Supplementary Information for

A pathogenic role for cystic fibrosis transmembrane conductance regulator in celiac disease

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Appendix Supplementary Methods

Barrier function measurements: Caco-2 cells and mouse

Caco-2

The test was performed as previously described (Rauhairta *et al*, 2013). Caco-2 cells were plated at a density of 1·7 to 2 x 10^5 cells/cm2 on Transwells with a pore size of 0.4 μm (12 mm diameter on 24-well plates). Cells were maintained until steady-state transepithelial electrical resistance (TER) was 1000 to 1500 W/cm2. TER was measured using a Millicell® ERS volt-ohm meter (Millipore). Confluent monolayers were equilibrated overnight in DMEM supplemented with 1% FBS, non-essential amino acids, sodium pyruvate, sodium bicarbonate and penicillin. Thereafter, the cultures were treated with the different study compounds for up to 5 h and measurements were made at 0, 1, 3 and 5 h. The TER was given as the percentage of initial TER values to normalize for variation in absolute values between individual monolayers. All experiments were performed in triplicate at least three independent times.

Mice

Portions of intestine tissues were stripped of seromuscular layers, mounted in Ussing chambers, and tested for basal ion transport, transepithelial resistance (TER), The spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short circuited through Ag-AgCl electrodes using a voltage clamp that corrected for fluid resistance and (TER; Ω·cm2) was calculated from the spontaneous PD and short-circuit current. Permeability using methods we have described previously (Pemeability assay: Fluorescein Isothiocyanate-Dextran 4000 (FITCD4000) Test). Stable TER was used as an indicator of tissue viability for ion-transport studies.

Mice and treatments

BALB/c mice (background BALB/cAnNCrl) (Galipeau *et al*, 2011) were purchased from Charles River (Varese, Italy). Three-generation gluten-free diet (Mucedola srl, Milan),

male and female, were challenged with gliadin for one or two days, to evaluate the effects of gliadin on TER, permability and CFTR function.

The mice were challenged via gavage for with i) vehicle alone or ii) gliadin (Sigma-Aldich, G3375) (daily somministration, 20 mg/mice in 100ul saline) (Moon *et al*, 2016) (n=10 mice per group of treatment).

At the end of the last daily treatment, mice were anesthetized with Avertine (tribromoethanol, 250 mg/kg, Sigma Aldrich, T48402) and then killed; the intestines were collected for CFTR function analysis or stored for all described techniques.

These studies and procedures were approved by the local Ethics Committee for Animal Welfare (IACUC No 583,849) and conformed to the European Community regulations for animal use in research (2010/63 UE).

Halide efflux analysis

The analysis of halide efflux was performed by the iodide-sensitive fluorescent indicator, SPQ (Molecular Probes/Invitrogen), as previously described (Villella *et al*, 2013; Luciani *et al*, 2010b; Maiuri *et al*, 2008; Luciani *et al*, 2009; Luciani *et al*, 2010a). The peak of halide efflux rate (usually after Fsk plus IBMX adding) of cells was calculated in accordance with the Stern-Volmer relationship. The rates were calculated using SigmaPlot Version 7.1 for each mean fluorescence trace for each time point generated from the 50 cells examined per population per coverslip.

Transient transfection and RNA interference

Cells were transfected with TG2 siRNA or CFTR siRNA or scrambled oligonucleotides by using Lipo RnaiMax (ThermoFischer) as described (Villella *et al*, 2013; Luciani *et al*, 2010b; Maiuri *et al*, 2008). Cell lines were also transfected with pCMV-Tag2bFLAG-SQSTM1-ΔUBA (E396X) (kind gift of Dr. Lynne J. Hocking, University of Aberdeen, UK), and GFP-tagged $FYVE_{SARA}$ domain (PtdIns3P probe) (kindly provided by S. Corvera) expression vectors as described (Villella et al, 2013). Empty vectors were used as control.

Reticulocyte Lysate System

Briefly, 1 ug of pcDNA-CFTR-wilde type plasmide was incubated with mixed reagents of TnT® Coupled Reticulocyte Lysate Systems kit (Promega, L4611). To ensure the glycosilation of full lenght CFTR, the system was added with Canine Microsomal Membranes

The reaction was performed as described:

- TnT® Reticulocytes Lysate 25µl
- TnT® Reaction Buffer 2µl
- TnT® RNA Polymerase (T7) 1µl
- Amino Acid Mixture, Minus Leucine, 1mM 0.5µl
- Amino Acid Mixture, Minus Methionine, 1mM 0.5µl
- RNasin® Ribonuclease Inhibitor, 40u/µl 1µl
- pcDNA CFTR WT or Control DNA, (0.5µg/µl) 2µl
- Canine Microsomal Membranes 3 µl
- Nuclease-Free Water to a final volume of 50µl

Then, the reaction was incubated at 30-33°C for 90 min to favour the translation processing of protein. At the end of the reaction, the samples were incubated with biotinilyted P31-43 (20 µg\ml) in the presence or absence of VX-770 (10 µM) for 2 hours. The total lysates were resolved onto 8% polyacrilammide gel, after immunoprecipitation with CFTR antibody. The gel was transfered on PVDF filter and blotted with HRP-Streptavidin or CFTR antibody. Empty control pcDNA plasmide was used as control.

Limited proteolysis of CFTR.

Membran fraction (1–1.5 mg\ml protein) were digested in PBS buffer at the indicated concentration of trypsin for 15 min on ice as described (Okiyoneda et al, 2010; Du et al, 2005). Proteolysis was terminated by 0.4 mg\ml soybean trypsin inhibitor (Sigma), 2 mM MgCl2, 1 mM PMSF, 5 µg\ml leupeptin and pepstain. Digested membrane protein were either dissolved in Laemmli's sample buffer for immunoblot analysis. The protease susceptibility of the full-length CFTR was measured by immunoblotting, densitometry and expressed as the percentage of remaining CFTR relative to the non-digested sample.

