Cdc42, reinforcing the specificity of the effects observed for RhoA and Rac1. To confirm these results biochemically, cell surface proteins on transfected cells were labeled with soluble sulfo-NHS-SS-biotin and isolated from cell lysates using Streptavidin beads. Analysis of pulled-down and input CFTR levels by Western blot (Figure 1B) confirmed that expression of Rac1 and RhoA CA mutants produced a significant enrichment in the amount of Golgi-processed, mature CFTR glycoform (band C) at the cell surface, whereas their DN mutants induced a reciprocal decrease in CFTR levels at the cell surface. Again, no significant effect was observed for Cdc42 CA or DN variants. Moreover, none of the mutants significantly affected the overall levels of either the mature (band C) or the immature (faster migrating, core-glycosylated, ER-associated band B) forms of CFTR in these cells (Figure1B, input protein). These data suggest that the observed effects were not caused by changes in CFTR synthesis, ER-to-Golgi trafficking, or turnover but rather by interference with either the channel delivery from the trans-Golgi network (TGN) to the PM or its retention at the PM. Because changes in cell surface channel levels are expected to impact CFTR-mediated ion transport, we tested the effect of Rac1 and RhoA mutants on cAMP-stimulated iodide efflux in these cells. Consistent with the above findings, expression of NHERF-1, Rac1-L61, and RhoA-L63 produced a ∼2-fold increase in CFTR-dependent iodide release, whereas transfection of the DN GTPases induced a reciprocal decrease (Figure 1C).

Rac1 and RhoA Affect CFTR Cell Surface Levels through Different Mechanisms. Using a bronchial epithelial cell line that stably expresses wt-CFTR (HBE41o-),[21] we then confirmed that the phenotypes induced by Rac1 and RhoA mutants in BHK cells also clearly occurred, even more pronouncedly, in this more physiologically relevant system (Figure 2A). Analysis of glucose transporter 1 (Glut-1) surface levels in the same cells confirmed the CFTR selectivity of the GTPase-induced effects (Figure 2A). We next assessed CFTR internalization kinetics to determine whether CA RhoA and Rac1-induced steady-state increments in CFTR surface levels were due to enhanced channel retention at the cell surface. Whereas mock-transfected cells presented steady-state internal-

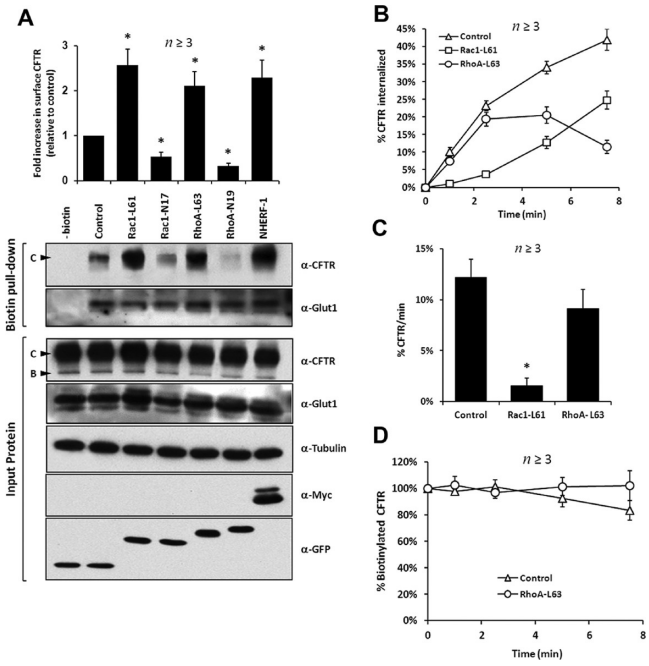

Figure 2. Rac1 and RhoA active mutants interfere, respectively, with CFTR internalization and recycling. (A) HBE41o- cells transfected as indicated were analyzed by surface protein biotinylation (quantitated in the upper graph). Shown are representative WBs of CFTR protein in either the pulled-down fraction (surface) or the correspondent whole cell lysates (input). These were further probed with anti-Glut-1 (surface protein pull-down control), anti-α-tubulin (loading control), and anti-tag antibodies. (B) CFTR at the surface of cells transfected as indicated was biotinylated, and the amount of biotin-CFTR internalized at the indicated time points was determined by densitometric analysis of WBs and expressed in percentage of total surface biotinylated CFTR (see also Supplementary Figure S2A). (C) Graph shows internalization rate constants (k_{IN}) as percent of internalized CFTR per minute, determined by the IN/SUR method (see Supporting Information). (D) Internalization assays using non-cleavable sulfo-NHS-LC-Biotin to discriminate between recycling and degradation in RhoA-L63 expressing cells (details in Supporting Information). Data points were determined and expressed as in panel B. All panels show means ± SEM. Asterisk indicates $p < 0.05$ relative to control cells.

ization kinetics as described,[9,22] CA-Rac1-expressing cells showed a clear delay in CFTR internalization, consistent with channel retention at the cell surface (Figure 2B, see also Supplementary Figure S2A). Using a previously described approach, the IN/SUR method,[23] we estimated the rate constants for the early steps of CFTR endocytosis (Figure 2C) and showed that Rac1-L61 slowed CFTR internalization to about one-sixth of that in control cells (1.6% *vs* 12.2% internalized CFTR min^{-1}). The effect of RhoA-L63 on the early steps of CFTR internalization was not significant (9.1% internalized CFTR min^{-1}). However, its expression led to a marked decrease in biotin-CFTR levels after 5 min at 37 °C (Figure 2B). The observed RhoA-L63-induced kinetics suggested either an increased degradation of internalized CFTR or the surface stripping of faster recycling biotin-CFTR at these later time points. To distinguish between both possibilities, we used nonhydrolyzable sulfo-NHS-LC-biotin, to prevent recycling-mediated biotin-CFTR stripping and analyze its degradation. RhoA-L63 expression did not promote CFTR degradation (Figure 2D), suggesting that RhoA acts by favoring

