The protein chaperones heat shock protein 70 (Hsp70) and Hsp90 are required for de novo folding of proteins and protect against misfolding-related cellular stresses by directing misfolded or slowly folding proteins to the ubiquitin/proteasome system (UPS) or autophagy/lysosomal degradation pathways. Here, we examined the role of the Bcl2-associated athanogene (BAG) family of Hsp70-specific nucleotide-exchange factors in the biogenesis and functional correction of genetic variants of the cystic fibrosis transmembrane conductance regulator (CFTR) whose mutations cause cystic fibrosis (CF). We show that siRNA-mediated silencing of BAG1 and -3, two BAG members linked to the clearance of misfolded proteins via the UPS and autophagy pathways, respectively, leads to functional correction of F508del-CFTR and other disease-associated CFTR variants. BAG3 silencing was the most effective, leading to improved F508del-CFTR stability, trafficking, and restoration of cell-surface function, both alone and in combination with the FDA-approved CFTR corrector, VX-809. We also found that the BAG3 silencing–mediated correction of F508del-CFTR restores the autophagy pathway, which is defective in F508del-CFTR-expressing cells, likely because of the maladaptive stress response in CF pathophysiology. These results highlight the potential therapeutic benefits of targeting the cellular chaperone system to improve the functional folding of CFTR variants contributing to CF and possibly other protein-misfolding–associated diseases.

The acquisition of a functional protein fold is determined by the balance between the energetic requirements of the polypeptide chain (1) and the function of the prevailing proteostatic environment (2–5). The heat shock proteins 70 (Hsp70) and 90 (Hsp90) play a central role in the de novo folding of proteins as well in protecting against misfolding-related stress and toxicity by directing misfolded or slowly folding proteins to the ubiquitin/proteasome and autophagy/lysosomal degradation systems (6–8). Misfolding diseases can occur as a result of alterations of the protein fold in response to inherited and sporadic causes, leading to either loss-of-function or gain-of-toxic function variants that trigger human pathophysiology.

Cystic fibrosis (CF), the most common lethal genetic disease in the Caucasian population, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which codes for a cAMP-regulated chloride channel expressed at the apical surface of epithelial cells (9). The most prevalent disease-causing mutation results from a 3-bp deletion (delCTT) resulting in the loss of phenylalanine at position 508 (F508del), with more than 70% of patients carrying at least one F508del allele (F508del-CFTR) (10). This CFTR variant is characterized by altered folding energetics resulting in misfolding and endoplasmic reticulum (ER)-associated degradation (7, 11–13). There are currently more than 2000 mutations reported in the CFTR gene (10, 14, 15), which can be separated into six functional classes, characterized by the loss of synthesis (I), folding (II), regulation (III), channel conductance (IV), cell surface density (V), and recycling (VI) (16). The loss of a functional CFTR chloride channel at the cell surface leads to loss of hydration of the epithelial lining of the lung and other tissues, triggering the progressive pathology characteristic of the disease (15, 17–19).

The folding of CFTR has previously been shown to be dependent on the activity of heat shock proteins and their associated co-chaperones (20–22). Whereas the WT-CFTR variant is capable of properly navigating Hsp-associated folding intermediates, the altered energetics of the polypeptide chain arising from the F508del mutation results in its accumulation in a stalled, on-pathway, Hsp70–Hsp90–bound folding intermediate in the endoplasmic reticulum referred to as the "chaperone
BAG family of proteins (BAG1–6) and are characterized by the presence of a C-terminal BAG domain, shown to be critical for its binding to and modulation of the ATPase domain of the molecular chaperone, Hsp70 (26–28). These BAG proteins act as nucleotide-exchange factors in the functional cycle of Hsp70, which alternates between its low-peptide-binding, ATP-bound state and the high-peptide-binding, ADP-bound state, mediated by Hsp40 activation of its ATPase activity. The subsequent action of a BAG protein mediates the ADP/ATP exchange to complete the chaperoning cycle. Therefore, BAG proteins act as inhibitors of the chaperone activity of Hsp70 (28). Whereas all BAG proteins share a common C-terminal BAG domain and Hsp70 regulatory function, they are divergent in their N-terminal domains, which also dictate their cellular function(s). BAG1, which contains a ubiquitin-like domain (UBL), is ubiquitinated by the action of the Hsp70-interacting E3 ubiquitin ligase, C terminus of Hsp70-interacting protein (CHIP), that links Hsp70 complexes to the proteasome, where released Hsp70 clients can be delivered for degradation by the ubiquitin proteosomal system (UPS) (29–32). Conversely, BAG2 exhibits an inhibitory function on CHIP, allowing for productive chaperoning of Hsp70 substrates such as CFTR (33–35). The BAG3 co-chaperone plays an active role in the autophagosomal-lysosomal degradation pathway, where it directly interacts with dynein motors (36) to deliver Hsp70 substrates to the aggresomal compartment, where they can be cleared via autophagy (36–40). BAG4, also known as the silencer of death domain (SODD), recruits Hsp70 to death receptors, TNF-R1 and DR3, which induces conformational changes in these domains to maintain them in their inactive monomeric states to prevent cell death (41). BAG5 is a driver of neuronal cell death, where it acts to inhibit the function of E3-ubiquitin ligase, Parkin. This distinguishes BAG5 from other family members, which are commonly considered as prosurvival factors (42–45). The largest member of the BAG family, BAG6, is part of the major histocompatibility complex (46, 47) and Hrd1-linked degradation (48), and whereas it contains a BAG domain and exhibits Hsp70-inhibitory activity, it is unclear whether it represents a true member of the BAG family.

