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Correctors of the major Cystic Fibrosis mutant interact through membrane spanning domains

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Running Title: Interacting effect of correctors in combination

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Abbreviations: CFTR, Cystic Fibrosis Transmembrane Regulator; F508del, deletion of F508; WT, wild type; MSD, membrane spanning domain; NBD, nucleotide binding domain; ICL, intracellular loops; DMSO, dimethylsulfoxide; TMA, 4,6,4'-Trimethylangelicin.

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ABSTRACT

The most common Cystic Fibrosis causing mutation is deletion of phenylalanine 508 (F508del), a mutation that leads to protein misassembly with defective processing. Small molecule corrector compounds: VX-809 or Corr-4a (C4) partially restore processing of the major mutant. These two prototypical corrector compounds cause an additive effect on F508del-CFTR processing and hence were proposed to act through distinct mechanisms: VX-809 stabilizing the first membrane spanning domain 1 (MSD1) and C4, acting on the second half of the molecule (consisting of MSD2 and/or nucleotide binding domain 2 (NBD2)). We confirmed the effect of VX-809 in enhancing the stability of MSD1 and showed that it also allosterically modulates MSD2 when co-expressed with MSD1. We showed for the first time that C4 stabilizes the second half of the CFTR protein through its action on MSD2. Given the allosteric effect of VX-809 on MSD2- we were prompted to test the hypothesis that the two correctors interact in the full length mutant protein. We did see evidence supporting their interaction in the full-length F508del-CFTR protein bearing secondary mutations targeting domain:domain interfaces. Disruption of the MSD1:F508del-NBD1 interaction (R170G) prevented correction by both compounds pointing to the importance of this interface in processing. On the other hand, stabilization of the MSD2: F508del-NBD1 interface (by introducing R1070W) led to a synergistic effect of the compound combination on the total abundance of both the immature and mature form of the protein. Together- these findings suggest that the two correctors interact in stabilizing the complex of MSDs in F508del-CFTR.

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INTRODUCTION

Cystic Fibrosis (CF) is caused by mutations in the *Cystic Fibrosis Transmembrane conductance Regulator* (*CFTR/ABCC7*) gene (Gadsby et al., 2006; Molinski et al., 2012; Riordan et al., 1989). The CFTR protein functions as an ATP- and phosphorylation-regulated chloride channel comprised of two membrane-spanning domains (MSD1 and MSD2, with a total of four intracellular loops: ICL1-4), two nucleotide-binding domains (NBD1 and NBD2) and a regulatory (R) domain (Molinski et al., 2012; Riordan, 2008). The physiological role of the CFTR channel is for maintenance of fluid transport across epithelial cells of the body's tubular organs, including the airways, intestines, pancreatic as well as bile ducts, and loss of this function causes CF with associated multi-system pathologies (Huang et al., 2004; Li and Naren, 2005).

Although over 2000 *CFTR* mutations have been described in the literature and mutation databases, most research efforts have been focused on CF patients bearing the most common disease-causing mutations: deletion of phenylalanine at position 508 (F508del) and the glycine to aspartate missense mutation at position 551 (G551D) (Cutting, 2015; Gadsby et al., 2006; Riordan et al., 1989) [(CFTR1 database: http://www.genet.sickkids.on.ca, CFTR2 database: http://cftr2.org/]. F508del causes aberrant assembly of the full-length protein, which is mediated through the NBD1:ICL4 interface. Misassembly caused by F508del impairs processing, trafficking and stability of the full-length protein at the cell surface, while G551D causes a channel 'gating' defect due to aberrant NBD1:NBD2 interdomain interaction (Mendoza et al., 2012; Mornon et al., 2008; Rabeh et al., 2012). However, two small molecule-based therapies, ORKAMBI™ and KALYDECO™, have recently been developed for patients bearing F508del and G551D, respectively. KALYDECO™, also known as ivacaftor (VX-770), is a small molecule 'potentiator' that promotes the open state of G551D-CFTR channels, while ORKAMBI™ is comprised of both VX-770 and lumacaftor (VX-

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809), a small molecule 'corrector' that promotes protein stability and forward trafficking of F508del-CFTR (Van Goor et al., 2009; Van Goor et al., 2011).

The mechanism of action of VX-809 (recently categorized as a Class I corrector) has been interrogated by several groups, yet uncertainties remain (Hudson et al., 2017; Laselva et al., 2016; Loo et al., 2013; Loo and Clarke, 2017; Ren et al., 2013). Previous studies using isolated domains of CFTR showed that the N-terminal domain of CFTR, MSD1, was important for the stabilizing effect of VX-809 (Loo et al., 2013; Ren et al., 2013). In the context of the full-length mutant protein: F508del-CFTR bearing strategically substituted cysteines at the interface between NBD1 and the coupling helix of MSD2, VX-809 promoted chemical crosslinking- supporting the hypothesis that modification of this interdomain interface constituted its major mechanism of action (Loo and Clarke, 2017). However, it is not clear how the interaction of VX-809 with MSD1 mediates such long-range interdomain conformational changes involving MSD2.

Another pharmacological corrector (Class II), Corr-4a (C4) also partially ameliorate the processing defect of F508del-CFTR, and as its rescue effects are additive with VX-809, C4 is thought to act via a distinct mechanism (Okiyoneda et al., 2013). Okiyoneda and colleagues suggested that C4 proposed that the C4 binding site may be located in NBD2, however, the binding site, as well as the intramolecular consequences on CFTR structure-function, of this small molecule has not yet been defined (Okiyoneda et al., 2013).

The goal of the current study was to provide insight into the mechanism of action of VX-809 and C4. We used several experimental approaches toward this goal, including the use of CFTR fragments to define the domains modified by these small molecules and studies of the full-length F508del-CFTR bearing interface-modifying amino acid substitutions.

