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## **Evaluation of Aminopyrrolidine Amide to improve chloride transport in CFTR-defective cells**

Florentin Huguet<sup>1</sup>, Julie Guellec<sup>1</sup>, Mathieu Kerbiriou<sup>1</sup>, Maxime Gandy<sup>1</sup>, Johan Thomas<sup>1</sup>, Claude Férec<sup>1</sup>, Nathalie Benz<sup>1</sup>, Pascal Trouvé<sup>1\*</sup>

<sup>1</sup> Inserm, Univ Brest, EFS, UMR 1078, GGB, F-29200 Brest, France

**\* Corresponding author:**

Pascal Trouvé (ORCID ID: <https://orcid.org/0000-0002-4675-6394>)  
Inserm UMR 1078, Génétique, Génomique Fonctionnelle et  
Biotechnologies.  
Faculté de médecine, bâtiment IBRBS  
22 avenue Camille Desmoulins  
29238 BREST Cedex 3  
France  
Tel: +33 (0)2.98.01.82.17  
[pascal.trouve@inserm.fr](mailto:pascal.trouve@inserm.fr)

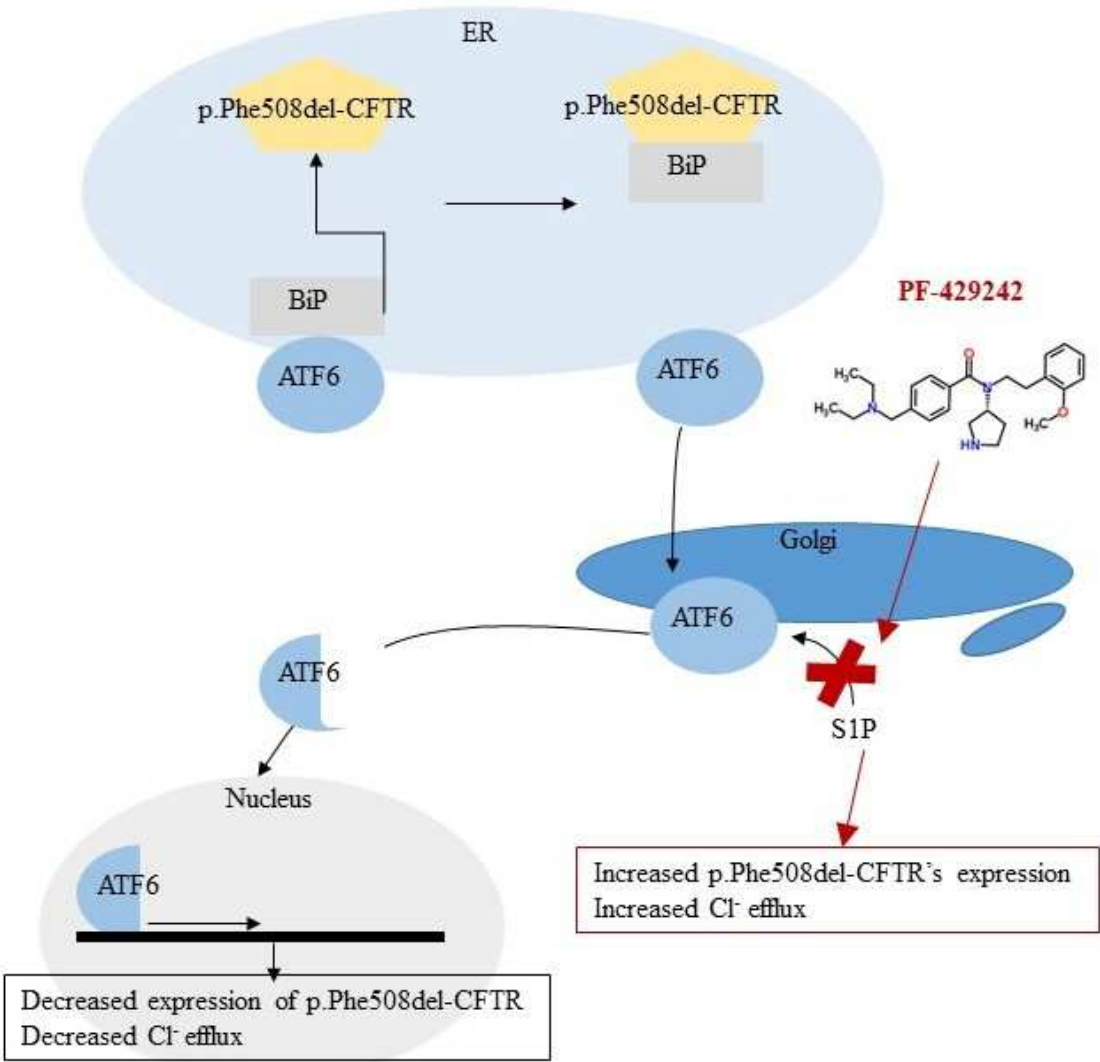
**Key words:** Aminopyrrolidine amide (PF-429242), ATF6, S1P, UPR, CFTR

**Abbreviations:** Activating Transcription Factor 6: ATF6; Site-1 Protease: S1P; Unfolded Protein Response: UPR; Cystic Fibrosis: CF; Cystic Fibrosis Transmembrane conductance Regulator: CFTR

## **Abstract**

The aminopyrrolidine amide PF-429242 is a specific inhibitor of the Site-1 Protease which is responsible for the cleavage, and thus the activation of the Activating Transcription Factor 6 that down regulates many genes, during the Unfolded Protein Response. We hypothesized that PF-429242 could be used to prevent the ATF6-dependent down regulation of some genes. We chose the *CFTR* gene encoding the CFTR chloride channel as a model because it is down-regulated by ATF6 in Cystic Fibrosis. We evaluated the action of PF-429242 in human bronchial cells expressing the most frequent mutation of *CFTR* (p.Phe508del) found in patients. We observed that PF-429242 increases the synthesis of the mRNA and the protein encoded by the *CFTR* gene harbouring the mutation. We also observed that PF-429242 alleviates the defects of the p.Phe508del-CFTR channel in human Cystic Fibrosis cells. Our results suggest that aminopyrrolidine amide is a potential therapeutic target for Cystic Fibrosis that could also have beneficial effects in other diseases involving CFTR, such as the Chronic Obstructive Pulmonary Disease.

