

Degradation of CFTR-F508del By the Ubiquitin E2 Conjugating Enzyme UBE 2L6 and the E3 Ligase RNF19B

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Abstract

Background Cystic Fibrosis (CF) is the most common, lethal autosomal-recessive disorder, and is caused by mutations in the cystic fibrosis transmembrane conductance regulator protein (CFTR), an anion channel that is found in epithelial cells lining multiple organs. The most common mutation of CFTR is deletion of phenylalanine at position 508 (CFTR-F508del), which produces a misfolded protein. This misfolded protein is confined in the endoplasmic reticulum (ER), ubiquitinated and signaled for degradation via the cytosolic proteasome. Previous studies demonstrating experimental restoration of CFTR-F508del (F508del) trafficking to the plasma membrane showed partial function of the chloride channel, raising therapeutic speculations. However, many mechanisms that underly its degradation is still unclear. Results We discovered that the E2 conjugating enzyme, UBE 2L6, aids in the degradation of F508del. We also show that UBE 2L6's interacting E3 ligase, RNF19B, also assists in F508del degradation. We used siRNA-mediated silencing of endogenous UBE 2L6 and RNF19B in the CF human bronchial epithelial (HBE) cell line CFBE-F508del to demonstrate that there is an increase in F508del-CFTR expression. We also co-expressed UBE 2L6 and RNF 19B with F508del in HEK293 cells that demonstrated a decrease in F508del compared to control. Cycloheximide-chase (CHX) experiments using HEK 293 cells overexpressing RNF19B and F508del showed that there was a decrease in F508del half-life. Lastly, using siRNA-mediated silencing of endogenous UBE 2L6 and RNF19B in CFBE-F508del cells increased forskolin-stimulated short-circuit currents. Interestingly, there seemed to be cooperatively larger increases in short-circuit currents in CFBE-F508del cells that were silenced with UBE 2L6 or RNF19B were treated for 24 hours with VX-809. Conclusion These data suggest that there is an additional mechanism where the E2 conjugating enzyme, UBE 2L6, and the E3 ubiquitin ligase, RNF19B, is responsible for the ubiquitination and subsequent proteasomal degradation of the misfolded F508del. This study presents a novel therapeutic strategy to synergistically restore the function of F508del.

Background

Cystic fibrosis (CF) is the most common, lethal autosomal recessive disease caused by a mutation in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR)[1, 2]. CFTR is a cAMP regulated chloride channel that is a member of the ABC (ATP-binding cassette) superfamily. The channel is found at the apical membranes of epithelial cells lining the airway and other organs [3]. The most common mutation amongst CF patients is the deletion of the phenylalanine residue at position 508 (CFTR-F508del). CFTR-F508del (F508del) is unable to achieve a native, folded state that is required for the protein to export from the endoplasmic reticulum (ER) to the apical membrane of epithelial cells [4]. More than 90% of CF patients have at least one copy of the F508del allele. F508del is synthesized in the ER, and it is trapped in the ER because it is unable to obtain its native state [5-8]. This trapped F508del is then recognized by the ubiquitin-proteasome system (UPS), where it is polyubiquitinated, translocated to the cytosol and is eventually degraded by the 26S proteasome [8-10]. This network is generally called ER-associated degradation pathway (ERAD) [11]. Studies have previously shown that F508del has a folding defect that can be adjusted by chemicals to promote proper folding and introducing channel function at

the plasma membrane [8, 10, 12]. The ER folding pathway of CFTR is tightly coordinated with the ERAD pathway whereby misfolded CFTR are targeted to the cytosolic proteasome [13]. This is extremely important because pharmacological agents that may help with F508del folding or the blocking of its degradation may help CF patients.

A small amount of F508del can return to its proper folding pathway with the help of correctors, such as VX-809 [14, 15]. Experimental restoration of F508del trafficking to the plasma membrane results in partial function of the chloride channel, raising therapeutic speculations [10, 12]. The UPS is essential in the degradation of F508del. Ubiquitin is covalently attached to F508del by an isopeptide linkage between the c-terminal glycine of ubiquitin and amino group of lysine of the substrate (F508del). This is done through a cascade system, which consists of E1 activating enzyme, E2 conjugating enzymes, and E3 ligase enzymes [16]. Ubiquitination of F508del occurs when the E3 ligase binds to the F508del and E2 enzyme that is thioesterified with ubiquitin [17, 18], and brings both to close proximity so that ubiquitin is transferred from the E2 to the substrate. This process occurs several times to the point a polyubiquitin tail is formed on F508del, which is then a signal for the protein to be degraded by the proteasome [19]. Ubiquitin E3 ligases are important enzymes that transfer ubiquitin from an E2 ubiquitin-conjugating enzyme to the mutant CFTR. E3 ligases are important for they show substrate specificity for ubiquitin transfer to the target proteins. To date, there are little studies in mammalian cells that identify E2 conjugating enzymes and ER-localized E3 ubiquitin ligases that function in the degradation of F508del.

About 60% of newly synthesized wild type-CFTR (WT-CFTR) and almost 99% of F508del is degraded by the UPS [20]. Therefore, there seems to be specific checkpoints set in place within the ER that allows F508del to stray apart from its WT counterpart during early stages of CFTR biogenesis. We believe that ER resident E3 ligases and their corresponding E2 conjugating enzymes are the initial checkpoint proteins that may be responsible for the divergence of WT-CFTR and F508del during CFTR biogenesis.

Previous studies have identified the E3 RMA1 and the E2, UBC6e cooperate to degrade both CFTR and F508del. [21, 22] However, there seems to be other mechanisms that facilitate F508del degradation because these studies still illustrate a significant amount of F508del that is being degraded, even when the above-mentioned proteins were silenced. The E2 UBCH5 forms an E3 complex with CHIP and the E3 ligase Hdj2 to ubiquitinate F508del [5, 22, 23]. When this complex is destroyed, F508del is still not able to traffic out of the ER, but instead is degraded. Therefore, other E2/E3 complexes may exist in F508del degradation and other mechanisms need to be sorted out to determine other potential therapeutic approaches to help prevent F508del degradation.

Through a literature search of E2 conjugating enzymes that seem to interact with ER membrane-bound E3 ligases, we discovered the E2 conjugating enzyme UBE2L6, whom has its role in the ubiquitin conjugating pathway [24-26] This E2 interacts with RING E3 ligases that are bound to the ER such as RNF122, RNF19B, and also RNF19A [27]. This is important because it has already been shown that certain E3 ligases, such as RMA1, acts in the ER membrane to identify the misfolded F508del [21]. We were able to determine that the E2 UBE2L6 was a key player in F508del degradation, and its interacting E3 ligase,

RNF19B, also mediates F508del. In this study, we explore the possible roles of UBE 2L6 and the E3 ligase RNF19B in the proteasomal degradation of F508del. We determined that both proteins degraded F508del when overexpressed. Also, when both proteins were knocked down through siRNA-mediated silencing in HBE cells, there was an increase in expression of F508del, which was further enhanced when treated with VX-809. The same cells treated with VX-809 also had more chloride channel activity at apical membrane of the cells. Our results provide a clearer landscape of the molecular mechanism that underlies the proteasomal degradation of F508del through UBE 2L6 and RNF19B.

