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CFTR controls the activity of NF-kB by enhancing the degradation of tradd

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Abstract

Background—Chronic lung infection in cystic fibrosis leads to an inflammatory response that persists because of the chronic presence of bacterial and ultimately leads to a catastrophic failure of lung function.

Methods—We use a combination of biochemistry, cell and molecular biology to study the interaction of TRADD, a key adaptor molecule in TNFα signaling, with CFTR in the regulation of NFκB.

Results—We show that Wt CFTR binds to and colocalizes with TRADD. TRADD is a key signaling intermediate connecting TNFα with activation of NFκB. By contrast, ΔF508 CFTR does not bind to TRADD. NF-κB activation is higher in CFBE expressing ΔF508 CFTR than in cells expressing Wt CFTR. However, this differential effect is abolished when TRADD levels are knocked down. Consistently, transfecting Wt CFTR into CFBE cells reduces NF-κB activity. However the reduction is abolished by the CFTR chloride transport inhibitor-172. Consistently, transfecting in the correctly trafficked CFTR conduction mutants G551D or S341A also fail to reduce NFκB activity. Thus CFTR must be functional if it is to regulate NF-κB activity. We also found that TNFα produced a greater increase in NF-κB activity in CFBE cells than in the same cell when Wt CFTR-corrected. Consistently, the effect is also abolished when TRADD is knocked down by shRNA. Thus, Wt CFTR control of TRADD modulates the physiological activation of NF-κB by TNFα. Based on studies with proteosomal and lysosomal inhibitors, the mechanism by which Wt CFTR, but not ΔΔF508 CFTR, suppresses TRADD is by lysosomal degradation.

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Conclusion—we have uncovered a novel mechanism whereby Wt CFTR regulates TNFα signaling by enhancing TRADD degradation. Thus by reducing the levels of TRADD, Wt CFTR suppresses downstream proinflammatory NFκB signaling. By contrast, suppression of NF-κB activation fails in CF cells expressing ΔF508 CFTR.

INTRODUCTION

Cystic fibrosis, an autosomal recessive disease most prevalent in Caucasians, is caused by mutations in the cystic fibrosis conductance regulator, CFTR [1]. CFTR is an ATP-binding cassette protein that functions as a chloride channel, and is involved in generating and maintaining the proper composition and amount of fluid covering different mucosal membranes in the body [2]. One of the most common manifestations of CF is chronic lung infection, leading to the persistent presence of *Pseudomonas aeruginosa* bacteria [3]. Chronic lung infection leads to an inflammatory response in the CF airway that persists because of the presence of the bacterial challenge and ultimately leads to a catastrophic failure of lung function [4]. The connection between bacterial infection and inflammation seems simple to explain: the bacteria release LPS, which acts through Toll-like receptors (TLRs) to activate NFκB signaling [5]. A second source of induced NFκB activation is calcium accumulation in the endoplasmic reticulum (ER), leading to ER stress[6]. However, it is not just a bacterial effect alone, because NFκB-activated inflammation persists in the infection-suppressed CF airway, or in CF lung epithelial cells cultured in the explicit absence of bacteria [7, 8]. Therefore, whether inflammation is intrinsic, is caused by bacterial infection, or is caused by both processes, has remained consistently controversial.

Several studies have detected CFTR in immune cells such as neutrophils [8] macrophages [9], and lymphocytes [10], and have noted functional changes in the absence of CFTR function. For example, in zebrafish, reduced CFTR function is correlated with a lowered ability of neutrophils to phagocytose and kill bacteria [11]. It has also been shown that airway cells from CF patients release proinflammatory cytokines such as IL-8 in the absence of bacterial stimuli [12, 13]. For example, parental IB3–1 epithelial cells isolated from the lungs of a CF patient continue to release high levels of IL-8 in sterile culture. Elevated IL-8 secretion by these CF cells can be corrected to near wildtype levels by rescue with Wt CFTR [14]. However, this is not a universal finding, since other groups have reported that there is either no difference, or less cytokine release, when comparing cells containing mutant CFTR compared to cells expressing Wt CFTR [15–19]. Nonetheless, it is widely accepted that patients with CF have a more robust inflammatory response to bacterial infection compared to non-CF patients with similar infections [20–23]. Thus, the controversy regarding whether CF is associated with an enhanced immune response to a bacterial challenge pertains to studies attempting to reproduce the inflammatory response observed in CF patient's cells in culture.

A number of studies have associated components of the immune system with the severity of lung disease, suggesting clearly that components of the immune system do play a role in CF [20]. For example, a variant of tumor necrosis factor alpha (TNFα), 238 G/G vs. G/A, is associated with improved survival in CF patients, perhaps suggesting that lower levels of TNFα are protective for CF patients [24]. Functionally, the binding of TNFα to its receptor

stimulates the transcription factor NF-κB, P65, and the downstream release of cytokines IL-6 and IL-8 into the airways of CF patients [14]. Upon interaction with its ligand, the TNFα receptor forms trimers, which cause the disassociation of the inhibitory protein SODD from the TNFα receptor. This allows the adaptor protein TRADD to bind to the death domain of the receptor. TRADD then forms a multi-protein complex that ultimately causes phosphorylation of the inhibitory protein IkBα, allowing NF-kB to translocate to the nucleus, where it stimulates transcription of cytokines such as IL-8 [25].

In earlier studies we identified digitoxin as a Wt CFTR-gene therapy mimic which inhibits TNFα/NFκB signaling and IL-8 hyperexpression in CF lung epithelial cells [26]. Subsequently we showed that the target of digitoxin action was the interaction between TNFR1 and TRADD [27]. More recently, TRADD has emerged as a top “hit” in our high throughput screen for candidate proteins linking CFTR to the regulation of proinflammatory signaling [28]. Finally, preliminary data for this study indicated that TRADD was elevated in CF lung epithelial cells.

We therefore hypothesized that CFTR-dependent suppression of TRADD might lead to suppression of NFκB activation. To test this hypothesis we asked: (1) whether Wt CFTR, but not ΔF508 CFTR, suppressed TRADD dependent NFκB activation: (2) whether the interaction between Wt CFTR and TRADD resulted in TRADD degradation: though direct binding and dispatch of TRADD in the lysosome; (3) whether a functional chloride channel was required for Wt CFTR to suppress TRADD: and (4) whether destruction of TRADD by shRNA phenocopied the suppressive effect of Wt CFTR on NFκB activation. Our results suggest that TNFα, and the downstream adaptor protein TRADD, contribute to dysregulation of intrinsic proinflammatory signaling in CF lung epithelial cells.

METHODS

Cell culture and treatments

Parental CFBE cells used were CFBE41o$^-$ (a gift from Dr. Dieter Gruenert [29]) cells, a CF human bronchial epithelial cell line that is homozygous for the ΔF508 mutation. These cells were in some experiments transfected with wt-CFTR or mutant CFTR (a gift from Dr.Bruce Stanton). These cells were identified by a # symbol. Cells were maintained in MEM with 20 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. CFBE41o-N6.2KBWT cells (also a gift from Dr. Dieter Gruenert [29]) stably expressing wt-CFTR were also used an maintained in MEM with 20 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 300 μg/ml hygromycin, and 10% fetal calf serum. Plasmids were transfected into CFBE cell lines using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction. The proteasome inhibitor MG-132 (10 μM; CalBiochem) or endolysosomal inhibitor E-64d (50 μM; Calbiochem) was added to CFBE cells for 16h. To stimulate NF-κB activity, cells were treated with 30ng/ml TNFα for 15 min at 24 h post-transfection. When specified in the experimental protocol, 10 μM $CFTR_{inh172}$ (CalBiochem), for inhibiting the CFTR chloride conductance [30], was added to CFBE cells, together with 10 μM forskolin (Sigma) and 100 μM IBMX. The effect of the autophagosome inhibitor bafilomycin A (Sigma) was studied by applying 1μM bafilomycin A for 16 hours.

Immunoblotting

Cells were harvested and solubilized in lysis buffer (50 mM NaCl, 150 mM Tris-HCl pH 7.4, 1% Nonidet P-40, and Complete Protease Inhibitor Roche Applied Science). The cell lysates were centrifuged at 14,000×g for 15 min at 4°C. The protein concentrations of the supernatants were quantified with a BCA protein assay kit (Thermo). The normalized supernatants were subjected to SDS-PAGE and Western blotting, followed by SuperSignal West Dura (Thermo Scientific). The chemiluminescence signal on the PVDF membrane was directly captured by a FujiFilm LAS-3000 plus system with a cooled CCD camera. Quantification was carried out within the linear range using Scion Image 4.0 software. We used monoclonal anti-human CFTR antibody 217 (University of North Carolina), mouse anti-TRADD monoclonal antibody (mAb;1:500, BD Transduction Laboratories), and rabbit polyclonal anti-β-actin antibody (1:1000, SantaCruz Biotechnology). NFκB activity was detected with a rabbit polyclonal antibody (1:500 FIVEphoton Biochemicals). TBP was detected with a mouse monoclonal anti-TBP antibody (1:2000, Abcam) as a nuclear loading control. Graphs presented in the Figures are from the same gel when possible. If not appropriate then they represent data from the same experimental sample.

Immunoprecipitation

Cells were washed with cold DPBS three times on ice. Cell lysates were collected and mixed with rabbit polyclonal anti-TRADD antibody (10ug, Santa Cruz Biotechnology), and incubated overnight at 4°C. Protein A/G-agarose beads (Santa Cruz Biotechnology) were added to the lysate with the antibody and rotated for 4 h at 4°C. The beads were washed three times with lysis buffer and incubated in Laemmli sample buffer (50 μl) containing 5% β-mercaptoethanol at 42°C for 30 min, followed by SDS-PAGE with a 4–15% gel (Bio-Rad) and Western blot analysis.

Cell surface biotinylation analysis

CFBE cells were cultured in 10-cm dishes until confluent. The cells were incubated with 0.5 mg/ml of a membrane-impermeable biotin, EZ-link sulfo-NHS-SS-biotin (Pierce), at 4°C for 30 min. The reaction was quenched with three washes of 200 mM glycine (GibcoBRL) in PBS at 4°C. The cell-surface proteins were isolated from the lysate by incubation with immobilized NeutrAvidin beads (Pierce) at 4°C for 2 h. After incubation in Laemmli buffer at 42°C for 30 min, the protein samples were separated by SDS-PAGE and analyzed by Western blotting.