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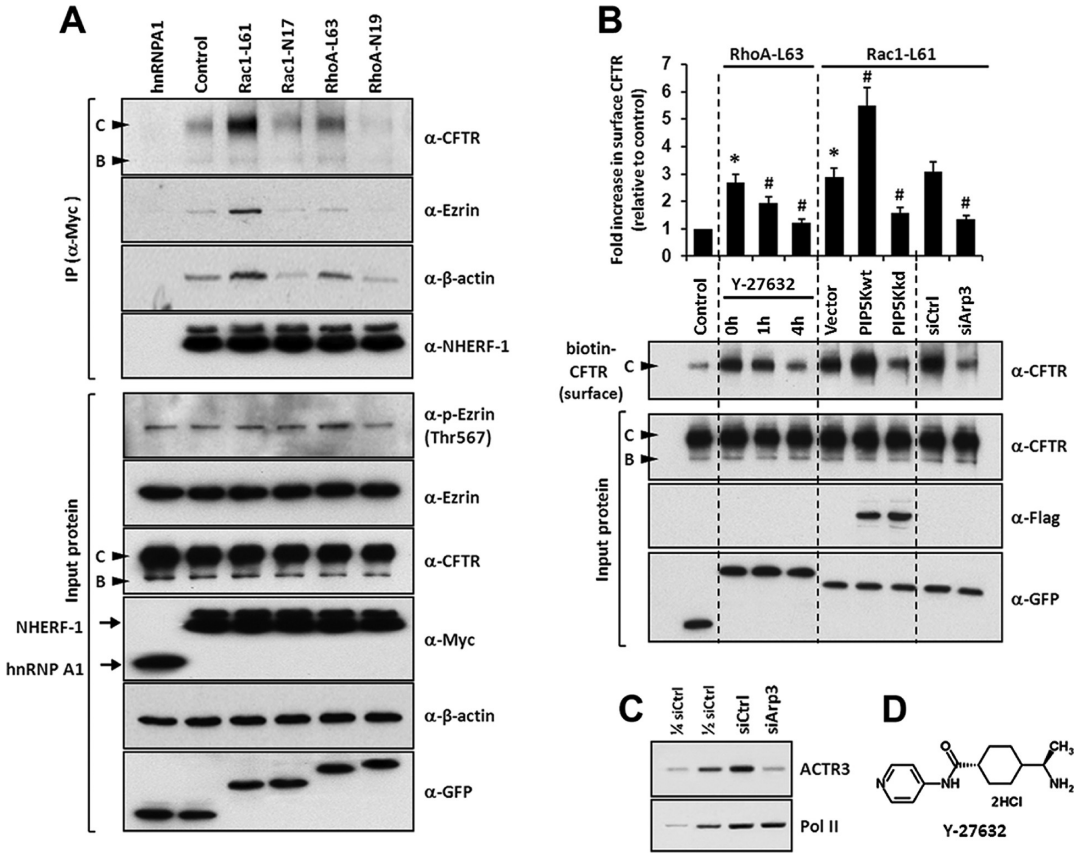

Figure 3. Rac1 signaling modulates CFTR surface anchoring and retention in human bronchial epithelial cells. (A) Analysis of CFTR/NHERF-1/Ezrin complex formation. WB analysis of Myc-NHERF-1 co-immunoprecipitated proteins (IP) in HBE41o- cells co-transfected as indicated (upper panels). Unrelated Myc-hnRNPA1 was used as a control for co-IP specificity. Lower panels show WB analysis of pre-IP lysates. Shown images are representative of three independent experiments. (B) RhoA and Rac1 downstream pathways affecting CFTR surface levels. HBE41o- cells expressing GFP-RhoA-L63 and treated with 10 μM ROCK inhibitor Y-27632 or co-transfected with GFP-Rac1-L61 and either wt or kinase dead (kd) human Flag-PIP5K, a nonspecific siRNA (siCtrl), or a siRNA mixture against Arp3 were analyzed by surface protein biotinylation (three independent assays are quantitated in the upper graph). Presented are means ± SEM. Asterisk and cardinal indicate $p < 0.05$ relative to, respectively, control cells and RhoA-L63 or Rac1-L61 alone. (C) Semi-quantitative RT-PCR for Arp3 mRNA levels (ACTR3 gene) after 48 h siRNA transfection and 20 h expression of GFP-Rac1-L61. Serial dilutions of the siCtrl cDNA were used to estimate Arp3 downregulation. RNA polymerase II (Pol II) was used as reference. (D) Chemical structure of Y-27632.

CFTR delivery to the PM. Altogether, these results highlighted Rac1 as the best candidate to modulate CFTR PM retention.