Methods

Antibodies and chemicals

Mouse monoclonal anti-CFTR antibody was obtained from University of North Carolina at Chapel Hill. Rabbit polyclonal actin and polyclonal anti-NA/K ATPase were purchased from Cell Signaling. Mouse monoclonal UBE 2L6 antibody were acquired from Research Diagnostics Inc. Mouse monoclonal Myc, HA, and His-tag antibodies were bought from Abcam. VX-809 and MG132 were obtained from Selleck Chemicals, GlyH101 was obtained from Calbiochem, Forskolin, 3-Isobutyl-1-methylxanthine (IBMX) and cycloheximide (CHX) were purchased from Sigma. siRNA was purchased through Dharmacon Inc.

Cell culture and drug treatment

CFBE-F508del expressing cell line was established from CFBE41o⁻ (CFBE), a well-characterized human airway epithelial cell line. CFBE cells was derived from the bronchial epithelial cells of a CF patient with *cftr* F508del/F508del genetic background and with no detectable expression of the mutant protein. CFBE-F508del cells were cultured in MEM with 10% fetal bovine serum addition of 0.5 µg/L puromycin. All cells were maintained in a humidified atmosphere containing 5% CO₂ at 37 °C. Confluent cells were treated with VX-809 in culture medium at the designated concentration for 24 h. Cells were harvested after 24 h treatment. HEK 293 cells were cultured in DMEM with 10% fetal bovine serum.

Plasmid constructs, DNA constructs.

F508del in pcDNA3.1 vector was described previously [35]. The UBE 2L6 and RNF19B cDNA sequence was amplified by PCR and inserted into pcDNA3.1(+) at EcoR1 and EcoRV sites. HA-ubiquitin was purchased from Addgene.

Immunoblotting

Cells were lysed by sonication in lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% NP40, 10% glycerol and protease inhibitors cocktail). Specifically, protein samples were resolved by SDS-PAGE and transferred to PVDF membranes, which were blocked at room temperature for 1 h with 5% (w/v) non-fat milk in TBST (10 mM Tris (pH 8.0), 150 mM NaCl, 0.05% Tween 20). The blots were incubated with primary antibodies in TBST with 10% fetal bovine serum at room temperature for 2-3 h. The blots were

then washed three times with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h, followed by three washes with TBST. The reactive bands were visualized by incubation with enhanced chemiluminescence substrates (PerkinElmer Products) and exposure to X-ray film (Eastman Kodak Co).

Co-immunoprecipitation (Co-IP) for ubiquitin assay

Cell lysate treated with MG132 in Lysis buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 10% glycerol) was precleared with protein A/G-Sepharose beads (Invitrogen). The precleared lysate was mixed with 10ug of the indicated antibody and 25ul of protein A/G-Sepharose beads and incubated overnight at 4°C with gentle rotation. Immunocomplexes were resuspended in SDS sample buffer and subjected to SDS-PAGE gel and immunoblotting.

Cycloheximide (CHX) chase analysis

After 24 h treatment with designated reagent, cells were continued to be cultured in medium supplemented with 50 µg/ml CHX and harvested at designated time points. Cell extracts were subjected to the immunoblotting analysis with appropriate antibodies.

Biotinylation and pulldown of CFTR on streptavidin beads

After treatment with specified reagent of 24 hours, cells were washed 3 times with PBS and exposed to 0.5mg/ml in PBS EZLink Sulfo-NHS-LC-biotin (Thermo Scientific) for 1 h on ice. Cells were washed 3 time with PBS, quenched with 100mM glycine in PBS, and rinsed 2 time again. Then cells were solubilized by sonication in RIPA buffer (150 mM NaCl, 1 mM Tris/HCl, 0.5% (w/v) deoxycholic acid, 1% (w/v) NP-40, 0.1% SDS, 2 mM EDTA, 50 mM NaF and protease inhibitors). The resulting lysate was centrifuged at 21100 *g* for 10 min at 4⁰C, and supernatant protein content was determined using protein assay dye reagent (Bio Rad). The supernatant was incubated with streptavidin beads for overnight at 4⁰C. After a brief centrifugation the supernatant was removed, the beads were washed 4 times with lysis buffer. Pull-downed proteins were resolved by SDS-PAGE, transferred to PVDF membranes and performed an immunoblot as described above.

Ussing Chamber experiments

CFBE-F508del cells grown on collagen-coated transwell filters (Costar, 0.33 cm², 0.4-µm pore) were polarized and treated with indicated reagent 24h. Filters were mounted in Ussing Chambers (Physiologic Instruments #P2300). The basolateral bathing solution consisted of 120 mmol/L NaCl, 25 mmol/L NaHCO₃, 3.3 mmol/L KH₂PO₄, 0.8 mmol/L K₂HPO₄, 1.2 mmol/L CaCl₂, 1.2 mmol/L MgCl₂ and 10 mmol/L d-glucose. The apical bathing solution replaced 120 mmol/L NaCl with 120 mmol/L Na glutamate to achieve a transepithelial chloride gradient. The bathing solutions were maintained at 37⁰C and gassed with 95% O₂, 5% CO₂ to retain a pH 7.4. Short-circuit current and transepithelial resistance

were measured continuously using a voltage-clamp (VCC-MC8) and Acquire and Analyze v2.3 data acquisition hardware and software (Physiological Instruments, San Diego, CA, USA).

Filters were equilibrated for approximately 15 min to permit electrical parameters to stabilize, and baseline short circuit current (*I*_{SC}) was measured. 10 μM forskolin and IBMX was added to the basolateral chamber to activate and remain CFTR-mediated anion secretion. After 3 min and currents had reached steady state, 10 μM/L CFTR inhibitor Glyh 101 was added to the apical solution. Stop record after currents had achieved steady-state. Changes in short-circuit currents were calculated from the mean currents obtained during the 20-s period.

Confocal Microscopy

Immunofluorescence staining of filter-grown cells was performed as described previously [35]. Briefly, CFBE-F508del cells were fixed in 4% paraformaldehyde and permeabilized with a mixture of 4% paraformaldehyde and 0.1% Triton X-100. The cells were then washed three times with buffer A (0.5% BSA and 0.15% glycine at pH 7.4 in phosphate-buffered saline). After blocking with purified goat serum, the monolayers were incubated in the appropriate primary antibodies (anti-Myc 1: 300, and anti-CFTR217 1:300) for 1 hour followed by three washes in buffer A and subsequent incubation with fluorescein isothiocyanate (green) or rhodamine (red)-labeled secondary antibodies (1:1000, Molecular Probes) for another hour. After washing with buffer A, the filters were mounted on glass coverslips using synthetic resin and subjected to confocal microscopy. Collected images were exported to ImageSpace (Molecular Dynamics) for subsequent reconstruction and processing.

Quantitative real-time reverse transcription PCR analysis

Total cellular RNA was extracted using TRIzol reagent and reverse-transcribed to cDNA using a random primer. The real-time PCR reaction mixture containing cDNA template, primers and SYBR Green PCR Master Mix (Invitrogen) was run in a 7,500 Fast Real-time PCR System (Applied Biosystems). Fold changes of mRNA levels were determined after normalization to internal control of β-actin RNA levels [36]. RNF19B Forward primer: TGCATGACTCAGCAAAGTGGA. RNF19B Reverse primer: GCATGAACATGGAGCGAGTCC

Data analysis.