Graphical abstract



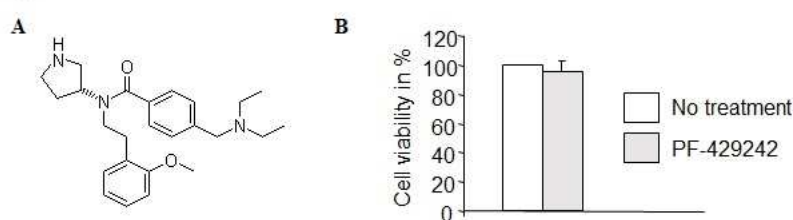
## 1. Introduction

Aminopyrrolidine amide (PF-429242) is a competitive and reversible inhibitor of the Site-1 Protease (S1P) which is a unique membrane-bound serine protease of the Golgi apparatus [1-3]. PF-429242 was obtained by high throughput screening of Pfizer's compounds [1, 2]. S1P is responsible for cholesterol-sensitive cleavage of the transcription factor sterol regulatory element binding protein (SREBP) that regulates numerous lipogenic genes involved in the cholesterol and fatty acid synthesis [4]. It also cleaves the Activating Transcription Factor 6 (ATF6) which is one of the three arms of the Unfolded Protein Response (UPR) that is triggered when cells are exposed to a stress such as the retention of misfolded proteins within the endoplasmic reticulum (ER), infections or inflammation [5, 6]. UPR is a normal physiological cellular recovery process regulating the protein load in the ER [7]. When it is triggered, ATF6 is cleaved by S1P and the cleaved form is translocated to the nucleus where it initiates a transcriptional program to restore ER homeostasis by promoting the synthesis of chaperone proteins, by regulating lipid synthesis, by stimulating ER-associated protein degradation and by enhancing N-glycosylation [8, 9]. Nevertheless, it also negatively regulates many genes. The *Cystic Fibrosis Transmembrane conductance Regulator* (*CFTR*) gene which is involved in Cystic Fibrosis (CF) is one of these down regulated genes [10-13]. It encodes the CFTR protein which acts as a chloride (Cl<sup>-</sup>) channel in the membrane of the cells, that maintains, in a reciprocal interaction with the Epithelial Sodium Channel (ENaC), the proper hydration of the epithelial mucus layer [14-16]. In CF, more than 2106 gene variants have been identified, the most frequent and clinically relevant being p.Phe508del. It affects the synthesis, the membrane localization and the function of the encoded protein (p.Phe508del-CFTR) [17-18]. The consequence in the lung, together with the lost inhibition of ENaC, is a more viscous mucus that diminishes the mucociliary clearance and enables pathogens to develop, inducing chronic infection and inflammation [19-22]. Whereas the altered lung function is the main cause of morbidity and mortality in CF, many organs are affected such as those from the digestive and the reproductive tracts.

Because PF-429242 inhibits S1P, we hypothesized that it could prevent the down-regulation of target genes of ATF6 such as *CFTR*. We chose *CFTR* as a model because it is involved in a lethal genetic disease, because ATF6 is cleaved in CF and because both the expression and the function of the CFTR channel can be classically studied.

Our hypothesis was tested in human bronchial cells expressing the p.Phe508del mutation. We used the CFBE41o- cells which are immortalized human bronchial epithelial cells from a CF homozygous patient for the P.Phe508del mutation [23-25]. They were transduced to express the wild-type CFTR (CFBE41o-/wt) or the mutated CFTR (CFBE41o-/P.Phe508del), in order to have control and CF cells with the same genetic background [25]. They are the main cells used in CF research and were thus cultured as previously described [24]. To inhibit the cleavage of ATF6, cells were incubated with 10 $\mu$ M Aminopyrrolidine amide (4-[[Diethylamino]methyl]-N-[2-[2-methoxyphenyl] ethyl]-N-[3R]-3-pyrrolidinylbenzamide, PF-429242, Tocris) (Fig. 1A). We tested its toxicity in our cell model despite it did not show any toxicity in mice [1]. We used a colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium bromide) test (EMD Millipore, USA), according to the manufacturer's instructions. The assay showed that PF-429242 did not induce any toxicity in our cells (Fig. 1B).