Rac1 Signals through PIP5K and Arp2/3 Enhances CFTR/NHERF-1/Ezrin Complex Formation. Tethering of CFTR to F-actin requires the formation of the CFTR/NHERF-1/Ezrin anchoring complex.[7−11] Thus, if the increase in CFTR surface retention by CA-Rac1 was indeed due to enhanced F-actin tethering, an enrichment of this very stable complex[7] should be detectable in cells. Thus, NHERF-1, the pivotal molecule in the complex, was co-expressed with the mutant GTPases and immunoprecipitated to allow the simultaneous detection of co-precipitated CFTR and Ezrin levels. CA-Rac1 co-expression remarkably enhanced CFTR/NHERF-1/Ezrin complex formation, as revealed by the increased levels of both CFTR and Ezrin co-precipitating with NHERF-1 in HBE41o- cells (Figure 3A, upper panel, lane 3). In contrast, DN Rac1-N17 (lane 4) clearly decreased the presence of Ezrin in the complex, when compared to mock-transfected cells. Consistent with an effect on Ezrin-mediated CFTR F-actin anchoring, the same pattern was observed on the levels of β-actin co-precipitating with the complex (Figure 3A, third image from top). Notably, neither of the Rac1 variants affected Ezrin phosphorylation on Thr567 (lower panel, first image lane 3), previously reported to promote Ezrin activation.[24] In contrast, Ezrin phosphorylation was moderately stimulated by CA-RhoA (lane 5); however, the levels of Ezrin and β-actin co-precipitating with NHERF-1 and CFTR were not significantly altered (upper panel, lane 5). Instead, RhoA-L63 only promoted CFTR/NHERF-1 binding, whereas DN RhoA-N19 impaired this interaction (upper panel, lane 6), consistent with a role for RhoA in delivering CFTR to the PM. Because RhoA signaling through Rho Kinase (ROCK) was previously implicated in NHERF-1 coaxing of CFTR to the PM,[24] we treated HBE41o- cells expressing RhoA-L63 for 1−4 h with 10 μM Y-27632, a selective ROCK inhibitor, and analyzed CFTR PM levels. Expectedly, inhibition of endogenous ROCK activity precluded CA RhoA effect on CFTR PM levels (Figure 3B, left lanes). Interestingly, Y-27632 is also a potent inhibitor of RhoA/ROCK-induced acto-myosin stress fibers (SF), and confocal IF analysis of the actin cytoskeleton in RhoA-L63 cells confirmed that CFTR localized to small vesicular dots aligned along SFs and accumulated at their PM anchoring points (Supplementary Figure S2B, lower panels). This suggests an involvement of these cytoskeletal structures in CA RhoA-induced CFTR PM delivery. In contrast, under Rac1-L61 most CFTR localized to F-actin-rich membrane ruffles

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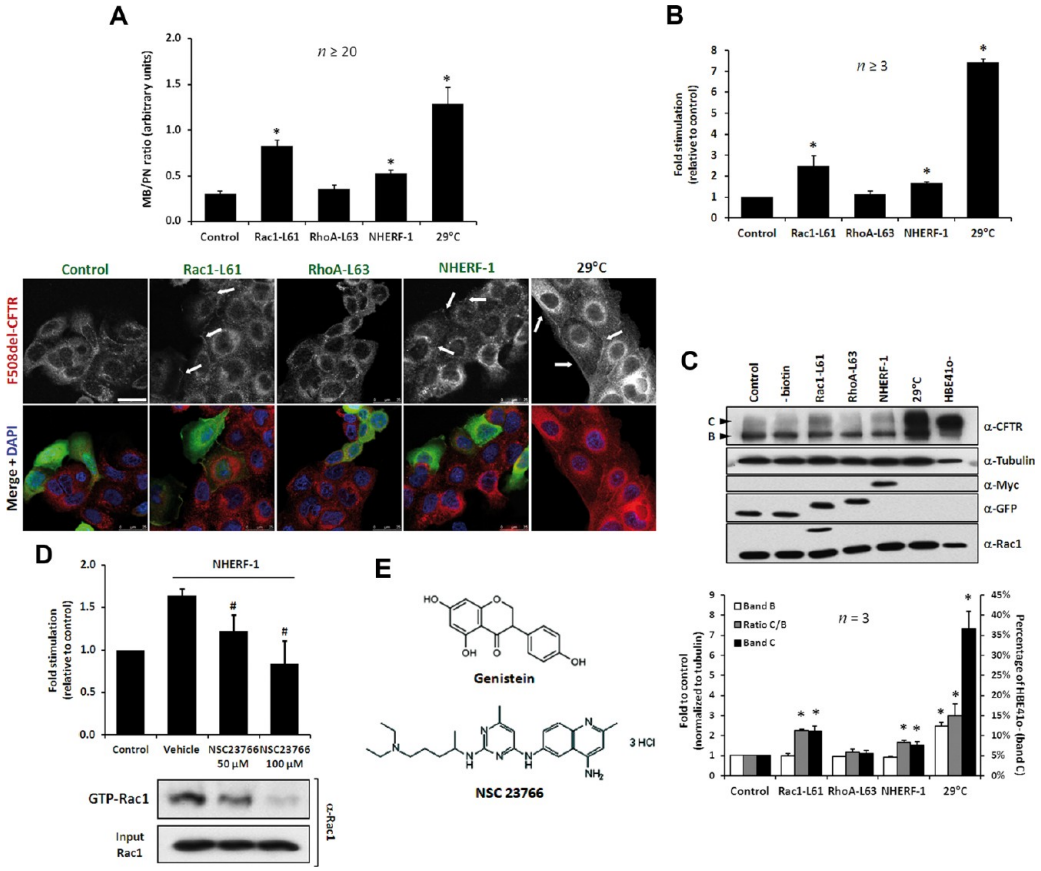

Figure 4. Active Rac1 partially restores F508del-CFTR expression and activity at the cell surface. CFBE41o- cells transfected as indicated or cultured for 24 h at 29 °C were analyzed by (A) confocal immunofluorescence microscopy (quantitated as in Figure 1A); (B) forskolin (10 μM) plus genistein (50 μM)-stimulated, CFTRInh172 (20 μM)-blocked iodide efflux; (C) WB analysis (quantitated in the lower graph) of band C and band B levels and ratio in whole cell lysates. (D) Iodide efflux, as in panel B, from NHERF-1 transfected cells treated for 1 h with 50−100 μM Rac1 inhibitor NSC23766 (three independent experiments quantitated in upper graph). Bottom images show representative WBs of endogenous, CRIB pull-down-captured active Rac1 (upper) and total Rac1 input (lower) from these cells. (E) Chemical structures of genistein and NSC23766. All panels show means ± SEM. Asterisk and cardinal indicate $p < 0.05$ relative to control and vehicle-treated cells, respectively.

