Syntaxin 8 and the Endoplasmic Reticulum Processing of ΔF508-CFTR

Inna Sabirzhanova, **Clément Boinot**, **William B. Guggino**, and **Liudmila Cebotaru**

Departments of Medicine and Physiology, Johns Hopkins University, Baltimore, USA

Abstract

Background/Aims: Cystic fibrosis (CF) is a lethal recessive disorder caused by mutations in the CF transmembrane conductance regulator (CFTR). ΔF508, the most common mutation, is a misfolded protein that is retained in the endoplasmic reticulum and degraded, precluding delivery to the cell surface [1].

Methods: Here we use a combination of western blotting, immunoprecipitation, and short circuit current techniques combined with confocal microscopy to address whether the SNARE attachment protein, STX8 plays a role in ΔF508's processing and movement out of the ER.

Results: Although the SNARE protein STX8 is thought to be functionally related and primarily localized to early endosomes, we show that silencing of STX8, particularly in the presence of the Vertex corrector molecule C18, rescues ΔF508-CFTR, allowing it to reach the cell surface and increasing CFTR-dependent chloride currents by approximately 2.5-fold over control values. STX8 silencing reduced the binding of quality control protein, Hsp 27, a protein that targets ΔF508-CFTR for sumoylation and subsequent degradation, to ΔF508-CFTR. STX8 silencing increased the levels of Hsp 60 a protein involving in early events in protein folding.

Conclusion: STX8 knockdown creates an environment favorable for mature ΔF508 to reach the cell surface. The data also suggest that when present at normal levels, STX8 functions as part of the cell's quality control mechanism.

Keywords

Snare; Processing; Degradation; Mutant; CFTR

Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a highly regulated chloride channel that functions at the plasma membrane [2]. While trafficking from the Golgi to the plasma membrane, CFTR interacts with several N-ethylmaleimide-sensitive

Dr. Liudmila Cebotaru, Johns Hopkins University School of Medicine, Hunterian 415, 725 North Wolfe Street, Baltimore (USA), lcebotaru@jhmi.edu.

Disclosure Statement
The authors declare that they have no conflicts of interest with the contents of this article.

factor attachment proteins (SNAREs) that play a role in CFTR-containing vesicular transport from early to late endosomes and ultimately to the plasma membrane [3]. One of the earliest-identified SNARE protein complexes involved the two t-SNAREs syntaxin 1A (STX1A) and synaptosome-associated protein of 23 kDA (SNAP-23). These two SNARES bind to each other, and also to CFTR. Both STX1A and SNAP-23 inhibit CFTR function. The functional inhibition by STX1A is relieved by the syntaxin-binding protein Munc18, which allows CFTR to function at the plasma membrane. SNAP23 also binds to CFTR and inhibits its activity, but only in the presence of STX1A. These data point to a complex regulation of CFTR within the vesicles that traffic it to the plasma membrane. The role played by these two SNAREs is interesting, in that they regulate gating rather than modulating CFTR's insertion into the plasma membrane [4-6]. It is possible that the SNAREs keep CFTR in a non-functional form until it reaches its ultimate location in the plasma membrane, where it normally functions.

Our group has described another SNARE, syntaxin 6 (STX6). STX6 is a localized to the trans-Golgi network (TGN), where it plays a role in TGN-to-endosome trafficking [7] and in the sorting of proteins from endosomes toward either the TGN or lysosome [8]. STX6 interacts with CFTR via the PDZ domain-containing protein known as CFTR-associated ligand (CAL). Interestingly, STX6 binds to both the N-terminal half of CFTR and to CAL, but it requires CAL in order to form a functional complex, CAL/STX6/CFTR, that directs CFTR trafficking to the lysosome [7].

Syntaxin 8 (STX8) is another SNARE protein that binds to CFTR [9, 10]. STX8 operates together with several other proteins, including vesicle associated protein 8 (VAMP8), STX7, and Vti1b, to support intracellular membrane fusion processes at the early endosome [9, 11]. Overexpression of STX8 also inhibits CFTR function, but unlike STX1A, it does so by sequestering it within intracellular vesicles and thereby reducing CFTR expression at the plasma membrane [10].

Taken together, all of these studies show that SNARE proteins play a critical role in modulating mature CFTR at the TGN/lysosome, early endosome, and plasma membrane. Tight regulation of CFTR trafficking is essential, given that the mature CFTR's role is to create the fluid that bathes mucosal membranes of the pancreas and the respiratory, intestinal, and reproductive tracts and maintain its proper composition [12]. Indeed, overactive CFTR leads to diarrheal diseases such as cholera, and underactive CFTR to cystic fibrosis [13].

Cystic fibrosis (CF) is a lethal autosomal recessive genetic illness caused by mutations in CFTR [14, 15]. CF patients experience recurrent pulmonary infections, resulting in lung inflammation and fibrosis and inexorably leading to respiratory failure and pancreatic insufficiency, which may be associated with diabetes mellitus [16] and several other disease-causing complications. The most common mutation is a missing phenylalanine, F508, in the NBD1 domain. F508-CFTR is a partially glycosylated and misfolded protein that is retained in the endoplasmic reticulum (ER) and degraded by the proteasome, precluding its delivery to the cell surface [1]. The question we have addressed here is whether SNARE proteins also play a role in CFTR's processing out of the ER. Although STX8 is thought to

be primarily localized to and functionally related to the early endosome, we show here that it is also plays a role in the ER trafficking of F508-CFTR.