Lentivirus production and infection

Viral particles were packaged in HEK 293T cells by cotransfection of the packaging plasmids pVSVG and D8.2R, together with pLKO.1containing the following target sequences for knockdown: TRADD shRNA (Invitrogen) CCGGCTGAAACTCCACTTGGCCTATCTCGAGATAGGCCAAGTGGAGTTTCAGTTTT and control knockdown sequence (Invitrogen). Supernatant containing viral particles was collected after 48 h. Cells in a 6-well plate were infected with lentivirus as 1:1 with medium supplemented with 8 μg/ml polybrene. After 24 h, 700 μl of fresh medium was added to

each well. At 48 h, the cells were trypsinized and transferred to a new 6-well dish. Infected CFBE cells were selected with 4 mg/ml puromycin.

Immunostaining and imaging

CFBE cells were plated on glass coverslips. One day after transfection, cells were washed with DPBS containing 1mM Ca^{2+} and1mM Mg^{2+} (DPBS-CaMg), fixed in 4% paraformaldehyde for 20 min, and permeabilized with 0.05% Triton X-100 in DPBS-CaMg for 10 min. Nonspecific binding was blocked with 5% normal goat serum in DPBS-CaMg. The cells were processed for the following double-immunostaining combinations: The cells were incubated in a solution containing mouse anti-TRADD mAb (1:100, BD BioSciences), rabbit anti-GFP polyclonal antibody (1:200, Invitrogen), and rabbit anti-CAL polyclonal antibody (1:1000). Cells were then washed with 1% bovine serum albumin in DPBS-CaMg containing 1% normal goat serum for 1 h at room temperature, washed with DPBS-CaMg, and then incubated with a mixture of two secondary antibodies: donkey anti-mouse IgG conjugated with Cy3 (1:200; Jackson ImmunoResearch) and donkey anti-rabbit IgG conjugated with Cy2 (1:200, Jackson ImmunoResearch). Cells were mounted with mounting medium containing DAPI. The samples were then observed by either conventional epifluorescence microscopy (Nikon 80i) or confocal microscopy (PerkinElmer Life Sciences).

NF-kB, p65 assay kit for the determination of NF-kB activity

Nuclear protein extracts were used to determine NF-kβ activation with an NF-kB activation assay kit (FIVEphoton Biochemicals) according to the manufacturer's protocols. Cells were fractionated into cytoplasmic and nuclear fractions, which were then subjected to Western blot analysis using an antibody selective for NFκB, p65.

Statistical Analysis—Statistical analysis was carried out using Microsoft Excel Data Analysis tool. Data are expressed as mean ± SE of three independent experiments. Statistical significance was determined by t test. $*P<0.05$.

RESULTS

TRADD binds to CFTR

As shown in Figure 1 A–C, TRADD binds to Wt, but not to ΔF508 CFTR. We also tested the binding of TRADD to the mutant G551D CFTR. G551D CFTR is a gating mutant that is fully processed to the plasma membrane [31–33]. We found that G551D CFTR also binds to TRADD. By contrast, no binding of TRADD was observed to TNR, a naturally occurring splice variant of CFTR, that contains only transmembrane domain 1, nucleotide binding domain 1, and the R domain [34]. We have previously shown that TNR, like ΔF508, resides in the endoplasmic reticulum (ER) [35]. From these data, it is clear that TRADD binds to CFTR that is fully processed.

Fig. 1B shows that both TRADD and Wt CFTR co-localize within the cells, whereas there is no co-localization of TRADD with ΔF508 CFTR (Fig. 1C). Again, this result is consistent with an interaction between TRADD and mature CFTR. To identify where the co-

localization of CFTR and TRADD is taking place, we utilized the PDZ binding protein CFTR-associated ligand (CAL), which we have shown to bind to and co-localize with CFTR in the trans-Golgi network (TGN). Fig. 2 shows that TRADD binds to and co-localizes with CAL, suggesting that CFTR, TRADD, and CAL are located together in the TGN.

The protein expressions of CFTR and TRADD are co-regulated

To begin to study the consequences of the binding of TRADD to CFTR, we transfected additional TRADD, into CFBE cells containing wt CFTR and into CFBE cells with parental levels of ΔF508 CFTR. Interestingly, TRADD transfection increased the CFTR wt protein levels approximately 5-fold but had no effect on ΔF508 CFTR (Fig. 3). We then tested the effect of TRADD on the surface expression of CFTR. Again, we saw a five-fold increase in wt CFTR at the cell surface when additional TRADD was transfected (Fig. 4). Consistent with ΔF508 CFTR residing in the ER, no ΔF508 CFTR could be detected at the plasma membrane, in either the control vector-transfected cells or the cells transfected with additional TRADD. These data show that elevating TRADD increases levels of wt CFTR, but does not increase levels of ΔF508 CFTR.

It has been shown recently that IL-1β upregulates CFTR mRNA and protein levels via NF-κB [36–37]. These publications suggest that one possible mechanism whereby TRADD upregulates CFTR protein levels is via TRADD-dependent upregulation of IL-1β.

The role of CFTR in regulating NFκB is dependent on TRADD

TRADD is a critical component in the signal transduction pathway that regulates NF-κB activity [25]. To address the role of CFTR in this process, we transfected additional TRADD into uncorrected parental CFBE cells containing endogenous ΔF508 CFTR, and into CFBE cells containing wt CFTR. Fig. 5 shows that in CFBE cells, transfecting in TRADD causes an approximately 40% increase in NF-κB activity. In sharp contrast, in Wt CFTR-containing cells, transfection of additional TRADD has no significant effect, suggesting that Wt CFTR dampens the ability of TRADD to regulate NF-κB activity. To corroborate this finding, we transfected Wt CFTR into CFBE cells. Fig. 6A–C shows that cells transfected with ΔF508-CFTR had a ~250% (2.5-fold) higher NF-κB activity than when wt-CFTR was transfected. In sharp contrast, the effect is abolished in when TRADD levels are reduced by shRNA. Thus NFκB levels in the nucleus are only reduced by transfecting in Wt CFTR, which lowers the levels of TRADD, or by knocking down TRADD directly with shRNA.

Regulation of NF-κB activity by TRADD is dependent on functional CFTR

One critical question is whether the interaction between CFTR and TRADD is essential, and physiologic; and conversely whether its absence is pathologic (as in CF symptoms). The conundrum is that both Wt CFTR and G551D CFTR bind to TRADD. If binding is playing a role, then how can people with Wt CFTR have a normal phenotype when patients bearing the G551D mutation, which also binds to TRADD, have severe CF, with potentially severe inflammation caused by hyperactivity of NFκB? We surmised that the key difference might be CFTR function. To address this question we used $CFTR_{inh172}$, a specific inhibitor of CFTR channel activity identified by high throughput screening [30]. Previous studies have

shown that this inhibitor phenocopies the inflammatory phenotype present in CF lungs when applied to primary Wt CFTR cells in culture [38].

In our experiments, Figs. 7A and B show that NFκB activity, which is low in CFBE cells containing Wt CFTR, is increased significantly when cells are treated with the $CFTR_{inh172}$. By contrast, in uncorrected parental CFBE cells (middle panel) and in uncorrected parental CFBE cells transfected with G551D, basal NF-κB activity was much higher and was unaffected by the inhibitor $CFTR_{inh172}$. This result suggests again that functional CFTR is regulating TRADD. To eliminate the possibility that the results were due to potential side effects of the CFTR inhibitor, we took a genetic approach. The key experiment involved testing the S341A CFTR conduction mutant, which produces mature CFTR with a greatly reduced function [39]. Consistent with the results of the experiments utilizing $CFTR_{inh172}$ to inhibit function, this conduction mutant could not dampen NF-κB activity when transfected into parental CFBE cells. The mutant TNR also failed to reduce NF-κB activity: Fig 7C and D. To provide further evidence for the validity of this effect, Fig. 8–A–B shows that the effect of the CFTR inhibitor is abolished when TRADD levels are reduced by shRNA. These experiments thus demonstrate that the effect of the inhibitor on NFκB activation is TRADD-dependent and that the inhibitor does not have a direct effect on NFκB activation. Taken together, these data show that both the functional activity of CFTR and the binding of CFTR to TRADD regulate NF-κB activity.

TNFα-mediated regulation of NF-κB is dependent on both TRADD and functional CFTR

TNFα is well known to activate NF-κB through formation of a complex including TNFα, TNFR and TRADD. Fig. 9 shows that in both parental CFBE cells and CFBE containing Wt CFTR, the addition of TNFα increased NF-κB activity. Noticeable, however, is that the effect of TNFα on NF-κB activity was much greater in cells lacking functional CFTR. Knockdown of TRADD not only reduced NF-κB activity, but also abolished the ability of TNFα to increase NF-κB activity. These observations clearly demonstrate that functional CFTR down-regulates the ability of TNFα to stimulate NF-κB activity, and that this effect is dependent upon TRADD.

Functional CFTR regulates the degradation of TRADD

In order to address how CFTR is regulating TRADD, we transfected CFBE cells with Wt CFTR, ΔF508 CFTR, or G551D CFTR. As shown in Figs. 10A & B, the steady-state levels of TRADD were lower when the cells were transfected with Wt CFTR than when they were transfected with ΔF508 or G551D. Importantly, when cells transfected with Wt CFTR were treated with $CFTR_{inh172}$, the steady-state levels of TRADD were significantly increased. This result suggests that functional CFTR is regulating the steady-state levels of TRADD.

To test the hypothesis that TRADD regulation might be mediated by a proteolytic mechanism, we treated cells with MG132, or E64 to inhibit proteosomal or lysosomal degradation, respectively (see [40] for a description of both inhibitors). Figs. 10 C & D shows that in cells transfected with Wt CFTR, there is a significant increase in TRADD protein in response to either MG132 or E64; the greater increase was noted with MG132. By

contrast, there is no increase when the cells were transfected with either ΔF508 or CFTR missing the terminal three amino acids (ΔTRL).

In order to pinpoint the location of the TRADD degradation, we treated cells with cycloheximide to measure the rate of disappearance of TRADD in the presence of either MG132, to block the proteasome, or bafilomycin to block lysosomal degradation. Figure 11 shows that in presence of CFTR, TRADD disappearance is faster than in the absence of CFTR when the proteasome is blocked. In sharp contrast, when the lysosomal degradation is blocked TRADD protein expression remains stable over the experimental period. Taken together, these data suggest that functional CFTR is enhancing the degradation of TRADD in the lysosome.