Herein, we have examined the role of BAG family members in the biogenesis and functional correction of CF-causing variants. We observed that the silencing of BAG1 and -3, two family members linked to clearance of misfolded proteins via the UPS and autophagy, respectively, leads to functional correction of F508del-CFTR and other disease-associated variants. Knockdown of BAG3 proved to be the most effective, leading to improved stability and trafficking and restoration of cell surface function both alone and in combination with the Food and Drug Administration–approved CFTR corrector, VX809. We determined that the siBAG3-mediated correction of F508del-CFTR is acting through restoration of the autophagy pathway, which is defective in F508del-CFTR–expressing cells (49), likely due to an overload response associated with the maladaptive stress response (MSR) that is present in F508del-expressing cells (18, 50). These data highlight the therapeutic benefits of targeting the chaperone-folding system to improve the functional fold in CF and possibly other protein-misfolding diseases.

Results

The silencing of BAGs improves the trafficking of F508del-CFTR

The characterization of the CFTR-interacting proteins for both WT- and F508del-CFTR has revealed that a number of proteins exhibit differing affinities for these two CFTR variants (22, 24). These data suggest that differentially bound proteins either could be playing an active regulatory role in the ER retention of the F508del variant or could be passively associated with the ER-restricted variant as part of a trapped folding intermediate, possibly targeting it to the ER-associated degradation pathway.

Although we have previously documented the increased recovery of the heat shock protein/cognate 70 (Hsp/c70) and heat shock protein 90 (Hsp90) with F508del-CFTR, relative to WT-CFTR, in a chaperone trap (23), we have not examined the differential binding affinity of Hsp/c70 co-chaperone proteins and the consequences of their differential affinities for CFTR variants. Both iterations of the CFTR interactomes (22, 24) have shown increased recovery of BAG proteins, specifically BAG2 and -3, with F508del-CFTR relative to WT-CFTR, even though both interactomes were performed from different cell lines (BHK versus CFBE410−) and with two different CFTR antibodies (M3A7 (NBD2 domain epitope) versus 3G11 (NBD1 domain epitope)). These data highlight the importance of these BAG proteins for the biogenesis of CFTR; however, their precise contribution to the stability, trafficking, and function of F508del-CFTR remains to be determined.

We first performed an siRNA-mediated silencing of all six BAG family members to assess their impact on the stability and trafficking of the F508del variant in F508del-CFBE airway cells. We observed that the silencing of BAG1, -3, -4, and -5 increased the level of the ER-resident glycoform of CFTR, referred to as band B, and corrected the trafficking defect associated with the F508del variants, exemplified by the appearance of the post-Golgi glycoform, referred to as band C (Fig. 1, A and B). These data reveal that the silencing of BAG3 provides the greatest improvement in F508del-CFTR stability and trafficking. Furthermore, only the silencing of BAG3 and -4 yielded a statistically significant increase in the ratio of band C to band B (C/B ratio) (Fig. 1B), a trafficking index used to assess the efficiency of ER export. We also observed that the silencing of BAG2 destabilizes F508del-CFTR (Fig. 1, A and B), suggesting that this isoform is critical for the biogenesis of the F508del variant.

