

**Title: Oxidative stress modulates the expression of genes involved in cell survival in  $\Delta$ F508 cystic fibrosis airway epithelial cells**

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## Abstract

Although, cystic fibrosis (CF) pathophysiology is explained by a defect in CF transmembrane conductance regulator (CFTR) protein, the broad spectrum of disease severity is the consequence of environmental and genetic factors. Among them, oxidative stress has been demonstrated to play an important role in the evolution of this disease, with susceptibility to oxidative damage, decline of pulmonary function and impaired lung anti-oxidant defense. Although oxidative stress has been implicated in the regulation of inflammation, its molecular outcomes in CF cells remain to be evaluated. To address the question, we compared the gene expression profile in *NuLi-1* cells, with wild-type CFTR and *CuFi-1* cells homozygous for  $\Delta F508$  mutation cultured at air-liquid interface. We analyzed the transcriptomic response of these cell lines with microarray technology, under basal culture conditions and after 24-h oxidative stress induced by 15  $\mu\text{M}$  2, 3-dimethoxy-1, 4-naphtoquinone (DMNQ). In the absence of oxidative conditions, *CuFi-1* gene profiling showed typical dysregulated inflammatory responses compared to *NuLi-1*. In the presence of oxidative conditions, the transcriptome of *CuFi-1* cells reflected apoptotic transcript modulation. These results were confirmed in the *CFBE41o-* and *corrCFBE41o-* cell lines as well as in primary culture of human CF airway epithelial cells. Altogether, our data point to the influence of oxidative stress on cell survival functions in CF and identify several genes that could be implicated in the inflammation response observed in CF patients.

## Introduction

Cystic fibrosis (CF) is the most frequent autosomal recessive genetic disorder in Caucasians (40). Respiratory manifestations predominate, with chronic airway infection and inflammation beginning in early childhood and leading to progressive loss of lung functions (40). Although the pathophysiology of CF lung disease is not fully understood, it is relatively well established that lung epithelial cells are central to this process. The key role of the epithelium has been emphasized by experimental data showing that CF airway ion transport is abnormal, including reduced transepithelial chloride conductance and increased basal sodium absorption (46). Multiple and controversial hypotheses have been proposed to explain CF pathophysiology, such as the modulation of cAMP-dependant chloride transport driven by CFTR (14), pH dysregulation of airway surface liquid (10), or dysregulation of other ion channels and of intracellular membrane transport processes (49). A number of studies also support the concept that there is an excessive intrinsic inflammatory response in CF lungs. Although the mechanisms underlying this response are still under investigation, the epithelial cells are thought to be a central player in this response (38). Several hypotheses have been proposed to explain the proinflammatory conditions prevalent in CF environment, including increased nuclear factor-kappa B signaling (26, 33), annexin A1 depletion (5) and aggresome formation in the cells (32). However, the implication of CFTR in regulating the inflammatory response in epithelial cells (20, 33) is still controversial.

Gene expression profiling is an interesting unbiased approach to explore the exaggerated inflammatory response in CF. Several investigations of CF mouse models have reported overexpression of inflammatory genes in CF lungs (19, 23, 53) and

intestinal tissue (36). However, these observations could not be consistently reproduced in CF bronchial epithelial cell lines or primary culture of CF epithelial cells (20). Increased modulation of inflammatory genes (interleukin-1 (IL-1), IL-6 and IL-8) has been encountered in CFT-2 cells, an immortalized human tracheal fetal ( $\Delta F508$ ) cell line (50), but has not been found in similar experiments on primary airway epithelial cell culture (56) or in cells obtained by nasal brushing (52). The most striking feature of gene profiling of CF epithelial cells is the lack of consistency in terms of pathways and genes reported to be regulated in CF. It stimulated Hampton and Stanton (21) to reanalyze, via a recently-developed statistical approach, the original data from 4 studies that evaluated the effect of  $\Delta F508$ -CFTR mutation on gene expression in human airway epithelial cells (HAE). Their work suggested that CF genotype may impair immune functions in airway epithelial cells, but is not associated with overexpression of genes involved in the inflammatory response. However, it might be difficult to identify a common gene expression pattern among all experiments conducted on different cells, tissues or culture conditions (air-liquid interface or liquid-liquid interface).