Materials and Methods

Cell culture

Cystic fibrosis bronchial epithelial cells (CFBE) stably expressing WT-CFTR, provided by Dr. Dieter Gruenert [17], were maintained in minimum essential medium (MEM) with penicillin (100 U/ml), streptomycin (100 μg/ml), hygromycin B (100 μg/ml), and 10% fetal bovine serum. CFBE stably expressing F508 CFTR [18] were cultured in MEM with penicillin (100 U/ml), streptomycin (100 μg/ml), puromycin (10 μg/ml), and 10% fetal bovine serum.

Plasmids and transfections

To overexpress STX8 in CFBE cells, transient transfection with the STX8 pCMV-6 plasmid (Origene) was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For knockdown experiments, the cells were transfected with predesigned siRNA directed against human STX8 (Qiagen, Cat#SI03109610) using INTERFERin® (Polyplus transfection) according to manufacturer's manual. In brief, STX8 siRNA was diluted in serum-free medium (SFM), and INTERFERin was added. The siRNA-INTERFERin-SFM mixture was incubated at room temperature for 10 min and then applied to the cells for 72 h.

Western blotting

The cells were harvested using lysis buffer (50 mM NaCl, 150 mM Tris-HCl, pH 7.4, 1% Nonidet P-40) with protease inhibitors (Halt protease inhibitor cocktail, Thermo Scientific), and proteins were extracted by rotation of the cell lysates for 30 min at 4°C. The cell lysates were then centrifuged on a high-speed tabletop centrifuge for 15 min, and the supernatants were collected to perform western blotting. CFTR was detected with IgG2b 596 (596 obtained from Dr. Jack Riordan at the U. of North Carolina at Chapel Hill), diluted 1:1000. STX8 was detected using a mouse monoclonal anti-STX8 antibody (BD Transduction Laboratories 611352). GAPDH protein was used as the loading control and detected with mouse monoclonal anti-GAPDH antibody (Santa Cruz Biotechnology). Peroxidase-labeled sheep anti-mouse IgG (Amersham) was used as the secondaiy antibody. The signal was enhanced with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific). Chemoluminescence was captured by a Fuji Film LAS-4000 plus system with a cooled CCD camera. Quantification was carried out within the linear range, using Image Gauge version 3.2 software (Fuji Film).

Co-Immunoprecipitation

Co-immunoprecipitation was performed by incubating 2000 μg of cell lysates prepared as described above with anti-CFTR M3A7antibody (Millipore) and A/G agarose beads (Santa Cruz Biotechnology) for 4 h at 4°C. After incubation, the A/G beads were washed four times with lysis buffer plus protease inhibitors and resuspended in 2X Sample Buffer (Bio-Rad) with β-mercaptoethanol. Samples were used for western blotting as described above, using

the appropriate antibodies for each individual protein co-immunoprecipitated. CFTR precipitation (pull-down with antibody M3A7 and blotting with antibody 596) was used as a control. Rabbit polyclonal antibodies were used for VCP and HDAC6 (1:200); and mouse monoclonal antibodies for HSP27, and HSP60 (1:500) (Santa Cruz Biotechnology).

Biotinylation

Surface proteins were labeled with sulfo-NHS-SS-biotin (Thermo Scientific) for 20 min at 4°C; thereafter, the cells were washed three times with glycine buffer (200mM glycine and 25mM Tris/HCl, pH 8.0, in DPBS with calcium and magnesium) and then lysed in lysis buffer (150 mM NaCl, 50 mM Tris/HCl, 1% Nonidet P-40) and protease inhibitors). For each sample, 2 mg of protein was incubated with avidin beads (NeutrAvidin Ultra Link Resin, Thermo Scientific) for 1 h at 4°C to isolate surface-labeled proteins. After incubation, the avidin beads were washed four times with lysis buffer. The supernatants were discarded, and 2X Laemmli sample buffer plus β-mercaptoethanol was added 1:1 to the beads. The samples were then used for western blotting. As an internal control, GAPDH was detected with mouse monoclonal antibody. Blots from biotinylation experiments were also probed for GAPDH and STX8 to verify that the cells were intact.

Small-molecule corrector

We used the small-molecule corrector compound C18, obtained from the CFFT panel library (www.cftrfolding.org) applied to the cells for 16 h.

Short-circuit current measurements

Short-circuit current (SCC) measurements were made using a six-channel Easy-Mount chamber system (Physiologic Instruments, San Diego, CA). CFBE cells stably transfected with F508-CFTR were plated on Snapwell filters (Corning Costar, Acton, MA; 3407) and at the same time transfected with STX8 siRNA as described above. Transfected cells were grown for 72 h on Snapwell filters and treated with compound C18 for 16 h before SCC measurements were performed. SCC was measured with a VCCMC6 multichannel voltage-current clamp amplifier (Physiologic Instruments). Data were acquired on an 1.71-GHz PC equipped with DI-720 (DATAQ Instruments, Akron, OH), with Acquire and Analyze version 2.3.159 (Physiologic Instruments) software. The cell monolayers were bathed on both sides with a solution containing 120 mM NaCl, 5 mMKCl, 2 mMMgCl$_2$, 2 mM CaCl$_2$, 10 mM D-glucose, and 10 mM HEPES (adjusted to pH 7.3 with NaOH). The solution was maintained at 37°C and bubbled gently with air. After stabilization, 10 μM forskolin and 50 μM genistein) were added to both chambers, followed by the CFTR channel inhibitor [19] CFTRinh-172 (10 μM).