The immunoblot images were scanned at 600 dpi for densitometry analysis using Image J software. The Values are means ± SEM. Statistical significance of differences was determined using paired two-tailed Student's t-test.

Results

Overexpressing UBE 2L6 degrades F508del

Through a literature search, we were able to discover UBE 2L6 interacts with many E3 ligases that were found in the ER [27]. This interaction with E3 ligases in the ER may mediate F508del degradation; therefore we wanted to observe what happens to CFTR when we overexpress UBE 2L6. We used HEK 293 cells and co-transfected with F508del and UBE 2L6 for 48 hours. 48-hours post-transfection, total protein lysates were harvested and probed for F508del levels by immunoblot analysis. Overexpressing UBE 2L6 decreased F508del expression by about 2.5-fold (**Fig. 1A,1C**).

To further confirm UBE 2L6 effects on degrading F508del, we developed a dominant-negative mutant of UBE 2L6 where a cysteine residue at position 86 had been mutated to a serine residue (UBE 2L6 DN). This mutation will destroy the catalytic activity of UBE 2L6 and therefore the activated ubiquitin is not able to transfer onto the E2. Hence, theoretically the ubiquitin is not able to transfer to the misfolded protein. We transiently transfected HEK 293 cells with F508del and with UBE 2L6 or UBE2L6 DN or a control (pCMV) (**Fig. 1B**). Consistent to previous finding, overexpressing UBE 2L6 decreases F508del. When UBE 2L6 DN is overexpressed, F508del expression increases 1.9-fold (**Fig. 1B, 1C**). These data suggest that UBE 2L6 facilitates F508del degradation by ubiquitination.

Silencing UBE 2L6 in HBE cells with VX-809 rescues F508del

We next wanted to determine if we can rescue F508del if we silenced UBE 2L6 in human bronchiole epithelial (HBE) cells. We used cystic fibrosis HBE cells that have the F508 mutation (CFBE-F508del) and knockdown UBE 2L6 with siRNA. 48 hours post-transfection, the cells were treated with VX-809 or DMSO as control for 24 hours. VX-809 is a drug used in the CF field that folds F508del so that the protein is to traffic to the plasma membrane [14]. 24 hours post-treatment, the cells were harvested and probed for CFTR and actin. When UBE 2L6 was knocked down with siRNA, there is a 2.8-fold increase in F508del expression, which is illustrated as Band-B (**Fig. 2A, 2C**). Band B is the misfolded CFTR that is trapped in the ER where it will be eventually degraded by the proteasome in the cytosol. Interestingly, with VX-809 treatment, there seems to be a 2.7-fold increase in C-band expression of CFTR compared to control (**Fig.2A, 2D**). This is critical in that this shows that with UBE 2L6 knocked down, there seems to be a greater pool of B-band CFTR. With this increased pool of B-band, VX-809 helps more F508del to traffic to the plasma membrane.

F508del function is increased with UBE 2L6 knockdown and VX-809 treatment

To investigate how UBE 2L6 affect CFTR function, we used the Ussing chamber to measure F508del function by measuring forskolin-stimulated short-circuit current in HBE cells. We transfected CFBE-F508del cells with siRNA against UBE 2L6 or a scramble siRNA as a control. 48 hours post-transfection, we treated the cells with either 3 μ M VX-809 or with DMSO for 24 hours. 24 hours post-treatment, cells were set up in the Ussing chamber to measure short-circuit currents. A representative trace of the short-

circuit currents demonstrates that compared to control, UBE 2L6 knocked down in CFBE-F508del cells, there is a slight, but significant increase in CFTR function by about $2.9 \mu\text{A}/\text{cm}^2$ when stimulated by forskolin and IBMX (**Fig. 3A, 3B**). When CFBE-F508del cells have UBE 2L6 knocked down and treated with VX-809, there is an increase in CFTR function by about $5.4 \mu\text{A}/\text{cm}^2$ compared to cell transfected with scramble siRNA and treated with VX-809 (**Fig. 3A, 3B**). The quantification of three experiments illustrates that knockdown of UBE 2L6 and VX-809 treatment had a significantly greater increase in F508del function compared to controls (**Fig. 3B**).

RNF19B is a candidate in F508del degradation

To identify which E3 Ligase may cooperate with UBE 2L6 to promote F508del degradation, we performed an siRNA screen on ER resident E3 ligases on HBE cells. We determined that RNF19B may be a candidate in F508del degradation and it shown that RNF19B is a partner of UBE 2L6 [28]. RNF19B is a Ring finger protein family of E3 ligases, which interacts with E2 conjugating enzyme(s) and substrates, facilitating the transfer of an activated ubiquitin to the substrate[29, 30]. RING finger E3 ligases have a RING structure that is involved in ubiquitinating its substrate. To show that this site is involved in ubiquitinating F508del, we generated a catalytically non-functioning RNF19B RING mutant where we mutated a cysteine residue at position 119 to a serine residue (c119s) to disable the RING domain. HEK 293 cells were co-transfected with F508del and with Myc-RNF19B or with Myc-RNF19B c119s (**Fig. 4A**). Overexpressing Myc-RNF19B causes F508del degradation. When transfected with the catalytically non-functioning mutant, Myc-RNF19B c119s, F508del is expressed to similar levels as control (**Fig. 4A, 4B**). To determine if RNF19B ubiquitinates F508del, HEK293 cells were first transfected with F508del and with Myc-RNF19B plasmid. 24 hours post-transfection, HA-ubiquitin was transfected into these cells. The cells were then harvested and CFTR was pulled down (**Fig. 4C**). The left panel demonstrates that there is more HA-ubiquitin immunoprecipitated with F508del when RNF19B was overexpressed compared to control. The right panel demonstrates that total CFTR was the same between both conditions under MG-132 treatment. If RNF19B regulates F508del, they should be located in the same compartment. To determine if these proteins are localized within the same area of the cell, CFBE-F508del cells were transfected with Myc-RNF19B and then immunostained with Myc (green) and F508del (red) (**Fig. 4D**). The immunostaining shows that Myc-RNF19B is localized with F508del in the perinuclear area, which is indicative of the ER. To determine how RNF19B affects the half-life of F508del, we performed a cycloheximide-chase where HEK 293 cells were co-transfected with F508del and with the control or with Flag-RNF19B for 48 hours. The cells were then treated with $50 \mu\text{g}/\text{ml}$ cycloheximide for the different indicated time periods. (**Fig. 5A, 5B**). Overexpressing RNF19B decreased the half-life of F508del from 60 minutes to about 20 minutes, suggesting RNF19B participates F508del degradation.

Silencing RNF19B in HBE cells rescues F508del

To determine if RNF19B has any effect on F508del, CFBE-F508del cells were transfected siRNA against RNF19B. When knocking down RNF19B, there is an increase in F508del protein expression almost 3-fold (**Fig. 6A**). Due to the lack of a sufficient antibody to RNF19B, silencing of RNF19B expression was confirmed by RT-PCR (**Fig. 6B**). We overexpressed a Myc tagged RNF19B (Myc-RNF19B) plasmid into CFBE-F508del cells and transfected these cells with siRNA against RNF19B (**Fig. 6C, 6D**) to determine knockdown of RNF19B in HBE cells. The knockdown of RNF19B increased endogenous F508del by about 3-fold in CFBE-F508del cells (**Fig. 6C, 6D**). Next, HEK 293 cells were co-transfected with Myc-RNF19B and F508del (**Fig. 6E**). 0.5 μg of RNF19B decreases F508del by about 1.4 fold, whereas transfection with 1.0 μg of RNF19B decreases F508del by about 4.7 fold (**Fig. 6E, 6F**), indicating RNF19B degrades F508del in a dose-dependent manner.