(Supplementary Figure S2B, top panels). Because CFTR anchoring requires Ezrin activation, which can be induced by Rac1 *via* phosphatidylinositol-4-phosphate-5-kinase (PIP5K),[25] we tested whether this pathway mediated Rac1-induced CFTR PM retention. Co-expression of a kinase-dead mutant of human PIP5K-alpha (kd-PIP5K) was sufficient to prevent Rac1-L61 from increasing CFTR PM levels. Conversely, overexpression of wt-PIP5K significantly enhanced CA-Rac1 effect, almost doubling the amount of surface CFTR (Figure 3B, middle lanes). Because active Ezrin binds to local F-actin, we tested whether Rac1-induced *de novo* F-actin polymerization and branching *via* Arp2/3 complex[26] was also involved. Knockdown of Arp3 subunit expression by siRNA (~75% reduction, Figure 3C) was in fact sufficient to drastically impair Rac1-L61-induced CFTR PM retention in HBE41o- cells (Figure 3B, right lanes).

Active Rac1, but not RhoA, Increases F508del-CFTR Levels and Activity at the Plasma Membrane. Next, we investigated whether the effects of Rac1 on wt-CFTR PM tethering and retention would also apply in a bronchial epithelial cell line stably expressing the pathological F508del-CFTR mutant (CFBE41o-).[21] We therefore expressed Rac1-L61 in these cells and compared its effects to those of expressing RhoA-L63, NHERF-1 [reported to partially rescue Cl^- transport and PM expression in these cells[20,24]] and of low temperature (29 °C), well-known to rescue F508del-CFTR processing. Confocal qIF showed that Rac1-L61 induced a ~3-fold increase in CFTR staining at the PM region (Figure 4A, upper graph) localizing with the active GTPase at membrane ruffles (lower images). Interestingly, Rac1 had a stronger effect than NHERF-1 (1.7-fold) in these cells, and RhoA-L63 induced no detectable PM-associated F508del-CFTR staining (Figure 4A). CFTR functional measurements by cAMP-induced iodide efflux under the same experimental conditions (Figure 4B) confirmed these results for Rac1, RhoA, and NHERF-1. The effect of low temperature was more expressive (~7-fold), reflecting, as expected, the influence of increased overall CFTR levels on qIF values (Figure 4A). We next attempted to analyze the levels of F508del-CFTR at the surface of CFBE41o- cells. An enrichment of mature F508del-CFTR (band C) in biotin-captured versus input fractions was apparent in Rac1- and NHERF-1-transfected cells, as well as in cells at low temperature (Supplementary Figure S3, top panels). However, as previously reported,[19] the scale-up conditions required to detect surface F508del-CFTR signals (see Supporting Information) generated considerable intracellular protein contamination from residual permeable cells, precluding an accurate estimation of its levels. The same technical limitation impeded also the analysis of F508del-CFTR internalization kinetics. Notwithstanding, quantitation of steady-state levels of

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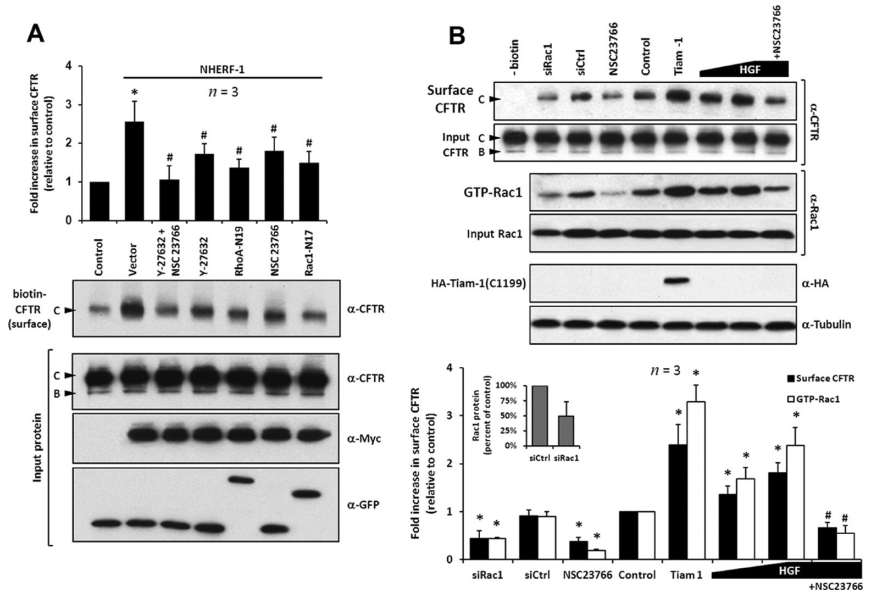

Figure 5. Endogenous Rac1 and RhoA signaling are required for NHERF-1-induced CFTR surface expression. (A) HBE41o- cells transfected with Myc-NHERF-1 were co-transfected as indicated and treated or not (control) with 100 μM NSC23766 (1 h) or 10 μM Y-27632 (4 h) or with a combination of both drugs. Shown are representative WBs (lower panels) and quantitation (upper graph) of CFTR protein either biotinylated at the cell surface or in whole cell lysates (Input). (B) WB analysis (upper panels) and quantitation (lower graph) of biotinylated CFTR at the cell surface and CRIB-pull-down-captured active Rac1 (GTP-Rac1) in HBE41o- cells transfected (16 h) with either control (siCtrl) or Rac1-specific (siRac1) siRNAs or treated with 100 μM NSC23766 (1 h) or transfected with an active form of the Rac1 GEF Tiam-1 (C1199-Tiam-1) or treated with 20–50 ng mL^{-1} of human recombinant HGF (4 h) or co-treated with 50 ng mL^{-1} HGF (4 h) and 100 μM NSC23766 (1 h). Insert shows quantitation of endogenous Rac1 knockdown from WBs (input Rac1). All panels show means ± SEM. Asterisk and cardinal indicate $p < 0.05$ relative to control or HGF (alone)-treated cells, respectively.