**Figure 1**



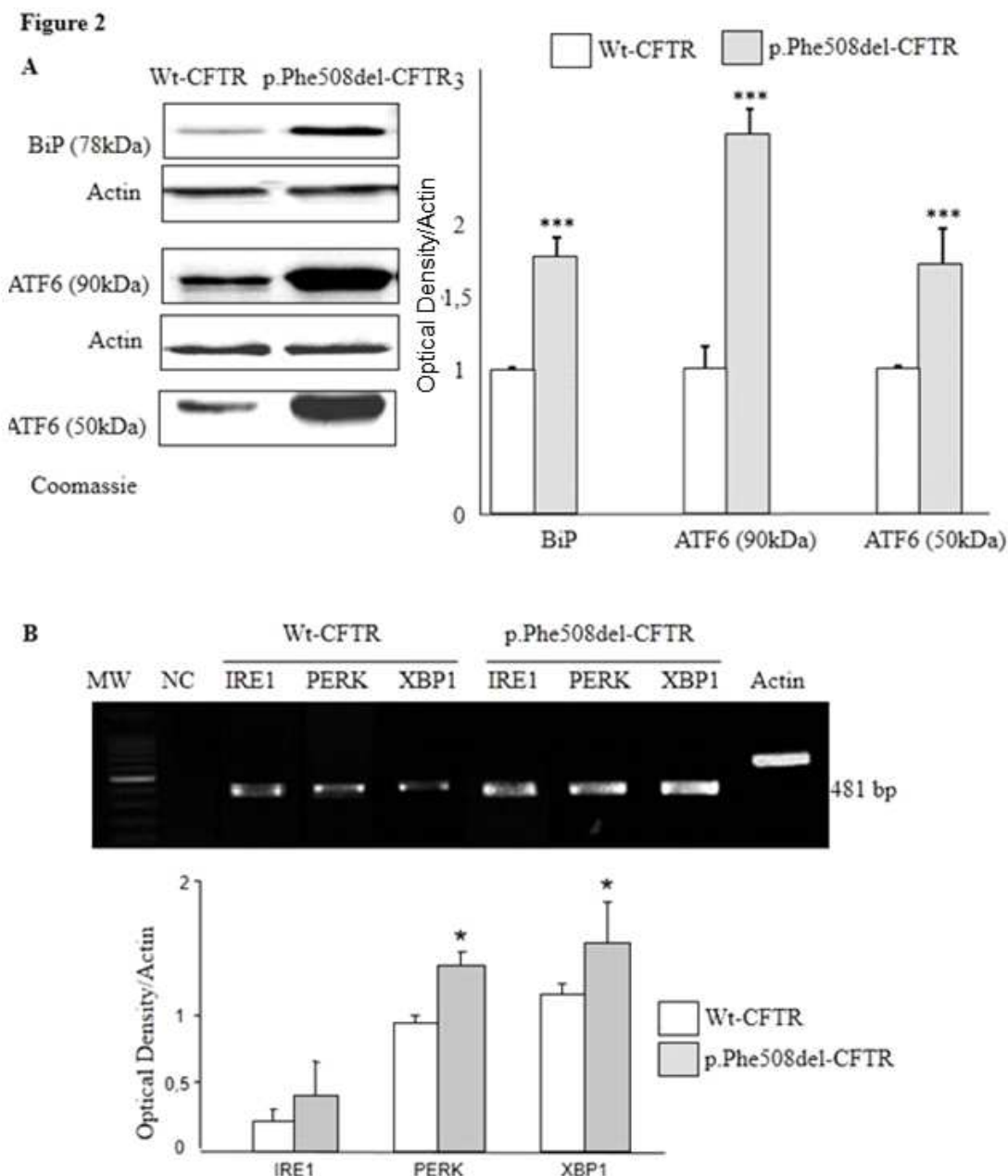
**Figure 1. Cell viability**

**A.** Molecular representation of PF-429242. **B.** Cells were treated (10  $\mu$ M, 48 hours; n=5) and their viability was assessed and compared to that of untreated cells (n=5). The bar graph is the representation of the statistical analysis which indicates that PF-429242 has no toxic effect in our cell model.

In eukaryotic cells the ER is involved in the synthesis and assembly of membrane and secreted proteins. These processes are tightly controlled by the ER quality control system to ensure the proper conformation of proteins [26]. This is important because the accumulation of misfolded proteins in the ER represents a cellular threat called ER stress [27]. To cope with the ER stress that can also be due to inflammation or infection, the cells trigger a physiological signalling increasing the folding, the maturation and/or the degradation of the proteins overloading the ER. This process is called the UPR [6, 28]. The main sensor of the UPR is the molecular chaperone immunoglobulin heavy chain-binding protein/glucose regulated protein of 78 kDa (BiP/GRP78) which detects the presence of unfolded proteins [29]. Three ER membrane proteins convey this information to the two primary effectors systems, the ER stress response element (ERSE) and the UPR response element (UPRE). These three proteins are the protein kinase RNA-like ER kinase (PERK) [30], the inositol-requiring enzyme 1 (IRE1  $\alpha/\beta$ ) [31] and ATF6 [32]. They define the three axes of the UPR in mammals. BiP is therefore considered as the main regulator of the UPR and is commonly used as a marker of the UPR [33].

BiP expression was assessed in cell lysates of CFBE41o/wt and CFBE41o-/P.Phe508del cells by western blots (Fig. 2A, left panel) which were performed as previously described, using a rabbit polyclonal anti-GRP78 primary antibody (SC-13968, SantaCruz Biotechnology) and a horseradish peroxidase conjugated secondary antibody (1/20,000, Goat anti-rabbit A0545, Servilab) [11]. We also assessed the expression of the full-length ATF6 (90 kDa) using a rabbit polyclonal anti-ATF6 antibody (09-069, EMD Millipore) (Fig. 2A, left panel). Because during UPR ATF6 is cleaved by S1P and its active form (50 kDa) is translocated to the nucleus, we assessed the presence of this active form by western blottings, in nuclear fractions (Fig. 2A, left panel) which were prepared as previously described [11]. The statistical analysis of the signals showed that the expression of BiP, ATF6 and of the cleaved form of ATF6 were significantly increased in CFBE41o-/P.Phe508del cells, comparatively to the cells expressing the wt-CFTR (Fig. 2A, right panel). The triggering of UPR in our cells was further shown by the increased expression of the mRNA of IRE1, PERK and XBP1 which showed that IRE1 is active [34], by RT-PCR (Fig. 2B, upper panel). The primers were for IRE1 $\alpha$ , forward: 5'-gaaaaggaatccctggatgg and reverse: 5'-tcagagggcgtctggagtc; for XBP1, forward: 5'-cagcgcttggggatggatgc and reverse: 5'-gggcttggtatatatgtgg; for PERK, forward: gactacatatggactcagtc5'- and reverse:

agatgtcctcccttcttac 5'-; for Actin, forward: 5'- gttgctatccaggctgtg and reverse: 5'- cactgtgttggtacag. The thermal cycling conditions were 95 °C for 15 min followed by 40 cycles at 94 °C for 30 sec, 61 °C for 30 sec and 72 °C for 1 min and a final extension at 72 °C for 10 min. The housekeeping  $\beta$ -actin gene was used as a control. We observed that all mRNA were significantly increased in the cells expressing P.Phe508del-CFTR (Fig. 2B, lower panel). Therefore, UPR was activated in our CFTR-defective cell model, as previously suggested [11, 35, 36].