GFP-tagged $FYVE_{SARA}$ detection

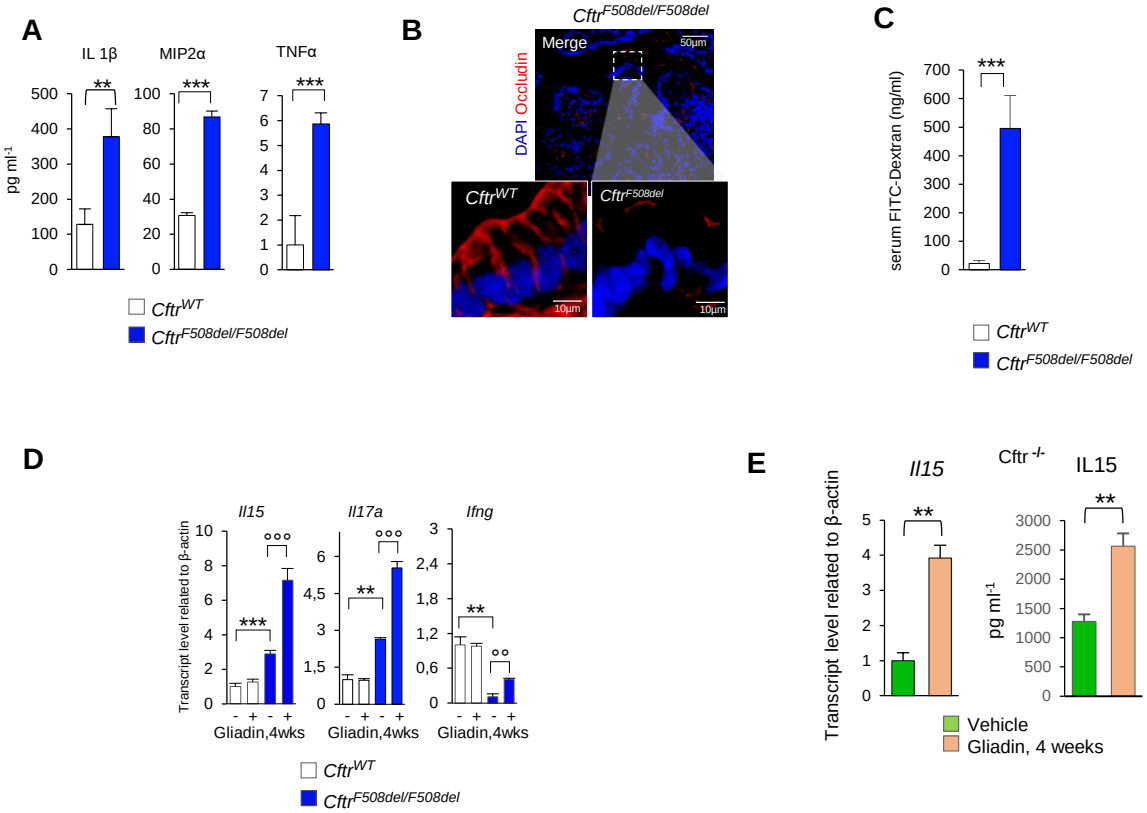
Caco-2 cells were transfected with GFP-tagged $FYVE_{SARA}$ domain plasmids and after 24 hours the cell were challenged with P31-43 in presence or absence of VX-770. After fixation with 4%PFA and saponin 0.2% permabilization the cells were incubated with

EEA1 (Abcam.ab2900) 1:300 over night at 4°C; after washing the Alexa-Fluor546 secondary antibody was added. Zeiss LSM 510 confocal laser-scanning microscope (Carl Zeiss MicroImaging) equipped with x63 oil-immersion or x40 objective were used and quantification of number of GFP-FYVESARA spots per cell was performed using the AnalySIS software (Soft Imaging Systems GmbH, Muenster, Germany) (Villella et al, 2013).

Appendix Supplementary References

Du K, Sharma M & Lukacs GL (2005) The ΔF508 cystic fibrosis mutation impairs domain-domain interactions and arrests post-translational folding of CFTR. *Nat Struct Mol Biol.* 12: 17-25

Okiyoneda T, Barrière H, Bagdány M, Rabeh WM, Du K, Höhfeld J, Young JC, Lukacs GL (2010) Peripheral Protein Quality Control Removes Unfolded CFTR from the Plasma Membrane. *Science*. 329:805-10



Appendix Figure S1

Figure S1. Gliadin responsiveness in CFTR defective mice

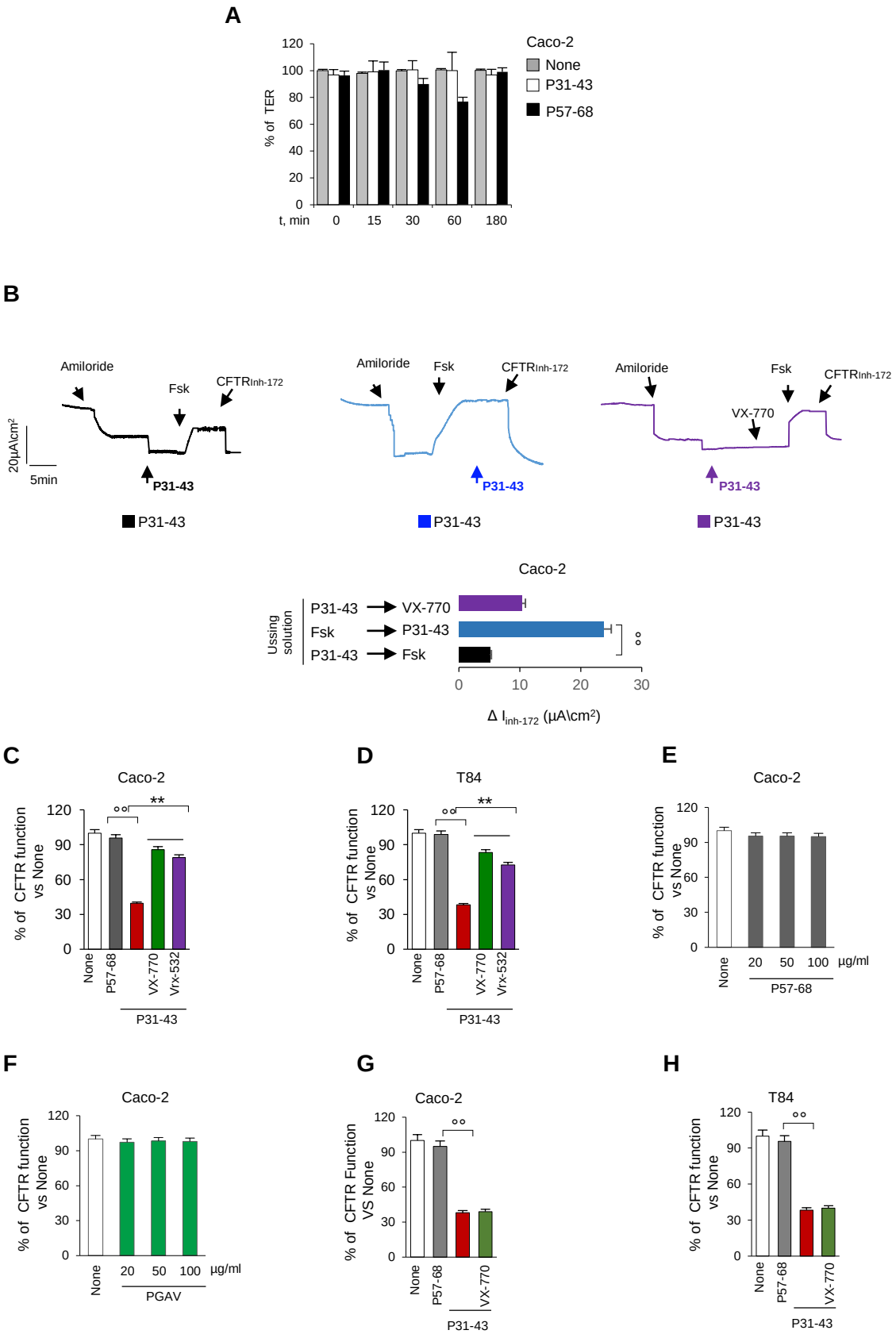
(A) IL-1β, MIP2α and TNFα production (by ELISA assay) in whole lysates from small intestine homogenates of *Cftr*$^{F508del/F508del}$ and *Cftr*^{WT}-mice. Mean ± SD of triplicates of independent pooled samples.***p<0.001 vs *Cftr*$^{F508del/F508del}$, (Student's t test).