DISCUSSION

The data reported here offer a solution to a basic clinical conundrum in CF: why inflammation in the airways of a CF patient is so severe and unremitting over the patient's lifespan. The signaling pathway in Figure 12 summarizes the data in support of the hypothesis that CFTR-dependent suppression of TRADD leads to suppression of NFκB activation. These data clearly show that the suppression of TRADD by Wt CFTR occurs by sending TRADD to the lysosome for degradation. In addition, Wt CFTR must have functional capacity to transport anions, since inhibition of wt CFTR chloride channel function by $CFTR_{inh172}$ inhibits the intrinsic suppressive effects of wt CFTR on TRADD activity. This effect mimics the biology of non-conducting mutant G551D CFTR which can bind to TRADD, but not inhibit TRADD activity. The same suppressive effect on NKκB activation can be achieved by shRNA against TRADD. Thus when TNFα binds to TNFR, the TNFα/TNFR complex binds TRADD and drives NFκB activation. By contrast, in the absence of Wt CFTR, or in the presence of either ΔF508 CFTR or G551D CFTR expression, TRADD is elevated and unopposed. Uncontrolled NFκB activation therefore ensues, leading to downstream hyperexpression of IL-8 and other proinflammatory mediators. Finally, we find that increased TRADD feeds back to raise levels of WT CFTR, which in turn suppresses increased levels of TRADD. We suggest therefore that the suppressive effect of WT CFTR on TRADD, whether in the additional presence of bacteria, or not, is at least one mechanism contributing to the severe and unremitting inflammation in cases of CF caused either by ΔF508 or G551D CFTR mutations.

Biological basis for differential disease severity in the CF lung

It has been known for decades that cystic fibrosis is a Mendelian genetic disease. Yet patients that are homozygous for the ΔF508 genotype have disease phenotypes that range from "mild" to "severe". Thus other biological factors may contribute. For example, the failure of mutant CFTR to create and maintain normal airway surface liquid may result in variably ineffective mucociliary clearance, and thus failure of bacteria to be cleared from the CF lungs [41]. It is also possible that prolonged inflammation may be due to the sustained release of substances from dead or dying bacteria, such as lipopolysaccharide (LPS) [42], that are differentially sensed by the patient's immune system. For example, CF patients do not seem to be able to develop tolerance to a bacterial infection or to limit the neutrophil

infiltration [20]. In CF, instead of tolerance, significant lung damage develops from repeated lung infections [20].

Another possibility is that the unfolded protein response (UPR), emanating from the endoplasmic reticulum (ER), may be caused by misfolded mutant CFTR accumulating within the ER (54). Activating the UPR activates ER stress sensors such as IRE1, PERK, and ATF6, which mediate activation of NFκB and the JNK/JUN pathways [43]. It has been further shown that in the additional presence of infection, calcium would be accumulated in the ER, thereby exacerbating ER stress [44]. However, a severely inactivating CFTR mutation that does not conduct chloride across the membrane, G551D CFTR, does manage to traffic correctly to the plasma membrane [33, 45]. Yet this mutation is also associated with severe pro-inflammatory lung disease. Therefore, the UPR may not be an exclusive proinflammatory mechanism.

It has also recently been reported that ΔF508 CFTR causes a hyperexpression of proinflammatory microRNA-155 (miR-155; [45]). miR-155 suppresses the phosphatase SHIP1, which itself drives the AKT and MAPK pathways to stabilize IL-8 mRNA. Rescue of ΔF508 CFTR by Wt CFTR results in suppression of miR-155, destabilization of IL-8 mRNA, and a profound reduction in IL-8 protein expression.

Finally, ΔF508 CFTR has been shown to cause dysfunctional, proinflammatory defects in: monocytes and macrophages [9, 46–48]; invariant natural killer T (iNKT) cells [49]; neutrophils [8]; dendritic cells [50, 51]; T helper type 2 lymphocytes [52]; and B lymphocytes [53]. Bioinformatics analysis of gene expression data from the NCBI Gene Expression Omnibus indicates that suppressive effects on antigen presentation is through repression of the MHC class I antigen presentation genes in CF epithelia [54]. Suppression of phagocytotic bacterial cell killing in macrophages and neutrophils in the low chloride concentration CF airway occurs by reduction of chloride conductance into the phagosome to produce bacterial killing with hypochloric acid.

Inhibition of TRADD proinflammatory signaling depends on the functionality of CFTR

We have shown here that the ability of CFTR to inhibit proinflammatory signaling by TRADD depends on the property of a *functional* CFTR chloride channel activity. It does not occur with the correctly trafficking, but chloride-transport-inactive G551D mutant, which also causes a severe CF phenotype [32]. Neither does it occur with the correctly trafficked S341A mutant, which also shows altered chloride conductance [55]. Like Wt CFTR, G551D CFTR can bind to TRADD. However, the ΔF508 mutant, the most commonly associated with severe disease [56], also does not modulate TRADD because it resides in the ER and does not bind to CFTR. Thus, when CFTR is either sequestered in the ER, or is non-functional, TRADD levels are higher, and the response to TNFα-mediated stimulation of NF-kB activity is greater.

CFTR interaction with TRADD via the PDZ domain leads to TRADD degradation

The interaction of CFTR with TRADD may depend on an interaction involving the PDZ domain in the CFTR molecule. The evidence for this conclusion is that CFTR that is missing the terminal three amino acids (ΔTRL), which comprise the PDZ binding domain, does not

promote the degradation of TRADD. However, for the interaction to lead to degradation, the CFTR must also be functional.

With respect to the PDZ interaction mechanism, CFTR has been previously shown to bind to the PDZ domain-containing protein CAL [57–59]. We have also shown that CAL binds to the C-terminal domain of CFTR and regulates its post-Golgi trafficking. CAL also binds to the small molecular weight protein, TC10, and to the SNARE protein, syntaxin 6 [59, 60]. When activated, TC10 promotes the trafficking of CFTR to the plasma membrane [60]. On the other hand, it has also been previously shown that when STX6 binds to CAL, CFTR is targeted for degradation in the lysosome by ubiquitination via the ligase MarchII [59]. Interestingly, CFTR that is missing the terminal three amino acids (ΔTRL), which comprise the PDZ binding domain, does not promote the degradation of TRADD (see Fig. 10), suggesting the involvement of the PDZ domain of CFTR in this process. Since CAL and TRADD colocalize to a perinuclear location within the cell, and since CAL is in the TGN, it appears that CAL and TRADD are both located within the TGN. The majority of TRADD immunoreactivity is in a similar perinuclear location to that of CAL. Taken together, these data suggest that by forming a macromolecular complex, CFTR regulates the expression of TRADD at this location. However, binding is necessary, but not sufficient, to accomplish the regulation, because CFTR must also be functional in order to regulate TRADD degradation.

How the functional state of CFTR that permits the ultimate degradative effect on TRADD is more problematic. Perhaps the permissive effect may depend not only on binding to the PDZ domain, but also on the conformational state assumed by CFTR when it is competent to transport anions. I has been recently shown [61] that in order for G551D CFTR to be rendered functional by VX-770 (Ivacaftor) the two nucleotide binding fold domains must be brought into close apposition. The detailed relevance of this process to regulation of TRADD degradation is not known. However, it is clear that anion transport by CFTR depends on a unique conformational state for the CFTR protein, and that this conformation, or processes associated with the conformation, control the TRADD degradation process.

There are additional or alternative explanations which our experiments do not exclude. For example, CFTR is involved in regulating the pH of intracellular vesicles such as the TGN, leading to changes in the composition of mucous in the airway [62]. However, the latter is highly controversial. Perhaps functional CFTR also influences TRADD's fate upon exiting the TGN by either allowing TRADD to proceed toward its site of complex formation with the TNFα receptor, or by being targeted for degradation. Evidence for this hypothesis comes from recent reports showing that IL-8 secretion can be suppressed by the expression of functional CFTR [14]) or by pharmacological activation of another chloride channel, TMEM16A [63]. As further shown here, transport does not occur with the mutant, G551D, which is not functional but nonetheless reaches the plasma membrane [33]. Although other chloride channels can substitute for CFTR, clearly the key player is CFTR, as evidenced by the inflammation induced in CF. We suggest that whichever of these mechanism(s) turns out to be correct, the data reported here directly correlate proinflammatory cytokine secretion, for example, IL-8, with anion transport activity.

Conclusion

The data shown here provide insight into the centrality of TRADD expression in cultured CF lung epithelial cells for mediating the ability of CFTR to constitutively suppress NFκB activation. These data thus support our previous studies with the gene-therapy-mimic drug digitoxin in CF lung epithelial cells, showing suppression of proinflammatory NFκB signaling [26] by blocking the TNFR1/TRADD interaction [27]. It has not escaped our attention that our results may not only be relevant to CF, but may also be pertinent to other disorders in which CFTR levels are suppressed, including chronic obstructive pulmonary disease (COPD) [64].

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Abbreviations

CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
CFBE	CF bronchial epithelial cells
TRADD	tumor necrosis factor receptor type 1-associated death domain protein
shRNA	short hairpin RNA
wt	wild type
TGN	trans-Golgi network
TBA	TATA binding protein
TNFα	the tumor necrosis factor alpha