The F508del mutation of CFTR represents a temperature-sensitive mutation where correction of the trafficking defect is...
BAG3 silencing corrects F508del-CFTR
observed after incubation at reduced temperature (27–30 °C) (51). In light of the observed effects of siBAGs described above, we focused on the effect of siRNA-mediated silencing of the BAG isoforms with the greatest impact, namely that of siBAG1, -2, and -3, on the improved stability and trafficking seen following incubation of F508del-CFBE cells at 30 °C for 24 h. Relative to the stabilization of F508del-CFTR observed at 30 °C, we found that silencing of BAG1 and -3 further stabilized band B of the F508del variant and improved the trafficking to band C (Fig. 1C), whereas siBAG2 caused a partial inhibition of the observed temperature correction (Fig. 1C). We made similar observations in WT-CFBE cells, where siBAG1 and -3 promoted improved trafficking and siBAG2 partially reduced the amount of band C observed (Fig. 1D) for the WT variant. The WT-CFTR results support the conclusion that the siBAG1- and siBAG3-mediated correction of F508del-CFTR is on the normal folding pathway.

Whereas the F508del variant is the most common CF-associated mutation (10), more than 2000 mutations have been mapped to both the coding and noncoding regions of the CFTR gene, of which ~300 have been validated as disease-causing (10, 14–16). We examined whether the observed effects associated with the silencing of BAG proteins were unique to F508del or whether they are generally applicable to other CF-causing mutations. To address this question, we screened three additional class II CF-associated variants, G85E (N-terminal domain), R560T (NBD1 domain), and N1303K (NBD2 domain), which exhibit an ER-retention phenotype similar to that seen for F508del-CFTR. Consistent with the observations made with the F508del variant, we observed increased stability and trafficking with siBAG1 and -3 (Fig. 1, E and F) in CFBE41o− cells transduced with these three CFTR variants. Whereas we also observed variable levels of correction with siBAG4 (G85E/N1303K), siBAG5 (R560T), and siBAG6 (G85E), only the silencing of BAG1 and -3 was able to target all of the variants tested. These data highlight a central role for BAG1 and BAG3 in managing CFTR for folding, stability, and export from the ER.

To further characterize the siBAG-mediated changes in CFTR protein levels and glycoform distribution, we also looked into the impact of silencing BAG1, -2, and -3 on the mRNA level of CFTR. We observed that the silencing of BAG2 results in an approximate 50% reduction in F508del-CFTR mRNA (Fig. 2). Conversely, we noted that the silencing of BAG1 and BAG3 induced an approximate 2- and 8-fold increase in F508del-CFTR mRNA, respectively (Fig. 2). In light of these observed results, we interrogated whether these mRNA changes were unique to the F508del variant or whether similar changes could be observed for WT-CFTR in response to the silencing of these BAG proteins. We noted that the silencing of BAG isoforms had nearly identical effects on WT-CFTR mRNA, indicating that the effects are not isoform-specific. Given that the mRNA changes observed in response to the silencing of BAG2 are concomitant with the amount of decrease in total CFTR protein observed in Fig. 1 and that we observed no changes in the trafficking index for the resulting protein, it is likely that the observed effect of siBAG2 on the CFTR protein is directly related to the decreased CFTR mRNA seen in the cells. Although the exact mechanism associated with the siBAG-mediated increase in CFTR mRNA is unclear, we hypothesize that it is a direct result of correction of the trafficking defect of the F508del-CFTR protein. The chronic expression of the misfolded F508del variant has been shown to induce a chronic heat shock-like state referred to as the maladaptive stress response (50), which is associated with transcriptional and translational repression. We have shown that correction of the trafficking defect of F508del-CFTR alleviates the chronic activation of this stress pathway, which restores a more WT-like proteostasis environment, allowing for restoration of normal transcription and translation. This is consistent with the observed corrective effect of siBAG3 and, to a lesser extent, siBAG1, on the trafficking-
Concomitantly, the expression of these BAG3 variants also reduced the amount of Hsp70 recovered in CFTR immunoprecipitations (Fig. 3A), suggesting that the binding of this folding chaperone depends on the presence of a functional BAG3 protein. When we performed the inverse experiment and immunoprecipitated BAG3, we observed that only the band B glycoform of WT- and F508del-CFTR were recovered, providing direct evidence that BAG3 was acting on the ER-pool of these CFTR variants (Fig. 3B). A more detailed analysis of the lysates revealed that the overexpression of WT-BAG3 resulted in increased conversion of the nonglycosylated band A to the band B glycoform for both WT- and F508del-CFTR without improving the trafficking of these variants (Fig. 3B). The expression of BAG3-D or BAG3-R480A not only prevented this improved band B maturation, but also destabilized the total amount of F508del-CFTR (Fig. 3B), demonstrating that the maturation to and stabilization of the band B glycoform is Hsp70-dependent.