(B) Confocal image staining with anti-occludin in the small intestine from Cftr$^{F508del/F508del}$ and WT mice. DAPI (blue), nuclear counterstaining. Scale bar, 50 µm.

(C) Plasma markers of intestinal permeability in *Cftr*$^{F508del/F508del}$ and *Cftr*^{WT} mice. Plasma concentration of FITC-dextran 4000 (FITC-D4000) measured 1 h after gavage of a single dose of 600 mg FITCD4000 per kg body weight. Quantification of plasma concentration from n=10 mice per group of treatment expressed as mean±SD .***p<0.001 vs *Cftr*$^{F508del/F508del}$, (Student's t test).

(D) Effects of 4 weeks of oral administration of gliadin (5 mg/daily for one week and then 5 mg/daily thrice a week for 3 weeks) on IL-15, IL-17A and IFN-γ transcript levels in small intestine homogenates from *Cftr*$^{F508del/F508del}$ and *Cftr*^{WT} *mice* (n =10 mice per group of treatment). Mean ± SD of triplicates of independent pooled samples; **p<0.01, ***p<0.001 (*Cftr*$^{F508del/F508del}$ vs *Cftr*^{WT} mice prior gliadin challenge), °°p<0.01, °°°p<0.001 (*Cftr*$^{F508del/F508del}$ mice vs *Cftr*$^{F508del/F508del}$ mice after gliadin challenge), (ANOVA, Bonferroni post-hoc test)**.**

(E) *Cftr*$^{-/-}$ mice treated for 4 weeks with gliadin (5 mg/daily for one week and then 5 mg/daily thrice a week for 3 weeks (n =10 mice per group of treatment). Transcript (*left*) or protein (*right*) level of IL-15 in small intestine homogenates. Mean ± SD of triplicates of independent pooled samples **p<0.01 vs gliadin treatment (Student t test).



Appendix Figure S2

Figure S2. Effect of gliadin peptides on CFTR function in intestinal epithelial cells

(A) Transepithelial resistance (TER) of Caco-2 cells measured at appropriate time-points after treatment with P31–43 or P57–68 or under untreated condition.

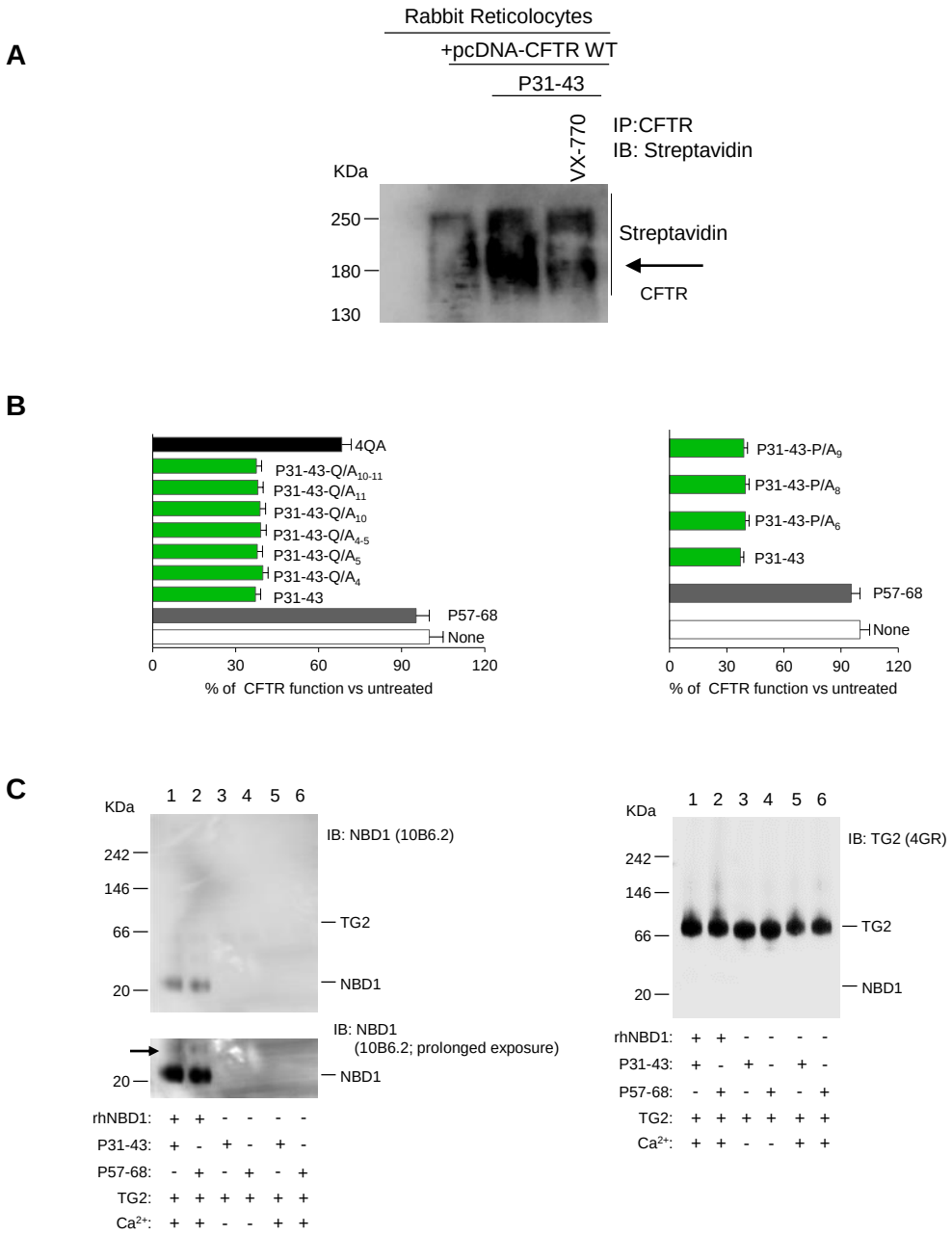
(B) Representative traces of CFTR-dependent Cl- secretion measured by forskolin (Fsk)-inducible chloride current (Isc (μA/cm2)) in Caco-2 cells mounted in Ussing chambers: effects of P31-43 (100uM) directly added to the bathing solution. After amiloride, the solutioin was directly supplied with P31-43 (100uM) (black traces) for a few minutes (as detailed in Methods) and then pulsed with forskolin (Fsk) (20uM) and finally with the CFTR inhibitor 172 (CFTRinh172). Blue traces: after amiloride, the solutioin was first pulsed with Fsk and then with P31-43 (100uM) followed by CFTRinh172 . Violet traces: after amiloride, the solutioin was first supplied with P31-43 followed by VX-770 (10uM) and then pulsed with forskolin (20uM) followed by CFTRinh172. Quantification of the peak CFTR Inhibitor 172 (CFTRinh172)-sensitive Isc (ΔIsc) in Caco-2 cells (n= 3 independent experiments). Mean ± SD of samples assayed. °°p<0.01 Fsk and then with P31-43, (ANOVA, Bonferroni post hoc test**).**