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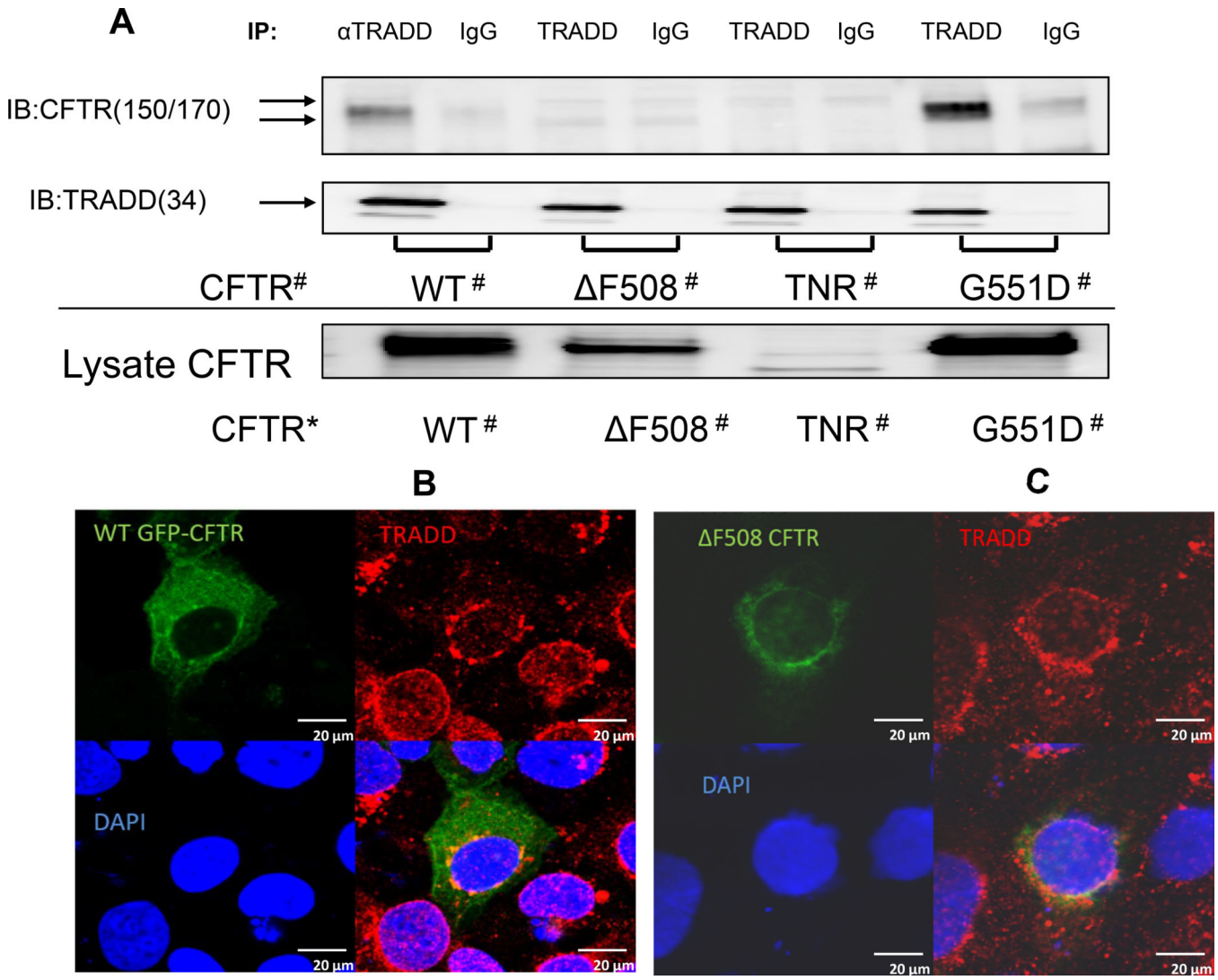

Figure 1. TRADD binds to mature CFTR.

1A) CFBE cells were transfected with GFP-wt CFTR, GFP-ΔF508 CFTR, GFP-TNR CFTR, or GFP-G551D CFTR. Forty-eight hours after transfection, cells were lysed, and co-immunoprecipitation was performed using either anti-TRADD or mouse IgG (immunoglobulin G) antibodies. CFTR expression in the total cell lysates is shown, as well as CFTR, which is co-precipitated with TRADD. The results show that TRADD binds to wt and G551D but not to ΔF508 and TNR CFTR, both of which remain in the ER, suggesting that TRADD binds only to CFTR that is processed to the plasma membrane. **(1B-C)** CFBE cells were transfected with GFP-wt CFTR **(1B)** or GFP-ΔF508 CFTR **(1C)**. Antibodies recognizing GFP (green) and TRADD (red) were used in immunofluorescence-based detection. wt CFTR and TRADD were colocalized in the perinuclear region of the cells. Importantly, TRADD did not colocalize with ΔF508 CFTR, consistent with the lack of binding of this mutant. # - denotes that CFBE cells were transfected CFTR.

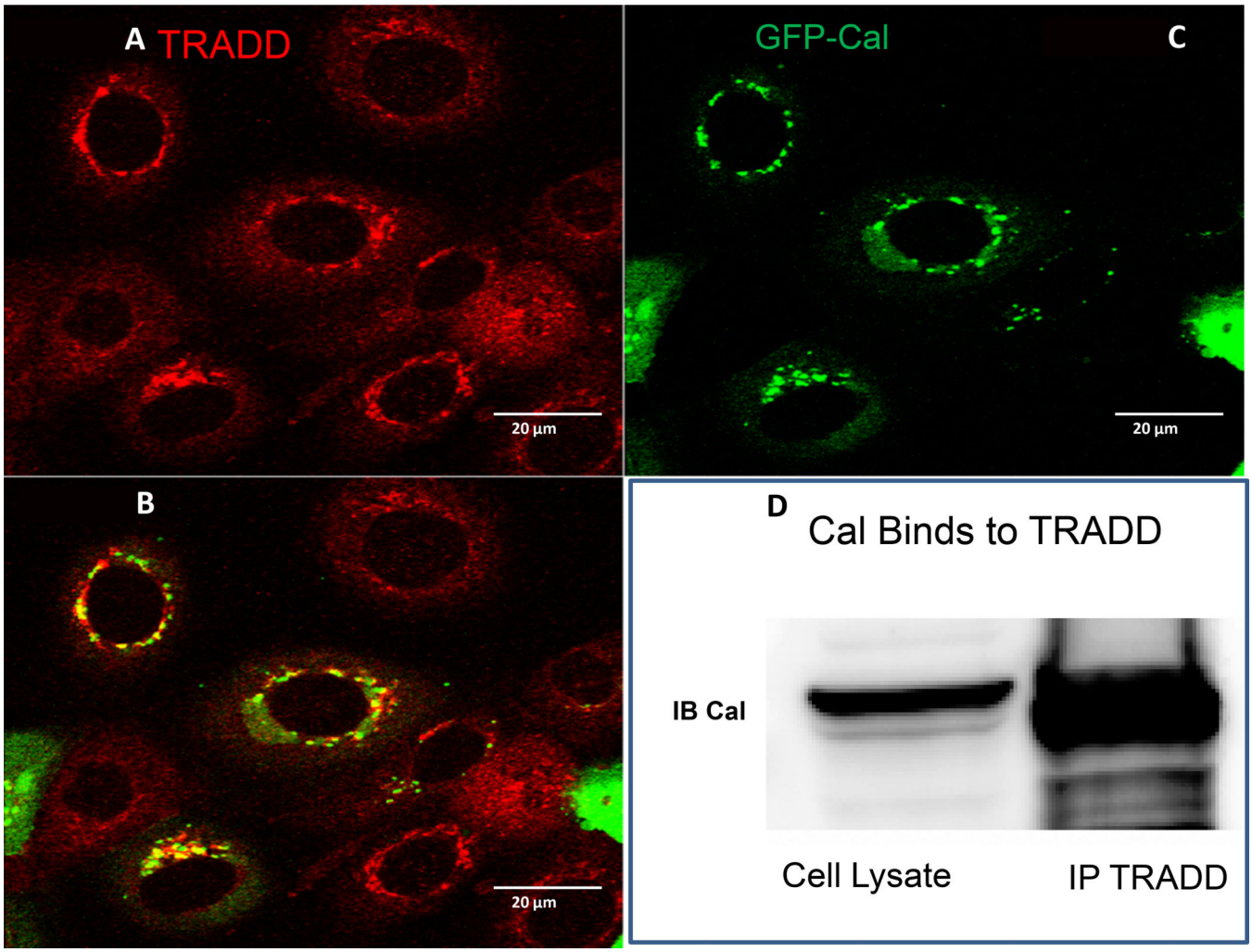

Figure 2. TRADD binds to and colocalizes with CAL.

CFBE cells were transfected with GFP-CAL. **(2A).** TRADD alone. (**2B).** Cal alone. **(2C)** is the merged image. Note that CAL colocalized with TRADD and bound to TRADD in co-immunoprecipitation experiments **(2D)**. Since we have shown previously [28] that CAL is localized at the TGN and also binds CFTR via the PDZ domain, these data suggest that TRADD and wt CFTR colocalize in the TGN.

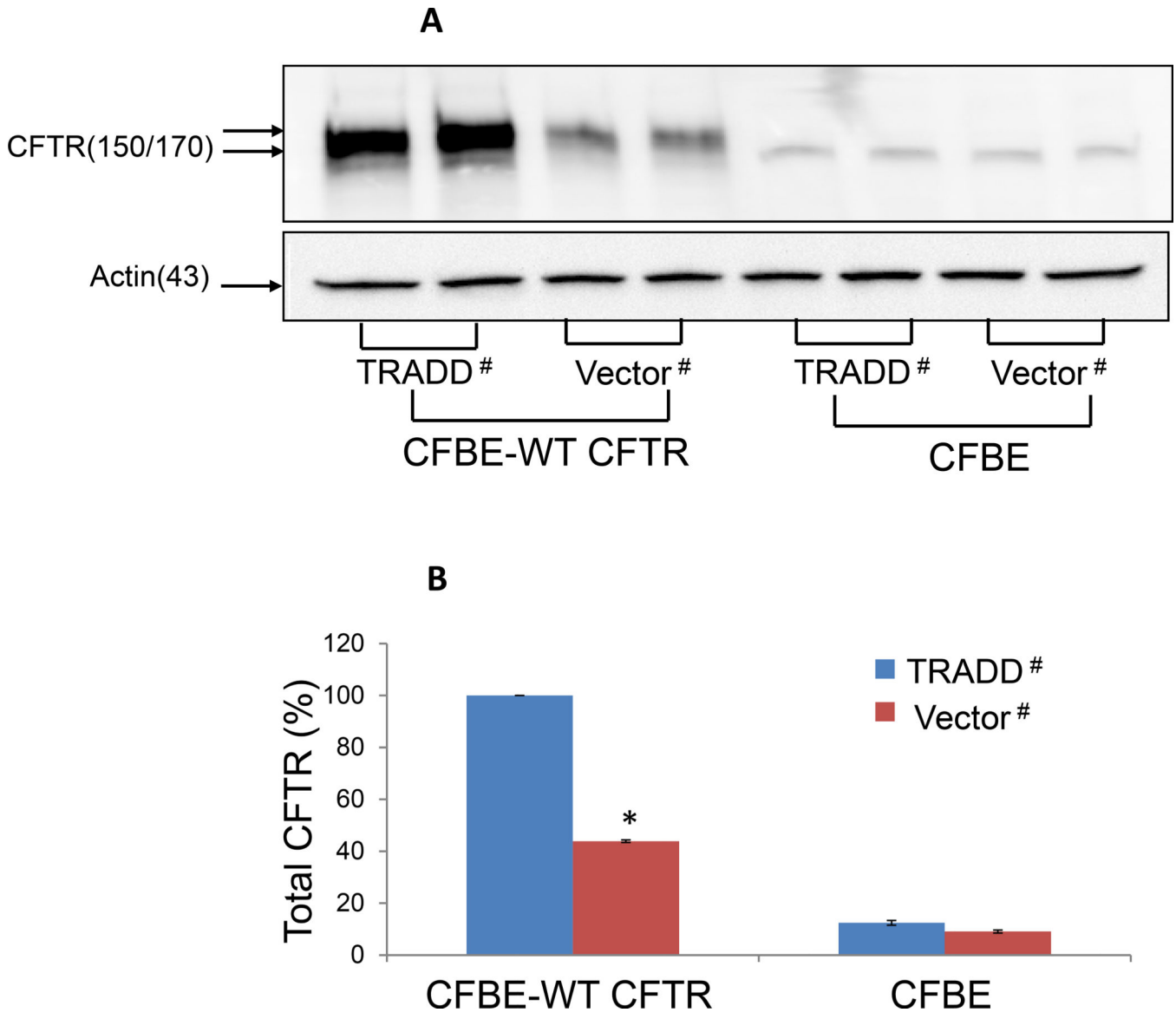

Figure 3. TRADD increases the expression of mature CFTR.