**Figure 2. UPR is triggered in CFBE41o-/P.Phe508del cells.**

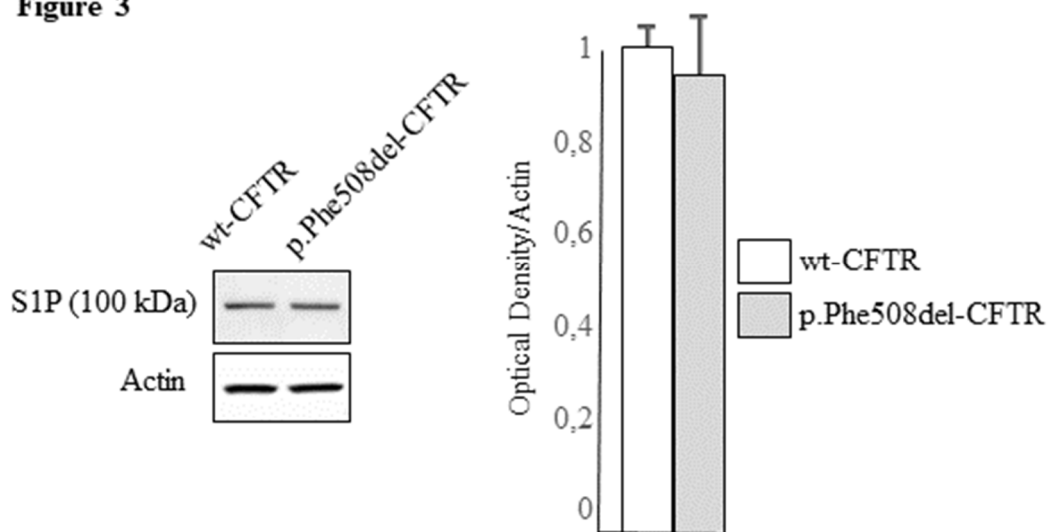
**A.** The left panel shows examples of the detection of BiP and ATF6 proteins in total lysates of CFBE41o- cells overexpressing either the wt- or the P.Phe508del-CFTR. The cleaved form of ATF6 (50kDa) was detected in the nuclear fractions from the same cells. The right panel is the bar graph representation of the statistical analysis of the expression of BiP (n = 6), ATF6 90 kDa (n = 6) and ATF6 50 kDa (n = 5) in CFBE41o-/wt and in CFBE41o-/P.Phe508del cells. The optical densities of the bands shown in the left panel were normalized by the optical density of Actin. The normalized optical densities in wt- and in the P.Phe508del-CFTR cells were then compared (Student's t test). A significant increased expression of BiP and ATF6 (90 kDa form) was observed in the cells expressing the mutated CFTR. The active form of ATF6 (50 kDa) was increased in the nuclear fractions of cells expressing the P.Phe508del-CFTR protein. We observed a residual presence of this form of ATF6 in CFBE41o-/wt cells. **B.** The upper image is a representative mRNA detection of IRE1, PERK and XBP1. The lower panel is the bar graph representation of the statistical



analysis. The mRNA expression of PERK and XBP1 were significantly increased in P.Phe508del-CFTR cells. The augmented expression of IRE1 was not significant but the expression of XBP1 shows that it is activated. The results are expressed as mean  $\pm$  standard error of the mean (SEM). Differences between experimental groups were evaluated by a two-tailed unpaired Student's t test and were considered statistically significant when  $p < 0.05$  (\*);  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*)).

Finally, because S1P is the target of PF-429242, we compared its expression between cells expressing wt-CFTR and p.Phe508del-CFTR by western blots using a polyclonal anti-S1P antibody (SC-20757, SantaCruz Biotechnology). No significant difference in the expression of S1P was observed, indicating that the observed effects of PF-429242 are due to the drug itself and not to a decreased expression of S1P in our model. (Fig. 3).

**Figure 3**



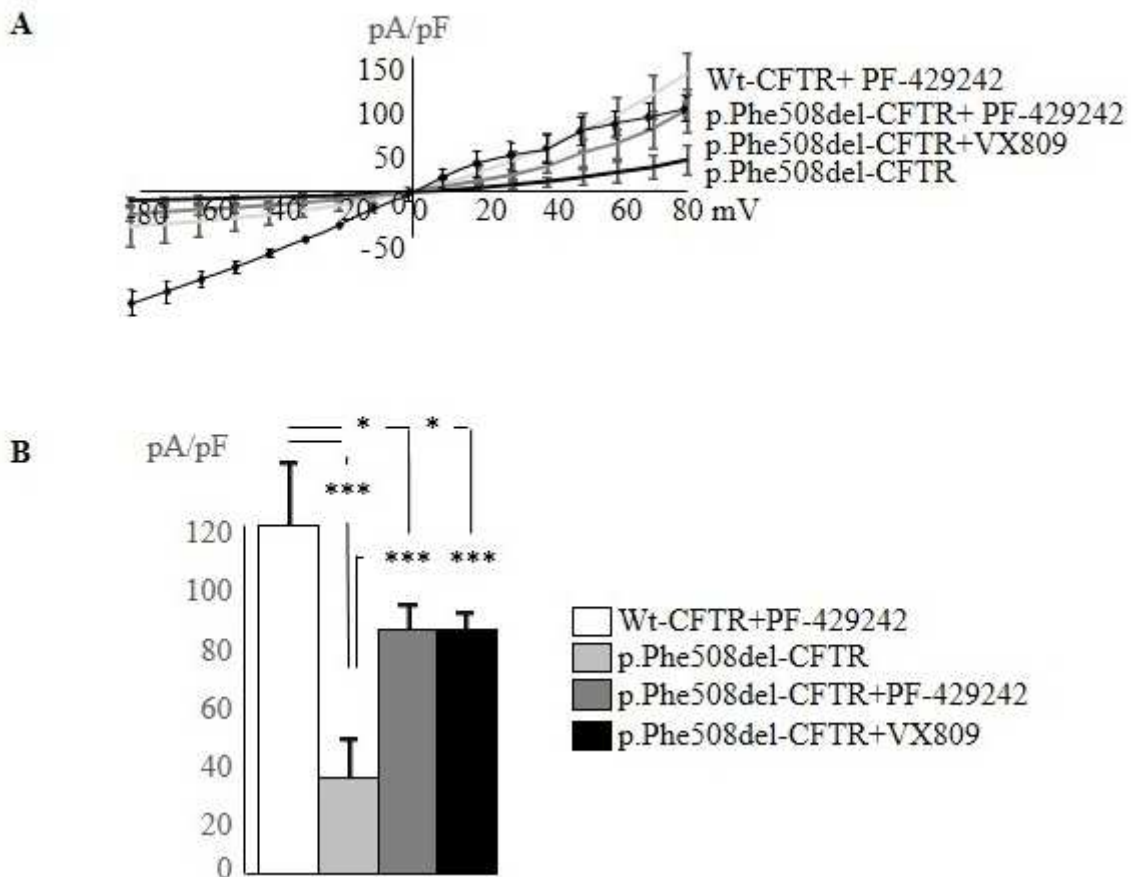
**Figure 3. Expression of S1P protease in cells expressing Wt- and P.Phe508del-CFTR**

The left image shows a representative immunodetection of S1P (110 kDa) in cells expressing either wt- or P.Phe508del-CFTR. The optical density of the bands for S1P were normalized by the optical density of Actin. The normalized values obtained in cells expressing the wt- or P.Phe508del-CFTR were compared using a two-tailed unpaired Student's t test. The right image shows the corresponding statistical analysis of the expression of S1P (n = 6). No significant difference was observed between the two cell lines.