(C-D) Treatment of of Caco-2 (**C**) or T84 (**D**) cells with P57-68 or PGAV or P31-43(20 μg/ml) (3h) in the presence or absence of pre-treatment with VX-770 or Vrx-532 (20 min). Assessment of iodide efflux by SPQ fluorescent probe upon stimulation with forskolin (Fsk) expressed as percentage of CFTR function in the presence or absence of peptide challenge; °°p<0.01 vs P31-43, **p<0.01 vs VX-770 and then with P31-43 (ANOVA, Bonferroni post hoc test**)**.

(E-F) Assessment of iodide efflux, as in E-F, in Caco-2 cells treated with different concentrations of P57-68 (**E**) or PGAV (**F**)**.**

(G-H) Treatment of Caco-2 (G) and T84 (H) cells with P57-68 or P31-43 (3h) followed by 20 min of treatment with VX-770. Assessment of iodide efflux by SPQ fluorescent probe upon stimulation with forskolin (Fsk) expressed as percentage of CFTR function. °°p<0.01 vs P31-43, (ANOVA, Bonferroni post hoc test**)**.

For all experiments *n*=3 independent experiments.



Appendix Figure S3

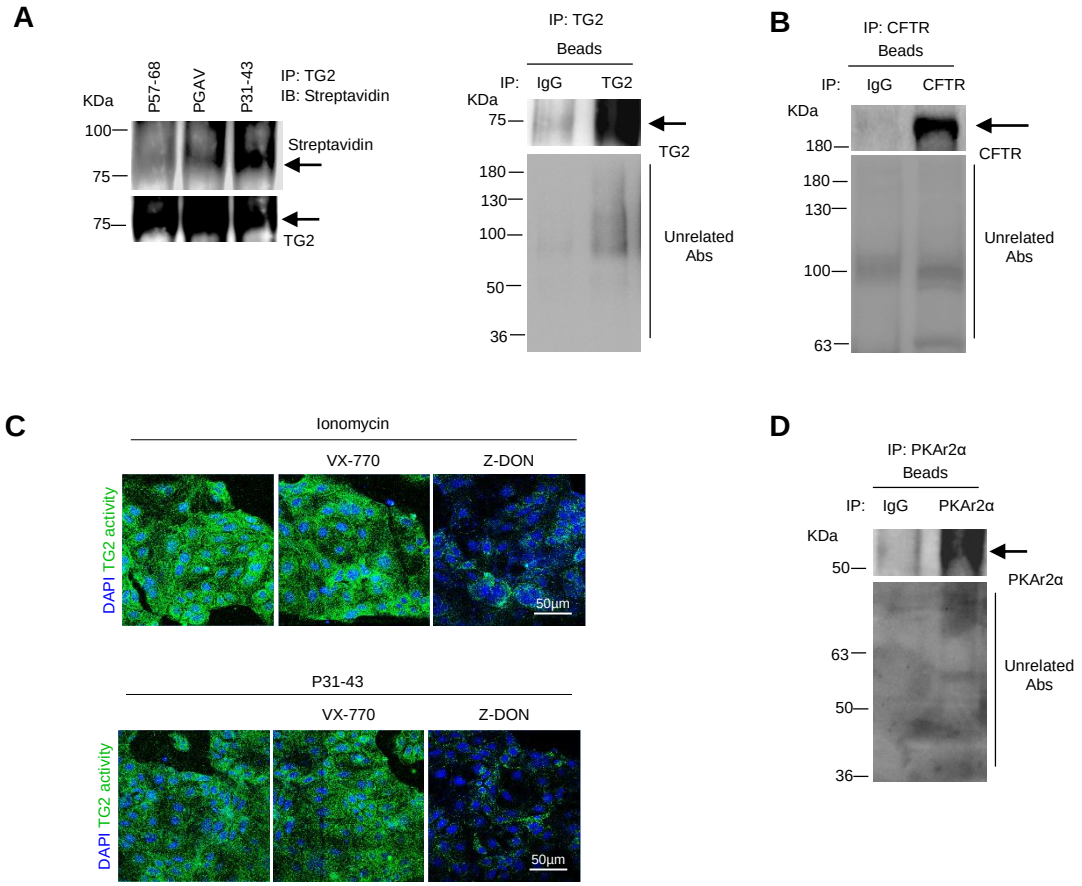
Figure S3 Interaction between TG2, P31-43 and CFTR

(A) Interaction between P31-43 and full lenght CFTR protein in vitro in a heterologous expression system. pcDNA CFTR-WT plasmide trasduced in a rabbit reticulocyte system (Promega) followed by a challenged with P31-43 in presence or absence of VX-770. Total reaction immuprecipitated with anti-CFTR antibody and blotted with HRP-Streptavidin or CFTR antibody. Empty pcDNA plasmide used as control (first line).