Microscopy

A Zeiss LSM 700 laser scanning system and 63x oil-immersion lens was used. CFBE41o⁻ cells were seeded onto cover glasses. The following steps were done at room temperature to prepare slides for assessment: After two washes with cold DPBS, the cells were fixed with 4% paraformaldehyde for 15 min. Subsequently, the cells were permeabilized with 0.3% Triton X-100 in DPBS for 5-7 min and blocked with 3% bovine albumin serum (BSA) for

45 min. After blocking, the cells were washed once with DPBS and then incubated with primary antibody in 3% BSA for 1 h. CFTR was detected using anti-CFTR antibody and either AlexaFluor 488 goat anti-mouse or AlexaFluor 594 goat anti-mouse secondary antibody. The cells were incubated with a 1:1000 dilution of the nuclear stain, DAPI for 5 min and washed three times with DPBS, then mounted using ProLong Gold Antifade (Invitrogen). All image processing was performed using Imaris Imaging.

Results

STX8 silencing increases the maturation of F508-CFTR

To determine whether STX8 plays a role in the ER, we utilized CF bronchial epithelial cells (CFBE41o⁻) stably expressing additional F508-CFTR. Fig. 1 shows that knocking down STX8 with siRNA increased both the immature band B and mature band C of F508-CFTR, with an optimal dose of 20 nM, which was subsequently used in further experiments. Silencing STX8 caused an approximately 1.5-fold increase in both mature and immature CFTR (Fig. 2), resembling the effect of the corrector compound C18, developed by Vertex to rescue F508-CFTR [18]. Importantly, silencing of STX8 was additive with that of C18, resulting in a more than 2-fold increase in the mature C band.

STX8 silencing restores the function of F508-CFTR

F508-CFTR is both a processing and functional mutant [20]; thus, the ultimate test of rescue is whether CFTR function is restored. To determine whether rescue of function had indeed occurred, we grew the cells on permeable supports and measured the short-circuit current produced. Fig. 3 shows that silencing STX8 increased the CFTR-dependent chloride currents by approximately 1.8-fold when compared to control currents. This increase was equal to that of C18, which is known to rescue F508-CFTR currents [18]. The combination of knockdown and corrector treatment caused an even greater increase in current, to approximately 2.5-fold higher than control. The magnitude of the increase in current mirrored the increased processing of F508-CFTR to the mature C band (Fig. 2), indicating that the silencing of STX8 stabilizes the immature B band in a form that is functionally rescued.

STX8 alters quality control proteins

The interaction of the mutant F508 with proteins involved in recognizing it as a mutant protein and targeting it for degradation has been studied intensively [21]. To explore whether STX8 knockdown, alone or combined with corrector, rescues CFTR by reducing its interaction with quality control proteins, we performed co-immunoprecipitation (co-IP) experiments with the heat shock proteins (Hsp) 27 and 60. Hsp27 (HspB1) is a member of the small heat shock protein family that targets F508 for degradation by connecting CFTR with the small ubiquitin-like modifier (SUMO)-2 [22, 23]. Hsp60 is a mitochondrial chaperone that is also found in the cytoplasm [24]. An increased interaction of Hsp60 with F508-CFTR has been associated with rescue by 4-phenylbutyrate [25].

The results in Fig. 4 are particularly interesting. Note that there is sharp drop in the binding of Hsp27 to CFTR when STX8 is silenced. The decrease in binding is similar to what occurs

when cells are treated with the corrector C18 with no further decrease noted in the combined treatment group. We have shown previously that silencing Hsp27 rescues F508-CFTR, consistent with our findings here. In contrast, binding of Hsp60 to F508-CFTR was increased significantly only when C18 is applied when STX8 is silenced. This result is entirely consistent with previous reports of an increase in Hsp60 binding following treatment with 4-phenylbutyrate [25].

STX8 and F508 are colocalized in the ER

F508 is an ER resident protein [26]. STX8 on the other hand has been colocalized with wt-CFTR with Rab11 associated with recycling endosomes [10, 11]. With these data in mind, the question is does STX8 interact with F508-CFTR in the ER. To address this question, we utilized confocal microscopy to ascertain whether STX8 and F508 were indeed in the same compartments. Fig. 5 shows strong colocalization between STX8 and F508. Fig. 5 also shows that the colocalization is indeed within the ER in CFBE41o⁻ a widely used bronchial CF epithelial cell model [27]. These results show conclusively that STX8 is in the proper location, i.e. the ER, to affect F508-CFTR.

Discussion

In F508-CFTR, the missing phenylalanine at position 508 in the NBD1 domain destabilizes this domain and disrupts its interactions with the transmembrane domains. The cell recognizes these defects and prevents the movement of F508-CFTR out of the ER [28]. During this process of recognition and sequestration, CFTR interacts with a network of proteins that include chaperones, ubiquitin, and SUMO ligases, as well as various components of the proteasomal and aggresomal degradative pathways [29]. Our data show that silencing STX8 increases the expression of the mature band of F508-CFTR, thereby increasing its function. This improvement in expression and function is accompanied by affecting the binding to CFTR to two key chaperones. Specifically, STX8 silencing reduces the binding of Hsp 27, a protein that targets F508-CFTR for sumoylation and subsequent degradation [22]. On the other hand it increases the binding of CFTR to Hsp60 which acts at the early stage of protein folding [30]. Thus, a decrease in Hsp27 and an increase in Hsp60 by knockdown of STX8 creates an environment favorable to increasing mature F508-CFTR at the cell surface. The data also suggest than when present at normal levels, STX8 is a component of the cell's quality control mechanism. The question is by what mechanism is this occurring? Examination of Fig. 4 shows that the effects of STX8 silencing in the presence of the corrector, C18, on the binding of Hsp 27 to CFTR are not additive suggesting that they are occurring via a common mechanism. This is exactly the pattern we noticed previously, but in a slightly different way [18]. In the published study, we silenced Hsp27 and applied CFTR correctors and showed that these maneuvers did rescue F508-CFTR individually but were not additive. The data reported here combined with our previous report suggest that the corrector, C18, STX8 and Hsp27 are acting via a common pathway.