The silencing of BAG3 stabilizes CFTR

To further clarify the role of BAG3 in the stability of CFTR, we performed a pulse-chase analysis of WT- and F508del-CFTR in response to the silencing of BAG3. The silencing of BAG3 decreased the clearance rate of band B of F508del-CFTR (Fig. 4A and B) and promoted trafficking to band C (Fig. 4A (4 h)).
The silencing of BAG1, which also provided an increase in the expression level of band B and correction of trafficking to band C, did not affect the rate of decay of band B in F508del-CFBE cells (Fig. 4, A and B) relative to that seen with a nontargeting siRNA (siScr). The silencing of BAG3 also promoted increased stability of the band B glycoform and increased the rate of maturation to band C for WT-CFTR relative to that seen with siScr (Fig. 4, C and D). The silencing of BAG1, which also improved the expression of band B and increased trafficking to band C for WT-CFTR, also promoted the trafficking of WT-CFTR relative to that seen with siScr (Fig. 4, A and B). The silencing of BAG3, which also improved the expression of band B and increased trafficking to band C for WT-CFTR, also promoted the trafficking of WT-CFTR relative to that seen with siScr (Fig. 4, C and D), consistent with its ability to increase the band C glycoform of both variants of CFTR shown above (Fig. 1, A–D). These data suggest that BAG3 is optimally expressed to maintain the proper biogenesis of CFTR. Reducing the expression level of BAG3 allows for the F508del variant to bypass the Hsp70-dependent chaperone trap (23), which promotes its ER retention and degradation, to achieve the necessary stability to become export-competent and exit the ER. Conversely, increasing the expression of WT-BAG3, which acts as a nucleotide-exchange factor, accelerates the Hsp70 chaperoning cycle, leading to accelerated glycosylation of the ER-restricted CFTR band A. Also, the WT-BAG3 overexpression prevents the ability of the F508del to be included in ER exit sites for trafficking, possibly due to decreased Hsp70 residency time required to fold and stabilize the CFTR variant (23). These data are in agreement with the numerous reports showing that modulation of the activity of heat shock proteins can provide benefit to disease-associated misfolded proteins by providing a proteostatic environment more tuned with the folding kinetics and/or thermodynamics of the challenged polypeptide fold (53–55).

**The silencing of BAG3 restores a functional F508del-CFTR to the cell surface**

Whereas the silencing of BAG3 corrects the trafficking defect associated with the F508del variant of CFTR, we wanted to assess whether the resulting band C glycoform represented a functional chloride channel in F508del-CFBE cells. To assess the function of the rescued F508del-CFTR, we employed the YFP-quenching assay commonly used to monitor CFTR function (56). Briefly, we examined WT- or F508del-expressing CFBE cells stably expressing the halide-sensitive H148Q/I152L-YFP variant whose fluorescence is quenched in response to the influx of extracellular iodide through a functional CFTR anion channel when properly located at the cell surface. The silencing of BAG1, -3, and -5 all led to an increase in YFP quenching in F508del-CFBE-YFP cells (Fig. 5A), with siBAG3 being the most effective, reaching a level of CFTR function approaching 50% of that seen with WT-CFTR, a result like that seen with siHDAC7, a corrector of F508del-CFTR (57). Conversely, the silencing of BAG2 led a decrease in the basal YFP quenching (Fig. 5A). These data are consistent with the trafficking data associated with the respective BAG silencing (Fig. 1, A and B).

To ensure that the YFP quenching observed was in fact due to correction of F508del-CFTR and not the result of activation of a different route of entry for the exogenously added iodide, we performed the YFP quenching assay in the presence of the CFTR-specific inhibitor, CFInh172. We observed that the siBAG1- and siBAG3-mediated increases in YFP quenching were completely blocked in the presence of CFInh172 (Fig. 5B), supporting the conclusion that the silencing of these BAG proteins does in fact promote the functional correction of F508del-CFTR. The level of F508del-CFTR activity seen in response to siBAG3 treatment is also similar to the level of activity seen following treatment of F508del-CFBE-YFP cells with the small molecule corrector of F508del-CFTR, VX809 (Fig. 5C). Strikingly, we observed that combining the silencing of BAG3 with VX809 led to a synergistic improvement in F508del channel activity (Fig. 5C). This observation is in agreement with our measurements of cell surface–localized F508del-CFTR in response to the siBAG3, VX809, and siBAG3 + VX809 treatments in F508del-CFBE cells, where a synergistic increase in cell surface–localized F508del-CFTR is observed when combining BAG3 knockdown with VX809 treatment (Fig. 5D) without changing the amount of total F508del-CFTR in these F508del-CFBE cells (Fig. 5E). These data suggest that these two corrective approaches have nonoverlapping mechanisms of action despite the fact that both are acting directly on the folding and/or stability of CFTR.