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MATERIAL AND METHODS

Cell culture and transfection

All CFTR variants and fragments used in this study were transiently expressed in human embryonic kidney (HEK)293 GripTite™ cells (HEK293) (a gift from Dr. Daniela Rotin, Hospital for Sick Children, Toronto, Ontario, Canada). HEK-293 cells were maintained in DMEM (Wisent, St-Bruno, QC) supplemented with non-essential amino acids (Life Technologies, Waltham, MA) and 10% fetal bovine serum (FBS; Wisent, St-Bruno, QC) at 37 °C and processed with 5% CO_2 as previously described (D'Antonio et al., 2013; Molinski et al., 2015). Transient transfections were performed using PolyFect Transfection Reagent (Qiagen, Hilden, Germany), according to the manufacturer's protocol as previously described (Molinski et al., 2015).

Plasmids, antibodies and reagents

Briefly, mutant CFTR constructs were generated using the KAPA HiFi HotStart PCR Kit (KAPA BIOSYSTEMS, Woburn, MA) according to the manufacturer's Standard PCR protocol with high-quality (>300 ng/μL, 260/280 nm ratio of 1.8) plasmid DNA containing WT-CFTR of F508del-CFTR cDNA (in pcDNA3.1) as the template as previously described (Molinski et al., 2015).

The primary antibodies used in this study were mAb MM13-4 (EMD Millipore, Billerica, MA) for MSD1-containing constructs, mAb A52 (a kind gift from Dr. David Clarke, University of Toronto) (Loo et al., 2013) for MSD2 containing constructs and mAb 596 (University of North Carolina Chapel Hill) for NBD2-containing constructs (Cui et al., 2007). Calnexin was used as a protein loading control and detected with a Calnexin-specific rabbit pAb (Sigma-Aldrich, St. Louis, MO). The small molecule modulators of CFTR used in this study were: VX-770 and VX-809 (Selleck Chemicals, Houston, TX), Trimethylangelicin (TMA) (Dr. Roberto Gambari, University of Ferrara,

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Italy) (Abbattiscianni et al., 2016; Favia et al., 2014), C4 and CFTR-specific inhibitor 172 (CFTRinh-172) (Cystic Fibrosis Foundation Therapeutics), and glycerol (Sigma-Aldrich, St. Louis, MO).

Immunoblotting:

For the purpose of CFTR immunoblotting, transfected cells were lysed in modified RIPA buffer containing a protease inhibitor cocktail (Roche, Mannheim, Germany) for 10 min. Soluble fractions were analyzed by SDS-PAGE on 6% Tris-Glycine gels (Life Technologies). After electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad) and incubated in 5% milk and CFTR bands were detected with human CFTR-specific murine mAb 596 (1:5,000). The blots were developed with either ECL (Amersham) and exposure to film for 0.5 to 5 min as required, or with ECL (Amersham) on the Li-Cor Odyssey Fc (LI-COR Biosciences, Lincoln, NE) in a linear range of exposure (Laselva et al., 2016; Molinski et al., 2017).

Measurement of CFTR fragment stability and glycosylation status

Steady-state levels of CFTR fragments, including MSD1 (containing residues 1-380, or K381X), A52-tagged MSD2 (containing residues 837–1196) and MSD2-NBD2 (containing residues 850-1480), were determined by Western Blot analysis. HEK293 cells were transiently transfected with the plasmids as described above. After 18 h of transfection, the cells were treated with 3 μM VX-809, 500 nM TMA, 10 μM C4 or vehicle control (DMSO) and 24 h after correction the cells were lysed in modified radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4, 0.2% SDS, and 0.1% Triton X-100) containing a protease inhibitor cocktail (Roche, Mannheim, Germany) for 10 min, and the soluble fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 4-12% Tris-Glycine gels (Life

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Technologies). After electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad) and incubated in 5% milk and CFTR bands were detected with human CFTR-NBD2-specific murine mAb 596 (1:10,000) or with human CFTR-MSD1-specific murine mAb MM13-4 (1:10,000) or with monoclonal antibody A52 for MSD2 (1:1,000) (Dr. David Clarke, University of Toronto) (Laselva et al., 2016; Loo et al., 2013; Molinski et al., 2015).

To evaluate protein glycosylation status of MSD2, we first transfected HEK293 cells, and then after 48 h cells were lysed with modified RIPA buffer as described above. Lysates were treated with either endoglycosidase H (endoH) or peptide-N-glycosidase F (PNGaseF) (both from New England Biolabs, Ipswich, MA) according to the manufacturer's protocol. Western blots were obtained using the murine monoclonal antibody A52 as described by Loo and colleagues (Loo et al., 2013). The blots were developed with enhanced chemiluminescence (ECL) (Amersham) and exposure to film for 0.5 to 5 min as required. Relative expression level of CFTR proteins were quantitated by densitometry of Western blots using ImageJ software version 1.46 (National Institutes of Health).

Cycloheximide chase assay

To test the effect of C4 on the stability of MSD1, MSD2 or MSD2-NBD2 fragments expressed as separate polypeptides, HEK293 cells were transfected as described above, and after 18 h media was replaced with fresh media containing either vehicle (DMSO) or C4 (10 μM). After 24 h at 27 °C, protein synthesis was stopped by addition of medium containing 0.5 mg/mL cycloheximide and either vehicle (DMSO) or C4 (10 μM). The cells were then incubated at 37 °C, lysed at various time periods (0-8 h), and whole cell extracts were subjected to Western Blot analysis as described above (Cui et al., 2007; Loo et al., 2013).

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Statistical analysis

Data are represented as mean ± S.D. unless otherwise noted. GraphPad Prism 7.0 software (San Diego, CA) was used for all statistical tests. One-way/two-way ANOVA were conducted as appropriate, and p-values < 0.05 was considered significant. Data with multiple comparison were assessed with Tukey's multiple-comparison test with $\alpha=0.05$. Each experiment is defined as a separate biological study on independently plated cells.