Parental CFBE cells and CFBE stably expressing wt CFTR were transfected with either TRADD or a control vector. (**3A)** CFTR was detected by Western blotting using an anti-CFTR monoclonal antibody. Note that there is much less endogenous CFTR in the untransfected CFBE cells compared to those stably expressing wt-CFTR. Thus, in this western blot containing wt-CFTR the endogenous ΔF508 CFTR is not evident. β-actin was used as a loading control. TRADD overexpression dramatically increased the expression of mature C-band of wt CFTR but had no effect on the immature B-band, nor did it rescue ΔF508 CFTR. (**3B)** Results are means ± S.E. *, $p<0.05$ versus the control. (n=3), Data was normalized to CFBE-WT CFTR TRADD transfected.

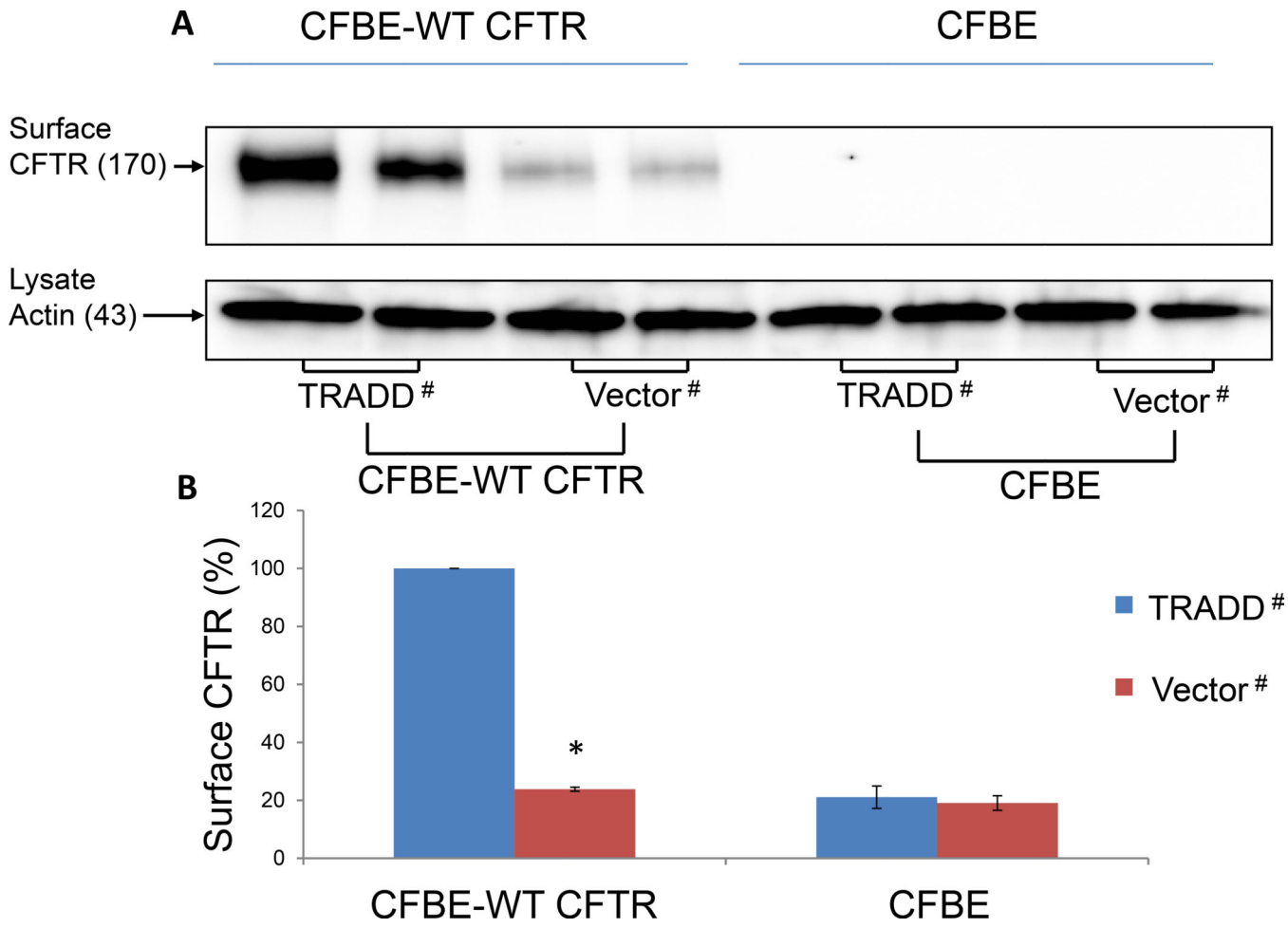

Figure 4. TRADD increases the expression of mature CFTR on the cell surface.

Parental CFBE cells and CFBE stably expressing wt CFTR were transfected with either TRADD or a control vector. (**4A)** Cell-surface CFTR was detected using a cell-surface biotinylation assay. CFTR was detected by Western blotting using an anti-CFTR monoclonal antibody. β-actin was used as a loading control. TRADD overexpression increased the cell-surface expression of wt CFTR but had no effect on ΔF508 CFTR. Note that there is no surface expression of ΔF508 CFTR in the parental CFBE cells as expected because this mutant does not reach the plasma membrane. (**4B)** Results are means ± S.E. *, $p<0.05$) (n=3).versus the control.

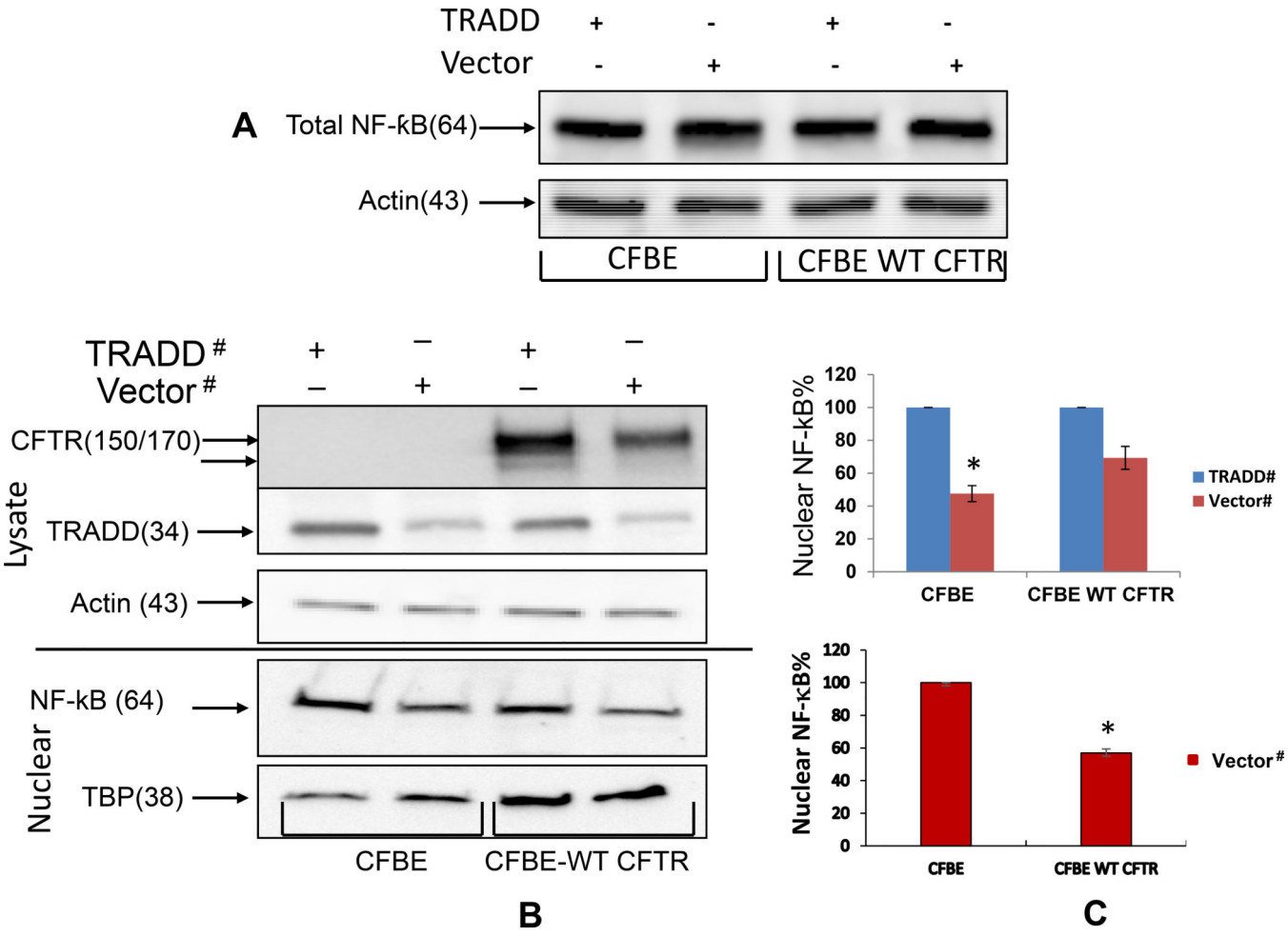

Figure 5: TRADD increases NF-κB activity in CFBE cells.

There was no difference in the total NF-κB protein in either the parental CFBE cells or those stably expressing wt CFTR **(5A)**. In **(5B)** and summarized in **(5C)**, NF-κB activity was measured with an NF-κB assay kit (see Methods) using an antibody against the p65 (RelA) NF-κB transcription factor subunit. The assay takes advantage of the process of activation of NF-κB. When activated, the IkB inhibitory subunit is degraded, allowing the transcription factor subunits of the complex to translocate to the nucleus [65]. In the assay, p65 reactivity was measured in the nuclear extracts by detecting the chemiluminescence of the sample using FujiFilm LAS-3000. Since there was no change in total NF-kB, the p65 reactivity was measured in the nuclear extract and compared across the experimental maneuvers. Results are normalized to control. Results are means ± S.E. (n=3). *, $p<0.05$. Transfection of TRADD increased NF-kB activity only in CFBE cells containing ΔF508 CFTR, and not in cells expressing wt CFTR. NF-κB was measured in CFBE cells and CFBE stably expressing wt CFTR with and without the transfection of additional TRADD.