To appreciate the effect of PF-429242 upon the Cl<sup>-</sup> channel function of CFTR, we performed patch-clamp experiments in the whole cell configuration, in untreated and treated cells expressing p.Phe508del-CFTR. The methodology was previously described [37-39]. The specificity of the responses were assessed using the CFTR's activators forskolin (10 $\mu$ M, Sigma) and genistein (30 $\mu$ M, Sigma) and the CFTR's inhibitor CFTRinh172 (10  $\mu$ M, Sigma). We used the corrector of p.Phe508del-CFTR, VX-809 (Lumacaftor, Euromedex) as a positive control [40]. The recorded I/V curves after normalization of the currents by capacitances are shown in Fig. 4A. Unsurprisingly, the currents in CFBE41o-/wt cells (118,8 $\pm$ 21,09 pA/pF) were

significantly higher than in CFBE41o-/*P.Phe508del* cells ( $32,98 \pm 14,71$  pA/pF). We observed that the  $\text{Cl}^-$  currents were higher in treated cells than in untreated cells. This was confirmed by the statistical analysis (Fig. 4B) showing that PF-429242 treatment leads to a significant increased channel function of *P.Phe508del*-CFTR ( $82,67 \pm 22,26$  pA/pF). Because VX-809 is a recognized corrector of *p.Phe508del*-CFTR [41, 42], we used it to compare its effects to the PF-429242 treatment. No difference was observed between PF-429242 and VX-809, regarding the  $\text{Cl}^-$  efflux. This point is of importance because VX-809 is used in patients [42]

**Figure 4**

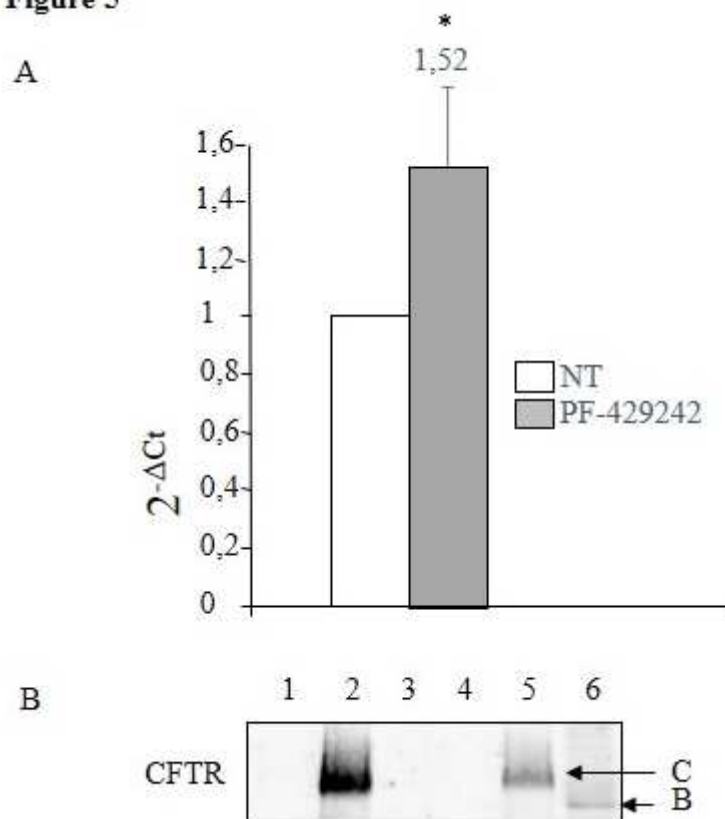


**Figure 4. The  $\text{Cl}^-$  channel activity of *P.Phe508del*-CFTR is partially restored by PF-429242.**

**A.** The top image shows a representative current-voltage (I-V) relationships in treated wt-CFTR cells (light grey), *P.Phe508del*-CFTR cells (dark grey), *P.Phe508del*-CFTR cells treated with VX-809 (medium grey) and in treated *P.Phe508del*-CFTR cells treated with PF-429242 ( $10 \mu\text{M}$ , 48 hours). **B.** The bar graphs show the statistical analysis of CFTR function in wt-CFTR cells ( $n = 5$ ), *P.Phe508del*-CFTR cells ( $n = 6$ ), *P.Phe508del*-CFTR cells treated with VX-809 cells ( $n = 3$ ) and in *P.Phe508del*-CFTR cells treated with PF-429242 ( $n = 5$ ). The analysis was performed using pA/pF values at +80mV. An important and significant increase (+152%) was observed in PF-429242 treated cells when compared to untreated cells. The  $\text{Cl}^-$  channel function of *P.Phe508del*-CFTR was increased to the same extend with PF-429242 or VX-809. The results obtained in cells expressing wt-CFTR were not different in the presence or in the absence of PF-429242.

In order to explain the restoration of the Cl<sup>-</sup> channel function of P.Phe508del-CFTR by aminopyrrolidine amide, the transcription of the channel was studied by real-time quantitative PCR, using the forward 5'-atgcccttcggcgatgtttt and reverse 5'-tgattcttcccagtaagagagc primers. Figure 5A is the bar graph representation of the statistical analysis of the 2<sup>-ΔCt</sup> using β-Actin as a housekeeping gene and non-treated cells as a reference [43]. PF-429242 significantly increased the mutated gene expression. Because the expression of P.Phe508del-CFTR is very low in CFBE41o-cells, we used the Chinese hamster ovary cell model (BHK) in which the membrane localisation of CFTR can be studied [44]. The cells were expressing a CFTR with a 3-Haemagglutinin (HA)-epitope tag that was recognized by an anti-HA antibody which was recovered by magnetic beads and detected by immunoblot, as previously described [44]. As expected, because it is the hallmark of the p.Phe508del mutation, the mature form of CFTR (C band, 170 kDa) was observed in the cells expressing the wt-CFTR and was absent in P.Phe508del-CFTR cells (Fig. 5B) [15]. In cells expressing p.Phe508del-CFTR, the mature form of CFTR was only observed after PF-429242 treatment (lane 5), indicating that PF-429242 induces the exportation of the mutated CFTR to the membrane. This explains the observed increased Cl<sup>-</sup> currents. We show here that PF-429242 does not improve the intrinsic function of CFTR but increases the quantity of channels within membranes.