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RESULTS

VX-809 and C4 differentially modify the stability of the membrane spanning domains: MSD1 and MSD2

It has previously been reported that the Class I corrector VX-809 stabilizes a protein fragment comprising MSD1 (residues 1-380) of CFTR but not domains comprising the second half of the CFTR protein (i.e. MSD2-NBD2, residues 850-1480) (Laselva et al., 2016; Ren et al., 2013). These results were confirmed in the present study (**Figure 1, Ai and Aii**). However, as certain reports showed that VX-809 enhanced the interaction between MSD2 and F508del-NBD1 in the context of the full-length mutant protein, we hypothesized that the VX-809 may also modulate the second half of the protein if co-expressed with MSD1. Interestingly, when co-expressed with MSD1, the steady-state abundance of MSD2-NBD2 was significantly increased by 3μM VX-809 (**Figure 1Ai and Aiii**). Similarly, when co-expressed with MSD1, the steady-state abundance of MSD2 was significantly increased by VX-809 (**Figure 1Bi and Biii**). These data suggest that VX-809 allosterically modifies the stability of MSD2 through its interaction with MSD1.

We then assessed the effect of 10μ Corr4a (C4) on fragment stability in a similar set of studies. In contrast to VX-809, C4 treatment stabilized the MSD2-NBD2 fragment rather than MSD1 (**Figure 2Ai, 2Aii**). Interestingly, there was no positive (i.e. allosteric) effect of C4 on MSD1 when co-expressed with MSD2-NBD2. We interpret these findings to suggest that C4 interacts with the second half of CFTR and this interaction does not indirectly modify the abundance of MSD1.

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To test the hypothesis that C4 binding to MSD2-NBD2 enhances its stability, we conducted cycloheximide-chase experiments, wherein protein synthesis was inhibited after production of this fragment, and then the abundance monitored over time in the presence or absence of C4. We observed a modest increase in the stability of this fragment in the presence of C4 at one hour after initiation of the chase (**Supplemental Figure 1**).

To determine if C4 binds to the MSD2 component of this two-domain fragment, we studied the effect of C4 on MSD2 alone. We transfected HEK cells with MSD2 bearing an A52 tag, after treatment with C4 for 24hr, (see Methods) this CFTR fragment was expressed as three distinct bands **(Figure 3A)**. As indicated by the sensitivity to both EndoH and PNGaseF, the higher migrating species of MSD2 represent differentially glycosylated, high mannose forms of this domain (Du and Lukacs, 2009). Next, to determine if C4 interacts with MSD2, we pre-incubated HEK cells expressing MSD2 with 3 μM VX-809, 500 nM TMA (a compound thought to act similarly to VX-809), 10 μM C4 or vehicle control (DMSO) and found that only C4 significantly increased its abundance (**Figure 3B**).

In **Figure 3C-D**, we showed that in the absence of C4 the half-life of MSD1 and MSD2 was short after cyclohexamide addition. Interestingly however, treatment with C4 significantly increased the abundance of the MSD2 fragment but not the MSD1 fragment at two hours after initiation of the cycloheximide-chase, supporting the hypothesis that C4-dependent stabilization of CFTR is mediated via its interaction with MSD2.

Interaction between VX-809 and C4 revealed in full-length F508del-CFTR bearing MSD: NBD1 interface mutants

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Our findings that VX-809 stabilizes MSD2, allosterically via MSD1 and C4 also stabilizes MSD2 led us to hypothesize that the two correctors may interact to modulate the full length F508del-CFTR protein. However, previous (and the current studies, Figure 4A), suggest that VX-809 (3 μM) and C4 (10 μM) may not interact as their combined effect on F508del-CFTR abundance and processing was additive – not supporting cooperativity. We were prompted to determine if such interaction would be revealed in F508del-CFTR proteins bearing secondary mutations in regions previously shown to modulate the assembly and correction of the major mutant.

As previously reported, the disease-causing R170G mutation in coupling helix 1 (CH1- extended from MSD1 to interact with NBD1) impairs processing of WT-CFTR to a mature (Band C) CFTR protein product (Okiyoneda et al., 2013). Both VX-809 and C4 improved processing of R170G-CFTR and together, exerted an additive effect on processing to band C (**Supplemental Figure 2**). On the other hand, R170G/F508del-CFTR failed to show correction of processing in response to either corrector alone or in combination- emphasizing the important function of CH1 in F508del assembly. Interestingly, the abundance of the core glycosylated (i.e. Band B form) of CFTR protein was increased synergistically by the corrector combination, suggesting that these compounds interact with the full-length protein to improve stability (as in the case of MSD fragment studies) yet fail to induce improved assembly of F508del-CFTR when the coupling helix of ICL1 is disrupted (**Figure 4B**).

We were then prompted to study the consequences of corrector compounds alone and in combination in F508del-CFTR bearing a rescue mutation: R1070W, located in the coupling helix of ICL4. As previously shown (He et al., 2013; Okiyoneda et al., 2013), we confirmed that VX-809 and C4 rescued the processing of F508del/R1070W-CFTR individually and their effect on

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processing was additive (**Figure 4C and Supplemental Figure 3).** Interestingly and consistent with the concept that both correctors mediate interacting effects on protein stability, we observed a synergistic effect of VX-809 plus C4 on the steady state abundance of both the immature and mature forms of the F508del-CFTR-R1070W protein (**Figure 4C**). These findings are consistent with the concept that the two compounds interact to promote protein stability.

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DISCUSSION

In this study we showed that two corrector compounds, prototypical of Class I and Class II, modify different regions of CFTR protein as previously suggested by Okiyoneda and Groves (Grove et al., 2009; Okiyoneda et al., 2013). When individual domains were studied independently, VX-809 treatment stabilized MSD1 whereas C4 treatment stabilized MSD2. Interestingly, we also report the first evidence that there is an overlapping effect of the two compounds on the stability of the membrane spanning domains when the MSDs are co-expressed. VX-809 exerted an allosteric effect on MSD2, supporting a new hypothesis wherein the two compounds exert an interacting mechanism of action (**Figure 5**). The interacting effect of the two compounds was revealed in studies of full length F508del-CFTR bearing MSD:NBD1 interface mutants.