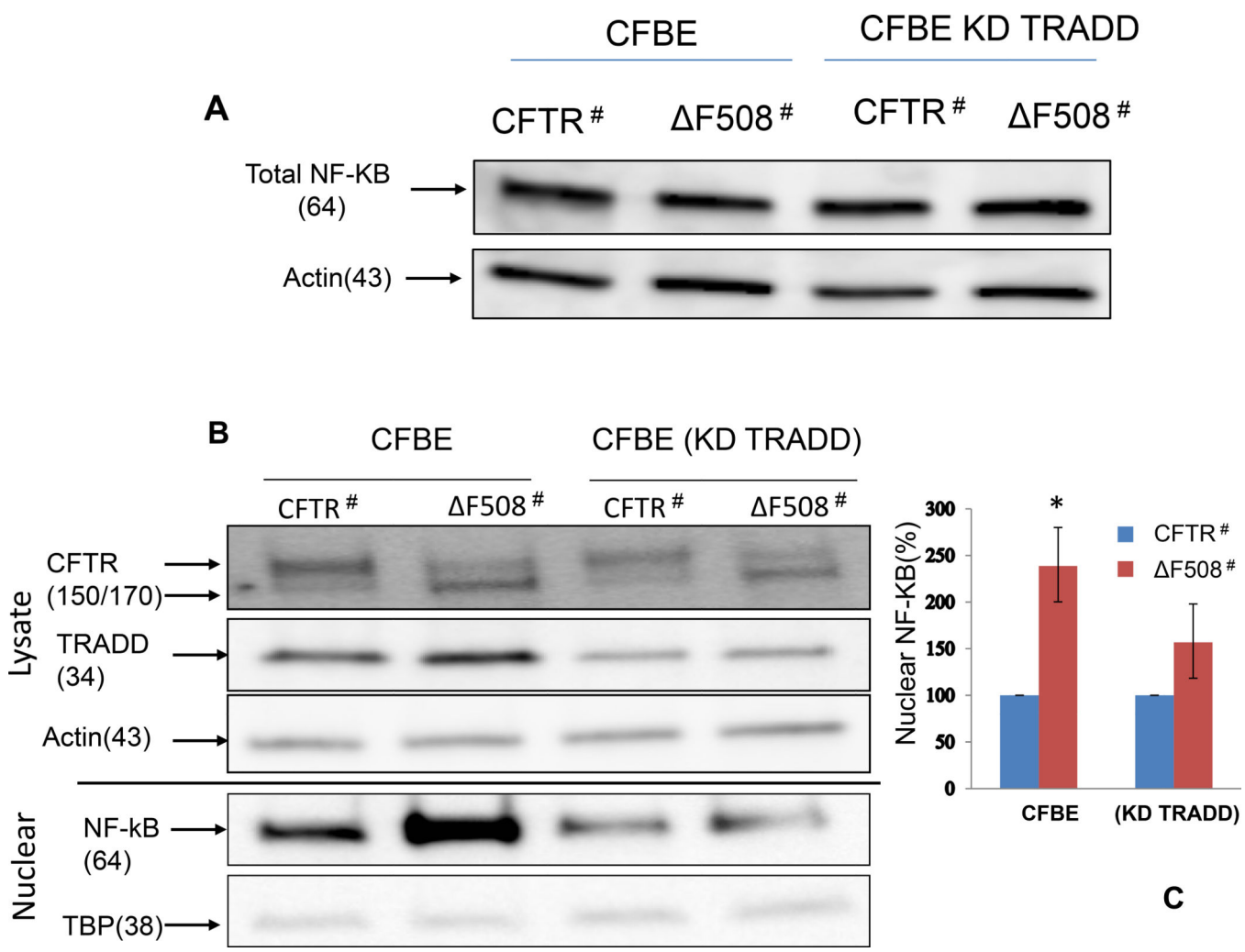

Figure 6: The increased NF-κB activity in ΔF508 when compared to wt CFTR-containing cells is dependent upon TRADD.

NF-κB was measured in CFBE cells transfected with either wt CFTR or ΔF508 CFTR, with and without knockdown (KD) of TRADD using shRNA. There was no difference in total NF-κB protein between the parental CFBE cells and those stably expressing wt CFTR **(6A)**. In **(6B)** and summarized in **(6C)**, transfection of wt CFTR into CFBE cells in TRADD-containing cells caused a reduction in NF-κB activity when compared to cells transfected with ΔF508 CFTR. The difference in nuclear NF-κB between wt and ΔF508 CFTR-expressing cells was less in cells in which TRADD levels were reduced by treatment with shRNA. Results are means ± S.E. (n=3). *, $p<0.05$.

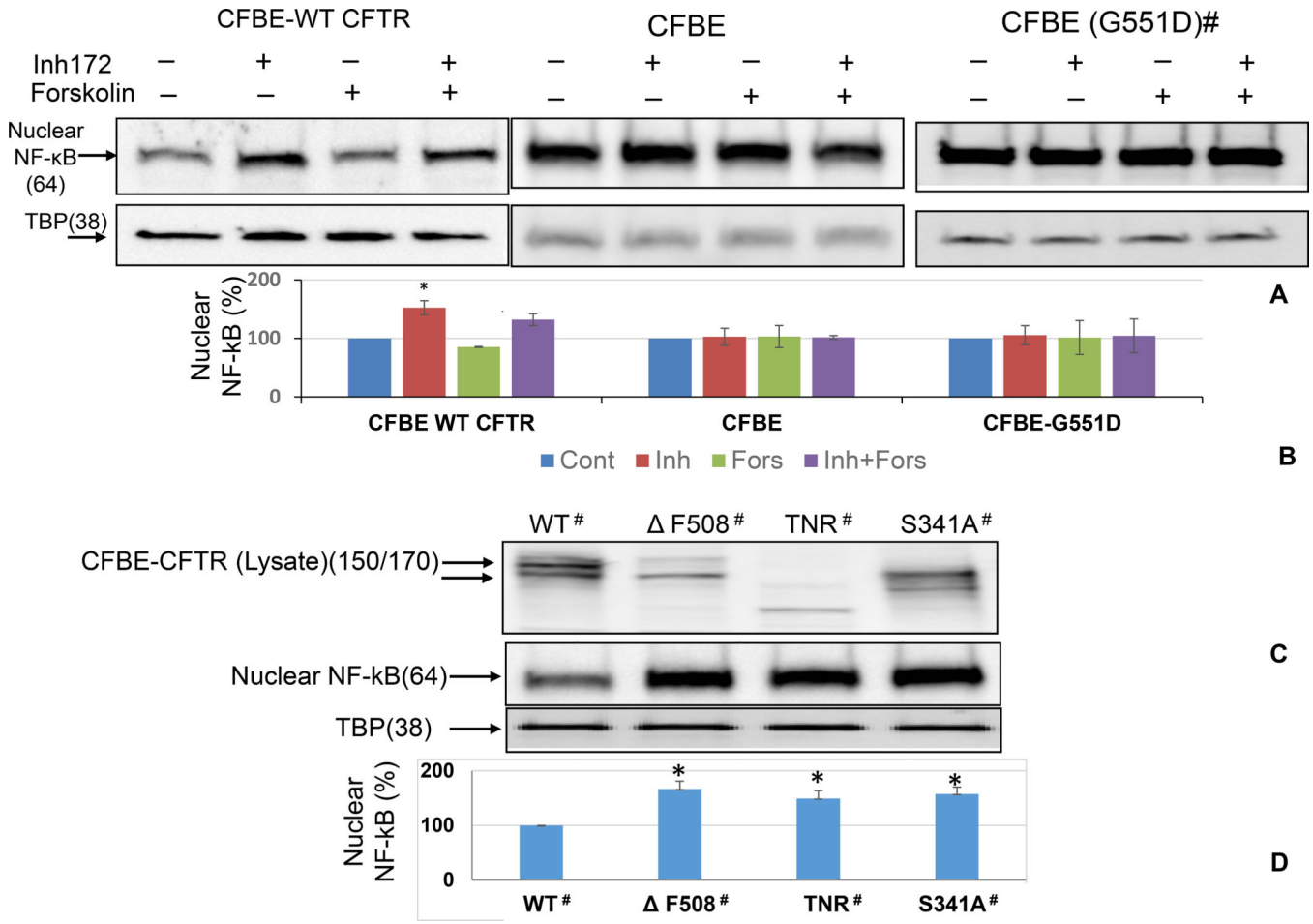

Figure 7. CFTR functional inhibition increases NF-κB activity.

CFBE-wt CFTR, CFBE, and CFBE cells transfected with G551D CFTR were treated with 10 μM $CFTR_{inh172}$, 10 μM forskolin, or 100 μM IBMX for 4 h as specified in the figure. **(7A)** $CFTR_{inh172}$ significantly increased NF-κB in the nucleus only in CFBE wt CFTR cells, and not in CFBE or CFBE cells transfected with G551D. TBP served as a nuclear loading control. **(7B).** Results are means ± S.E (n=3). *, $p<0.05$. (**7 C-D)** In CFBE cells, TRADD was co-transfected with GFP-wt CFTR, GFP-ΔF508 CFTR, GFP-TNR CFTR, S341A CFTR, or G551D CFTR. NF-kB activity was reduced by wt CFTR, but not by any of the other tested mutations of CFTR. TBP served as a nuclear loading control. **(7D).** Results are means ± S.E (*n=3*).