**The silencing of BAG3 restores autophagy in CFBE cells**

To shed light on the impact of silencing BAG3 on F508del-CFBE cells, we examined markers associated with the heat shock response (Hsp70, Hsp90, Hsf1, and p-Hsf1), the ER-stress response (p-eIF2α), and autophagy (p62 and LC3). We observed that the silencing of BAG3 caused a statistically significant increase in the phosphorylation of both HSF1 and eIF2α (Fig. 6, A, C, and D), possibly due to alterations in Hsp70 function expected in response to reduced BAG3 levels. We also observed an increase in the lipidation of the cytosolic LC3-I to the autophagosome–associated LC3-II species (Fig. 6A), a marker of increased autophagy in siBAG3-treated F508del-CFBE cells. This observation is significant because F508del-CFBE cells have been shown to be defective in autophagy (58), and treatments that block aggresome formation or that restore autophagy can correct the trafficking defect associated with the F508del variant (58).

Given the increased expression level of CFTR seen in response to the silencing of BAG3, we wanted to address whether the activation of these stress-related pathways was simply the result of increased misfolding stress. To address this possibility, we monitored the impact of F508del overexpression on these biomarkers. We observed that overexpressing F508del-CFTR did not result in the activation of any of these pathways (Fig. S1), suggesting that the observed increases in p-HSF1, p-eIF2α, and LC3 processing are not related to the increased pool of cellular F508del-CFTR. To confirm our hypothesis that the activation of these pathways is connected to disruption of the BAG3-Hsp70 axis in CFBE cells, we performed an siRNA-mediated silencing of BAG1 to -3 in parental CFBE cells, which do not express CFTR mRNA or protein. Here, we observed that the silencing of BAG3 led to a statistically significant increase in both p-HSF1 and p-eIF2α (Fig. S2). These data support the conclusion that the activation of these stress-related pathways
is associated with the silencing of BAG3 rather than with an increased load of F508del-CFTR expression.

To address whether the increase in autophagy is causal in the siBAG3-mediated correction of F508del-CFTR, we assessed the impact of blocking the ability of F508del-CFBE cells to induce autophagy by silencing ATG7, a critical component in the lipidation of LC3, on the trafficking of F508del-CFTR in the presence or absence of siBAG3. The silencing of ATG7 (siATG7) alone had no impact on the trafficking of F508del-CFTR when combined with a nontargeting siRNA (siScr) (Fig. 7, A, B, and E). However, the combined silencing of ATG7 and BAG3 resulted in 40–50% inhibition of the siBAG3-mediated correction of F508del-CFTR relative to that seen with siBAG3 alone (Fig. 7, A, B, and E). The silencing of ATG7 did not impact the siBAG1-mediated correction known for its link to proteasomal degradation of client proteins (29–32) (Fig. 7, A, B, and E), suggesting that it is specifically related to the BAG3-linked autophagosome pathway.

Additionally, we monitored the ability of the autophagy inhibitor ammonium chloride (NH₄Cl) (61) to inhibit the siBAG3-mediated correction of F508del-CFTR. Ammonium chloride is a cell-permeable base that can cross-vesicle membranes and neutralize the acidification of lysosomes, thereby preventing fusion of autophagosomes and subsequently inhibiting their ability to degrade autophagosome cargo proteins. The treatment with NH₄Cl caused a massive accumulation of lipidated LC3 (Fig. 7F), which could be indicative of either increased or decreased autophagy; however, we also observed an accumulation of p62 (Fig. 7F), an autophagosome-associated protein that is degraded by autophagy, suggesting that NH₄Cl did in fact block autophagy in these CFBE cells. We observed that NH₄Cl treatment was able to partially inhibit the siBAG3-mediated correction of F508del-CFTR.
mediated correction of F508del-CFTR (Fig. 7, C and D). These data agree with the role of BAG3 in delivering misfolded proteins to the aggresome, which are subsequently delivered to the autophagosome for degradation. These data suggest that the activation of the autophagy pathway generated by silencing BAG3 promotes partial rescue of CFTR in F508del-CFBE cells.

Discussion

Previously published efforts have emphasized the importance of autophagy in rescue of CFTR (58, 62–69). Given the central role of BAG3 in the management of autophagy pathways for multiple targets (70–74), we have demonstrated that reducing the cellular expression level of BAG3 generates a proteostatic environment that provides both chaperoning activity of F508del-CFTR, leading to improved biogenesis, and restoration of cell surface chloride channel activity. We observed that whereas the silencing of numerous BAG proteins could provide improved expression, stabilization, and trafficking, the silencing of BAG3 provided the most robust improvement in trafficking and functional correction of F508del-CFTR and other ER-restricted CF-causing mutations spanning multiple domains of the polypeptide.