The findings in the current work support several previous publications regarding the mechanism of action of Class I and Class II corrector compounds. Grove and colleagues were the first to show that the Class I compound: VX-809, enhanced the stability of a fragment of CFTR corresponding to MSD1. A recent publication by Loo et al., similarly showed that VX-809 corrector activity is dependent on MSD1 (Loo et al., 2013; Loo and Clarke, 2017). We confirmed these findings, and further, showed that VX-809 treatment also stabilized the MSD2 domain fragment but only when co-expressed with MSD1. Our results provide a greater understanding of mechanism underlying the well-described modulation by VX-809 on the interaction between ICL4 (MSD2) and NBD1 in the full-length mutant protein, namely, by promoting a stable MSD1:MSD2 complex.

Previous studies of the mechanism of action of C4 (the prototypical Class II corrector) supported a role for direct interaction with F508del-CFTR (Grove et al., 2009; Okiyoneda et al., 2013). A truncation mutant (F508del/K1218X-CFTR), shown to be misfolded, failed to show correction with C4, leading the authors of this paper to suggest that NBD2 (only partially removed in the mutant)

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constituted the C4 binding site (Okiyoneda et al., 2013). On the other hand, Grove et al studied a different NBD2 deletion mutant (E1172X-CFTR and F508del/E1172X-CFTR) and found that processing was enhanced with C4, arguing that NBD2 is not essential for C4 corrector activity (Grove et al., 2009). The findings in the current work, supported the Groves et al. study and their interpretation that NBD2 is not critical for C4 activity. Inspection of the recent CFTR protein structural models solved by the group of Chen and colleagues (Liu et al., 2017; Zhang et al., 2017), suggests that the E1172X-CFTR mutant better recapitulates an NBD2 deletion mutant than K1218X-CFTR (Okiyoneda et al., 2013).

Importantly, we showed that C4 interaction stabilized the MSD2 protein fragment, but not the MSD1 fragment, suggesting that MSD2 is required for C4 activity. Altogether, our findings suggest that the two correctors, VX-809 and C4 exert non-identical but potentially overlapping mechanisms of action.

The current work also highlights the essential role of ICL1 in mediating the corrector activity of VX-809, C4 and the combination of these two modulators. Although both correctors enhanced the interaction between ICL4 and NBD1 in WT-CFTR protein, in the context of F508del-CFTR this conformational change was not sufficient to account for the activity of VX-809 nor the corrector combination as the secondary mutation (R1070W) in ICL4 did not abolish their effect in improving processing. However, disruption of ICL1 completely abrogated the effect of correctors individually and in combination. Hence, there is a dominant role of the ICL1:NBD1 interaction in the "rescue" of F508del-CFTR (Loo and Clarke, 2017). Examination of the recent structures of the full length CFTR protein (Liu et al., 2017; Zhang et al., 2017), will enable the generation of new hypotheses regarding the effect of small molecule binding on the ICL1: NBD1 interface.

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As previously mentioned, our results suggest distinct binding sites for VX-809 and C4 on the CFTR protein, and supports the current hypothesis that the combination of compounds will lead to more effective therapies for subjects bearing the major mutation. Encouraging results are emerging from clinical trials of corrector combinations developed by Vertex Pharmaceuticals. One of the compounds is VX-809 (lumacaftor), however, the structure of the second compound is not known. The field is excited to learn in preliminary press releases that the combination is exerting improved clinical responses relative to those observed when a single corrector (i.e. lumacaftor alone) was administered. Soon, we will learn if this clinical promise persists and whether the second corrector resembles the mechanism of action that we have described for C4.

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Author contributions

Research design: Onofrio Laselva, Christine E Bear

Conducted experiments: Onofrio Laselva

Performed data analysis: Onofrio Laselva

Wrote the manuscript: Onofrio Laselva, Steven Molinski, Valeria Casavola, Christine E Bear and Jacqueline McCormack assisted in editing

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Footnotes

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Legends for Figure

FIGURE 1: Class I corrector VX-809 stabilize the interaction between MSD1 and MSD2. HEK293 cells were transiently transfected with (**Ai**) MSD1 or MSD2-NBD2, or co-transfected with MSD1 and MSD2-NBD2 domains and (**Bi**) MSD1 or MSD2, or co-transfected with MSD1 and MSD2- domains. Cells were treated for 24 h at 37 °C with: DMSO, VX-809 (3 μM). The MSD1 fragment was detected with an antibody against the N-terminus of CFTR (i.e. mAb MM13-4), the MSD2-NBD2 fragment was detected with an antibody against the C-terminus of CFTR (mAb 596) and MSD2 fragment with antibody A52. (**Aii**) Bar graphs show the mean (±SEM) of % abundance of MSD1 normalized to calnexin loading. Data are representative of 4 biological studies. (**P<0.01; ***P<0.001). (**Aiii**) Bar graphs show the mean (±SEM) of % abundance of MSD2-NBD2 normalized to calnexin loading. Data are representative of 4 biological studies. (****P<0.0001). (B**ii**) Bar graphs show the mean (±SEM) of % abundance of MSD1 normalized to calnexin loading. Data are representative of 4 experiments. (*P<0.05; ***P<0.001, ****P<0.0001). (**Biii**) Bar graphs show the mean (±SEM) of % abundance of MSD2 normalized to calnexin loading. Data are representative of 4 biological studies. (***P<0.001).