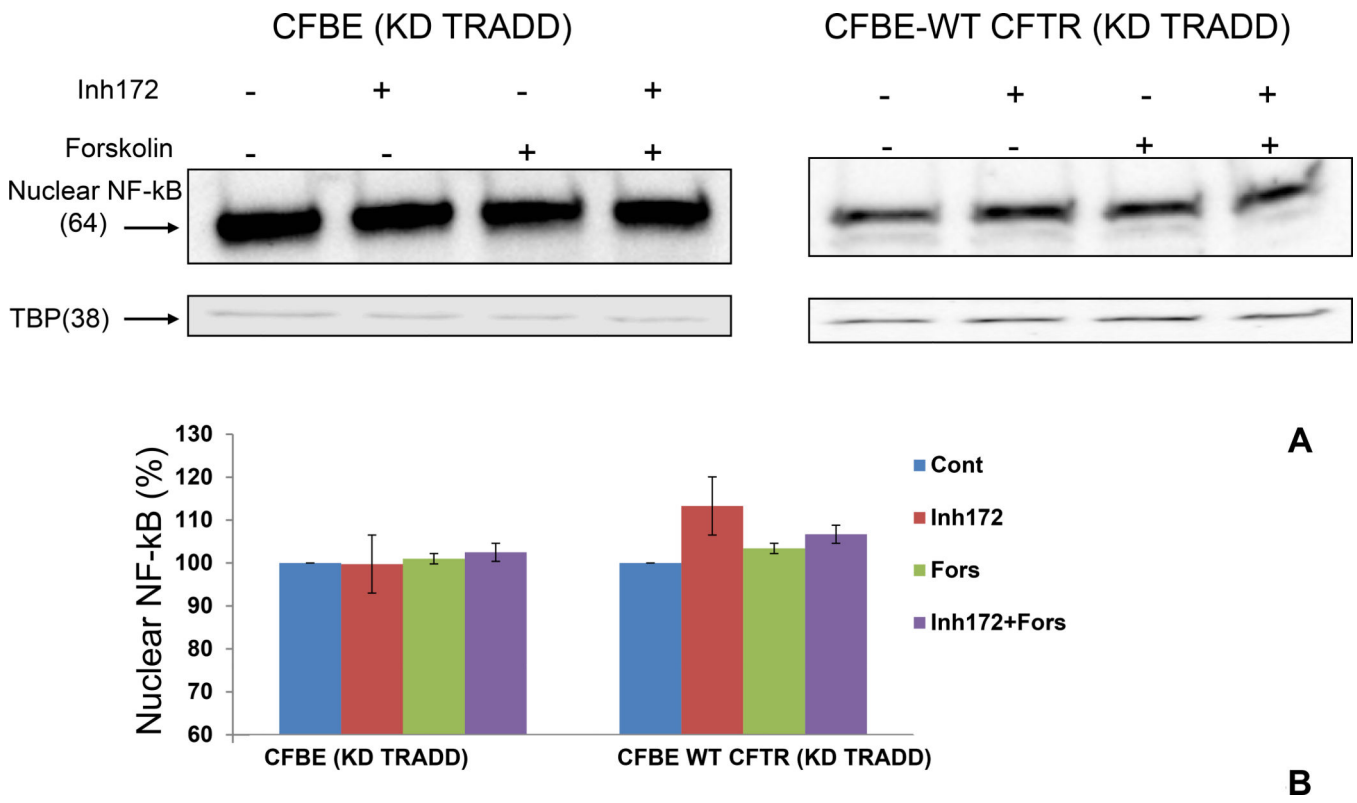

Figure 8. CFTR inhibitor increases NF-κB activity in a TRADD-dependent manner.

CFBE wt CFTR cells and CFBE cells were transfected with shRNA to knock down TRADD. The cells were treated with 10 μM $CFTR_{inh172}$, 10 μM forskolin, or 100 μM IBMX for 4 h as specified in the figure. **(8A)** When TRADD was knocked down, $CFTR_{inh172}$ did not change NF-κB located in the nucleus (*n=3*); *, $p<0.05$). TBP served as a nuclear loading control. **(8B)**. Results are means ± S.E.

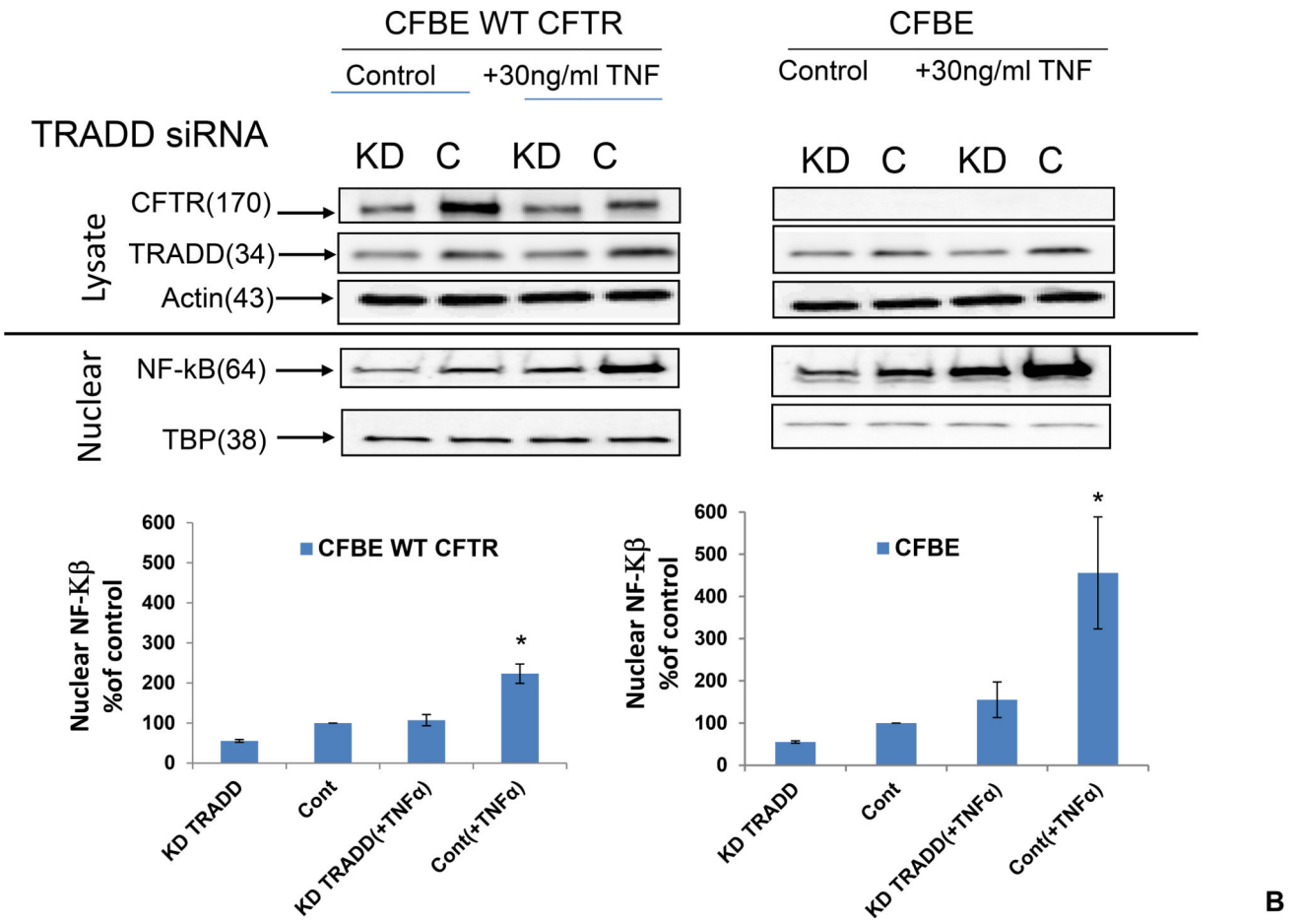

Figure 9. NF-κB activation by TNFα is dependent on TRADD and modulated by CFTR.

(9A) TRADD expression was significantly reduced after shRNA transfection. 30ng/ml TNF treatment increased NF-kB levels in CFBE-wt CFTR and CFBE cells, and this enhancement was most notable in CFBE cells containing TRADD. **(9B)** Results are means ± S.E. *, $p<0.05$ ($n=3$).

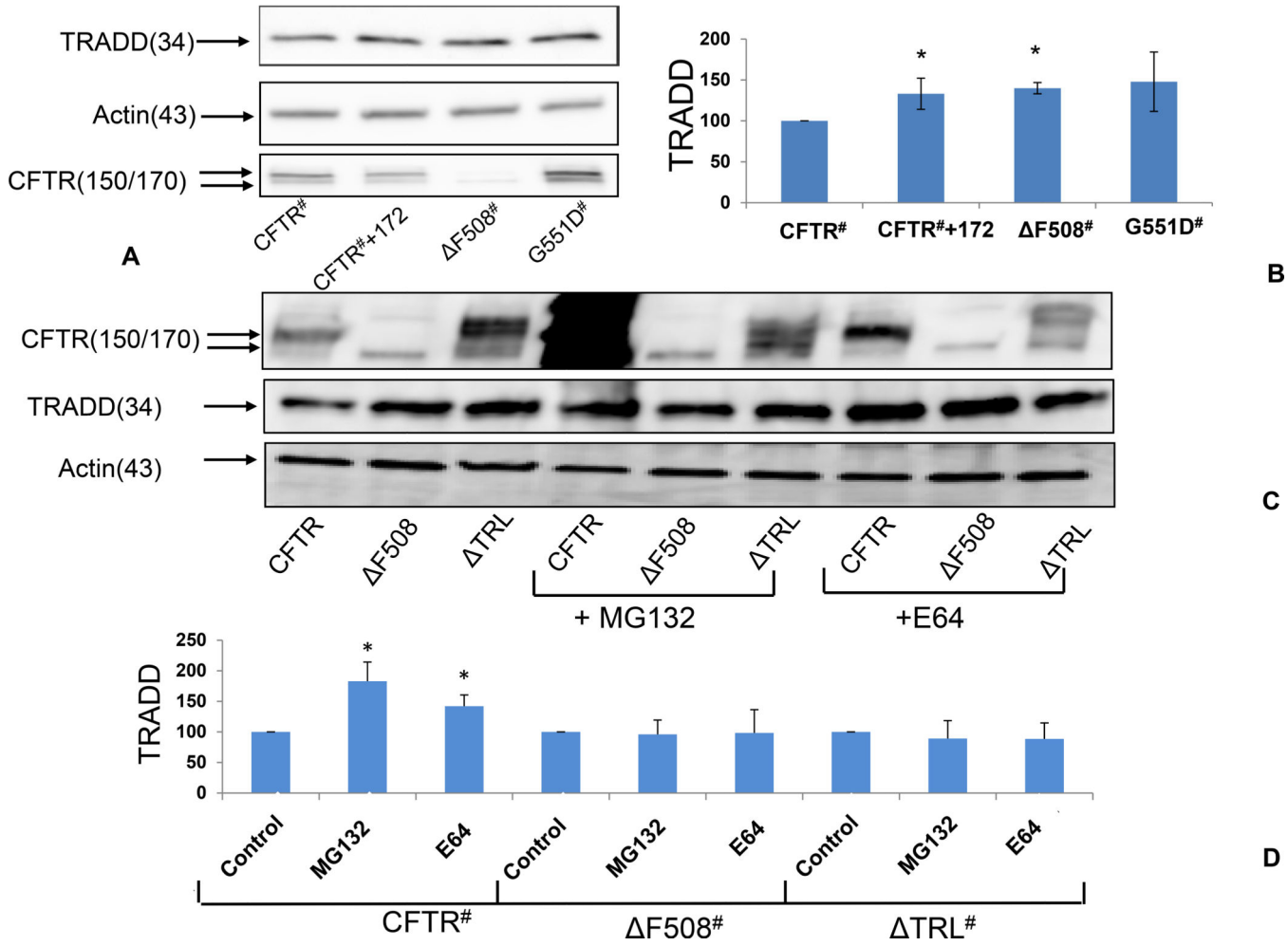

Figure 10. Functional CFTR regulates the degradation of TRADD.