F508del function is enhanced by RNF19B knockdown in the presence of VX-809 treatment

To further determine if RNF19B is involved F508del degradation and its function, we needed to determine if CFTR trafficking to the plasma membrane is affected by RNF19B. We performed biotinylation experiments to determine if more F508del traffics to the plasma membrane when RNF19B is silenced (**Fig. 7A**). Knockdown of RNF19B promotes F508del C-band to the plasma membrane to act as a chloride channel. When RNF19B is knocked down with VX-809 treatment, there is a 1.8 fold increase in C-Band (**Fig 7A, 7B**). To determine if there is enhanced F508del function when RNF19B is knocked down, we performed Ussing Chamber experiments. We transfected CFBE-F508del cells with siRNA against RNF19B or a scramble siRNA as a control. 48 hours post-transfection, we treated the cells with either 3 μM VX-809 or with DMSO for 24 hours. 24 hours post-treatment, cells were set up in the Ussing chamber to measure short-circuit currents. Short-circuit currents demonstrated that compared to control, RNF19B knocked down in CFBE-F508del cells increased short-circuit currents by about 3.25 $\mu\text{A}/\text{cm}^2$ compared to scramble controls when stimulated by forskolin and IBMX (**Fig. 7C, 7D**). There is an increase of about 7.7 $\mu\text{A}/\text{cm}^2$ in CFTR function when CFBE-F508del cells have RNF19B knocked down and treated with VX-809 compared to scramble control (**Fig. 7C,7D**). This set of data indicates that we are able to rescue F508del protein and function when RNF19B is silenced in HBE cells.

Discussion

In this paper we have identified an E2 conjugating enzyme, UBE 2L6 that mediates the proteasomal degradation of F508del. We've found that when UBE 2L6 is knocked down in human airway epithelial cells and treated with VX-809, there is an increase in expression of CFTR C-band, the functional form of CFTR. UBE 2L6 works as a conjugating enzyme, acting as a transient holder of activated ubiquitin to be ready to tag a substrate [31], in this case misfolded F508del. We have shown that by mutating UBE 2L6 so that it cannot carry that activated ubiquitin, there is an increase in expression of F508del, therefore demonstrating that UBE 2L6 does indeed mediate the degradation of F508del through the ubiquitin proteasome system. Also, this shows that UBE 2L6 is an important player in the F508del degradation

because the mutation to UBE 2L6 rescues F508del to similar levels as seen in control. Therefore, there is a greater pool of F508del that can be used on by VX-809 to allow for greater trafficking of the misfolded protein to the plasma membrane. Future studies will be focusing on developing and discovering small inhibitors to act as a drug to inhibit UBE 2L6 function so that there is a greater pool of F508del protein for VX-809 to act on. VX-809 will help fold F508del so that it can traffic to the plasma membrane.

With the increase in F508del pool, we also showed that there is an increase in CFTR function when UBE 2L6 is knocked down. Using human airway epithelial cells, we knocked down UBE 2L6 and determined that there was an increase in CFTR function when forskolin and IBMX was added to the cells. Importantly, when the same cells that had UBE 2L6 knocked down were treated with VX-809, there was a greater CFTR function compared to control. These experiments further support the idea that when UBE 2L6 is knocked down there is a greater pool of CFTR protein that VX-809 can work on.

Our data also illustrates an interacting partner of UBE 2L6 that mediates the degradation of F508del. We have shown that RNF19B, an E3 ligase that interacts with UBE 2L6, mediates the degradation of F508del. Interestingly, RNF19B is localized in the endoplasmic reticulum [32, 33], where F508del is localized. Our data also demonstrates that VX-809 treatment of RNF19B knocked down in HBE cells, there is an increase in F508del function at the plasma membrane. This is similar to what was seen when performing similar experiments with UBE 2L6. This is important in that not only does UBE 2L6 aid in the degradation of F508del, but that it interacts with and ER localized E3 ligases to assist in this degradation. Therefore, this is a potential early checkpoint in CFTR degradation because these two proteins that are involved in the ubiquitin proteasome system prevent F508del from trafficking to the plasma membrane to act as a chloride channel. If we are able to use small inhibitors to prevent these proteins from ubiquitinating F508del, we will be able to provide the cell with a greater pool of F508del that is not degraded by the proteasome but allow VX-809 to act on more F508del so that it is able to traffic to the plasma membrane. Synergy between the knockdown of UBE 2L6 and RNF19B with VX-809 treatment, which enhanced F508del chloride secretion, indicates that these two proteins may offer a therapeutic approach for CF. This allows for more chloride channels to the plasma membrane that will be extremely beneficial to CF patients.

Previous studies have shown that RMA1 and Ubc6e are involved in the proteasomal degradation of F508del [21], however we have also identified that RNF19B and its interacting partner, UBE 2L6, mediates F508del degradation. Based on the data presented, we propose the following mechanism that is involved in F508del degradation. Quality control checkpoints within the ER sense the misfolded F508del due to the F508del mutation. The misfolded F508del is brought into association with RNF19B and UBE 2L6 where both the E3 and E2 proteins cooperate to ubiquitinate F508del. The ubiquitinated F508del is then signaled to proceed to the proteasome for degradation.

F508del is the most common mutation amongst CF patients, where these patients lack the functional form of CFTR to act as an anion channel [34]. With this mutation, therapeutic strategies that create the mature form of CFTR partially restore the function of CFTR at the apical membrane of cells. Therefore,

understanding the early quality control checkpoints in CFTR degradation is important in revealing possible avenues in trying to rescue CFTR protein in CF patients.

Conclusions

This study reveals additional proteins that are involved in F508del degradation, and possible targets for therapeutic intervention. The E2 conjugating enzyme UBE 2L6, and its' interacting E3 ligase, RNF19B, both mediate the proteasomal degradation of F508del. Also, our data demonstrates that VX-809, an already established processing drug treatment, operates to enhance F508del function at the plasma membrane when UBE 2L6 and RNF19B are also silenced. Therefore, modulation of UBE 2L6/RNF19B mediated degradation of F508del offers a promising area for therapeutic intervention.