Two chemical compounds found by high-throughput screening have been approved as new drugs to treat CF patients. Ivacaftor is a CFTR potentiator that restores the function of

mutations with defective gating by increasing the probability that the channel will open and allow chloride ions to flow [31]. On the other hand, Lumacaftor (also known as VX-809) is a corrector that improves the conformational stability of F508-CFTR, resulting in increased processing and trafficking of the mature C band to the cell surface [20]. Since F508-CFTR is both a processing and gating mutant, a combination of both potentiator and corrector, known as Orkambi, is currently an approved regimen for CF patients. Although approved as a therapy, the clinical benefit of Orkambi for patients bearing the processing mutant F508-CFTR is much lower than that of Ivacaftor for patients with gating mutations. The reason for this difference is that in the case of a gating mutant such as G551D CFTR, Ivacaftor activates a channel that is already at the cell surface and only needs activation.

Lumacaftor (VX-809), is not fully effective in rescuing F508-CFTR because it targets only one of its defects, enhancing the interaction between NBD1 and the extracellular domains of the transmembrane segments [32, 33] but not affecting the instability of NBD1. Thus, there is no fully efficient processing of F508-CFTR to the cell surface. The question is whether silencing STX8 can improve the processing of F508-CFTR that has been partially rescued by correctors. To address this question, we studied the effects of the CFFT compound C18, originally identified by Vertex [18]. C18 and VX809 (Lumacaftor) belong to the same class of correctors targeting the interaction between NBD1 and the intracellular loops of the transmembrane domains [32]. Importantly, we found that STX8 silencing and C18 treatment were additive in restoring function, suggesting that STX8 is regulating a pool of F508-CFTR that can be further rescued by C18.

Conclusion

Our data provide evidence for a previously undefined role for STX8 in sequestering F508-CFTR molecules in the ER and promoting its interaction with two heat shock proteins involved the quality control mechanism. Movement of tubulovesicular clusters of the ER-Golgi in intermediate compartment involves tethering and fusion of vesicles. This process involves an ER-Golgi SNARE complex that includes syntaxin 5 [34]. One possibility is that STX8 is part of this complex and that it interacts abnormally with the mutant F508-CFTR, together with its attendant chaperone and proteasomal transport molecules, regulating the biosynthetic arrest of the F508-CFTR within the ER.

Acknowledgements

References

1. Jensen TJ, Loo MA, Pind S, Williams DB, Goldberg AL, Riordan JR: Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. Cell 1995;83:129–135. [PubMed: 7553864]

2. Fuller CM, Benos DJ: CFTR. Am J Physiol Cell Physiol 1992;263:C267–C286.

3. Peters KW, Qi J, Johnson JP, Watkins SC, Frizzell RA: Role of snare proteins in CFTR and ENaC trafficking. Pflugers Arch 2001;443 Suppl 1:S65–69. [PubMed: 11845306]

4. Cormet-Boyaka E, Di A, Chang SY, Naren AP, Tousson A, Nelson DJ, Kirk KL: CFTR chloride channels are regulated by a SNAP-23/syntaxin 1A complex. Proc Natl Acad Sci U S A 2002;99:12477–12482. [PubMed: 12209004]

5. Naren AP, Nelson DJ, Xie W, Jovov B, Pevsner J, Bennett MK, Benos DJ, Quick MW, Kirk KL: Regulation of CFTR chloride channels by syntaxin and Munc18 isoforms. Nature 1997;390:302–305. [PubMed: 9384384]

6. Naren AP, Quick MW, Collawn JF, Nelson DJ, Kirk KL: Syntaxin 1A inhibits CFTR chloride channels by means of domain-specific protein-protein interactions. Proc Natl Acad Sci U S A 1998;95:10972–10977. [PubMed: 9724814]

7. Cheng J, Cebotaru V, Cebotaru L, Guggino WB: Syntaxin 6 and CAL mediate the degradation of the cystic fibrosis transmembrane conductance regulator. Mol Biol Cell 2010;21:1178–1187. [PubMed: 20130090]

8. Kuliawat R, Kalinina E, Bock J, Fricker L, McGraw TE, Kim SR, Zhong J, Scheller R, Arvan P: Syntaxin-6 SNARE involvement in secretory and endocytic pathways of cultured pancreatic beta-cells. Mol Biol Cell 2004;15:1690–1701. [PubMed: 14742717]

9. Bilan F, Nacfer M, Fresquet F, Norez C, Melin P, Martin-Berge A, Costa de Beauregard MA, Becq F, Kitzis A, Thoreau V: Endosomal SNARE proteins regulate CFTR activity and trafficking in epithelial cells. Exp Cell Res 2008;314:2199–2211. [PubMed: 18570918]