(B) Caco-2 challanged up to 3 hours with P57-68 or P31-43 or P31-43 modified in different Q (left) or P (right) positions. Assessment of iodide efflux by SPQ fluorescent probe upon stimulation with forskolin (Fsk) expressed as percentage of CFTR function in the presence or absence of peptide challenge.

For all experiments $n=3$ independent experiments

(C) Controls blot of Figure 3E.



Appendix Figure S4

Figure S4 Effects of P31-43 on TG2 protein and activity

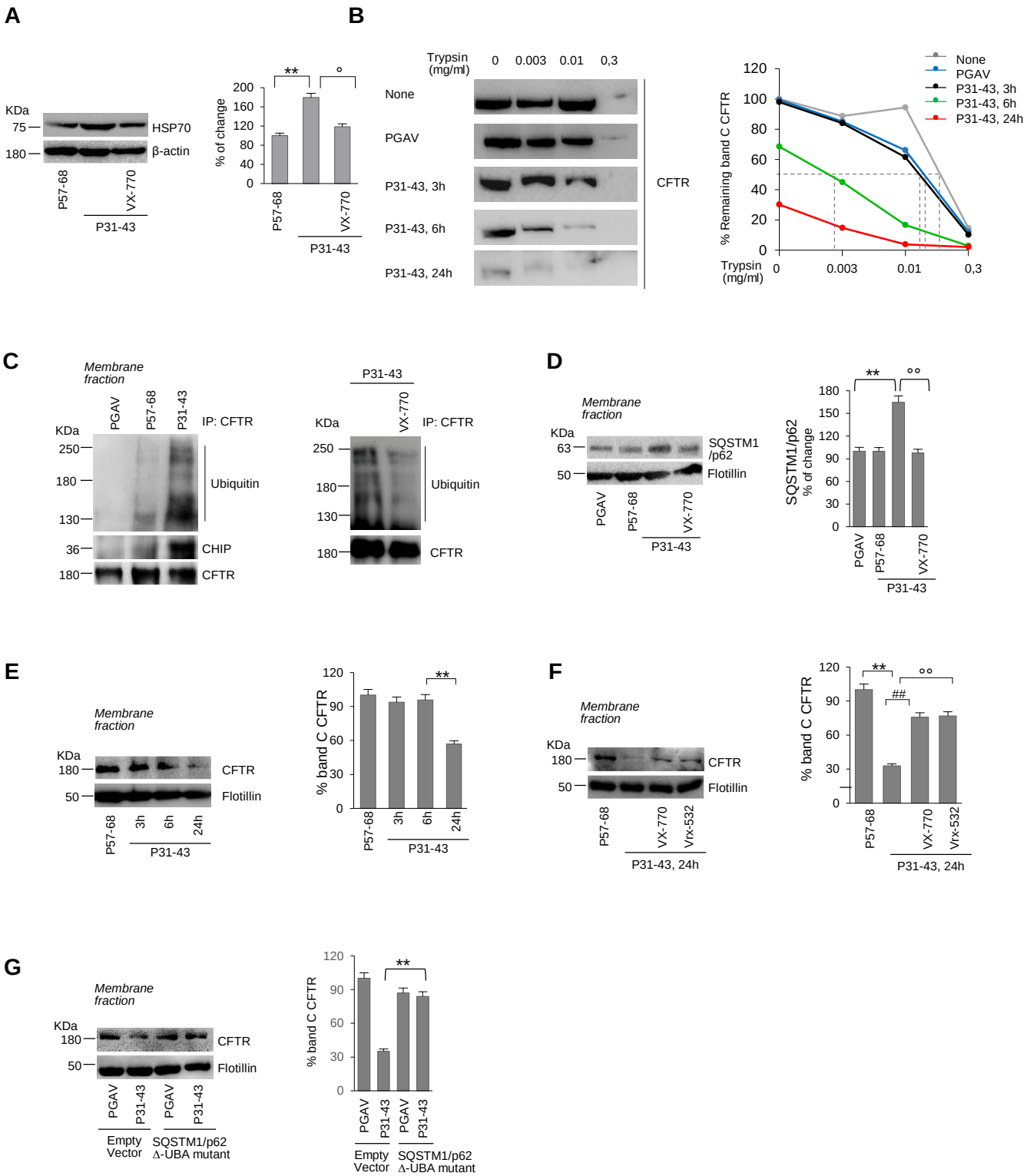
(A) Immunoprecipitation in non-reducing and non denaturing conditions of TG2 protein in Caco-2 cells challenged with P57-68, PGAV or P31-43 and blotted with HRP-streptavidin or TG2 antibody *(left*). Controls of Immunoprecipitation *(right)*.

(B) Controls of Immunoprecipitations of Figure 3 F, G, J.

(C) In situ TG2 activity assay of Caco-2 cells pre-treated with Ionomycin (*top*) or P31-43 *(bottom*) and then incubated with VX-770 or Z-DON. Activity assay by immunostaining of the TG2-catalyzed incorporation of monodansylcadaverin. Scale bar 50µm.

(D) Controls of Immunoprecipitation of Figure 3K.

For all experiments $n=3$ independent experiments.



Appendix Figure S5

Figure S5 Prevention by CFTR potentiators of P31-43 induced CFTR plasma membrane disposal

Caco-2 cells challenged with P57-68 or PGAV or P31-43 in the presence or absence of VX-770 or Vrx-532.

(A) Immunoblot of HSP-70 and β-actin (*left)*; densitometric analysis of protein levels (*right*). Mean ± SD of three independent experiments. **p<0.01 vs P31-43, °p<0.05 vs P31-43+VX-770 (ANOVA, Bonferroni Post-hoc test).