Abbreviations

CFTR: cystic fibrosis transmembrane conductance regulator; UBE: ubiquitin-conjugating enzyme; RNF: Ring Finger protein; CF: cystic fibrosis; wild-type CFTR: WT-CFTR; CFTR-F508del: F508del; PM: plasma membrane; UPS: ubiquitin-proteasome system; CFBE-F508del: F508del cystic fibrosis bronchial epithelial cells; IB: immunoblot; IP: immunoprecipitation; siRNA: small interfering RNA; ER: endoplasmic reticulum; FSK: Forskolin; IBMX: isobutylmethylxanthine. I_{sc} : Short-circuit current; CHX: cycloheximide-chase

Declarations

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Author Contributions

CR and FS were responsible for developing the project and experiment design. CR, HW, XH and MB performed cell and molecular biological experiments. CR, HW, and XH analyzed the experiments. CR, XH, and FS wrote the paper. All authors discussed the results and commented on the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

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Figures

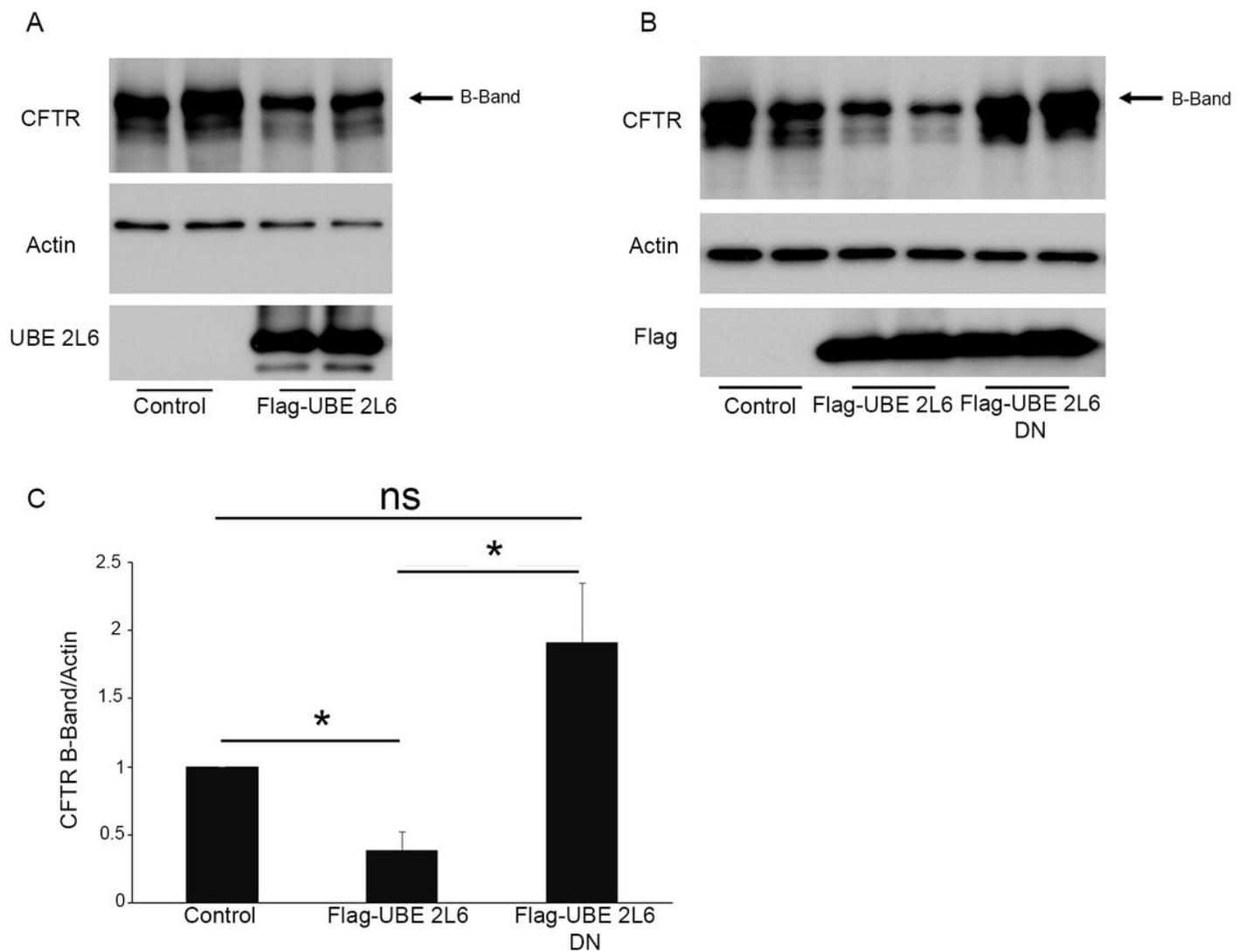


Figure 1

UBE 2L6 over-expression increase F508del degradation. A. HEK 293 cells were co-transfected with CFTR-F508del and control or Flag-UBE 2L6 plasmid. Cell lysates were subjected to immunoblot. Compared to the control, UBE 2L6 overexpression increased F508del degradation. B. HEK 293 cells were co-transfected with F508del and the plasmids that are listed above. Cell lysates were subjected to immunoblot. Compared to control, UBE 2L6 increased F508del. The dominant negative mutant, Flag-UBE 2L6-DN, decreased F508del degradation. Flag-UBE 2L6-DN plasmid was developed by mutating the cysteine at position 86 to a serine. C. Quantification of CFTR C-Band/Actin when Flag-UBE 2L6 or Flag-UBE 2L6 DN is overexpressed. (mean \pm SE, n = 3, p < 0.05).