Although gene array studies have been performed to ascertain gene expression differences between CF and non-CF cells, few experiments have examined the gene profiling response in CF cells under stress. We choose to explore the transcription response to oxidative stress since CF patients exhibit elevated markers of oxidative stress compared to non-CF healthy controls (9, 57). Furthermore, CFTR dysfunction drives mitochondrial defects, alterations in oxidative phosphorylation, calcium homeostasis, oxidative stress, apoptosis and innate immune response (48). We choose to work with 2, 3-dimethoxy-1, 4-naphthoquinone (DMNQ) to enhance oxidative stress in CF cells

without inducing major perturbations of epithelial functions. Investigations were conducted in 2 human immortalized bronchial epithelial cell lines, *NuLi-1* cells, isolated from a non-CF patient expressing wild-type (wt) CFTR, and *CuFi-1* cells, a CF cell line homozygous for  $\Delta F508$  mutation. Our results demonstrated that *CuFi-1* cells, under oxidative conditions, expressed more genes involved in cell survival than *NuLi-1* cells. To validate these results, the expression of these genes was also tested in  $\Delta F508$  CF (*CFBE41o-*) and non-CF CFTR corrected (*corrCFBE41o-*) cell lines. We found that they were modulated similarly as in *CuFi-1/NuLi-1*, which lends a more comprehensive dimension to our bioinformatic analysis. To complete our study, the internucleosomal degradation of genomic DNA was tested to determine if apoptosis was driven similarly by a DMNQ stress in CF and non-CF cell lines. Taken together, these experiments indicate that under oxidative stress, CF epithelial cells are resistant to apoptosis when compared to non-CF epithelial cells.

## Experimental procedures

### *Cell lines, primary bronchial cells, experimental conditions and RNA extraction*

Two CF and non-CF matched cell lines were studied: first, *CuFi-1* (homozygous  $\Delta F508/\Delta F508$ ) derived from CF bronchial epithelium and immortalized E6/E7 and hTERT expression and *NuLi-1* (non-CF), normal HAE cells, were a gift from Dr. J. Zabner, University of Iowa (55). Second, a  $\Delta F508$ -CFTR (homozygous  $\Delta F508/\Delta F508$ ) named *CFBE41o*- is derived from bronchial epithelial cells isolated from CF bronchus at the 1st bifurcation and immortalized with pSVori-plasmid (18) and a corrected version of *CFBE41o*- transduced with wt-CFTR, named *corrCFBE41o*- (for corrected *CFBE41o*-) were kindly provided by Dr. J. Hanrahan, McGill University. Finally, primary human bronchial cells were isolated from CF homozygous  $\Delta F508/\Delta F508$  lung transplants (median age 30 yrs, mean forced expiratory volume in 1 s (FEV1)  $22.6 \pm 1.9\%$ ) and non-CF donors. Briefly, After lung dissection, bronchi were digested overnight, at  $4^{\circ}\text{C}$ , under gentle agitation in MEM (Invitrogen) supplemented with 7.5%  $\text{NaHCO}_3$  (Sigma-Aldrich), 2 mM L-glutamine, 10 mM HEPES (Fisher, Ottawa, Canada), 0.05 mg/ml gentamycin, 25 U/ml penicillin-streptomycin, 0.25  $\mu\text{g/ml}$  Fongizone (Invitrogen), 0.1% pronase E (Sigma-Aldrich), 10  $\mu\text{g/ml}$  DNase (Sigma-Aldrich) and antibiotic specific from each patient. After pronase-DNase neutralization, the cells were gently scraped off the remaining tissue. The cell suspension was washed and seeded into flasks coated with Purecol (Cedarlane) and cultured in complete SAGM (LHC basal medium supplemented with the SAGM kit (Clonetics) and 25 ng/ml EGF, 100 U/ml of penicillin-streptomycin, 0.07  $\mu\text{g/ml}$  phosphorylethanolamine (Sigma-Aldrich), 1.86 ng/ml ethanolamine (Sigma-Aldrich), 0.05 nM retinoic acid (Sigma-Aldrich) and stock 11, stock 4 and trace elements