(B) Immunoblot of CFTR in Caco-2 cells treated with the indicated peptides up to 24 hours and exposed to trypsin dygestion (*left*); analysis of the percentage of remaining CFTR band C (*right).*

(C) Immunoprecipitation of CFTR protein in membrane fractions and immunblot with anti-CHIP or anti-CFTR (*left*) or anti-ubiquitin (*right*) antibodies.

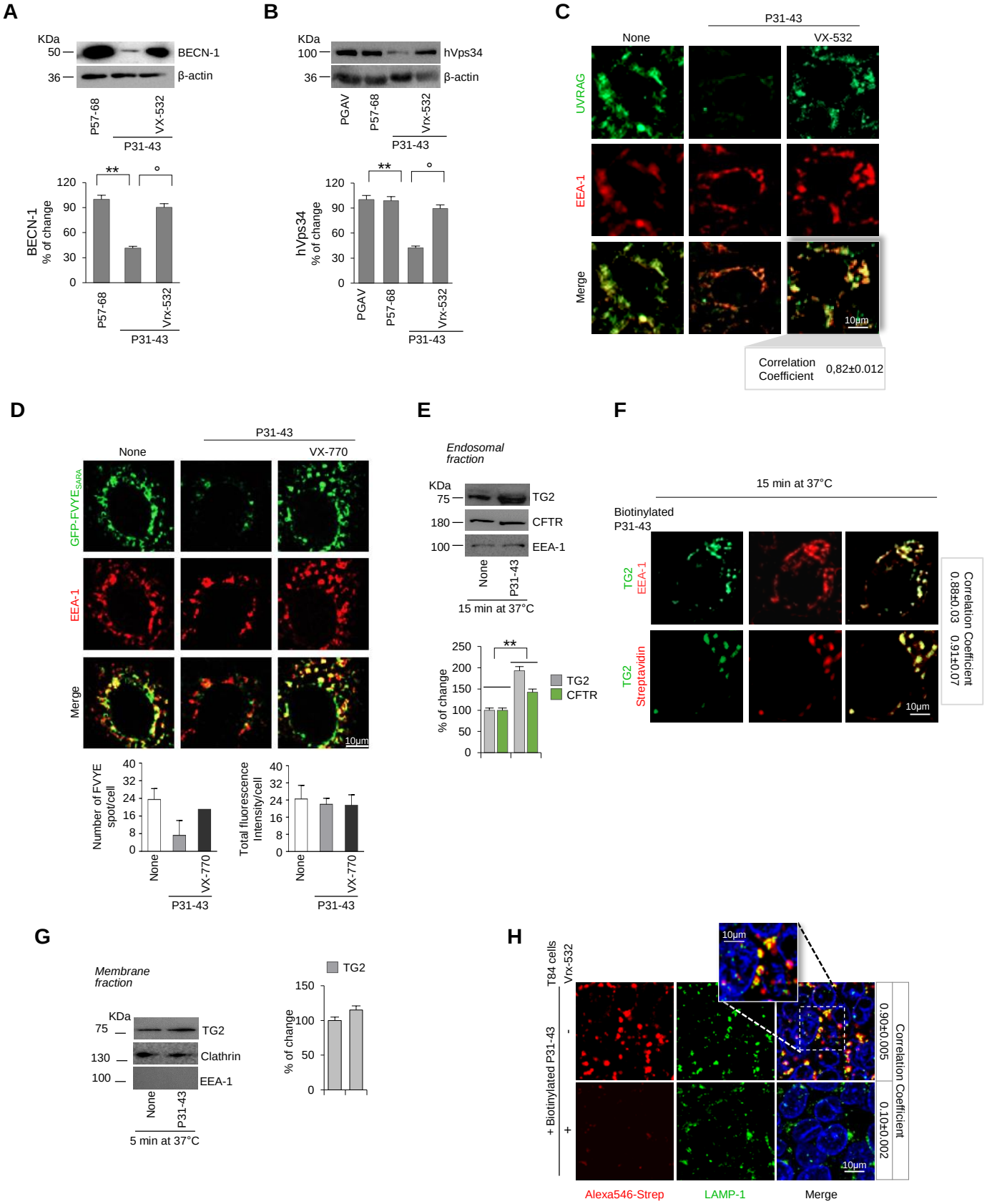
(D) Immunoblot of plasma membrane proteins with anti-SQSTM1\p62 and anti-flotillin antibodies; densitometric analysis of protein levels. Mean ± SD of three independent experiments,**p<0.01 vs P31-43 or °°p<0.01 vs p31-43+VX-770.

(E) Immunoblot of purified plasma membrane proteins at 3, 6 and 24 h of peptide challenge with anti-CFTR and Flotillin antibodies. Densitometric analysis are mean ± SD of three independent experiments; **p<0.01 P31-43 6h vs 24h.

(F) Immunoblot of CFTR and Flotillin at the PM. Densitometric analysis are mean ± SD of three independent experiments; **p<0.01 vs P31-43, ##p<0.01 vs VX-770,°°p<0.01 vs Vrx-532.

(G) Immunoblot of purified PM with anti-CFTR and anti-flotillin antibodies from cells transfected with SQSTM1-Δ-UBA mutant and challenged with peptides. Densitometric analysis of protein levels. Mean ± SD of three independent experiments,**p<0.01 vs P31-43 in presence of trasfection.

For all experiments n=3 independent experiments.



Appendix Figure S6

Figure S6 Prevention by CFTR potentiators of P31-43 induced derangement of endosomal trafficking

Caco-2 cells challenged with P57-68 or PGAV or P31-43 peptides in the presence or absence of pretreatment withVX-532 or VX-770.

(A-B) Immunoblot of BECN-1 (**A**) and phosphatidyl-inositol-3 (PI3)-kinase (hVps34) (**B**) and β-actin; densitometric analysis of protein levels (*bottom*). Mean ± SD of three independent experiments. **p<0.01 vs P31-43, °p<0.05 vs Vrx-532 (ANOVA, Bonferroni Post test).

(C) Confocal microscopy images UVRAG (green) and EEA-1 (red). Scale bar, 10 μm.

(D) Confocal microscopy images of Caco-2 cells transfected with a plasmid encoding EGFP-tagged-$FYVE_{SARA}$ after incubation with 3 hours of PGAV or P31-43 with/without Vrx-532 pre-incubation. Images of EEA1 (red) and EGFP-tagged-$FYVE_{SARA}$ (green). Scale bar, 10 μm. Number of $FYVE_{SARA}$ spots per cell were counted (*bottom*). Total fluorescence intensity per cell was used to monitor EGFP-tagged-FYVE transfection efficiency.