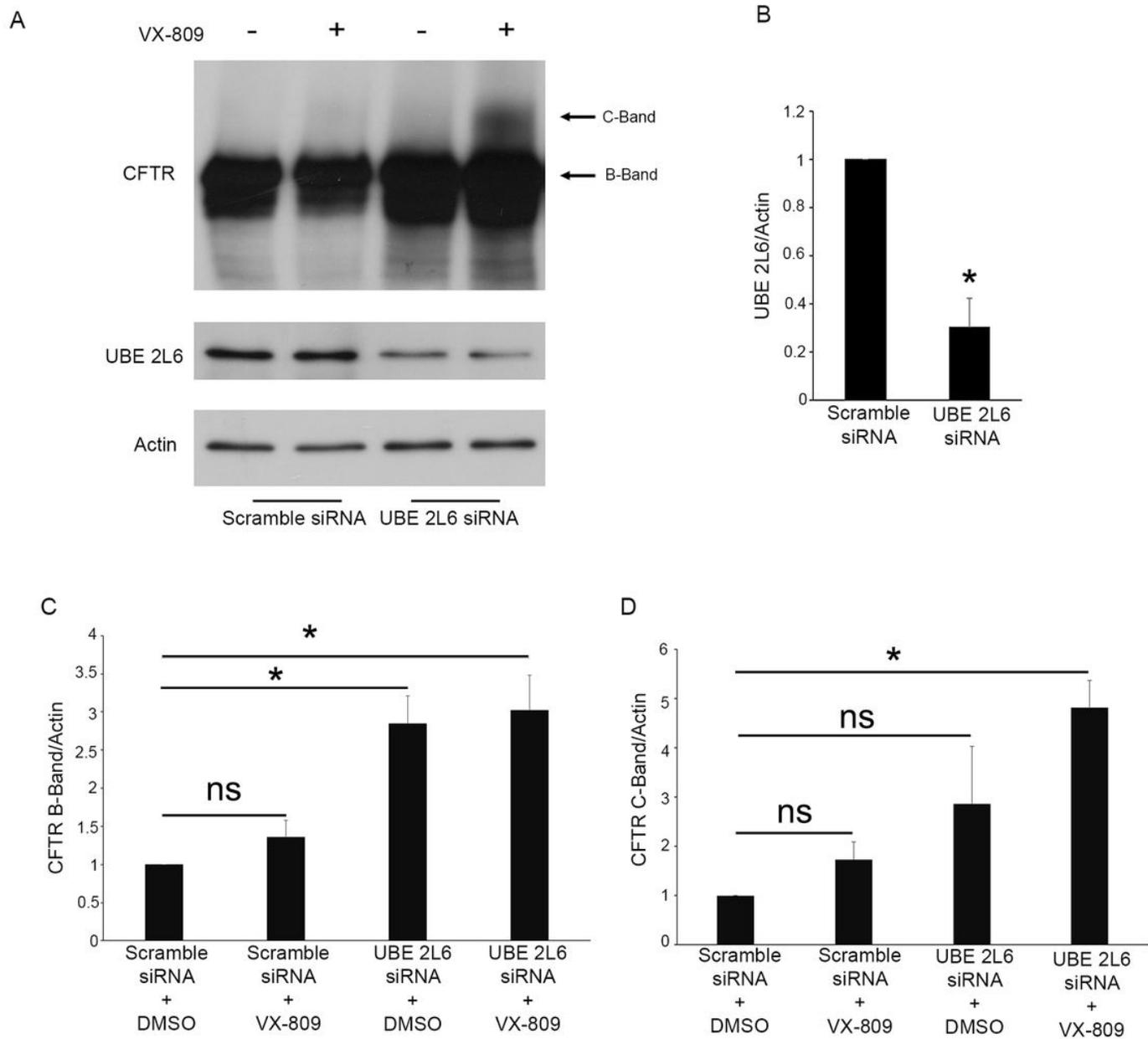


Figure 2

Silencing UBE 2L6 in F508del-CFBE cells decrease F508del degradation. A. Represented blot shows silence of endogenous UBE 2L6 expression using siRNA in cells CFBE-F508del following 3 μ M VX-809 treatment increased immature F508del (B band) and rescued mature F508del-CFTR (C Band). B. Quantification of knockdown of UBE 2L6. C. Quantification of CFTR B-Band/Actin when UBE 2L6 is knocked down with or without VX-809 treatment. D. Quantification of CFTR C-Band/Actin when UBE 2L6 is knocked down with or without VX-809 treatment. (mean \pm SE, n = 3, p < 0.05).

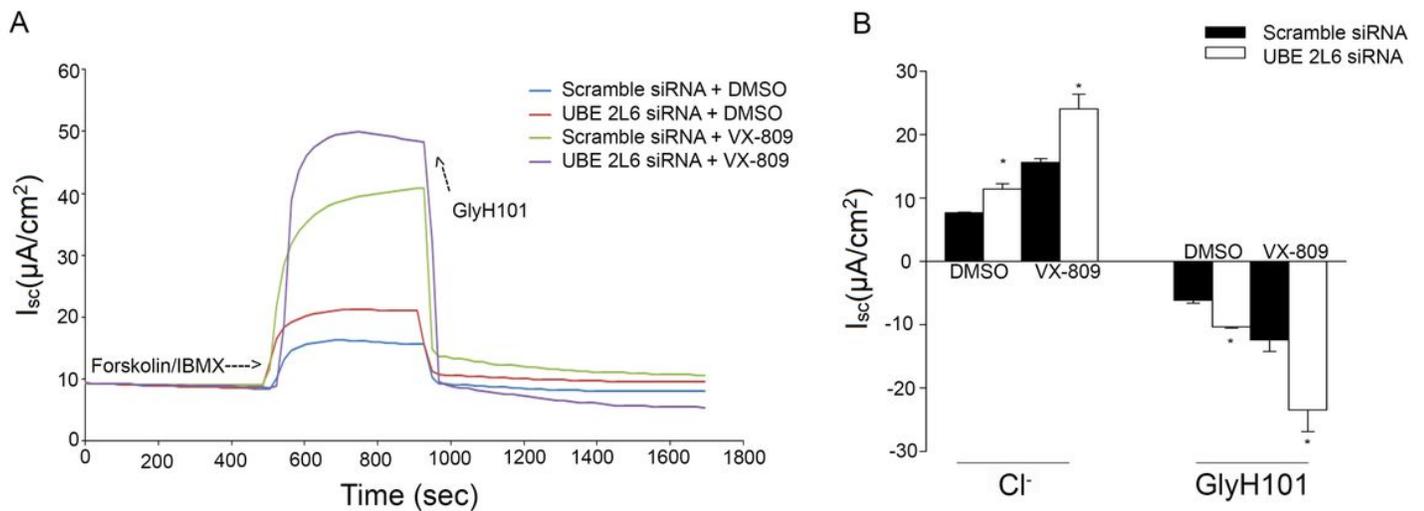
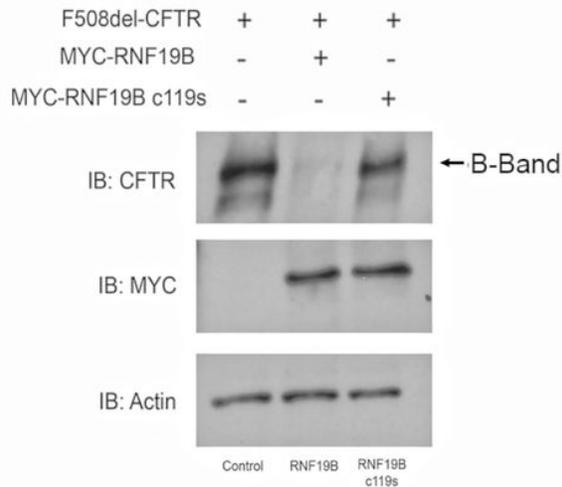
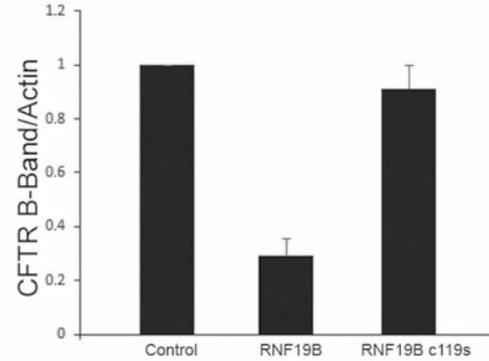
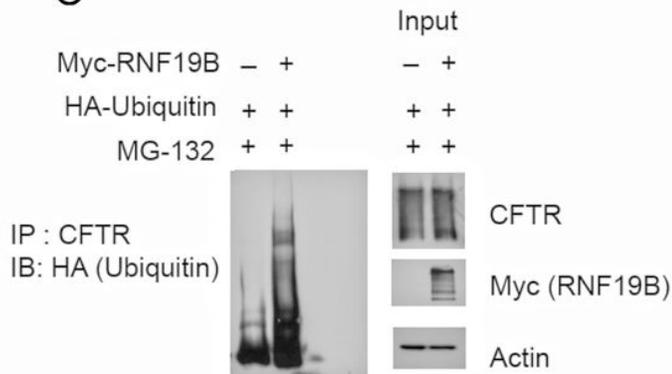
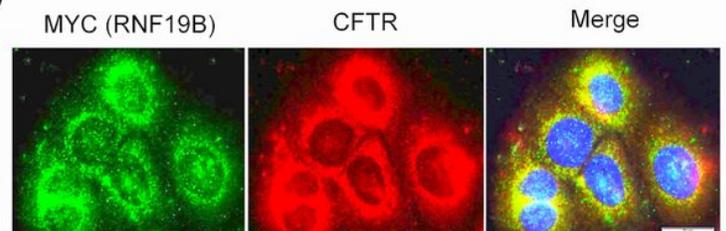


Figure 3

UBE 2L6 knockdown enhances F508del function. A. Ussing Chamber experiments. CFBE-F508del cells were first treated indicated siRNAs for 48 hrs and then incubated with 3 μM VX-809 for additional 24 hrs in 6.5 mm transwells. Short-circuit currents were retrieved through the Ussing chamber and F508del currents were determined through the treatment with the indicated conditions. Forskolin/IBMX (10 μM) and GlyH101 (10 μM). B. Quantification of short-circuit currents. (mean \pm SE, n = 3, p < 0.05).