(kindly provided by Dr. Scott Randell, University of North Carolina). Cultured under air-liquid conditions, the cells were coated on collagen plastic dishes with serum-free bronchial epithelial cell growth medium (BEGM) and supplements (Lonza, Allendale, NJ, USA). The passage number of each type was recorded, and the cells were analyzed at passage 14. After differentiation by cell culture at the air-liquid interface, the cellular monolayer was treated on the apical side with 15  $\mu$ M DMNQ for 24 h. These conditions were optimal for generating oxidative stress without affecting cellular integrity. At the beginning, 75000 cells were cultured per filter. The proliferation rate is around 10 fold after one week. The final cell number, although difficult to estimate on filter, should be around 7.5 millions. Total RNAs were isolated from each cell line after or no DMNQ treatment using Trizol (Invitrogen) and RNA was purified with RNeasy Mini Kit (Qiagen, Valencia, CA). The quality of total RNA was evaluated by Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). All 28s/18S ratios gave values between 1,9 and 2,1 and the RIN value (RNA integrity number), ranging from 9.8 to 9.9, where 10 indicates the highest possible RNA quality. We obtained an average of 700ng/ $\mu$ l eluted in 35 $\mu$ l, so a final concentration of 24,5 $\mu$ g of Total RNA. Only four (4)  $\mu$ g of total ARN were used for cDNA and hybridization.

#### ***Glutathione level***

Glutathione (GSH) and glutathione disulfide (GSSG) in bronchial epithelial cells were quantified with a previously described technique (2). In brief, samples were mixed with an equal amount of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid)/0.1 M potassium phosphate, pH 7.5/17.5 mM EDTA, then centrifuged at 2,000 xg for 10 min. A 50- $\mu$ l

sample of supernatant was added in a cuvette containing 0.5 unit of glutathione reductase in 0.1 M potassium phosphate, pH 7.5. After 1-min incubation at 25°C, the assay reaction was started by adding 200 nM NADPH/0.1 M potassium phosphate, pH 7.5/5 mM EDTA in a final volume of 1 ml. The rate of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) reduction was recorded spectrophotometrically at a wavelength of 412 nm using Envision multiplate reader (Perkin Elmer, Waltham, MA, USA). GSH/GSSG levels were quantified by standard curves generated from known GSSG concentrations (0.125  $\mu$ M) in PBS (pH 7.4).

To quantify GSSG, a sample of bronchial epithelial cells was mixed immediately after recovery with an equal volume of 10 mM N-ethylmaleimide/0.1 M potassium phosphate, pH 6.5/17.5 mM EDTA and centrifuged at 2,000xg for 10 min. Subsequently, 250  $\mu$ l of supernatant was passed through a Sep-Pak cartridge previously washed with 3 ml of methanol, followed by 3 ml of distilled water. GSSG was eluted from the column with 1 ml of 0.1 M potassium phosphate, pH 7.5/5 mM EDTA/800  $\mu$ M DTNB/glutathione reductase at 2 units/ml/1 mM NADPH, and the rate of DTNB reduction was measured by spectrometry at 412 nm. Standard curves were derived from dilutions of known GSSG concentrations (0.125-4  $\mu$ M) mixed with 10 mM N-ethylmaleimide and chromatographed in a Sep-Pak C18 column. GSH was quantified by subtracting GSSG from GSH/GSSG levels.

### ***Electrophysiology***

Short-circuit currents ( $I_{sc}$ ) were measured at 37°C in cell monolayers grown at the air-liquid interface on polycarbonate membrane inserts (24 mm diameter, Corning



Transwell, Tewksbury, MA, USA) placed horizontally in a modified Ussing chamber. For  $I_{sc}$  measurement, the monolayers were bathed in physiological buffer (140 mM NaCl, 5 mM KCl, 1 mM  $MgCl_2$ , 1 mM  $CaCl_2$ , 10 mM glucose, 10 mM TES, pH 7.4 at 315 mOsm/kg). Voltage (calomel) and current (Ag/AgCl) electrode pairs were in contact with the apical and basolateral bathing solutions and connected via 2 M KCl/5% agarose bridges.  $I_{sc}$  were generated by VCC MC2 voltage clamp amplifier (Physiological Instruments, San Diego, CA) linked to a 4sp PowerLab data acquisition and analysis system (ADInstruments, Grand Junction, CO). The currents were recorded and analyzed with PowerLabs Chart 5 software. Transepithelial resistance ( $R_{te}$ ) was calculated according to Ohm's law by recording the current jump obtained after applying a 1-mV square pulse for 1 s every 10 s.