**Figure 5**



**Figure 5. Detection of P.Phe508del-CFTR before and after treatment with PF-429242.**

**A.** The transcription of p.phe508del-CFTR before and after treatment with PF-429242 was assessed by real-time quantitative PCRs. The bar graph represents the statistical analysis and shows that PF-429242 significantly increases the gene expression of p.phe508del-CFTR (n=3). **B.** The

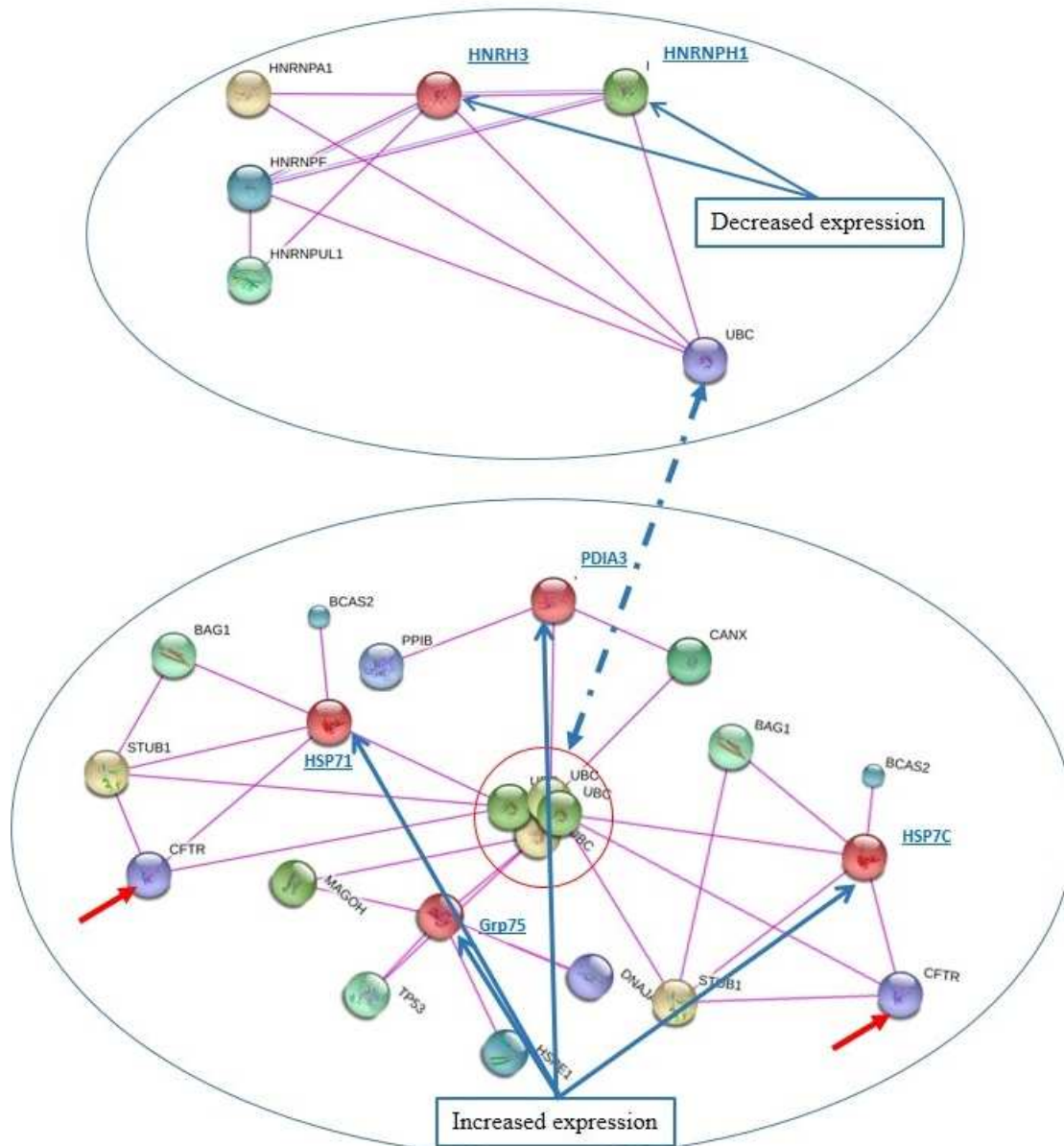
tagged wt- and P.Phe508del-CFTR present within cell membranes were detected with an anti-HA antibody. The image shows an example of the detection in the following samples: 1, Negative control (wt-CFTR cells not incubated with anti-HA antibody); 2, wt-CFTR cells incubated with anti-HA antibody; 3, P.Phe508del-CFTR cells not incubated with the anti-HA antibody; 4, P.Phe508del-CFTR cells incubated with anti-HA antibody; 5, PF-429242 treated P.Phe508del-CFTR cells incubated with anti-HA antibody and 6, Supernatant of the magnetic beads. No CFTR was detected in the negative control lane (1). The mature form of CFTR (C band) was observed in wt-CFTR cells and was absent in P.Phe508del-CFTR cells. In P.Phe508del-CFTR cells the mature form of CFTR, representing the CFTR within membrane, was only observed after PF-429242 treatment (lane 5).

We hypothesized that the inhibition of the cleavage of ATF6 by the inhibition of S1P by Aminopyrrolidine amide, could alleviate the down regulation of the *CFTR* gene. *CFTR* is involved in CF which is the most common inherited lethal genetic disease in the Caucasian population. Therefore, if our hypothesis was verified, it would be of interest for the patients, despite the great advances that have been made in the treatment of CF through the development of new therapies such as Trikafta [48]. Indeed, while 90% of patients with CF can now benefit of this drug with great clinical benefits, a fraction (10%) remains without any therapeutic option. Whereas the triggering of UPR in CF is still in debate (for review, [6]), we showed that ATF6 is cleaved in the most frequently used CFTR defective cell model, indicating that it was relevant in our study. We treated cells with PF-429242 and we observed that the function of the p.Phe508del-CFTR Cl<sup>-</sup> channel was increased in link with its significantly increased expression (mRNA and protein).