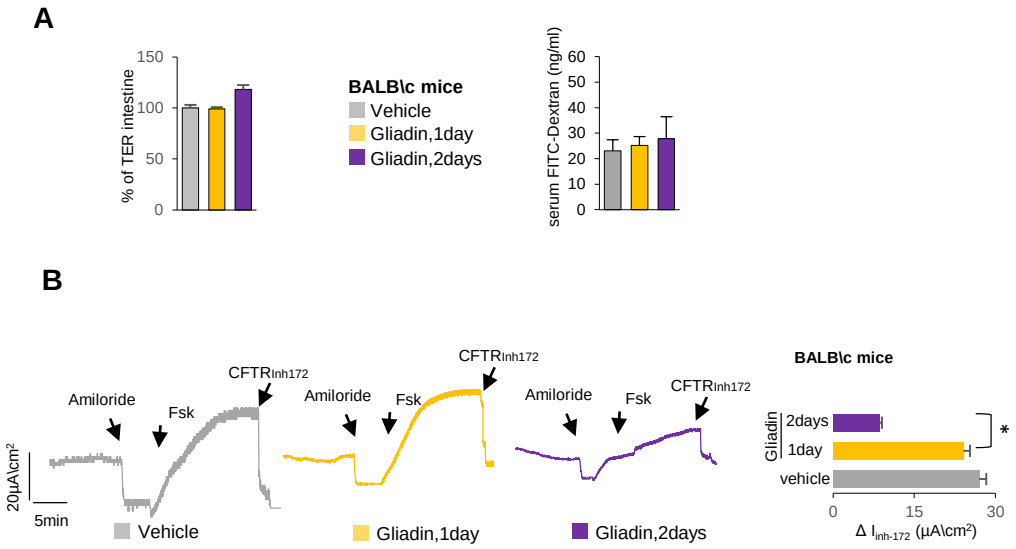
(E-F) Caco-2 cells challenged with biotinylated P31-43 for 15 minutes at 37°C.

(E) Immunoblot of purified protein from endosomal fractions with anti-TG2, anti-CFTR and anti-EEA-1 antibodies. EEA-1 was used as control of purification. Densitometric analysis of protein levels (*bottom*). Mean±SD of three independent experiments. **p<0.01 vs P31-43 (Student t Test).

(F) Confocal microscopy images, co-staining of TG2 (green) and EEA-1 (red) or TG2 (green) and Alexa-Fluor 546-Streptavidin (red) (*bottom*). Scale bar 10mm.

(G) Caco-2 cells challenged with biotinylated P31-43 for 5 minutes at 37°C. Immunoblot of purified protein from membrane fractions with anti-TG2, anti-clathrin and anti-EEA-1 antibodies. EEA-1 and clathrin used as control of purification of clathrin positive membrane fraction vesicles. Densitometric analysis of protein levels (*right*). Mean±SD of three independent experiments.

(**I**) Confocal microscopy images of T84 cells incubated with 3 hours of biotinylated-P31-43 in the presence or absence of pre-incubation of VX-532. Images of Alexa546-streptavidin (red) and LAMP-1 (green). Scale bar, 10 μm.



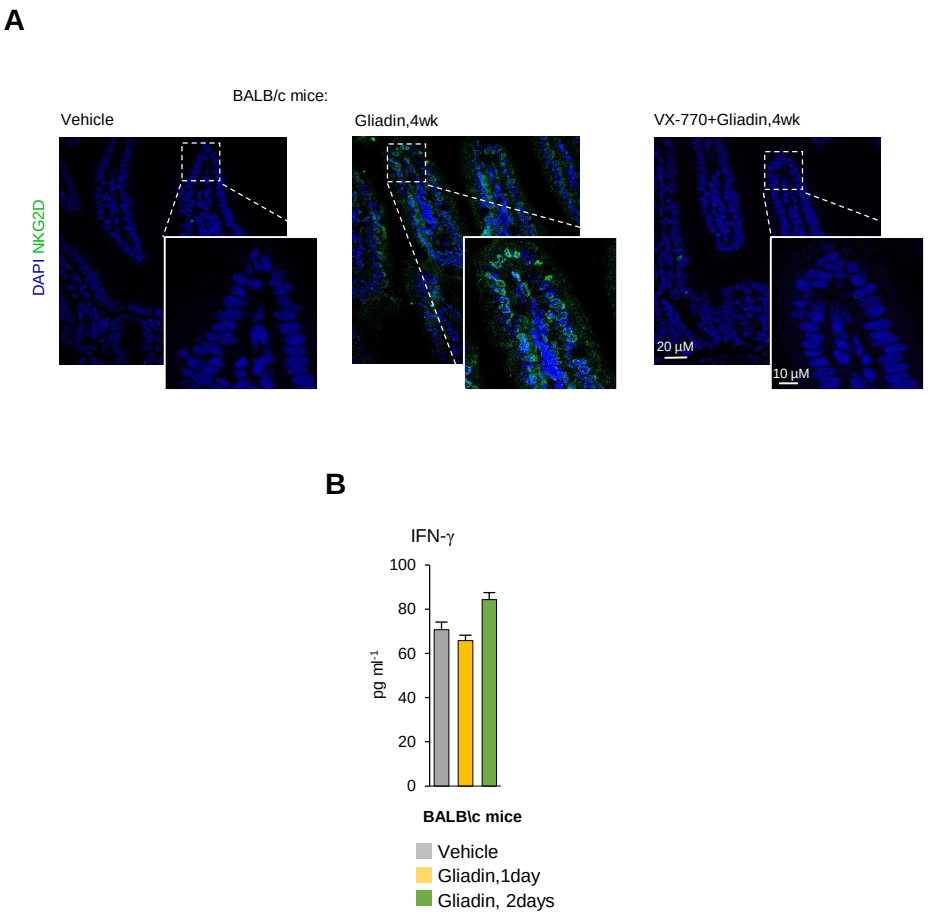
Appendix Figure S7

Figure S7 Effect of gliadin on intestinal permeability in BALB\c mice

BALB/c mice fed for at least 3 generations with a gluten-free diet, and orally challenged with vehicle or gliadin (20 mg/mice in 100 µl saline/day) for one or two days (*n*=10 mice per group of treatment).