mature and immature F508del-CFTR (Figure 4C) revealed a clear proportionality between the increase in F508del band C accumulation, reaching up to 11% of wt-CFTR in the case of CA-Rac1, and the amount of iodide released from these cells (Figure 4B). Moreover, neither CA-Rac1 nor NHERF-1 affected immature F508del band B levels, consistent with a decreased turnover of mature F508del-CFTR that reached the PM rather than an overall enhancement of CFTR production or processing. NHERF-1 was, however, substantially less effective than CA-Rac1 in these cells, which made us wonder whether NHERF-1 could be relying on endogenous Rac1 signaling to exert its effect on F508del-CFTR. We therefore treated NHERF-1-overexpressing CFBE41o- cells with increasing doses of the Rac1-specific inhibitor NSC23766[27] and assessed the outcome on CFTR-mediated ion transport. NSC23766 treatment resulted in a clear, dose-dependent inhibition of endogenous Rac1 activation, as determined by CRIB-pull-down assay[28,29] (Figure 4D, lower panels), which, consistent with our hypothesis, produced a proportional decrease in iodide efflux from these cells (upper graph).

NHERF-1 Requires Endogenous Rac1 and RhoA Signaling To Coax CFTR to the PM. As NHERF-1 was recently described to require endogenous RhoA/ROCK signaling to enable PM coaxing and tethering of CFTR[24] and we found no significant contribution from RhoA in increasing F508del-CFTR surface levels, we sought to assess NHERF-1 requirement for both endogenous RhoA and Rac1 signaling in HBE41o- cells. Cells were, therefore, transfected with NHERF-1 and either Rac1-N17 or RhoA-N19 or treated with the ROCK inhibitor Y-27632 or with the Rac1 inhibitor NSC23766, and CFTR surface levels were analyzed as before. As described,[24] co-expression of RhoA-N19 or treatment with Y-27632 clearly impaired NHERF-1-induced increase in CFTR PM levels (Figure 5A). Notably, both Rac1-N17 and NSC23766 treatment produced similar results, and co-treatment with Y-27632 and NSC23766 (co-expression of DN-Rac1 and DN-RhoA was cytotoxic) virtually abolished the effect of NHERF-1 on wt-CFTR. These data confirmed that endogenous signaling from both RhoA and Rac1 are required to mediate wt-CFTR PM coaxing by NHERF-1 and explained its dependence on Rac1 signaling to exert its effect on F508del-CFTR, which does not respond to active RhoA. Moreover, these results also explain why others have associated Ezrin phosphorylation *via* RhoA/ROCK, downstream of NHERF-1, to CFTR anchoring: stimulation of PIP5K by Rac1, downstream of NHERF-1, leads to the conversion of 1-phosphatidyl-1D-myo-inositol-4-phosphate (PIP) at the membrane to 1-phosphatidyl-1D-myo-inositol-4,5-bisphosphate (PIP2), a second messenger molecule that is required for both actin polymerization[30] and Ezrin activation.[25] In fact, PIP2 binding is sufficient to activate Ezrin,[31] disrupting a head-to-tail inhibitory conformation that prevents its interaction with actin and ERM-binding proteins such as NHERF-1. PIP2 binding is also required for, and precedes, Thr567-phosphorylation by kinases such as ROCK that further stabilizes the open conformation of Ezrin.[32] These observations with NHERF-1 suggested that interfering with endogenous Rac1 signaling might be sufficient to elicit a significant effect on CFTR surface levels. In fact, reducing endogenous levels of active Rac1, either by NSC23766 or through siRNA (~50% knockdown; higher efficiencies severely compromised cell viability), significantly decreased CFTR PM levels (Figure 5B), whereas expression of an active form of the Rac1 GEF (guanine exchange factor) Tiam-1 [C1199-Taim-1[16]] produced an over 2-fold increase in surface CFTR along with a proportional stimulation of endogenous Rac1 activity. Notably, treatment of HBE41o-

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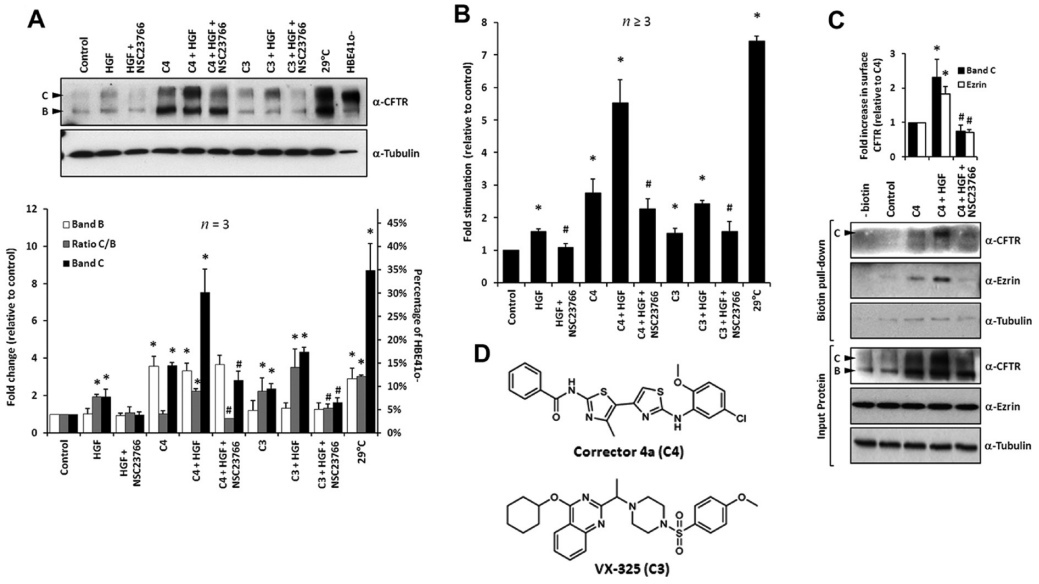