10. Bilan F, Thoreau V, Nacfer M, Derand R, Norez C, Cantereau A, Garcia M, Becq F, Kitzis A: Syntaxin 8 impairs trafficking of cystic fibrosis transmembrane conductance regulator (CFTR) and inhibits its channel activity. J Cell Sci 2004;117:1923–1935. [PubMed: 15039462]

11. Thoreau V, Berges T, Callebaut I, Guillier-Gencik Z, Gressin L, Bernheim A, Karst F, Mornon JP, Kitzis A, Chomel JC: Molecular cloning, expression analysis, and chromosomal localization of human syntaxin 8 (STX8). Biochem Biophys Res Commun 1999;257:577–583. [PubMed: 10198254]

12. Guggino WB: Cystic fibrosis and the salt controversy. Cell 1999;96:607–610. [PubMed: 10089875]

13. Goodman BE, Percy WH: CFTR in cystic fibrosis and cholera: from membrane transport to clinical practice. Adv Physiol Educ 2005;29:75–82. [PubMed: 15905150]

14. Abeliovich D, Pashut Lavon I, Lerer I, Cohen T, Springer C, Avital A, Cutting GR: Screening for five mutations detects 97% of cystic fibrosis (CF) chromosomes and predicts a carrier frequency of 1:29 in the Jewish Ashkenazi population. Am J Hum Genet 1992;51:951–956. [PubMed: 1384328]

15. Rosenstein BJ, Cutting GR: The diagnosis of cystic fibrosis: a consensus statement. Cystic Fibrosis Foundation Consensus Panel. J Pediatr 1998;132:589–595. [PubMed: 9580754]

16. Mickle JE, Cutting GR: Clinical implications of cystic fibrosis transmembrane conductance regulator mutations. Clin Chest Med 1998;19:443–458. [PubMed: 9759548]

17. Illek B, Lei D, Fischer H, Gruenert DC: Sensitivity of chloride efflux vs. transepithelial measurements in mixed CF and normal airway epithelial cell populations. Cell Physiol Biochem 2010;26:983–990. [PubMed: 21220929]

18. Lopes-Pacheco M, Boinot C, Sabirzhanova I, Morales MM, Guggino WB, Cebotaru L: Combination of Correctors Rescue F508-CFTR by Reducing Its Association with Hsp40 and Hsp27. J Biol Chem 2015;290:25636–25645. [PubMed: 26336106]

19. Ma T, Thiagarajah JR, Yang H, Sonawane ND, Folli C, Galietta LJ, Verkman AS: Thiazolidinone CFTR inhibitor identified by high-throughput screening blocks cholera toxin-induced intestinal fluid secretion. J Clin Invest 2002;110:1651–1658. [PubMed: 12464670]

20. Van GF, Straley KS, Cao D, Gonzalez J, Hadida S, Hazlewood A, Joubran J, Knapp T, Makings LR, Miller M, Neuberger T, Olson E, Panchenko V, Rader J, Singh A, Stack JH, Tung R, Grootenhuis PD, Negulescu P: Rescue of DeltaF508-CFTR trafficking and gating in human cystic fibrosis airway primary cultures by small molecules. Am J Physiol Lung Cell Mol Physiol 2006;290:L1117–L1130. [PubMed: 16443646]

21. Amaral MD: CFTR and chaperones: processing and degradation. J Mol Neurosci 2004;23:41–48. [PubMed: 15126691]

22. Ahner A, Gong X, Frizzell RA: Cystic fibrosis transmembrane conductance regulator degradation: cross-talk between the ubiquitylation and SUMOylation pathways. FEBS J 2013;280:4430–4438. [PubMed: 23809253]

23. Ahner A, Gong X, Schmidt BZ, Peters KW, Rabeh WM, Thibodeau PH, Lukacs GL, Frizzell RA: Small heat shock proteins target mutant cystic fibrosis transmembrane conductance regulator for degradation via a small ubiquitin-like modifier-dependent pathway. Mol Biol Cell 2013;24:74–84. [PubMed: 23155000]

24. Hartl FU, Hlodan R, Langer T: Molecular chaperones in protein folding: the art of avoiding sticky situations. Trends Biochem Sci 1994;19:20–25. [PubMed: 7908149]

25. Singh OV, Pollard HB, Zeitlin PL: Chemical rescue of Delta F508-CFTR mimics genetic repair in cystic fibrosis bronchial epithelial cells. Mol Cell Proteomics 2008;7:1099–1110. [PubMed: 18285607]

26. Cheng SH, Gregory RJ, Marshall J, Paul S, Souza DW, White GA, O'Riordan CR, Smith AE: Defective intracellular transport and processing of CFTR is the molecular basis of most of cystic fibrosis. Cell 1990;63:827–834. [PubMed: 1699669]

27. Illek B, Maurisse R, Wahler L, Kunzelmann K, Fischer H, Gruenert DC: Cl transport in complemented CF bronchial epithelial cells correlates with CFTR mRNA expression levels. Cell Physiol Biochem 2008;22:57–68. [PubMed: 18769032]

28. Mendoza JL, Schmidt A, Li Q, Nuvaga E, Barrett T, Bridges RJ, Feranchak AP, Brautigam CA, Thomas PJ: Requirements for efficient correction of DeltaF508 CFTR revealed by analyses of evolved sequences. Cell 2012;148:164–174. [PubMed: 22265409]

29. Balch WE, Roth DM, Hutt DM: Emergent properties of proteostasis in managing cystic fibrosis. Cold Spring Harb Perspect Biol 2011;3 pii: a004499 [PubMed: 21421917]