A**B****C****D****Figure 4**

RNF19B ubiquitinates F508del and colocalizes with F508del in the ER. Stable HEK293 cells were co-transfected with F508del and with either Myc tagged RNF19B or Myc tagged RNF19B mutant (RNF19B c119s). B. Quantification of panel A. C. HEK 293 cells were transfected with F508del plasmid and control or Myc-RNF19B plasmid. 24 hours later, these cells were transfected with HA-Ubiquitin. Cells were harvested and CFTR (217) was immunoprecipitated. HA-ubiquitin was immunoblotted. Input was immunoblotted with the indicated antibodies. Conditions were all under MG132 treatment. (mean \pm SE, n = 3, p < 0.05 D. F508del-CFBE cells were transfected with Myc tagged RNF19B and immunostained for Myc (green) and F508del (red). Nuclei were stained blue.

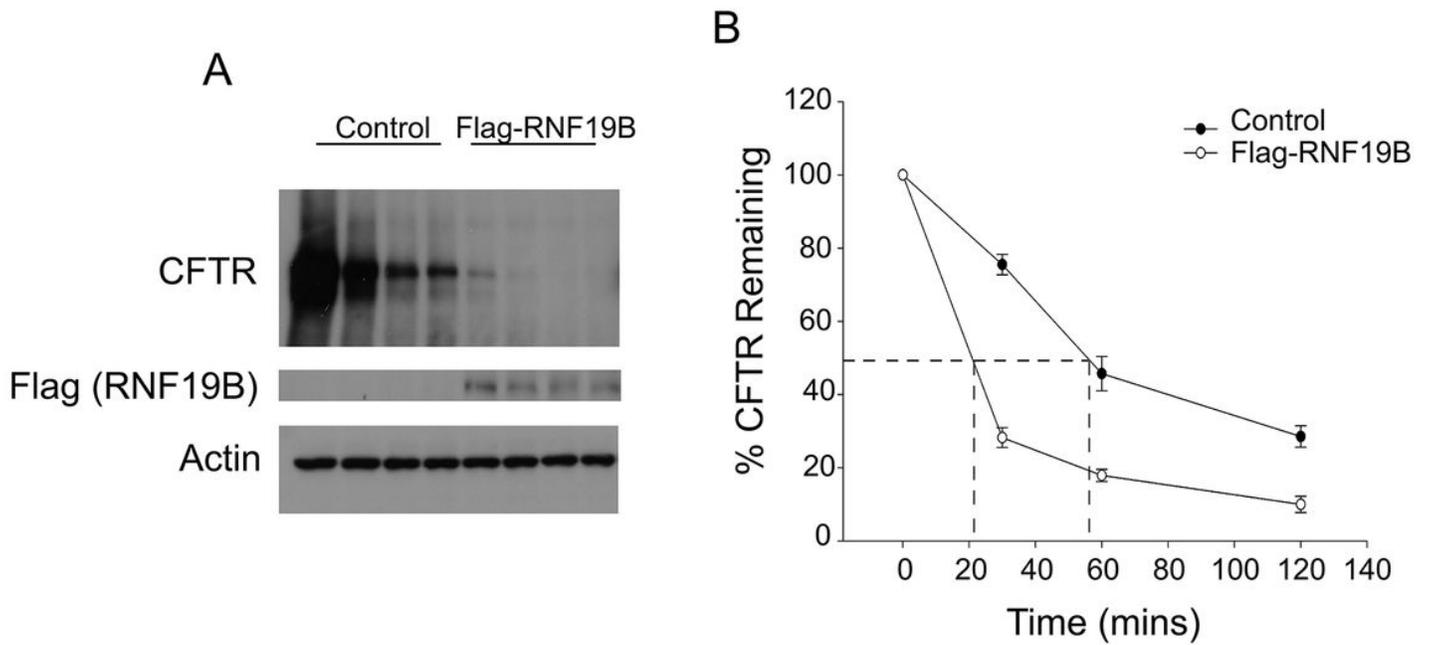


Figure 5

RNF19B over-expression accelerates F508del-CFTR degradation. A. Cyclohexamide (CHX) experiments were performed on HEK 293 cells co-transfected with F508del and with a control or with Flag tagged RNF19B for 48 hours. The cells were then treated with CHX (50 μ g/ml) for the indicated times. B. The level of remaining F508del at different time points was quantified as the percentage of initial F508del level (0 min of CHX treatment). RNF19B expression resulted in a shorter half-life of F508del. (mean \pm SE, n = 3, p < 0.05).