### ***Microarray experiment***

This experiment was designed to study 2 factors ( $\Delta F508$  mutation and oxidative stress) at 2 levels (absence and presence) in triplicate. Microarray hybridization was performed with human expression HG-U133 Plus 2.0 Genechip arrays (Affymetrix, Santa Clara, CA) following the manufacturer's recommendations. Briefly, for each experimental condition, 4  $\mu g$  of total RNA were reverse-transcribed using T7-(dT)<sub>24</sub> oligonucleotide as primer. The probes were labelled with biotin and fragmented by Affymetrix's reagents according to a standard protocol. After washing and staining with streptavidin-phycoerythrin (Invitrogen, Carlsbad, CA), 10  $\mu g$  of resulting cRNA were loaded on each chip and scanned in a Genechip Scanner 3000 workstation. Raw and processed microarray data were deposited in the publicly-accessible Gene Expression

Omnibus (GEO) database (3): accession number GSE39843 . Twelve Agilent chips were used to these experiments: 2 cell lines (CuFi-1 and NuLi-1) X two treatments (1- no treatment and 2- DMNQ treatment – 15µM for 24 hours) X 3 replicates per cell line and conditions.

### ***Microarray data processing***

For each hybridized chip, probe intensity levels were extracted by Affymetrix<sup>®</sup> Scanner 3000 7G and the data analyzed by Microarray Analysis Suite (version 5.0) algorithm. Quality of the microarray experiments was evaluated according to Affymetrix quality control criteria through internal controls in the Affymetrix platform. First, the absence of hybridization bias was controlled for each chip. Second, several features, including background correction, reduction factor, present percentage, 3'/5' ratio and internal controls, were evaluated as per Affymetrix recommendations. In the absence of bias for all these parameters, data from the different chips comprising our experiment were concluded to be of good quality and suitable for further analysis. The expression data had to be transformed by a normalization step to compare the chips to one another. The Robust Multichip Average method (25) was used for this purpose: 1. quantile transformation was applied, 2.data from the 1,352,569 probes were summarized to 54,675 probe sets, and 3. log<sub>2</sub> transformation was carried out. Twelve Agilent chips were used: 2 cell lines (CuFi-1 and NuLi-1) X two treatments (1- no treatment and 2- DMNQ treatment – 15µM for 24 hours) X 3 replicates per cell line and conditions.

## ***Differential analysis and ontology assessment***

Quality control, normalization, univariate and multivariate differential analysis were assessed by routines from Bioconductor version 2.7 (<http://www.bioconductor.org>). Differential expression between cell lines in the absence of DMNQ treatment was detected with linear models from the microarray data package (42). A list of genes expressed with significant statistical differences ( $P < 0.05$ ) was generated for *CuFi-1* in comparison to *NuLi-1*. To study the effect of DMNQ on the gene expression profile of both cell lines, we developed methodology to isolate transcripts that were modulated and provided relevant biological information. Because biological data rarely follow normal distribution, interquartile range (IQR), a nonparametric measure, identified the most relevant expression data. This value, more robust than variance, indicates dispersion around the median and includes 50% of expression level data. The *rda()* function of the *vegan* package in R statistical language, for canonical redundancy analysis (37), served as a form of 2-way multivariate analysis of variance (ANOVA) to evaluate the quantitative effect induced by mutation and oxidative stress (30, 31). This approach estimates fractions of variation in gene expression attributable to the experimental factors mutation and oxidative stress. With this method, tests of significance do not require normality because they use the permutation method. Ontological analysis was performed with function *hyperGTest* of the *GOstats* package (15). Only significantly over-represented biological functions ( $P \leq 0.05$ ), composed of a minimum of 20 genes, were taken into account.