(10 A-B) In CFBE cells, TRADD was co-transfected with GFP-wt CFTR, GFP-ΔF508, or GFP- G551D. WT-CFTR transfected cells were treated with vehicle or $CFTRi_{nh172}$. TRADD protein expression was increased when cells containing wt CFTR. **(10 C-D)** In CFBE cells, TRADD was co-transfected with GFP-wt CFTR, GFP-ΔF508, or GFP-ΔTRL CFTR. At 24 h after transfection, cells were treated with proteasome inhibitor (MG-132) or lysosome inhibitor (E64) for 16 h. MG132 and E64 treatment significantly increased TRADD protein expression only when wt CFTR was expressed. **(10 D)** Results are means ± S.E. *, $p<0.05$ ($n=3$).

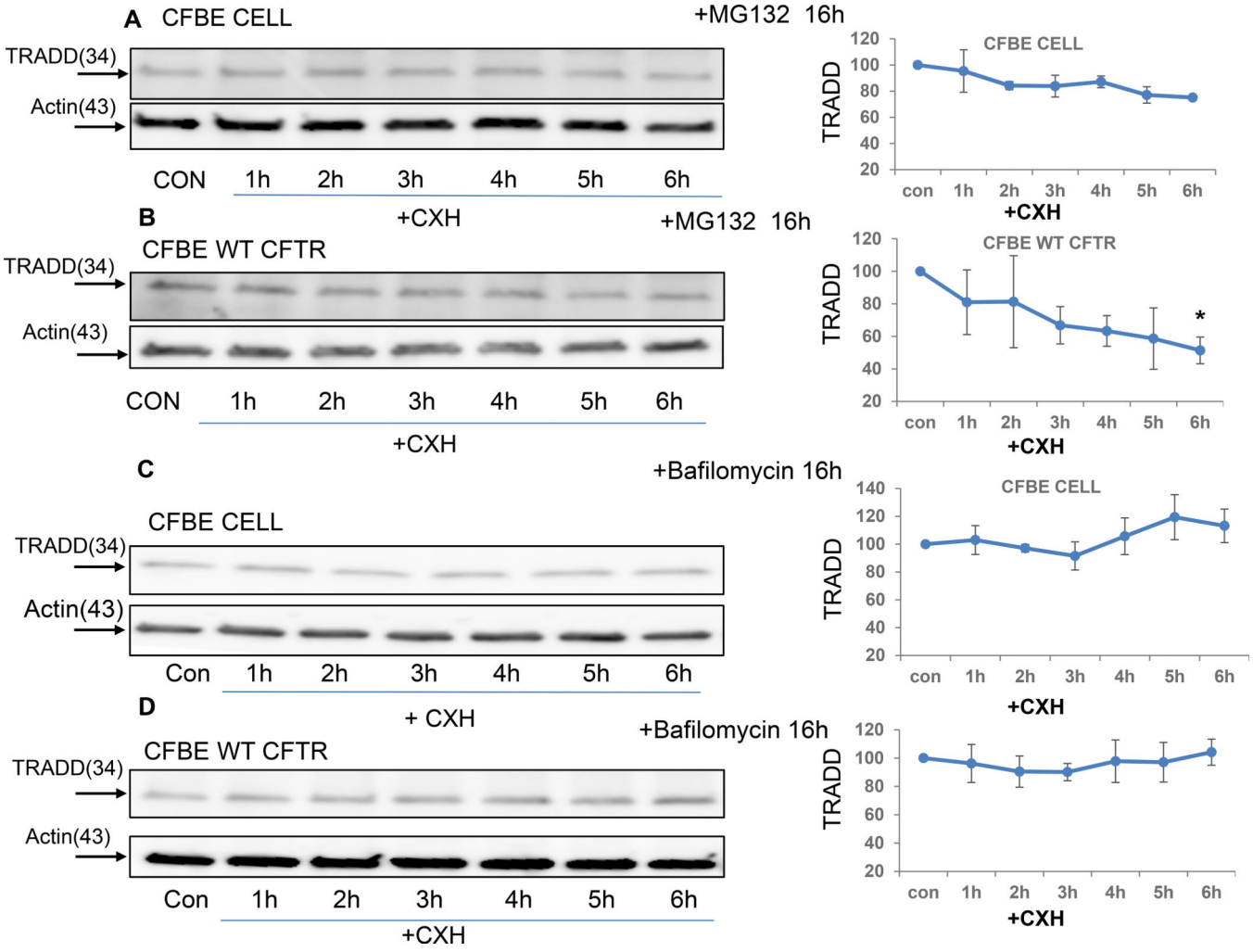

Figure 11. Functional CFTR reduces the residence time of TRADD.

(11A -B) Parental CFBE and cells stably expressing wt-CFTR were treated with proteasome inhibitor (MG-132) for 16 hours prior to exposure to cycloheximide to block protein translation. **(11C-D)** Parental CFBE and cells stably expressing wt-CFTR were treated with bafilomycin [66]which prevents degradation of proteins by the lysosome for 16 hours prior to exposure to cycloheximide to block protein translation[67]. Note that in the presence of CFTR that the disappearance of TRADD is greater than in the absence of CFTR when the proteasome is blocked. When the lysosome is blocked the expression of TRADD protein is stable over the experimental period indicating that CFTR enhances the degradation of TRADD by the lysosome. Results are means ± S.E. *, $p<0.05$ (*n=3*).

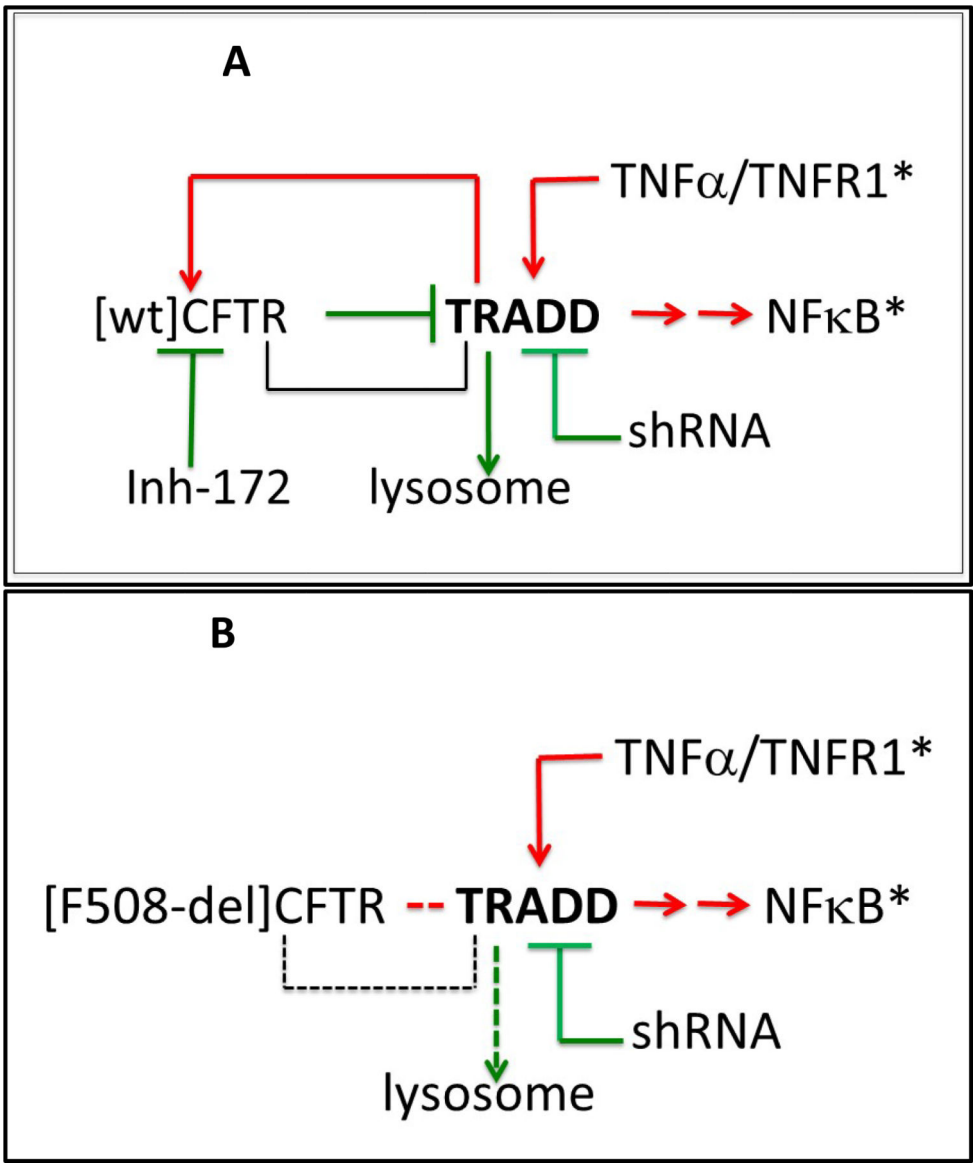

Figure 12. CFTR/TRADD/NFκB Signaling pathway.

12A. TNFα forms a complex with TNFR, which binds to its obligate intracellular adaptor TRADD. The TRADD complex drives proinflammatory signaling by activating NFκB. Wt CFTR inhibits TRADD by inducing degradation to the lysosome. The result is suppression of NFκB activation. ShRNA against TRADD reduces TRADD and blocks TNFα-activated NFκB activation. Elevation of TRADD expression feeds back to elevate Wt CFTR. $CFTRi_{nh172}$ blocks channel activity by Wt CFTR, and blocks inhibitor of TRADD by Wt CFTR. Color code: **red** = activation; **green** = inhibition. **12B).** In the presence of mutant ΔF508 CFTR, TRADD is not inhibited. TNFα/TNFR (R=receptor) can now bind to TRADD, and drive activation of NFκB. The "*" signs indicate "activation" of the TNFα/TNFR complex and the NFkB complexes, respectively.