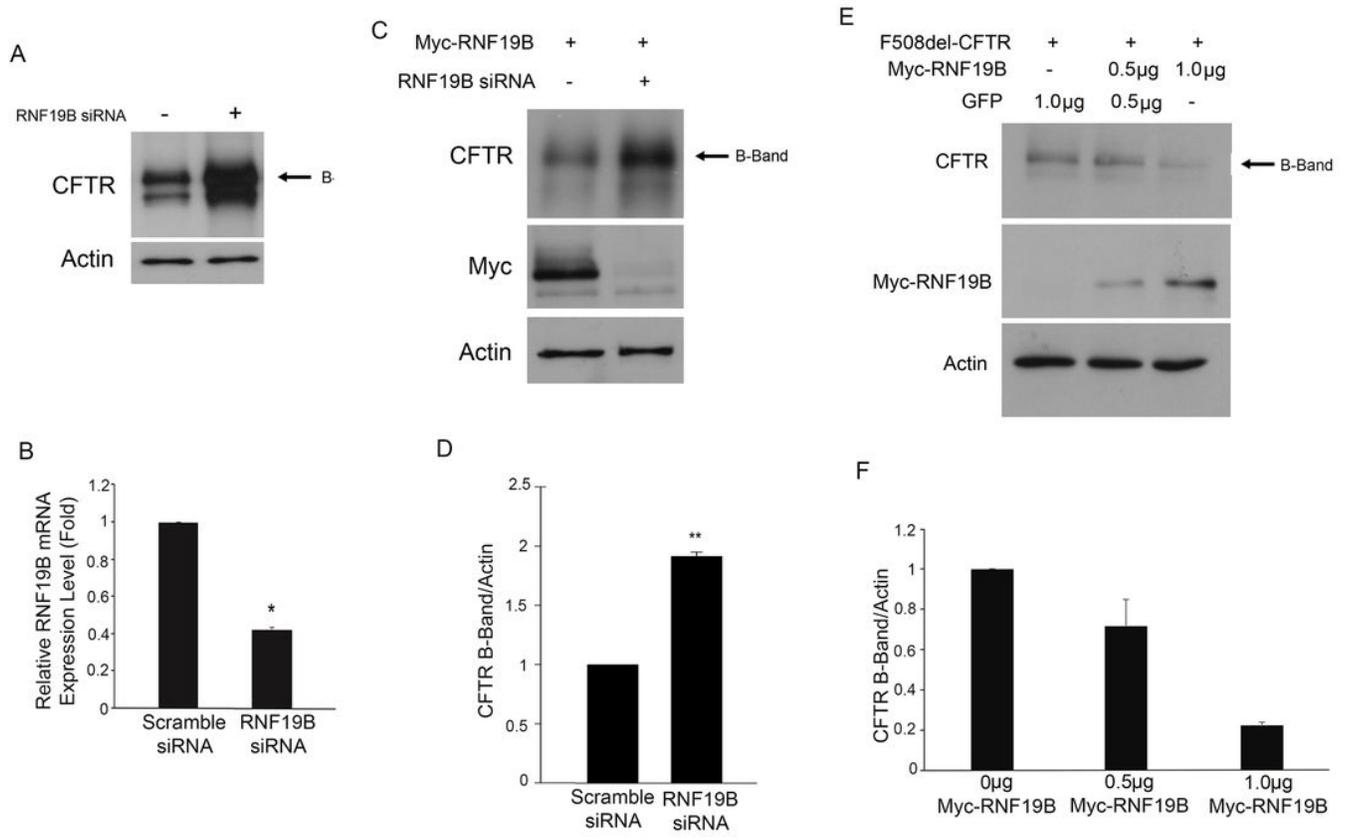


Figure 6

Silence of RNF19B increased F508del Expression and RNF19B mediates F508del degradation in a dose-dependent manner. A. Silence of endogenous RNF19B expression using siRNA increased F508del expression by about 3-fold in CFBE-F508del cells. B. Quantification of mRNA expression levels of RNF19B in CFBE-F508del cells with RNF19B knocked down using siRNA. C. Silence of overexpressed RNF19B expression using siRNA increased endogenous CFTR-F508del by approximately 3-fold in human airway epithelial cells. D. Quantification of panel C. E. HEK293 cells were stably transfected with Myc-tagged RNF19B. F. Quantification of CFTR-F508del expression level from panel E. (mean \pm SE, n = 3, * = p < 0.05, ** = p < 0.01).

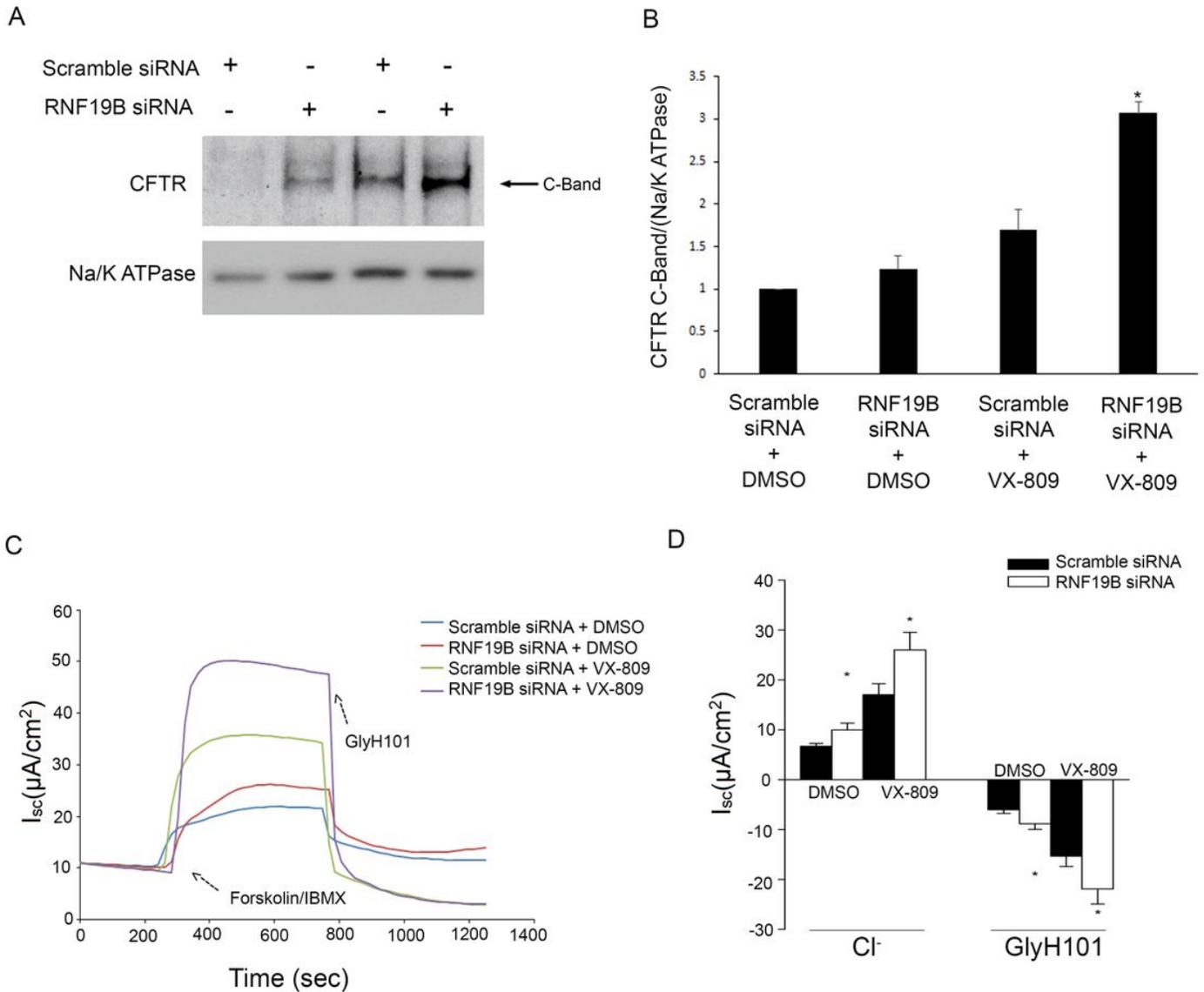


Figure 7

Silence of RNF19B promotes VX-809-mediated F508del trafficking to the plasma membrane and enhances F508del-CFTR function. A. Biotinylation experiment illustrating that silencing endogenous RNF19B in CFBE-F508del cells using siRNA increased F508del C-band expression to the plasma membrane compared to control and even more so with 3 μ M VX-809 treatment. B. Quantification of panel A. C. Ussing Chamber experiments. CFBE-F508del cells were first treated indicated siRNAs for 48hrs and then incubated with 3 μ M VX-809 for additional 24 hrs in 6.5 mm transwells. Short-circuit currents were retrieved through the Ussing chamber and F508del currents were determined through the treatment with the indicated conditions. Forskolin/IBMX (10 μ M) and GlyH101 (10 μ M). D. Quantification of short-circuit currents. (mean \pm SE, n = 3, p < 0.05).