241

242 ***mRNA quantification***

243 mRNA expression levels were quantified by assays designed with the Universal  
244 Probe Library from Roche ([www.universalprobelibrary.com](http://www.universalprobelibrary.com)). The high melting  
245 temperature characteristic of longer probes is retained by Locked Nucleic Acid nucleotide  
246 chemistry in these shorter probes. The Reverse transcription-quantitative polymerase  
247 chain reaction (RT-qPCR) of selected genes and glyceraldehyde-3-phosphate  
248 dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) was  
249 performed as described below. Reverse transcription was undertaken with Quantitect  
250 (Qiagen, Toronto, ON, CA) following the manufacturer's instructions and using either  
251 poly-TT or random primers. The PCR amplification was carried out with SYBR Green  
252 Master Mix (Applied Biosystems, Grand Island, NY, USA) according to the  
253 manufacturer's instructions on Rotor-Gene Q (Qiagen, Toronto, ON, CA). Reactions  
254 contained: 2X Universal PCR Master Mix, 2 µl of template cDNA, 200 nM of primers  
255 (all primers deployed in these studies are listed in Table 1, supplementary materials) in a  
256 final volume of 15 µl. Relative transcript quantity was measured by the comparative  
257 threshold (CT) cycle method and normalized with GAPDH or HPRT1 as internal control  
258 (Applied Biosystems) by Rotor-Gene 6000 software. cDNA ratio was calculated on the  
259 basis of CT values standardized by the amount of GAPDH/HPRT1 housekeeping gene  
260 expression. Each PCR reaction was performed in triplicate.

261

262 **Apoptosis measurements**

263 *Quantitative estimation of oligonucleosomes after DMNQ-induced stress.* Photometric  
264 enzyme immunoassay (Cell death detection ELISA<sup>plus</sup>, Roche Applied Science, Grand  
265 Island, NY, USA) quantified cytoplasmic-histone-associated DNA fragments *in vitro*  
266 after oxidative stress induced by DMNQ. The following protocol modification was  
267 suggested by the company for sample preparation: apical supernatants of *CuFi-1/NuLi-1*  
268 or *CFBE41o-/corrCFBE41o-* were aspirated and discarded. The monolayers were then  
269 rinsed twice with warm PBS before trypsin (0.25%) was added for 7-8 min at 37°C.  
270 Following trypsin treatment, cells from the monolayer are collected and resuspended in 2  
271 or 5 ml size in specific medium (DMEM-F-12 + USG + antibiotics), depending on pellet  
272 size. About 20,000 cells were counted and resuspended in 100 µl of culture medium  
273 (DMEM-F-12), then recentrifuged (10 min at 3,500 rpm at 4°C). After supernatant  
274 removal, the cell pellets was resuspended in 200 µl of lysis buffer for 30 min at room  
275 temperature. Finally, 20 µl of supernatant, which represent the cytosolic fraction, were  
276 subjected to ELISA according to a standard procedure. For immunofluorescence analysis,  
277 the absorbance was measured at 405 nm and subtracted from absorbance measured at 490  
278 nm after background correction. Two-way ANOVA was used to analyze the data.

279 *Caspase 3/7 activity.* Synergy HT Multi-Mode Microplate Reader (BioTek) in  
280 combination with Promega's Caspase-Glo Assay kit (Promega) provides a convenient  
281 procedure for measuring caspase-3 or -7 activities. Caspase 3/7 activity was measured in  
282 duplicate on *CuFi-1/NuLi-1* cell lines using 96-well lumitrac 200 (Biolyntx Inc.)

283 according to the standard protocol excepted for incubation time, which was optimized for  
284 90 min. at room temperature.