(A) *Left,* transepithelial resistance (TER) of mouse small intestine measured at appropriate time-points after treatment with vehicle or gliadin. *Right,* plasma concentrations of FITC-dextran 4000 (FITC-D4000) measured 1 h after gavage of a single dose of 600 mg FITCD4000 per kg body weight; data from 10 mice per group of treatment expressed as mean ± SD*;*

(B) Assessment in Ussing chambers of CFTR-dependent Cl^- secretion measured by forskolin-induced (Fsk) increase of the chloride current (Isc ($\mu A/cm^2$); quantification of the peak of CFTR inhibitor 172 (CFTRinh172)-sensitive Isc (ΔIsc). *p<0.05 vs one day.



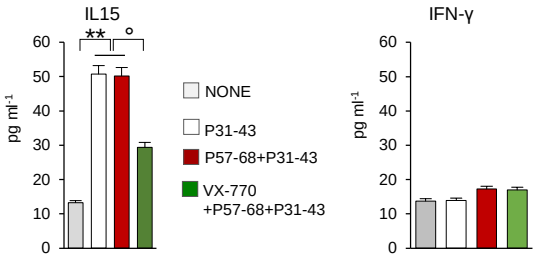
Appendix Figure S8

Figure S8 Effect of CFTR potentiators on gliadin induced immune dysregulation in BALB\c mice

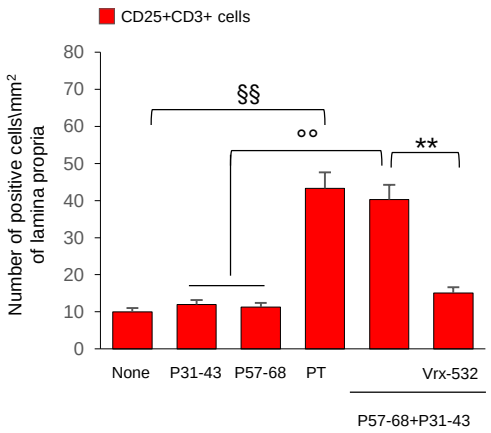
(A) BALB/c mice fed for at least 3 generations with a gluten-free diet, and orally challenged with vehicle or gliadin (20 mg/mice in 100 ul saline/day) for consecutive 4 weeks in the presence or absence of intraperitoneal administration of VX-770 15 minutes 15 minutes prior to gliadin challenge (*n*=10 mice per group of treatment). Confocal image staining with anti-NKG2D and nucleus (blue) in the small intestine. Scale bar, 20 µM.

(B) BALB/c mice fed for at least 3 generations with a gluten-free diet and orally challenged with vehicle or gliadin (20 mg/mice in 100ul saline/day) for one or two days (*n*=10 mice per group of treatment). IFNγ production (by ELISA assay) in whole lysates from small intestine homogenates. Mean ± SD of of triplicates of independent pooled samples.

A



B



Appendix Figure S9

Figure S9 Effect of P31-43 challange ex vivo in celiac cells and tissues

(A) Caco-2 cells cultured in a bidimentional system in absence of PBMC. Caco-2 challenged with P31-43 or combinantion P57-68+P31-43 with or without VX-770. IL-15 (*left*) or IFN-γ (*right*) protein release in the lower compartment. Mean ± SD of triplicates of independent pooled samples; **p<0.01 vs P31-43 or combination,°p<0.05 vs VX-770 treatment (ANOVA, Bonferroni post-hoc test).

(**B**) Duodenal biopsies from 5 celiac patients cultured ex vivo with medium or P31-43 or P57-68 or a PT-gliadin digest or with the combination of P31-43 or P57-68 with or without pretreatment with Vrx-532. Number of CD3+/CD25+ cells per mm^2 of lamina propria; §§p<0.01 PT vs None, °°p<0.01 P57-68 or P31-43 vs combination of P31-43 and P57-68; **p<0.01 peptide combination vs treatment with Vrx-532 (ANOVA, Bonferroni post test).

Patients	N°	Percentage
Cystic Fibrosis	288	
CF patients with:		
Specific antibody response to gluten	12	4.16%
Diagnosis of CD **(a)**	8	2.78%

a. Data from children with Cystic Fibrosis who have received diagnosis of Celiac Disease

Patient	CF Genotype	CI	tTG IgA	EMA IgA	Duodenal histology^	HLA-DQ
P1	F508del/F508del	104	76.1	+	T3b	DQ8
P2	2789+5G>A/Y849X	106	21.4	+	T3c	N.A.
P3	G542X/2184insA	94	17.9	+	T3a	DQ2
P4	F505del/D1152H	44	>200	+	T3c	DQA1*03
P5	F508del/L732X1	80	>200	+	T3c	DQ2
P6	171711G>A/D579G	67	23.3	+	T3b	DQ2
P7	D1152H/D579G	15	>200	+	T3c	DQ2
P8	F508del/F508del	128	161.6	+	T3a	DQ2

b. Data from children with Cystic Fibrosis who have shown fluctuant serological anti-tissue anti-transglutaminase and anti-endomysial antibodies above the cut-off level at least once.

Patient	CF Genotype	CI	tTG IgA*	EMA IgA*	Duodenal histology^	HLD-DQ2
P9	N1303K/G1244E	98	+	+	N.A	DQ2
P10	F508del/G542X	109	+	+	T0	DQ2
P11	F508del/F508del	85	+	+	N.A	DQA1*05
P12	G542X/4016insT	79	+	+	T0	DQB1*06

N.A. Not available; CF Cystic Fibrosis; tTG anti-tissue transglutaminase; EMA anti-endomysial antibodies ^, Marsh classification of intestinal lesion; *, highest antibody titer above the cut-off level registered at leat once during the screening

Appendix Table S1. Prevalence of Celiac Disease in a cohort of 288 patients with Cystic Fibrosis

A retrospective cohort study on a total of 288 participants < 18 years old attending the Pediatric Cystic Fibrosis Care Unit of the University of Naples Federico II during the average 6 years of follow-up detected 2.78% of CD diagnosis, according to the criteria of the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), and 4.16% of specific antibody response to gluten at least once. These data are consistent with those reported in a CF Polish cohort (2.13% prevalence of CD and an overall presence of 4.6% of positive anti-TG2-IgA autoantibodies, even in the absence of villous atrophy (Walkowiak et al, 2010).