The active form of ATF6 links the promoters of UPR target genes inducing the transcription of ER chaperones, proteins of the quality control of the ER and of the ER-associated degradation [45, 46, 49-51]. Therefore, it was possible that the observed effects upon P.Phe508del-CFTR were due to the modulation of the expression of ATF6-dependent genes. In a previous work (unpublished), we performed a proteomic analysis of differentially expressed proteins after treatment of the cell lines used in the present work, by a nonspecific S1P inhibitor (AEBSF). Two-dimensional Gel Electrophoresis (Ettan IPGphor 3, GE Healthcare) were performed to compare the protein expressions, before and after treatment. The proteins exhibiting variations of at least 2 fold in their expression, which was measured by PDQuest Basic-8.0.1 (BioRad), were identified by mass spectrometry (LC-MS/MS) using a LTQ-Orbitrap Spectrometer. Databases interrogation was performed using X!Tandem Piledriver (v2015.04.01.1) and X!Tandem Pipeline (v 3.4.2 << Elastine Durcie >>). We found that the expression of HNRNPH1 and HNRH3 were decreased and that the expression of PDIA3 (Grp58), HSP7C, GRP75 and HSP71 were increased, in treated cells. We performed a bibliographic analysis to characterise these proteins. A summary of their function and possible involvement in CF is given in Table 1. The H1 and H3 members of the HRNPs family are regulators of alternative splicing [52, 53]. They modulate the mRNAs processing and the alternative splicing of CFTR [54]

but in view of the bibliography it is not clear how they could explain the observed effects upon the function of P.Phe508del-CFTR. PDIA3 (Protein disulfide-isomerase A3) is a stress-responsive disulfide isomerase of the ER that in link with calnexin and calreticulin, corrects the folding of glycoproteins [55, 56]. Its increased expression in our cells favours the refolding of P.Phe508del-CFTR, likely explaining why an increased amount of P.Phe508del-CFTR was observed in the treated cells. Its overexpression also indicates that PF-429242 has a beneficial effect on P.Phe508del-CFTR because it was shown to be up-regulated when P.Phe508del-CFTR is chemically rescued [57]. HSP71 (Heat Shock Protein 71 kDa, HspA1A) belongs to the HSP70s family and binds misfolded proteins to prevent their aggregation [58]. The previously described P.Phe508del-CFTR/Hsp70 complex permits the degradation of P.Phe508del-CFTR [59]. Because we observed an increased expression of the HSP71 in the presence of the drug, it likely eliminates unfoldable P.Phe508del-CFTR and favours the refolding of some reversely unfolded P.Phe508del-CFTR. Among HSP70s, HSP7C is involved in the proper folding of P.Phe508del-CFTR by a direct PPI [60, 61]. Its increased expression is in favour of a greater folding and exportation of P.Phe508del-CFTR to the membrane. GRP75 (Glucose-regulated protein 75), is also a member of the HSP70s family involved in intracellular trafficking [62]. Because it ensures the final localization of proteins, protects them against stress and is up-regulated when P.Phe508del-CFTR is rescued [57, 63], its overexpression likely leads to the recovery of some mutated CFTR, explaining our results. By analogy, we propose that these differentially expressed proteins are involved in the positive effects of PF-429242.

The protein network in which a protein takes place is an indicator of the pathway in which it is involved. Thus, we search for Protein-Protein Interactions (PPI) involving PDIA3, HSP7C, GRP75 and HSP7, using STRING 10.0 (Search Tool for the Retrieval of Interacting Genes and Proteins, <http://string-db.org/>). The search parameters were as following: all PPI prediction methods were enabled except for High-throughput Lab Experiments; maximum of 5 interactions by node; cut-off criteria of combined score  $\geq 0.9$  (highest confidence permitted in STRING); Homo sapiens. Unexpectedly, we found that all the proteins that were modulated when S1P is inhibited have Ubiquitin C (UBC) as a common partner (Fig. 6).



**Figure 6. PPI networks of the differentially expressed proteins when S1P is inhibited.**

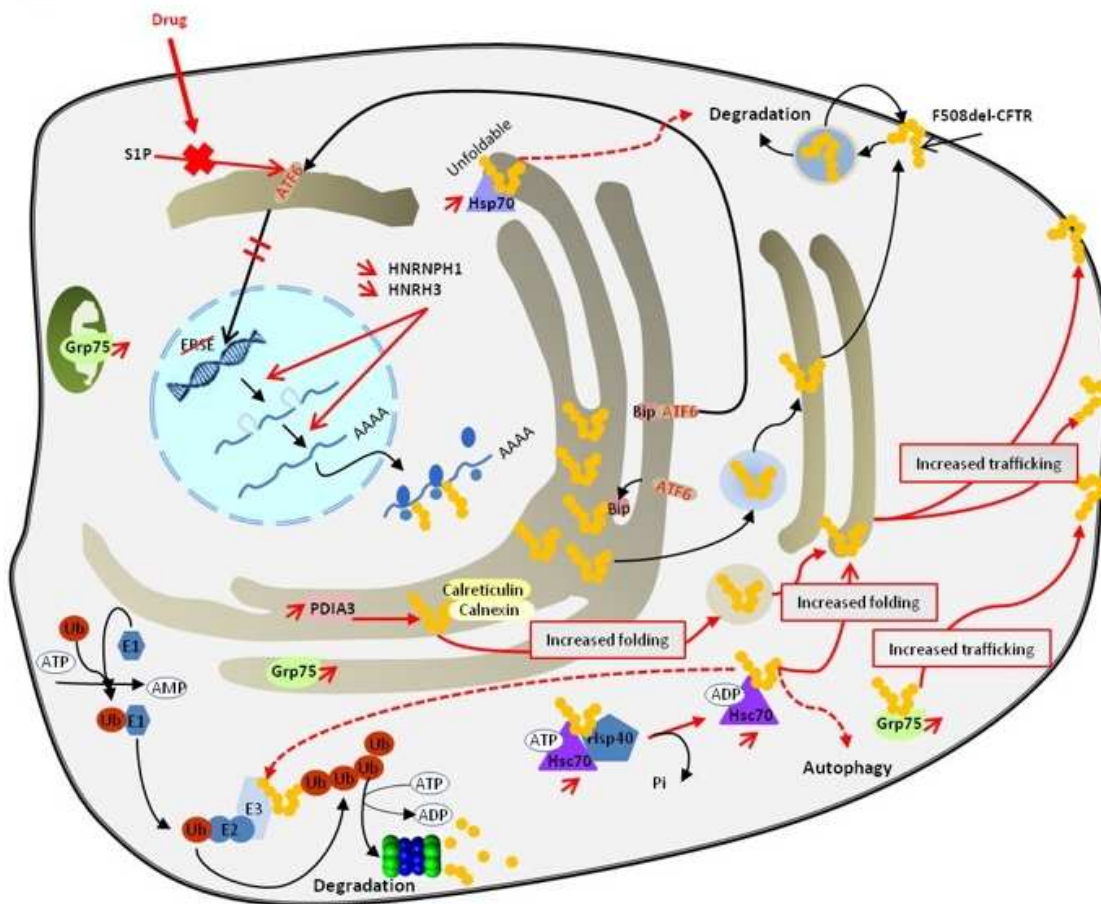
Graphs for HNRNPH1, HNRH3, PDIA3, HSP7C, GRP75 and HSP71 were retrieved. We gathered the graphs and drew a new graph showing that Ubiquitin C (UBC) is a common interacting protein. The graph shows the query proteins (red nodes) and the first shell of interactions. It can be noticed that HSP7C and HSP71, as well as UBC, do directly interact with CFTR. Edges represent experimentally determined PPI (pink). Blue edges represent protein homologies. CFTR in the network is shown by red arrows.