Figure 6. HGF potentiates chemical correction of F508del-CFTR in human bronchial epithelial cells. (A) WB analysis (upper panels) and quantitation (lower graph) of F508del-CFTR band C, band B, and C/B ratio in whole cell lysates of CFBE41o- cells cultured for 24 h at 29 °C or at 37 °C and treated with HGF (50 ng mL^{-1}) or correctors C3 (25 μM) or C4 (15 μM) or a combination of these agents, as indicated, in the presence or absence of 100 μM NSC23766 (1 h). (B) Fold change in cAMP-mediated iodide efflux (as described for Figure 4B) in CFBE41o- cells treated as in panel A. (C) Representative WBs of surface biotinylated CFTR (biotin pull-down) and CFTR levels in whole cell lysates (input) from cells treated as in panel A with the indicated compounds. Further staining of these membranes revealed co-enrichment of Ezrin in the pulled-down fraction (three independent experiments quantitated in upper graph). (D) Chemical structures of correctors C3(VX-325) and C4 (corrector 4a). All panels show means ± SEM. Asterisk and cardinal indicate $p < 0.05$ relative to control cells and cells not treated with NSC23766, respectively.

cells with increasing doses of human hepatocyte growth factor (HGF), a known physiological Rac1 activator[33] previously shown to enhance the regeneration of airway epithelia[34,35] and to repress the onset of fibrosis and inflammation,[36,37] produced similar results, in a dose-dependent manner, that were completely abolished by co-incubation with NSC23766 (Figure 5B, last three lanes).

HGF Potentiates Chemical Correction of F508del-CFTR through Rac1-Mediated Surface Anchoring. To determine whether HGF treatment would also enhance F508del-CFTR surface levels, we exposed CFBE41o- cells to the maximum stimulating dose used in HBE41o- cells. Indeed, HGF treatment promoted an increase in F508del-CFTR band C levels (Figure 6A, lane 2) and a stimulation of iodide efflux from these cells (Figure 6B, second bar) comparable to those achieved by CA-Rac1 (see Figure 4). Again, both effects were completely prevented by co-treatment with NSC23766 (lane 3 and third bar in Figure 6A and B, respectively), consistent with increased F508del-CFTR PM retention *via* Rac1-induced anchoring. To answer the core question of this study, we investigated whether HGF co-treatment would improve the efficacy of commonly used F508del-CFTR corrector compounds, available at the CFFT modulator library (http://www.cftrfolding.org/CFFTReagents.htm). Treatment of CFBE41o- cells with C4 (compound 4a) or C3 (VRT-325) alone yielded a ~3.5- and ~2.5-fold increase in F508del band C levels, respectively, which were also clearly reflected by increased membrane-associated CFTR IF signals (Supplementary Figure S4). Functionally, C4-treated cells responded with a proportional increase in iodide release, whereas cells exposed to C3 revealed a weaker response (Figure 6B), consistent with the reported inhibitory effect of this compound in cAMP-induced channel activity.[38] Significantly, HGF synergized with C4 in co-treated cells, yielding a near 8-fold increase in band C levels, compared to control cells, and nearly doubled the corrective effect of C3, leading to >4-fold increase in fully glycosylated F508del-CFTR levels (Figure 6A). These effects were also evident in IFs of co-treated cells (Supplementary Figure S4) and in cAMP-induced stimulation of channel activity (although attenuated in the case of C3), as measured by iodide efflux (Figure 6B). The robust response of CFBE41o- cells to HGF/C4 co-treatment, leading to a band C rescue of >30% of wt-CFTR levels (close to the ~35% observed with low temperature), allowed the scaling of surface biotinylation conditions to acceptable background levels and the confirmation that the observed accumulation in F508del band C was reflected by proportionally increased channel levels at the cell surface (Figure 6C, upper panels). Moreover, an equivalent enrichment was also detectable in co-captured Ezrin, and both effects were completely prevented by NSC23766 (Figure 6C), consistent with enhanced, Rac1-mediated F508del-CFTR anchoring at the cell surface.

HGF Treatment Enhances Apical Expression of Pharmacologically Rescued F508del-CFTR. Next, we used confocal IF to investigate the effect of HGF treatment on chemically rescued F508del-CFTR in polarized CFBE41o- cells. As observed for nonpolarized cells, treatment of polarized monolayers with standard concentrations of either C3 or C4 led to an overall enhancement of F508del-CFTR levels, compared to control cells (Figure 7A, first, third, and fifth images from top). However, although a significant increase was observable in apical signals, most of the rescued protein showed a basolateral accumulation, particularly with C4 treatment. Significantly, HGF co-treatment produced a dramatic inversion of this pattern, doubling the intensity of apical F508del-CFTR signals, which for C4 translated to a ~5-fold increase over control cells, again very close to the ~6-fold increment for low-temperature rescue (Figure 7A, right graph). Notably, HGF