30. Saibil H: Chaperone machines for protein folding, unfolding and disaggregation. Nat Rev Mol Cell Biol 2013;14:630–642. [PubMed: 24026055]

31. Van Goor F, Hadida S, Grootenhuis PDJ, Burton B, Cao D, Neuberger T, Turnbull A, Singh A, Joubran J, Hazlewood A, Zhou JL, McCartney J, Arumugam V, Decker C, Yang J, Young C, Olson ER, Wine JJ, Frizzell RA, Ashlock M, Negulescu P: Rescue of CF airway epithelial cell function *in vitro* by a CFTR potentiator, VX-770. Proc Natl Acad Sci U S A 2009;106:18825–18830. [PubMed: 19846789]

32. Okiyoneda T, Lukacs GL: Fixing cystic fibrosis by correcting CFTR domain assembly. J Cell Biol 2012;199:199–204. [PubMed: 23071149]

33. Okiyoneda T, Veit G, Dekkers JF, Bagdany M, Soya N, Xu H, Roldan A, Verkman AS, Kurth M, Simon A, Hegedus T, Beekman JM, Lukacs GL: Mechanism-based corrector combination restores DeltaF508-CFTR folding and function. Nat Chem Biol 2013;9:444–454. [PubMed: 23666117]

34. Appenzeller-Herzog C, Hauri H-P: The ER-Golgi intermediate compartment (ERGIC): in search of its identity and function. J Cell Sci 2006;119:2173–2183. [PubMed: 16723730]

35. Dunn KW, Kamocka MM, McDonald JH: A practical guide to evaluating colocalization in biological microscopy. Am J Physiol Cell Physiol 2011;300:C723–742. [PubMed: 21209361]
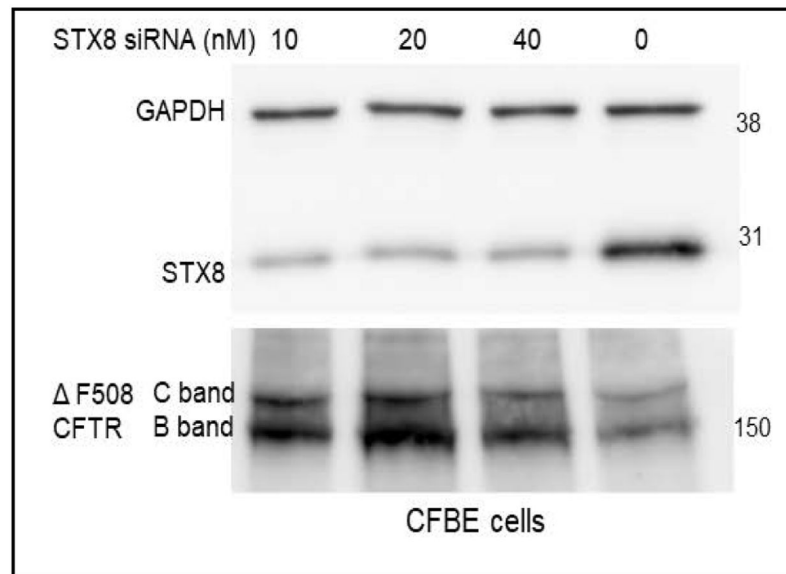
Fig. 1.