We have demonstrated that BAG3 associates with de novo synthesized CFTR in the endoplasmic reticulum in an Hsp70-dependent manner. The increased recovery of BAG3 bound to F508del-CFTR relative to WT-CFTR (22, 24) suggests that this disease-associated variant undergoes repeated failed cycles of Hsp70-linked chaperoning, leading to the eventual targeting of this misfolded polypeptide to one of two degradation systems in the cell, namely the UPS and the autophagy-lysosomal system. The former clearance system is linked to the chaperone system via BAG1, which associates with the proteasome to mediate the degradation of released Hsp70 client proteins, which fail to achieve a functional fold (28–31). Conversely, BAG3 has been linked to the delivery of Hsp70-associated client proteins to the perinuclear localized aggresomal compartment, a staging area for autophagic clearance of proteins by macroautophagy (75, 76). Improved autophagy is correlated with correction of disease through the indirect impact of cysteamine on autophagy.
pathways at the bench and bedside (58, 62–69). Our data reveal that disrupting the delivery of CFTR disease–associated variants to both the UPS, via silencing of BAG1, and autophagosome, via the silencing of BAG3, corrects the trafficking defect associated with F508del-CFTR, leading to restoration of cell-surface activity. However, targeting the autophagic pathway was significantly more effective in overcoming the kinetic and thermodynamic defects associated with this CF-causing mutation. We also noted that the silencing of BAG3 proved effective at correcting the trafficking defect associated with other class II CF mutations, namely G85E, R560T, and N1303K, suggesting a more global impact on management of the CFTR nascent fold.

Previously, a direct correlation between the increased expression level of BAG3 and the degree of autophagosome formation (74) was established. Here, the functional contribution of BAG3 to the autophagy-lysosomal degradation axis was suggested to be the delivery of proteins to the aggresome via direct interaction with dynein (36). Substantial evidence also exists for its role in interaction with p62 to initiate autophagosome formation through the phagophore client-collecting precursor (40, 77, 78). These results suggest that, in the normal
cellular state, the flow of aggresomal cargo to the autophagy pathway proceeds unimpeded. However, we have previously shown that F508del-expressing cells exhibit a state of chronic stress, referred to as the MSR (50), which could lead to altered kinetics of aggresomal clearance. In fact, F508del-expressing cells exhibit a defect in their ability to induce autophagy at steady state (58) (Fig. 6A), suggesting that the ability of these cells to normally induce autophagy in the face of a chronic misfolding stress is impaired, a state that can be reversed by cysteamine, which inhibits transglutaminase and relieves the inhibition of BECN1 (ATG6) involved in p62-mediated assembly of the autophagosome (49, 58, 62–69, 79–81).

Our data presented herein demonstrate that the silencing of BAG3, in part, operates through an autophagy-dependent pathway, which at first glance seems counterintuitive to the current understanding of BAG3 in autophagy assembly (40, 70, 82–86). The apparent contradiction needs to be balanced by addressing the question of whether the silencing of BAG3 improves rescue by increasing aggresome clearance by macroautophagy to correct F508del-CFTR, given the established role of up-regulation of BAG3 in improving delivery and generation of autophagosomes (40, 70, 82–86), or whether the silencing of BAG3 improves F508del-CFTR folding, which subsequently relieves the folding stress imposed by MSR, leading to a more normalized level of autophagy (i.e. too much autophagic stress is bad for you). To address this question, we monitored the impact of impeding the ability of F508del-CFBE cells to induce autophagy by using known autophagy inhibitor NH₄Cl as well as by silencing ATG7, a critical component involved in autophagosome formation. Cells contain three different autophagy pathways: macroautophagy, which is responsible for the degradation of organelles and unused proteins; microautophagy, which involves degradation of cytosolic material through invagination of the lysosomal membrane; and chaperone-mediated autophagy, which unfolds and translocates Hsp70-associated client proteins across the lysosomal membrane. The observation that NH₄Cl, which neutralizes the acidification of the lysosome thereby inhibiting lysosomal proteolytic enzymes, blocks the siBAG3-mediated correction of F508del-CFTR clearly demonstrates that the correction is, at least in part, mediated by activation of autophagic pathways. Because the three types of autophagy are sensitive to NH₄Cl, these data do not help us identify the specific autophagic pathway(s) responsible for the correction. However, because we also observed inhibition of the siBAG3-mediated correction of the F508del variant in response to the silencing of ATG7, which participates in autophagosome formation and maturation, the re-initiation of the macroautophagy pathway, which is defective in F508del-expressing CFBE cells, is responsible for the observed correction. The defect in macroautophagy, which is responsible for the degradation of the aggresomes, limits the ability of the cell to degrade misfolded proteins contributing to the misfolding stress leading to the MSR seen in F508del-expressing CFBE cells (50). These conclusions are supported by the reported correction of F508del-CFTR by impeding aggresome formation through siRNA-mediated or chemical inhibition of the aggresome-associated protein, p62/SQSTM1 (58). One possibility is that the MSR state (50) causes an imbalance in the critical components regulating the autophagic pathway of the cell that, in turn, impede the ability of the autophagy-lysosomal system to function properly, thereby contributing to disease progression. Reducing the flow of proteins into the aggresome via silencing of p62 (58) or BAG3 (this work) could provide the necessary relief to allow the system to clear the stress-related aggresome pool. We posit that this clearance could contribute to proper management of the F508del polypeptide, leading to improved channel activity as shown herein. Thus, it is not the increase in autophagosome activity but restoration of defective autophagy flow that contributes to rescue of CFTR from the MSR.