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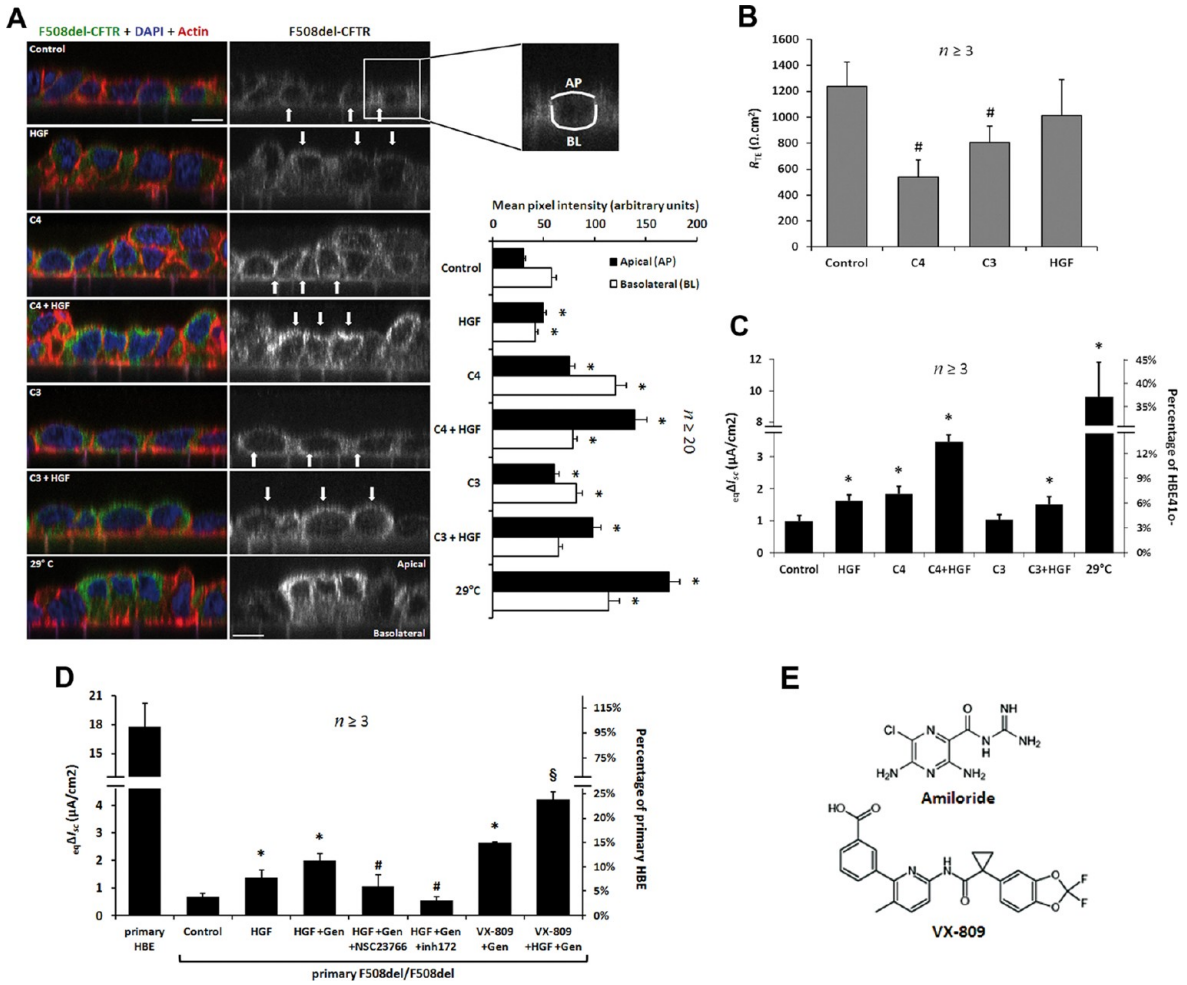

Figure 7. HGF treatment enhances apical expression of chemically rescued F508del-CFTR in polarized airway epithelia. (A) Polarized CFBE41o- cells cultured for 24 h at 29 °C or at 37 °C and treated with HGF (50 ng mL^{-1}) or correctors C3 (25 μM) or C4 (15 μM) or a combination of these agents, as indicated, were stained with anti-CFTR/Alexa 488, Phalloidin-TRITC, and DAPI and analyzed by confocal microscopy. Shown are overlay images (left) as well as isolated CFTR-staining (green channel) representative of all conditions. Upper right insert shows a zoomed image of a control cell, to exemplify apical (AP) and basolateral (BL) CFTR signal quantitation, plotted on the right for all conditions (bar graph). White arrows indicate basolateral to apical shift in CFTR staining upon HGF treatment. (B) Transepithelial resistance (R_{te}) of monolayers treated as in panel A. (C) Fsk/Gen-sensitive, CFTRinh172-blocked, equivalent short-circuit currents ($_{eq}\Delta I_{sc}$) on Ussing chamber recordings from monolayers treated as in panel A. (D) cAMP-induced (forskolin, 2 μM) $_{eq}\Delta I_{sc}$ on non-CF and CF (F508del/F508del) primary HBE polarized monolayers from three individuals, treated for 24 h at 37 °C with HGF (50 ng mL^{-1}) and/or VX-809 (3 μM) and potentiated or not with genistein (Gen, 50 μM) in the presence or absence of CFTRinh172 (30 μM) or the Rac1 inhibitor NSC23766 (100 μM, 2 h). (E) Chemical structures of corrector VX-809 and Amiloride. All panels show mean ± SEM. *, #, and § indicate $p < 0.05$ relative to control, NSC23766 or CFTRinh172 untreated, and VX-809-treated (alone) cells, respectively.