Effect of STX8 knockdown on F508. STX8 was silenced to determine the effect of this SNARE on F508-CFTR. CFBE cells stably expressing F508-CFTR were transfected with STX8 siRNA at 10, 20, or 40 nM. STX8 knockdown increased the amount of core glycosylated B band and the mature C band of F508-CFTR in CFBE cells stably expressing F508-CFTR.
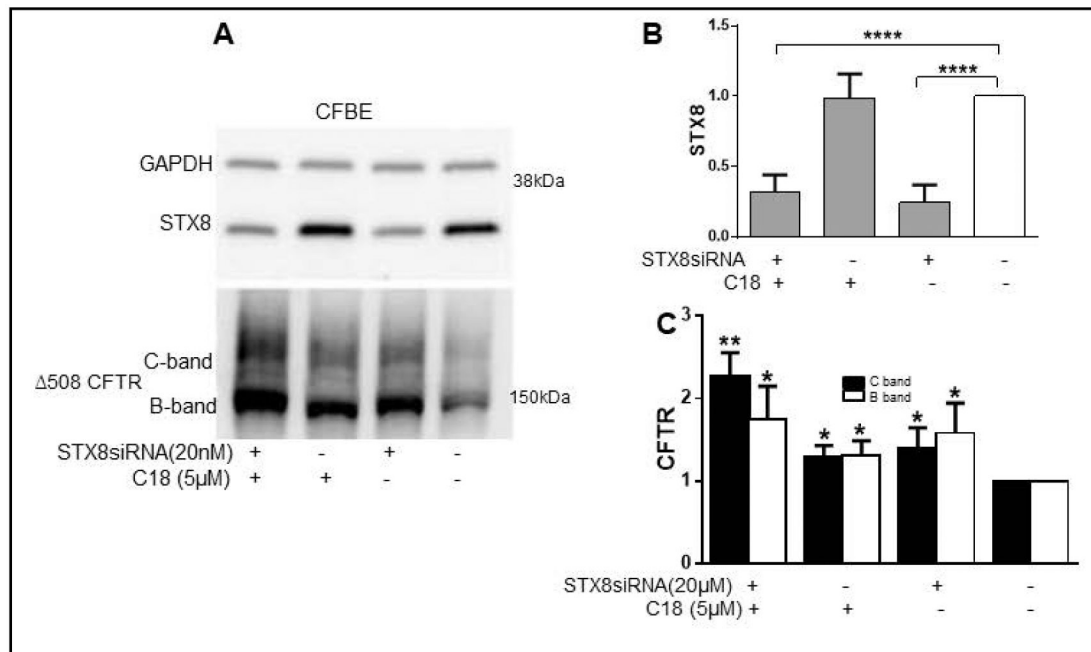
Fig. 2.
Effect of STX8 knockdown and C18 treatment on F508-CFTR. (A) Top: Western blot showing the GAPDH loading control and the amount of endogenous STX8 before and after silencing; Bottom: Western blot showing the amount of F508-CFTR B and C bands after silencing with STX8 siRNA and C18 treatment in CFBE cells stably transfected with F508-CFTR. B: Densitometry measurements of STX before and after silencing and treatment with C18. C: Densitometry measurements of CFTR bands B and C before and after silencing and treatment with C18. Protein quantification was normalized GAPDH. When STX8 was silenced in the presence of C18 it resulted in more rescued C band when compared to STX8 siRNAtreatment alone. Data from three individual experiments. Data are expressed as mean ± SD (n=3). *P<0.05; **P<0.01, ****p<0.0001.
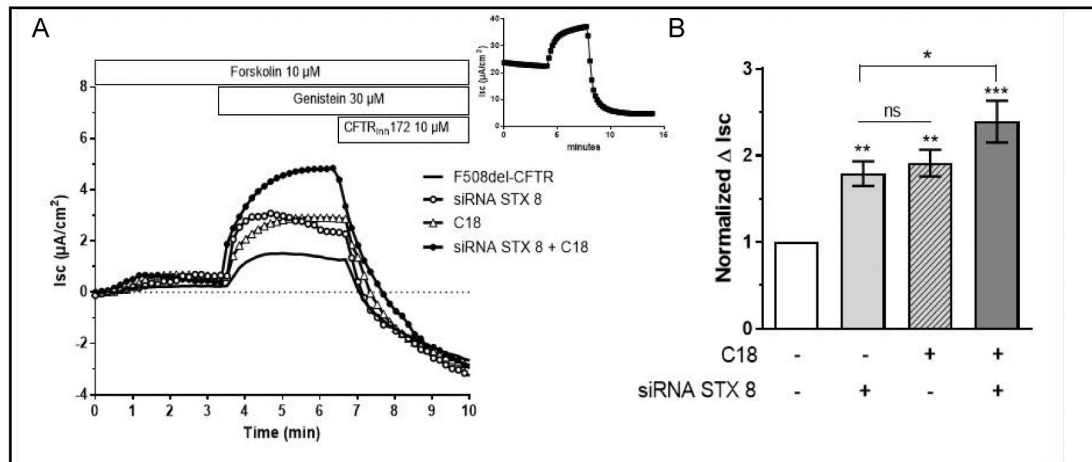
Fig. 3.