Experimental procedures

Reagents

Antibodies used for CFTR detection were 3G11, 596 (Cystic Fibrosis Therapeutics), and 217 (Cystic Fibrosis Therapeutics). The antibodies used to detect BAG proteins were BAG-1 (Santa Cruz Biotechnology, Inc.), BAG-2 (Abcam), and BAG-3 (Abcam and Proteintech). The epitope tag antibodies used were FLAG (Sigma-Aldrich and Thermo Fisher Scientific). The protein marker antibodies used were Hsp70 (BD Biosciences and Thermo Fisher Scientific (4g4)), Hsc70 (Enzo Bioscience), Hsp90 (Enzo), Hsp40 (Enzo), HSF1 (Abcam), phospho-(Ser326)-HSF1 (Abcam), phospho-ElF2A (Invitrogen), p62/SQSTM (Santa Cruz Biotechnology), LC3 (Novus), and ATG7 (Invitrogen).

WT- and F508del-CFTR expression vectors were constructed as described previously (90). FLAG-BAG3, FLAG-BAG3-R480A, and FLAG-BAG3-ΔC were kindly provided by Dr. Michael Sherman (Boston University School of Medicine, Boston, MA).

Cell lines

Human bronchial epithelial cells stably expressing F508del-CFTR (F508del-CFBE) or WT-CFTR (WT-CFBE) and parental CFTR null (CFBE41o−) cells were cultured in α-MEM supplemented with 2 mM l-glutamine, penicillin/streptomycin (Thermo Scientific), puromycin (F508del-CFBE), or blasticidin (WT-CFBE). For temperature-corrected experiments, F508del-CFBE cells were transferred to 30°C for 24 h.

siRNA knockdown

Cells were seeded at the appropriate cell density a day before transfection and transfected at 60% confluence using 50 nM final concentration of siRNA and RNAiMax transfection reagent as per the manufacturer’s instructions (Invitrogen). Cells were harvested 72 h after transfection.

Adenoviral delivery of CFTR variants

CFBE41o− cells were seeded at 1.25 × 10⁵ cells/well of a 12-well dish a day before transduction. Cells were transduced...
with adenovirus at a multiplicity of infection of 200 in growth medium for 18 h to deliver the indicated CFTR variant. The cells were subsequently transfected with the indicated siRNA as indicated above, and cells were harvested for Western blot analysis as described below.

**Co-transfection of BAG and CFTR**

For protein overexpression, CFBE41o- cells were transiently transfected with the indicated expression plasmids using Lipofectamine 2000 (Life Technologies, Inc.). After 36 h, the cells were lysed in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 10% glycerol, and 1% Triton X-100 and protease inhibitor (Roche Applied Science), and equal amounts of protein were resolved on SDS-PAGE and subjected to Western blot analysis as described below.

**Western blotting**

Cell lysates were prepared by adding 50 μl of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 2 μg/ml protease inhibitor mixture (Roche Applied Science)) directly into the well of a 12-well culture dish and incubating in ice for 30 min with occasional rocking. The lysates were scraped using a rubber policeman and transferred to an Eppendorf tube. The lysate was centrifuged at 20,000 × g for 20 min at 4 °C, and the post-nuclear supernatant was transferred to a fresh Eppendorf tube. The protein concentration of the lysate was determined using a Bradford assay, and 20 μg of total protein for each sample were separated on an 8% SDS-polyacrylamide gel. The samples were transferred to nitrocellulose and probed for the indicated proteins of interest using specific primary antibodies. Detection was performed by enhanced chemiluminescence and exposed to film or scanned on a LI-COR Odyssey IR scanner.