285

## Results

### *Expression profiling and gene ontology (GO) term analysis of CF ( $\Delta F508$ ) airway epithelial versus non-CF cell lines*

The gene expression profile of *CuFi-1* cells, a human airway epithelial cell line homozygous for the  $\Delta F508$  mutation of CFTR (the most common mutation present in CF), was compared to that of non-CF *NuLi-1* cells by HG-U133 Plus 2.0 Affymetrix microarray. The platform is composed of 54,675 probe sets, representing approximately 38,500 genes (estimated by UniGene identifiers). 9,755 transcripts were expressed differentially between *CuFi-1* and *NuLi-1* cells, as monitored by moderated *t*-test ( $P < 0.05$ , Figure 1). From these selected transcripts, 4,438 were upregulated and 5,317 downregulated in *CuFi-1* compared to *NuLi-1* cells. To define the biological processes modulated in *CuFi-1* cells, GO term analysis of all significantly altered transcripts was undertaken. As reported in Figure 2A, GO term analysis highlighted 3 preponderant groups of processes: cellular survival, ribonucleotide metabolism and regulation of signaling pathways. In a second step, we chose transcripts classified as “upregulated” (Figure 2B) and “downregulated” (Figure 2C), to identify biological processes specifically over-represented in these selections. Inflammation, regulation of signal transduction and cellular survival were also over-represented in upregulated transcripts, and ribonucleotide metabolism, chromatin modification and translational elongation were significantly over-represented in downregulated transcripts. To summarize, the transcriptomic response of *CuFi-1* cells can be divided into 4 groups of processes: cellular survival, ribonucleotide metabolism, signal transduction and inflammation.

***Modulation of inflammatory and immune response transcripts in CuFi-1 compared to NuLi-1 cells in culture***

After GO term analysis, we identified the over-representation of several processes associated with immune and inflammatory responses represented by 123 upregulated transcripts. From these 123 transcripts, 19 genes, described in the literature as being involved in CF, were validated by RT-qPCR to confirm the modulation observed in the microarray experiment. As shown in Figure 3, the 2 techniques gave similar *CuFi-1/NuLi-1* expression ratios, except for HLADRB1 and HLADQB1. Several transcripts, already reported to be upregulated in CF pathophysiology (IL-6, IL-8, IL-1B and IL-1A), were found to be significantly modulated in *CuFi-1* cells, and these changes were confirmed by RT-qPCR (Figure 3). Genes indirectly linked to inflammatory responses, such as signal transducer and activator of transcription 1 (STAT1, modulation confirmed by RT-qPCR) and FBJ murine osteosarcoma viral oncogene homolog (cFOS, modulation not confirmed by RT-qPCR), were also upregulated. Also related to inflammatory processes, genes of the alarmin family were shown to be upregulated in CF cells (in the microarray experiment), such as IL-33 (22), lactotransferrin (11), heat-shock protein 70, defensin beta 4 or calcium-chelating S100 family proteins (7) (also called calgranulins S100A8-A9-A12, confirmed by RT-qPCR). An Alarmin-like protein, chitinase-like 1 protein (29), was also upregulated.

***Gene modulation induced by oxidative stress in CF cell lines***

Several lines of evidence indicate that oxidative stress is increased in CF patients (9, 28, 57), impacting several biological functions involved in CF pathophysiology (9).



Nevertheless, its involvement in transcriptomic modulation has not yet been investigated. To evaluate this aspect, *CuFi-1* and *NuLi-1* cells were exposed for 24 h to DMNQ, an oxidative stress inducer. The cellular level of total GSH (pmol. $\mu\text{g}^{-1}$  of protein) was measured to assess the oxidative effect of DMNQ. 24-h DMNQ treatment elicited a significant increase of total GSH in *CuFi-1* and *NuLi-1* cells at all concentrations tested (Figure 4A). In addition to its oxidative action, DMNQ impacted the electrophysiological properties of *CuFi-1* cells in a dose-dependent manner. At high concentration (20  $\mu\text{M}$ ), DMNQ significantly decreased the total  $I_{\text{sc}}$  of *CuFi-1* (Figure 4B), but  $R_{\text{te}}$  remained relatively stable (Figure 4C). No significant modification of  $I_{\text{sc}}$  or  $R_{\text{te}}$  was evident in *NuLi-1* cells. After obtaining these preliminary results, we treated *CuFi-1* and *NuLi-1* cells with 15  $\mu\text{M}$  DMNQ in transcriptomic experiments, to evoke significant oxidative stress without affecting cellular integrity and bioelectrical properties.