treatment alone was sufficient to induce a significant basolateral to apical F508del-CFTR shift in control cells, reaching apical levels close to those induced by C3 alone. We then investigated how HGF treatment affected the recovery of channel function in polarized cells. Unfortunately, the reported toxicity of the corrector compounds used[38,39] severely compromised monolayer permeability (particularly C4), as determined by transepithelial resistance (R_{te}) measurements (Figure 7B). This impaired an accurate estimation of cAMP-induced Cl^- transport in corrector-treated cells. Nevertheless, equivalent short-circuit currents ($_{eq}\Delta I_{sc}$) derived from HGF co-treated monolayers showed an enhancement of corrector-induced, CFTR-mediated Cl^- transport compatible with the effects observed in nonpolarized cells (Figure 7C). Moreover, the increase in $_{eq}\Delta I_{sc}$ produced by HGF-treatment alone (which did not hinder R_{te}) was proportional to the observed increment in apical CFTR signals. We therefore tested HGF alone on primary human bronchial epithelial (HBE) cells homozygous for the F508del mutation, the "gold standard" preclinical model for the validation of CFTR modulators.[40] Relative to control monolayers, HGF treatment resulted in a ~2-fold increase in Cl^- transport in response to forskolin alone, with a clear additive effect by genistein, a well-characterized CFTR potentiator (Figure 7D and see also Supplementary Figure S5). This roughly 3-fold increase in channel activity represented a recovery of >11% in CFTR function relative to primary HBE cells from non-CF individuals, a level of CFTR restoration some authors believe to be already clinically relevant to attenuate disease severity.[2] A potential interference by the epithelial sodium (Na^+) channel (ENaC) on V_{te} recordings was excluded by luminal exposure of all primary monolayers to the ENaC inhibitor Amiloride. Moreover, HGF effect was blocked by coincubation with NSC23766, consistent with enhanced F508del-CFTR PM retention, whereas CFTRinh172 com-

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pletely abrogated Fsk/Gen-induced currents (Figure 7D). To further assess the clinical relevance of HGF treatment, we tested its effect in combination with the clinically relevant corrector VX-809. This investigational drug, reported to be more effective than C3 or C4 in restoring F508del-CFTR function with no significant toxicity,[41] recently entered clinical trials.[42] Treatment with VX-809 alone increased cAMP-induced Cl^- transport by 3.9-fold in primary F508del-CFTR HBE cells, corresponding to a ~15% restoration of wt-CFTR function (Figure 7D). When cells were co-treated with HGF, however, a clear additive effect arose, leading to a 6.3-fold increase in CFTR activity that translates to a ~24% restoration of CFTR function, compared to non-CF cells. Moreover, these data were biochemically supported, as HGF/VX-809 co-treatment led to a proportional increase in mature F508del-CFTR forms, compared to VX-809 alone (Supplementary Figure S5B).

We thus demonstrate that the improvement of F508del-CFTR surface retention can dramatically enhance the extent of functional restoration achieved in HBE cells through the use of pharmacological correctors, namely, VX-809, which alone had modest results in clinical trials.[42] These results strongly support the idea that maximal functional rescue of F508del-CFTR in CF patients will probably require the combination of low-toxicity drugs that (i) promote its folding at the ER, (ii) improve F508del-channel activity, and importantly, (iii) enhance its anchoring and retention at the cell surface. Moreover, our findings reveal Rac1 and its effector PIP5K as novel targets for pharmacological intervention in CF. Although the pharmacological modulation of these molecules may prove challenging, given their pleiotropy and the association of their deregulated activity with certain pathologies,[43] our results with HGF attest its plausibility. Moreover, since HGF is already in the clinical trial setting for skin, renal and neuronal diseases,[37] it is tempting to suggest that it could be easily transposed to the CF field to be used along with CFTR modulators (correctors and potentiators) already in clinical trial (http://www.clinicaltrials.gov/ct2/show/NCT01225211?term=vertex). In conclusion, our data provide strong evidence that molecular strategies achieving surface retention and actin anchoring of F508del-CFTR can be of high therapeutic relevance for CF, opening new avenues for pharmacological intervention in correcting the basic cellular defect underlying this disease. Importantly, these data may also have repercussions on other diseases caused by a trafficking defect of surface proteins.

METHODS

Cell Culture, Treatment, and Transfection. Primary non-CF and CF (F508del/F508del-CFTR) airway epithelial cells were isolated as previously described.[44] CFBE41o-, HBE41o-, and BHK-21 cells were cultured as previously described.[18,21] Cells were transfected as indicated with LipofectAMINE 2000 (Invitrogen) and treated as described (details in Supporting Information).

Immunoprecipitation, CRIB-Pull-Down, and Western Blot Procedures. GTP-Rac1 CRIB-pull-down was performed as previously described.[28,29] For co-immunoprecipitation cell lysates were incubated for 2 h at 4 °C with rabbit anti-Myc (clone A14, Santa Cruz), then 1 h with protein G-Agarose beads (Roche). Proteins were solubilized from beads in 2x SDS Buffer, separated on 10% (w/v) SDS-PAGE gels, transferred onto PVDF membranes (BioRad), and probed as indicated (details in Supporting Information).

Immunofluorescence Microscopy. Cells grown on membranes or coverslips were transfected or/and treated as described, fixed, permeabilized, and immunolabeled as indicated (details in Supporting Information). Images recorded on a Leica TCS-SPE confocal microscope. Fluorescence data quantitation was performed on a minimum of 20 individual cells from at least two independent experiments using Leica in-built quantitation software.

Iodide Efflux. Iodide efflux assay was adapted from ref 45 (details in Supporting Information).

Surface Protein Biotinylation. Cell surface proteins were labeled on ice with EZlink-sulfo-NHS-SS-biotin (Pierce) in nonpermeabilizing conditions and captured with Streptavidin-agarose beads (Invitrogen). Captured proteins were analyzed by WB as above.

Micro-Ussing Chamber Recordings. Monolayers were mounted in modified micro-Ussing chambers and analyzed under open circuit conditions. Transepithelial resistance (R_{te}) was determined by applying short (1 s) current pulses (I = 0.5 μA), and changes in transepithelial voltage (V_{te}) were recorded continuously (details in Supporting Information). Equivalent short-circuit currents ($_{eq}\Delta I_{sc}$) were calculated by Ohm's law from V_{te} and R_{te} ($I_{sc} = V_{te}/R_{te}$).

Statistical Analysis. Statistical comparisons were made using two tailed Student's t tests and nonweighted linear regressions.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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