FIGURE 2: The Class II corrector C4 stabilizes only MSD2-NBD2. (**Ai**) HEK293 cells were transiently transfected with MSD1 or MSD2-NBD2, or co-transfected with MSD1 and MSD2-NBD2 domains and treated for 24 h at 37 °C with: DMSO or C4 (10 μM). The MSD1 fragment was detected with an antibody against the N-terminus of CFTR (i.e. mAb MM13-4), and the MSD2-NBD2 fragment was detected with an antibody against the C-terminus of CFTR (mAb 596). (**Aii**) Bar graphs show the mean (±SEM) of % abundance of MSD1 normalized to calnexin loading. Data are representative of 4 biological studies. (**Aiii**) Bar graphs show the mean (±SEM) of % abundance of MSD2-NBD2 normalized to calnexin loading. Data are representative of 3 biological studies (****P<0.0001).

FIGURE 3: C4 directly stabilizes MSD2 of CFTR. (**A**) Endoglycosidase sensitivity of the MSD2-CFTR fragment. Whole cell extracts of HEK293 cells transfected with A52-tagged MSD2-CFTR cDNA were treated with endoglycosidase H (H), peptide-N-glycosidase F (F), or untreated (–). **(B)** Effect of C4 (10 μM), TMA (500 nM) or VX-809 (3 μM) on the abundance of A52-tagged MSD2-CFTR in HEK293 cells. (**C-D**) MSD1 or MSD2 domains of CFTR were expressed in

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HEK293 cells in the presence or absence of C4 (10 μM) at 27 °C. After 24 h, protein synthesis was inhibited by addition of cycloheximide (0.5 mg/mL) with or without C4 (10 μM), and cells collected after the indicated times for Western blot analysis of whole cell extracts. (**Cii,Dii**) The amount of CFTR protein at each time point was quantitated and expressed relative to that at time 0 and calnexin loading. Data are representative of 3 biological studies (**p<0.01).

FIGURE 4: Additive effect of VX-809 and C4 on correction of F508del-CFTR. (A-C) HEK293 cells were transiently transfected with F508del-CFTR, R170G/F508del, or F508del/R1070W-CFTR, and treated for 24 h at 37 °C with: DMSO, VX-809 (3 μM) and/or C4 (10 μM). F508del-CFTR, R170G/F508del-CFTR and F508del/R1070W-CFTR were detected with an antibody against the N-terminus of CFTR (MM13-4). C: mature, complex-glycosylated CFTR; B: immature, core-glycosylated CFTR. (**Ai,Bi,Ci**) Bars represent the mean (±SEM) of the ratio C/(C+B) and are representative of 3 biological studies (*p<0.05; **p<0.01; ***p<0.001). (**Aii, Bii, Cii**) Bars represent the Band C and band B forms, normalized to DMSO and are representative of 3 biological studies (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001). Stippled horizontal lines in **A** and **C** show prediction for additive effect of VX-809 plus C4 on C/C+B (left bar graph) or band C (right bar graph). Dotted horizontal line in B shows prediction for additive effect on band B (right bar graph).

FIGURE 5. Graphic hypothesis depicting putative interaction between VX-809 and C4 and key role of CL1 in corrector activity. Upper cartoon shows the MSD1 and F508del-NBD1 as peach coloured and MSD2 and NBD2 as cyan coloured. Lower panel, left cartoon highlights the putative stabilizing effect of VX-809 (red) on MSD1 with its allosteric effect on MSD2. Middle cartoon shows putative stabilizing effect of C4 (green) on MSD2. Right cartoon shows putative interaction of VX-809 plus C4 on MSD2 (orange) and the primary role of CL1 on mediating correction by the combination.

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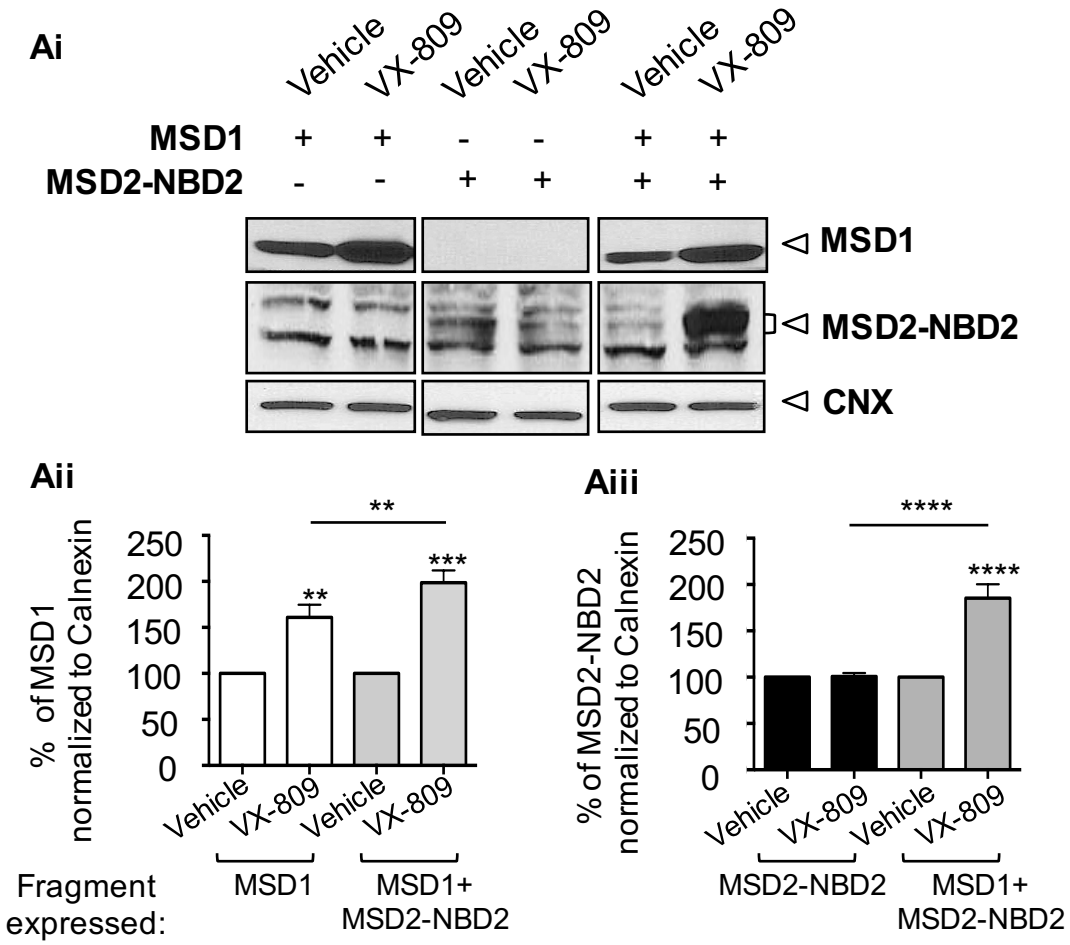
FIGURE 1