To investigate the effects of oxidative stress in these cell lines, we studied gene expression as previously explained. Because we needed to consider 2 conditions, CF mutation and oxidative stress, to select modulated transcripts, we proceeded in 2 steps: first, we retained those genes, with an IQR of 0.5, where the expression was modulated significantly by mutation and/or oxidative stress (see ***Experimental procedures: Differential analysis and ontology assessment***). Second, the selected transcripts underwent Rao's redundancy analysis (RDA), to quantify the percentage of the variance of the gene expression levels that can be attributed to each factor (mutation and oxidative stress). Using an IQR threshold of at least 0.5 and a percentage of explained variance of at least 20% for each experimental condition, we isolated 687 interesting transcripts (figure 5) (see ***Experimental Procedures: Differential analysis and ontology***

*assessment*). We then apply a GO term analysis to determine the biological processes that were over-represented processes in these transcripts. Applying IQR filter, RDA and GO term analyses, three significant biological processes were identified: oxidation/reduction, cell death and apoptosis (Figure 5). Supported by the GO database, 59 transcripts were found to participate in the cell death process (GO: 0008219); they included 29 transcripts associated with apoptosis (GO: 0006915). For this reason, we only assessed the expression of these 59 transcripts in the next analysis, to examine the impact of mutation and oxidative stress on the processes implicated in cell death.

To investigate the expression profiles of the 59 transcripts in *CuFi-1* and *NuLi-1* cells and highlight the modulation induced by DMNQ, we applied supervised classification based on the Mfuzz c-means algorithm (16). Among the 59 transcripts selected, only 44 showed DMNQ-dependent expression profiles, which were segregated into 2 tendencies: downregulation of 25 transcripts (Figure 6A) and upregulation of 19 transcripts (Figure 6B). Based on the RDA analysis, we were able to rank these transcripts as a function of the ratio variance explained by experimental conditions ( $\Delta F508$  mutation and oxidative stress)/total explained variance. Other genes indirectly involved in the modulation of survival were also present. The vascular endothelial growth factor A (VEGFA) is implicated in activation of apoptosis and associated with hyperinflammation in CF. In addition, stratifin is involved in VEGFA signaling and in regulation of the mitogen-activated protein kinase (MAPK) cascade. Finally, many RDA-selected transcripts are involved in MAPK cascade regulation, such as the MAP-kinases MAP3K5, MAPK1 and some indirect regulators (ERBB3, CASP1, CD14, TNFRSF10A).

Finally, we noted the presence of BCL2A1, an element that is important in the regulation of apoptosis.

### ***Validation of targeted genes after oxidative stress in CFBE cell lines***

Based on the above results, we decided to validate the expression of MAP3K5, VEGFA and BCL2A1 in another cell model, CFBE (homozygous  $\Delta F508$ ) and *corrCFBE41o-*, a corrected version of *CFBE41o-* transduced with wt-CFTR (see Mat. & Met. section). As illustrated in Figure 7A, MAP3K5 expression was significantly decreased after DMNQ treatment in the *CFBE41o-* and the *corrCFBE41o-* whereas VEGFA and BCL2A1 were increased. These results are comparable to those obtained in *CuFi-1/NuLi-1* with microarray and RDA selection. The results were also validated in CF and non-CF primary bronchial cells. Results demonstrated an upregulation of BCL2A1 and VEGFA as shown in cell lines. However, we could not show any significant modulation of expression of MAP3K5 in primary bronchial CF cells (Figure 7B).