Cell short-circuit current of CFBE cells stably transfected with ΔF508-CFTR and treated with STX8 siRNA and C18. Rescue of CFTR-mediated Cl– secretion across the apical plasma membrane of epithelial cells after STX8 knockdown and its potentiation with C18 treatment. (A) Original short-circuit current recordings in untreated CFBE F508del-CFTR cells or after incubation with C18 (5 μM, 16 h), with or without incubation with STX8 siRNA (72h), as indicated. Inset shows a sample tracing of Isc recorded in CFBE cells containing wide-type CFTR. The experimental maneuvers are the same as in the main figure. (B) Corresponding ΔIsc response. Data are expressed as the CFTRinh172-sensitive short-circuit current (ΔIsc), calculated by subtracting the Isc after CFTRinh172 treatment from the peak forskolin-genistein-stimulated Isc. Amiloride (100 μM) was present during the whole experiment to avoid interference by ENaC-mediated Na+ currents. ns, no significant difference; *P<0.05; **P<0.01; ***P<0.001 (n=10-15 for each condition) compared with control condition (n=15).
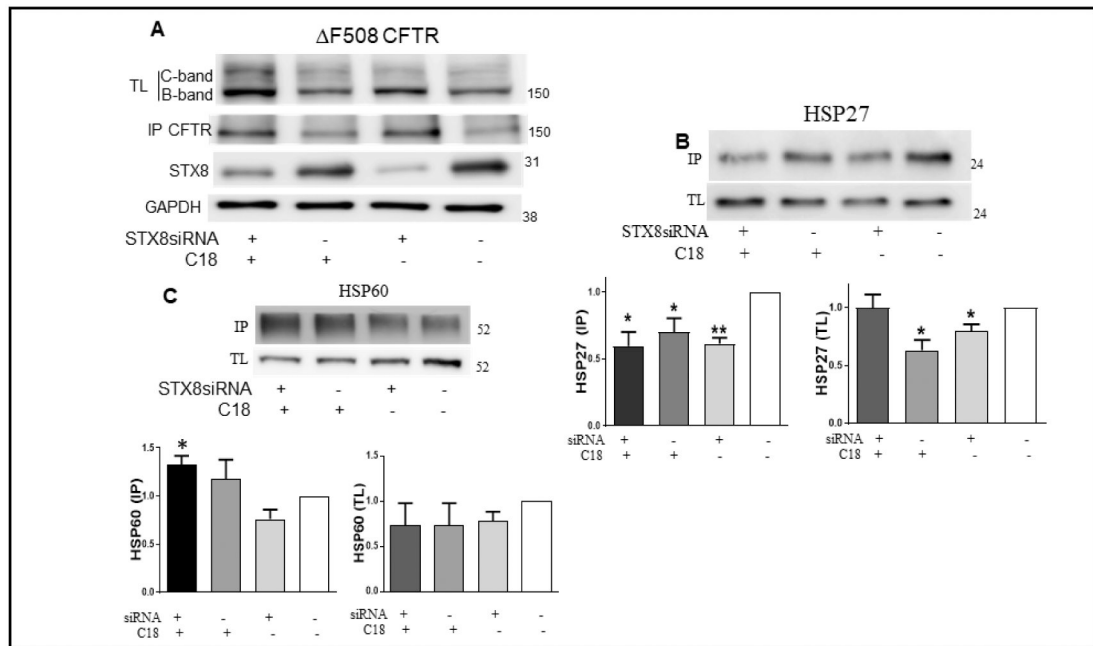
Fig. 4.
Impact of STX8 knockdown on the binding of Hsp27 and 60 to F508. Co-immunoprecipitation with anti-CFTR M3A7 antibody (Millipore) after STX8 siRNA and C18 treatment. (A) Western blot of immunoprecipitated and total lysate of CFTR. (B) Western blot and densitometry of co-immunoprecipitated and total lysate of HSP27. (C) Western blot and densitometry of co-immunoprecipitated and total lysate of HSP60. Combined silencing and C18 treatment reduced the binding of Hsp27 but increased that of Hsp60. Protein quantification in IP experiments was normalized to CFTR and in total lysate the quantification was normalized GAPDH. *P<0.05, **P<0.01.
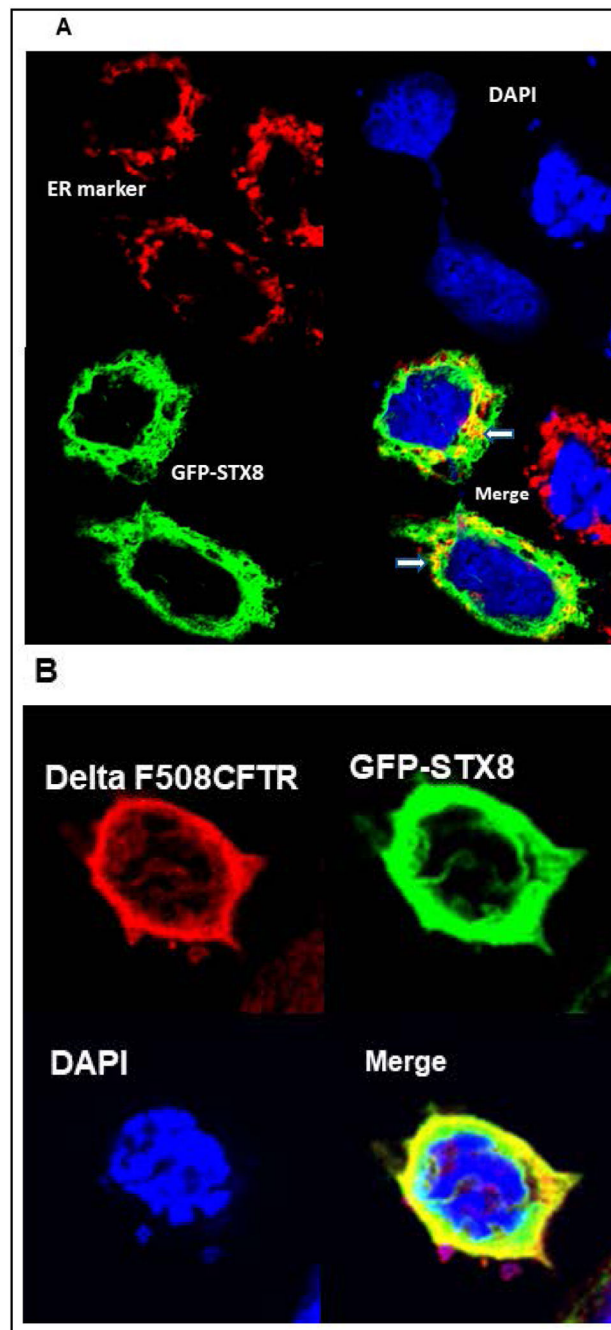
Fig. 5.
Colocalization of F508 and STX8 in the ER. (A) Colocalization of STX8 and the ER maker Calreticulin. Note that STX8 strongly colocalizes within the ER. (B). Colocalization of F508 and STX8. Note that F508-CFTR and STX8 strongly colocalize. Methods: For colocalization, anti-CFTR-596 antibody (aa 1204-1211) obtained from University of North Carolina - Chapel Hill was used to detect CFTR. GFP-STX8 (OriGene RG202177) was transfected into CFBE41o⁻ cells containing F508 CFTR and detected with corresponding GFP antibody (TA1590041) from OriGene. The ER Marker used was the Anti-Calreticulin

Antibody(ab39897). The Secondary antibody (Far-Red) was Zenon Alexa Fluor 647 Rabbit IgG Secondary Antibody. (Red) was Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 594. DAPI was used to stain the cell nuclei Images were taken with a 63X objective. Measured over the entire image the Pearson's correlation coefficient (PCC) is 0.39 ± 0.07 average$\pm$S.E., (n=3) representing a high degree of overlap [35] between STX8 and the ER marker calreticulin.