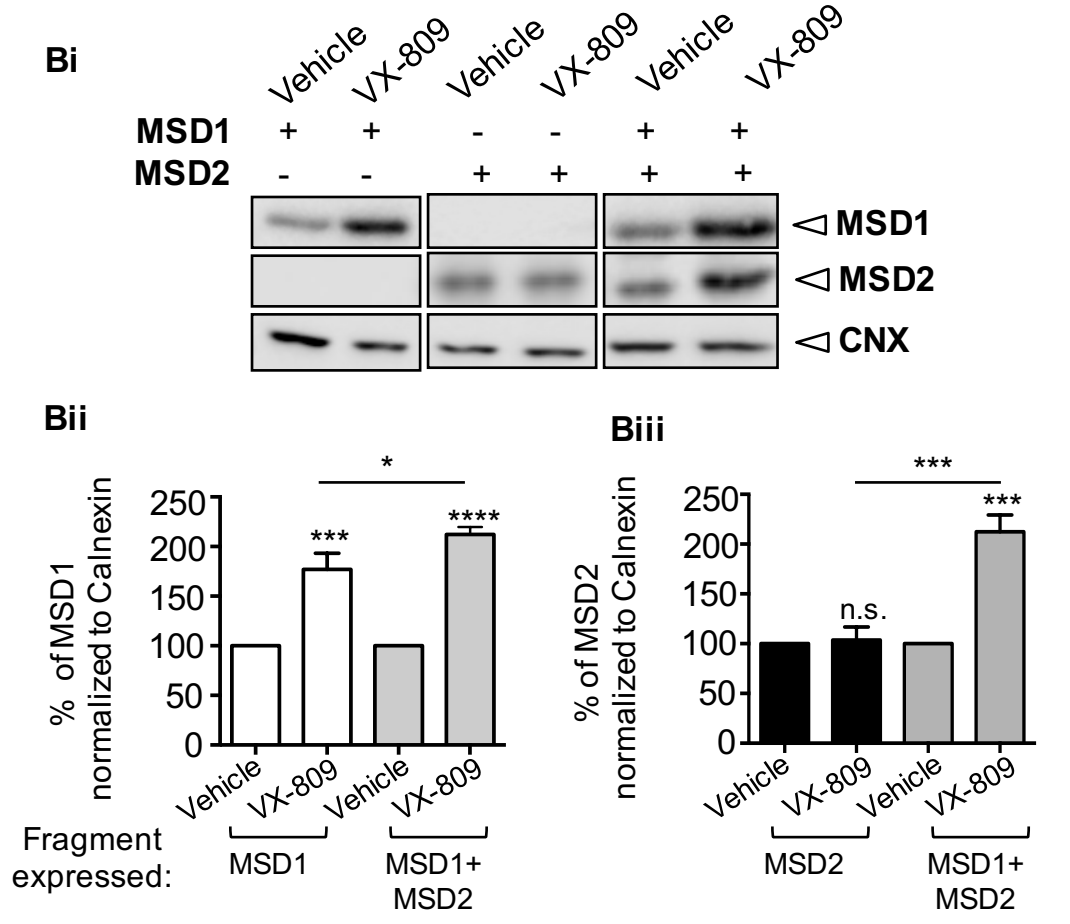


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FIGURE 2

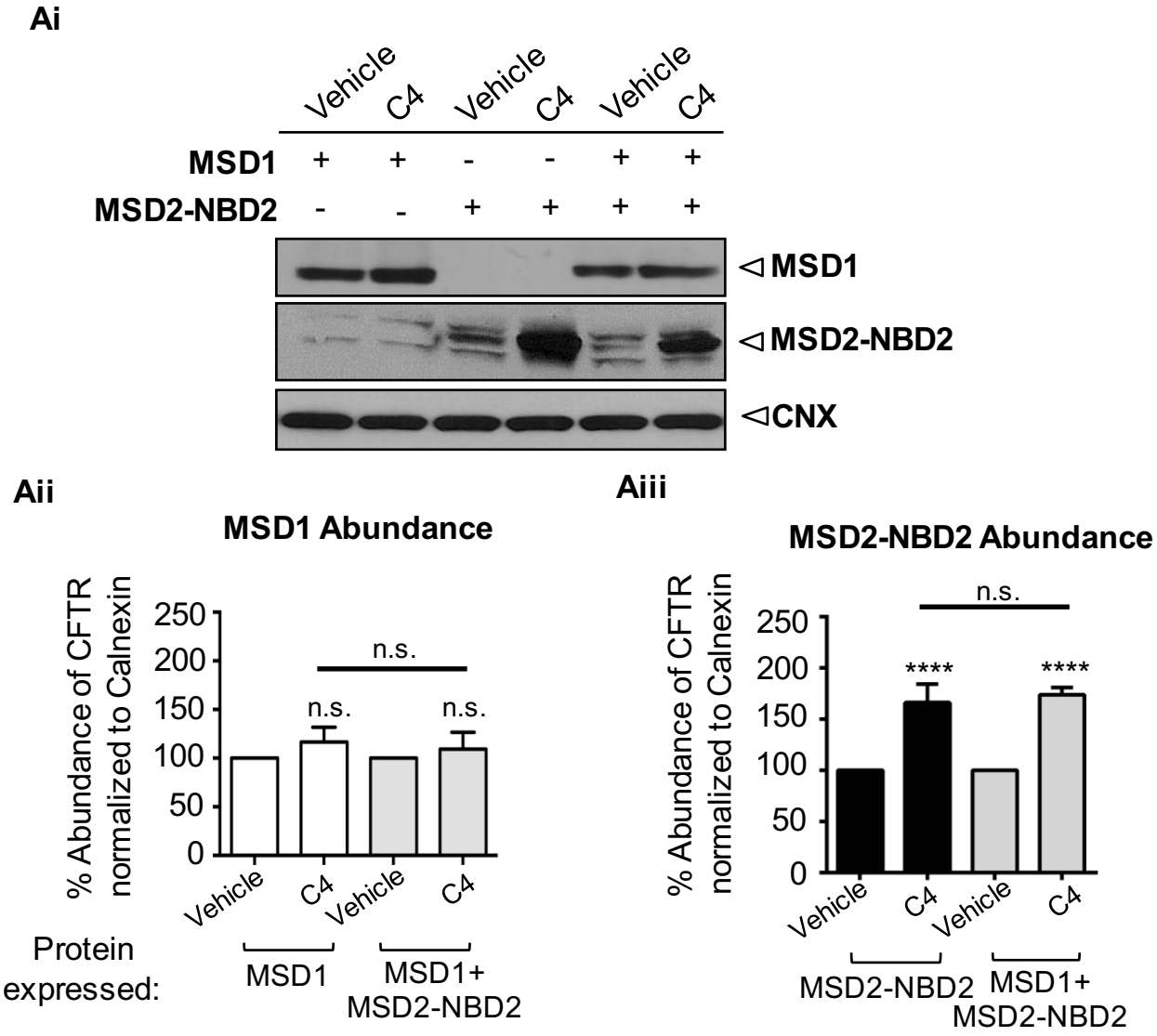

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FIGURE 3

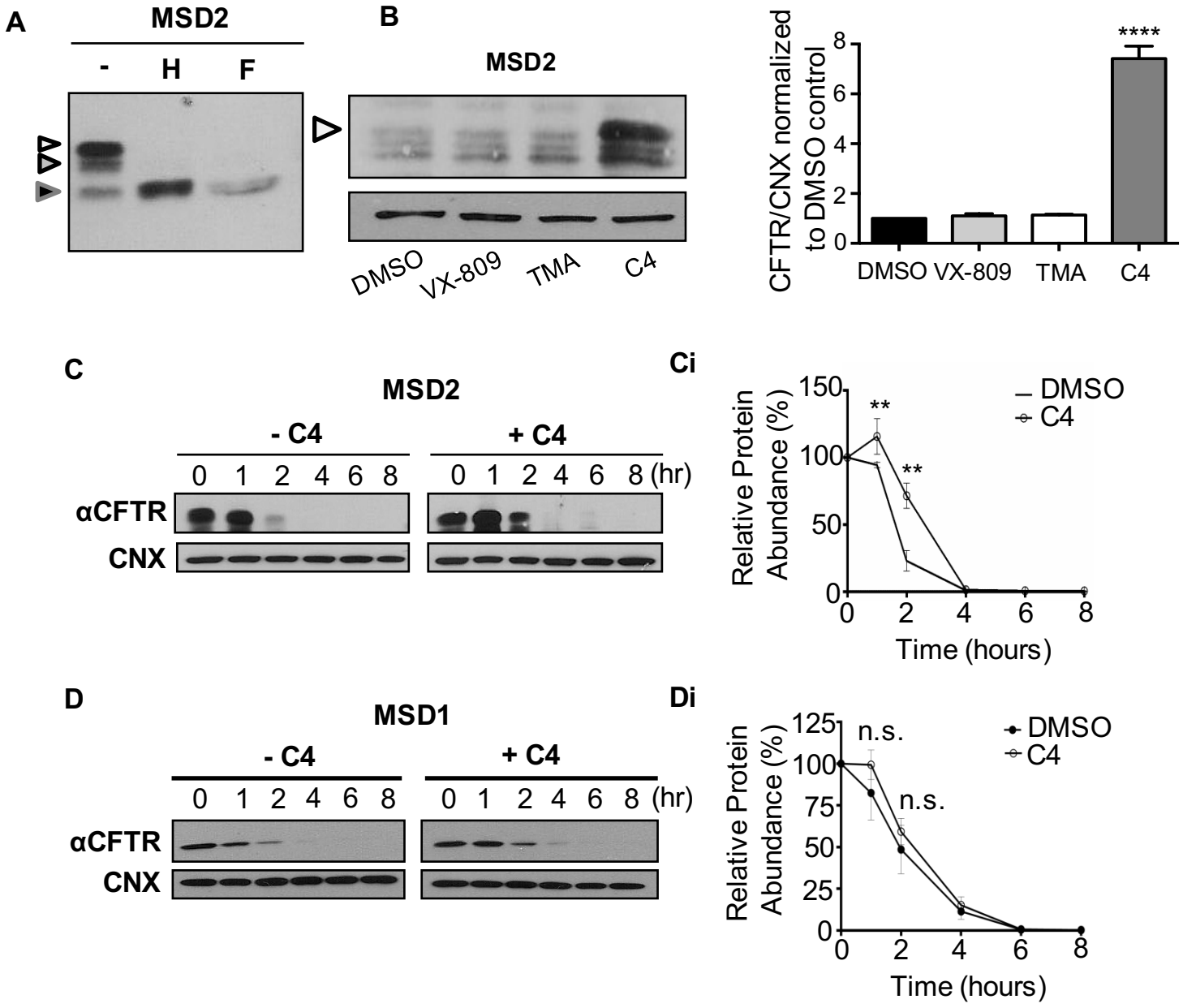