**Quantitative RT-PCR**

RNA was isolated from the indicated cells using an RNasey RNA isolation kit (Qiagen). Quantitative RT-PCR was performed on 12 ng of RNA using the iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad). RNA was standardized by quantification of glucorondase (GUS) mRNA, and all values were expressed relative to GUS. Statistical analysis was performed on three independent technical replicates for each RNA sample, where error bars represent S.E.

**Pulse-chase analysis**

Analysis of CFTR stability by pulse chase was performed as described previously (57). Briefly, cells were starved in methionine-free MEM (Sigma) for 30 min, pulse-labeled for 4 h with [35S]methionine (0.1 mCi/well in a 6-well plate), and chased for the indicated time. Cells were lysed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 2 μg/ml protease inhibitor mixture (Roche Applied Science)), and CFTR immunoprecipitation was performed using 40 μl of the 3G11 CFTR antibody pre-cross-linked to γ-bind beads (GE Healthcare) and incubated overnight at 4 °C. The beads were washed three times with lysis buffer without protease inhibitor mixture, and bound proteins were eluted by the addition of 20 μl of SDS sample buffer containing DTT and incubation at 37 °C for 15 min. The recovered radiolabeled proteins were then visualized by autoradiography.

**CFBE-YFP quenching assay**

F508del-CFBE cells stably expressing the halide-sensitive YFP-H148Q/I152L (CFBE-YFP) were reverse-transfected with a 50 nM final concentration of siRNA using Lipofectamine RNAiMax (Invitrogen) as per the manufacturer’s protocol. Cells were trypsinized and resuspended in Opti-MEM with 10% FBS, and 2.5 × 10^4 cells were added per well of a black-walled, clear bottom 96-well view plate (PerkinElmer Life Sciences). Opti-MEM was replaced with growth medium 24 h after transfection. Cells were subsequently washed three times with 200 μl of PBS, pH 7.4 (137 mM NaCl, 2.7 mM KCl, 0.7 mM CaCl2, 1.1 mM MgCl2, 1.5 mM KH2PO4, 8.1 mM Na2HPO4) and equilibrated in 40 μl of PBS, pH 7.4, and maintained at 37 °C throughout. Cells were stimulated with a final concentration of 10 μM forskolin and 50 μM genistein for 15 min before the addition of PBS + NaI (replacement of NaCl with 137 mM NaI). Fluorescence was monitored every second for a total of 30 s (3 s before the addition of NaI and 27 s after the addition of NaI). Data were normalized to the initial fluorescence to account for variations in the overall starting fluorescence. To ensure that the observed H148Q/I152L-YFP fluorescence quenching was the result of ΔF508-CFTR activation and not the action of additional halide channels, the CFTR-specific inhibitor (CFIinh172) was used.

**Fluorogen detection of cell-surface CFTR expression**

A clone of CFBE cells stably expressing a fluorogen-activated peptide (FAP)-tagged F508del-CFTR (FAP-F508del-CFTR) (FAP-F508del-CFBE) construct was generated as described previously for a 293A stable cell line (59, 91). The FAP-F508del-CFBE cells were reverse-transfected with either nontargeted or BAG3-targeted siRNA during plating into an optically clear-bottom 96-well plate using Dharmafect 1 (0.2 l of Dharmafect 1/well, 40 nM siRNA). CFBE41o- cells were transfected and plated in parallel in the same 96-well plate to serve as nonspecific background signal for the FAP assay. Two days after the initial plating, the medium was replaced with medium containing either DMSO or 5 μM VX-809, and the cells were returned to incubate at 37 °C for an additional 24 h before commencing the FAP assay. The FAP assay for FAP-F508del-CFTR surface expression was performed as described previously (59), with the exception that the cells were incubated at 37 °C for the current assay, instead of the 27 °C described previously. To measure total FAP-F508del CFTR expression, 100 nM membrane-permeant FAP dye MGNBu (60) was added to the wells of the 96-well plate after measuring FAP-F508del-CFTR surface expression and incubated for 1 h at 37 °C to allow for labeling of all intracellular FAP-F508del-CFTR. The plate was then assayed for FAP/MGNBu signal as described for MG-B-Tau (59). The FAP-F508del-CFTR CFBE cells were maintained in a humidified incubator at 37 °C, 5% CO2 in MEM supplemented with 1% penicillin/streptomycin, 10% FBS, and 20 μg/ml blasticidin. The FAP dyes MG-B-Tau and MGNBu
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