This result was further confirmed by the analysis of the relevant bibliography for each interaction HNRNPH1-UBC [64]; HNRH3-UBC [65]; PDIA3 (Grp58)-UBC [66]; HSP7C- HSP71- and GRP75- UBC [67]. As any proteins, both wt- and p.Phe508del-CFTR undergo ubiquitination and degradation [67]. The overexpression of UBC would not explain the increased quantity of P.Phe508del-CFTR in cell membranes because it is involved in the degradation of CFTR despite the fact that Ubiquitin C-



terminal hydrolase-L1, is highly expressed in CF airway epithelial cells, with a positive correlation between its expression and the steady state levels of P.Phe508del-CFTR cells. Nevertheless, the promoter of the stress-regulated polyubiquitin gene contains heat shock elements (HSEs) which mediate UBC induction upon stress. Three HSEs exist that all bind transcription factors belonging to the heat shock factor (HSF) family. The proximal HSE is a negative regulator of the stress-induced transcription [68]. Therefore, if a HSE with a negative role on the transcription is overexpressed, it could explain why in our hands, more P.Phe508del-CFTR is present in the membrane. This is reinforced by our previous results showing a pivotal role of UBC as a modifier gene in CF [69]. The summary of our findings is schematically represented in Fig. 7.

**Figure 7**



**Figure 7. Schematic representation of the effect of Aminopyrrolidine amide in cells expressing P.Phe508del-CFTR.** Black arrows show the normal processing of P.Phe508del-CFTR and normal ubiquitination process. Red arrows show the positive effects of the inhibition of the cleavage of ATF6 upon P.Phe508del-CFTR, according with the known effects of each protein which were highlighted in the present work. Red dashed lines indicate the possible side effects of Hsc70 and Hsp70.

## 2. Conclusion

Our result indicate that aminopyrrolidine amide restores the expression of a gene which is down-regulated by the active form of ATF6. In our model, it alleviates the p.Phe508del-CFTR defects through the modulation of the expression of its mRNA maturation, protein folding and degradation, as previously reported in ATF6  $-/-$  cells [70]. Interestingly, the CFTR's modifier gene UBC is involved [69]. Finally, we show

here that the serine protease inhibitor PF-429242 is a potential drug candidate that can restore the expression of genes that are down-regulated by the active form of ATF6. Based on the present results, it can be postulated that the drug could be a new drug candidate in disease involving CFTR such as CF or chronic obstructive pulmonary disease [71].

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**Table 1. Spots identification by MS.**

Accession number UniProtKB	Description	MW	Pi	Coverage %	log(E value)
P31943	HNRNPH1_HUMAN Heterogeneous nuclear ribonucleoprotein	49,1	5.89	38	-102,2701645
P31942-2	HNRH3_HUMAN Isoform 2 of heterogeneous nuclear ribonucleoprotein H3	35,1	6.36	28	-48,68246078
P30101	PDIA3_HUMAN Protein disulfide-isomerase A3	56,7	5.98	45	-172,0068665
P11142	HSP7C_HUMAN Heat shock cognate 71 kDa protein (HspA8)	70,8	5.87	37	-132,8794556
P38646	GRP75_HUMAN Stress-70 protein	73,5	5.87	18	-58,63391113
P0DMV8 (New Nomenclature)	HSP71_HUMAN Heat shock 70 kDa protein HspA1A/HspA1B	69,9	5.47	29	-126,2914658

**Table 2. Summary of the highlighted protein analysis.**

<b>Protein</b>	<b>Behaviour when ATF6 is inhibited</b>	<b>Protein function</b>	<b>Involvement in CF</b>
HNRNPH1	Decreased	HNRNPH1 belongs to heterogeneous nuclear ribonucleoprotein complexes and mediates pre-mRNA alternative splicing regulation. It inhibits alternative splicing in neurons [55].	Involved in the post-transcriptional mRNA processing of CFTR [72].
HNRH3	Decreased	Ribonucleoprotein are responsible for RNA splicing and processing [55].	Involved in the post-transcriptional mRNA processing of CFTR [72].
PDIA3 (Grp58)	Increased	PDIA3 is a member of the protein disulfide isomerase family mainly present in the ER. It is a chaperone and a disulfide-reshuffling enzyme [56]. Together with calnexin and calreticulin, it is involved in the correct folding of glycoproteins within the ER [73].	Grp 58 kDa/PDIA3 is present in CFTR's complex and is up-regulated when P.Phe508del-CFTR is chemically or genetically rescued [57].
HSP7C (HspA8)	Increased	Hsc70 forms a cytosolic complex with CHIP E3 and cooperates with the ER membrane associated ubiquitin ligase complex to favour triage of proteins [60].	Hsc70 together with Hsp70, is involved in the folding of P.Phe508del-CFTR [60].
GRP75	Increased	GRP75 is involved in the stress response, the intracellular trafficking and the cell differentiation and proliferation [74].	GRP75 is up-regulated when P.Phe508del-CFTR is chemically or genetically rescued [75].
HSP71 (HspA1A) (HspA1B)	Increased	Protects the proteome from stress. It is involved in the folding and transport of polypeptides, the activation of proteolysis of misfolded proteins. It has a pivotal role in the protein quality control system of misfolded proteins and in their targeting to degradation [76].	Hsp70 associates with both Wt-CFTR and P.Phe508del-CFTR. It dissociates from Wt-CFTR when it is transported from the ER to the Golgi. P.Phe508del - CFTR/Hsp70 forms a stable complex permitting the degradation of P.Phe508del-CFTR in the pre-Golgi, [60].