FIGURE 4

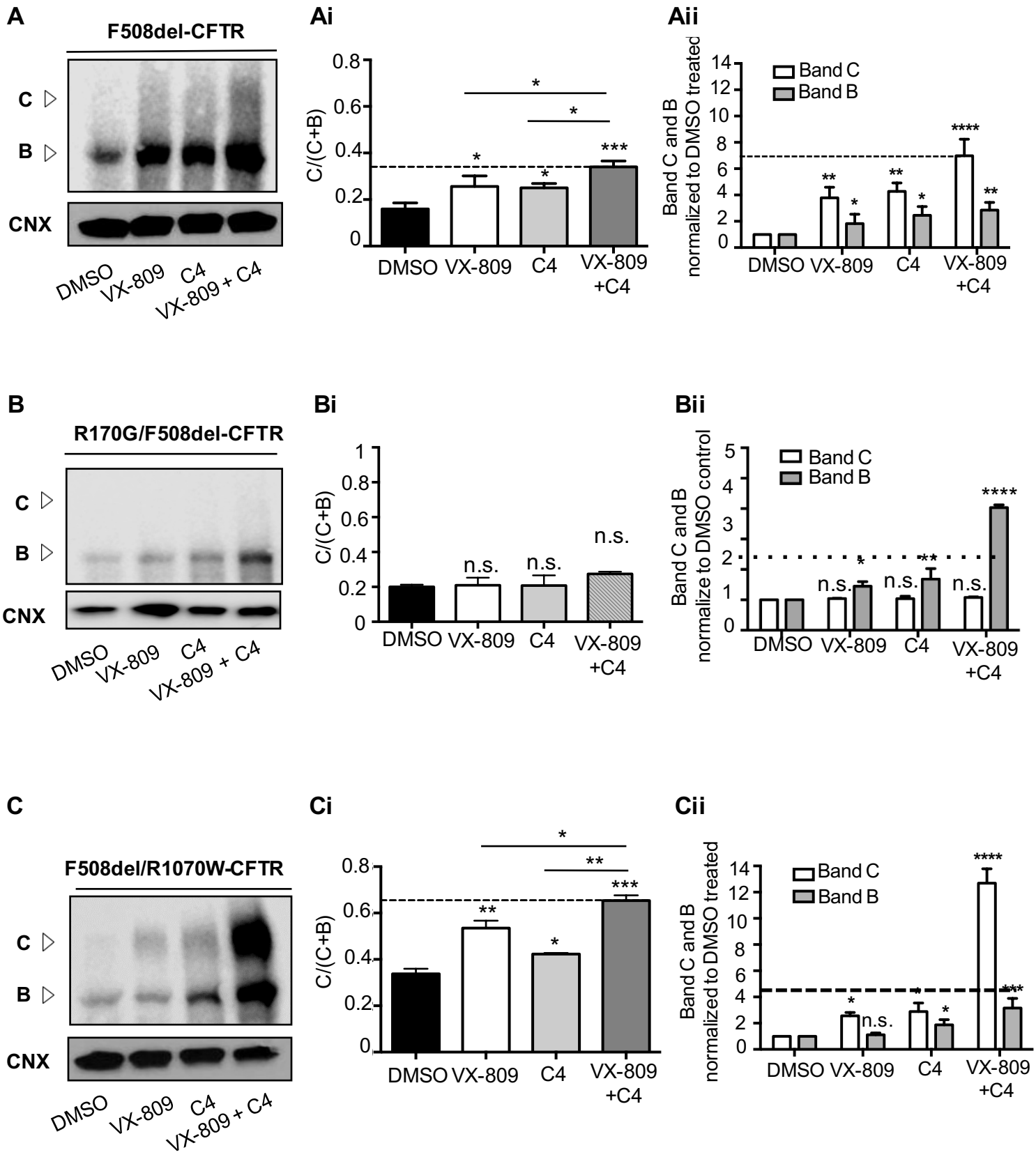
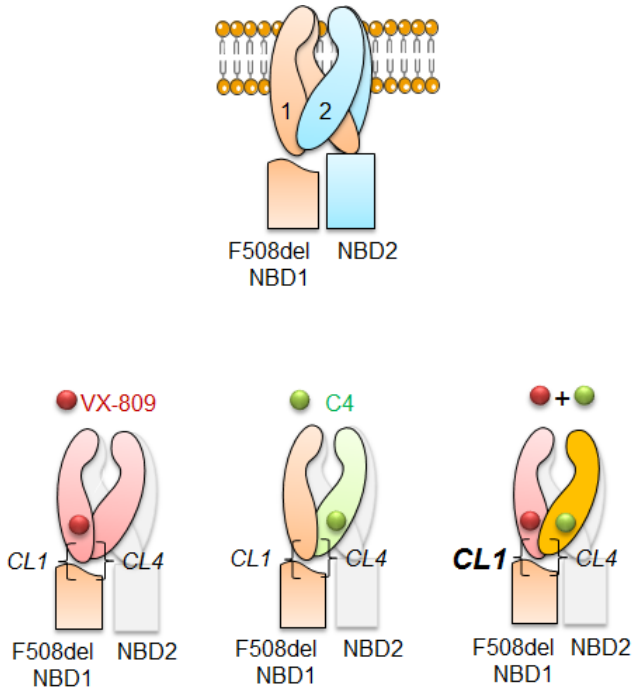

A
F508del-CFTR
C
B
CNX
DMSO
VX-809
C4
VX-809 + C4
Ai
C/(C+B)
0.8
0.6
0.4
0.2
0
DMSO
VX-809
C4
VX-809 +C4
Aii
Band C and B normalized to DMSO treated
Band C
Band B
14
12
10
8
6
4
2
0
****
B
R170G/F508del-CFTR
n.s.
Bi
Bii
Band C and B normalize to DMSO control
5
4
3
2
1
C
F508del/R1070W-CFTR
Ci
Cii

FIGURE 5


1
2
F508del
NBD1
NBD2
VX-809
CL1
CL4
F508del
NBD1
NBD2
C4
CL1
CL4
F508del
NBD1
NBD2
+
CL1
CL4
F508del
NBD1
NBD2