### ***Absence of apoptosis induction in CF cell lines after oxidative stress***

The internucleosomal degradation of genomic DNA to oligonucleosomal fragments generated by  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonucleases is well accepted as a biochemical marker of apoptosis. Based on our transcriptional results, we hypothesized that apoptosis would be modulated in CF cell line. Therefore, we quantified oligonucleosome fractions in CF (*CuFi-1/CFBE41o-*) and non-CF (*NuLi-1/corrCFBE41o-*) cell lines before and after DMNQ stress (Figure 8). In absence of DMNQ, in *CuFi-1* cells, we did not observe a significant increase in the release of

oligonucleosomal fragments compared the *NuLi-1* cells. However, after DMNQ-induced stress, there is a significant increase in the release of oligonucleosomes in the *NuLi-1* cells compared to the *CuFi-1* cells. *CFBE41o-* and *corrCFBE41o-* cell lines were tested to confirm defective apoptosis in another CF cell type background. Cells were treated with 10  $\mu$ M DMNQ and the results were similar to those obtained with oligonucleosomes in *CuFi-1/NuLi-1*: in both cell types, DMNQ induced an increase of oligonucleosome release. However, the response of *corrCFBE41o-* was stronger in comparison to *CFBE41o-* cells, again indicating a tendency of CF cell lines to resist apoptosis.

To confirm these observations, Caspase 3/7 activity was measured in *CuFi-1/NuLi-1* in presence or not of DMNQ. Results show no increase in Caspase 3/7 activity confirming a decreased in apoptotic activity in CF cells as observed with the oligonucleosomes assay (Figure 9).

## Discussion

Based on the complexity of CF pathophysiology, influenced by environmental conditions as well as genetic background, we adopted a whole ORFeome microarray approach to study the transcriptional adaptation of CF cells exposed to oxidative stress. First, we demonstrated that there is an over-representation of several processes associated with immune and inflammatory responses in the CF cell line studied (*CuFi-1*). Second, using a novel approach based on canonical analysis, which estimates the contribution of 2 experimental conditions to the variance of each gene, we show that oxidative stress induced by DMNQ treatment elicited upregulation of genes involved in inflammatory responses and apoptosis. Our results pointed to the involvement of important molecules, such as BCL2A1 and VEGFA, in the mechanisms of apoptotic modulation of CF cell lines under oxidative stress.

### *Pro-inflammatory profile of CuFi-1/NuLi-1 cell lines*

In the present study, the CF model tested was derived from HAE cells carrying the  $\Delta F508$  mutation (*CuFi-1*) (55). This cell line constitutes a polarized monolayer with expected transepithelial activity, in air-liquid interface conditions that mimic the behavior of airway epithelial cells *in vivo*. The CuFi-1/NuLi-1 model has been investigated in many CF studies to explore the CF inflammatory response (6, 12), the CF epithelial repair processes (47), and to compare the transcriptomic response in CF epithelia (17). Our transcriptome analysis of *CuFi-1* and *NuLi-1* cells demonstrated over-representation of genes associated with the inflammatory response, such as of interleukins (IL-1A, IL-1B, IL-6 and IL-8) and chemokines (CXCL1, CXCL10, CXCL11, CXCL2, CXCL3,

CXCL5 and CXCL6) in *CuFi* cells. We were also able to confirm the modulation of selected genes (Figure 3) by RT-qPCR. Furthermore, many of the upregulated genes identified in our study, such as IL-6, IL-8, IL-1B and IL-1A, have also been identified in other CF tissues (19, 23, 36, 53). However, in a recently-reported experiments on CFBEs, the authors reported no compelling evidence that mutations in CFTR induce a hyperinflammatory response in CF epithelial cells (20). This discrepancy in the expression of inflammatory genes could be explained by the diversity of experimental models of CF tissues or CF epithelial cells to study this question (1, 4, 20, 33). Second, the difference between our data and theirs could argue in favor of other regulatory mechanisms involved in the regulation of inflammation in CF cell lines. One of these mechanisms could be epigenetic regulation. Indeed, DNA methylation or histone tail modification profiles, that are well known to influence gene expression, could be cell line-specific, independent of  $\Delta F508$  mutation or dependent on cell culture conditions. We have recently demonstrated different DNA methylation profiles of *CFBE* and *CuFi-1/NuLi-1* cell lines (8). Further studies are necessary to understand the role of these epigenetic modifications in CF cells.

#### ***RDA analysis highlights pathways modulated by oxidative stress in CF cell lines***

To understand the impact of oxidative stress on the transcriptome of CF cells, 2 factors (experimental conditions: cell lines and oxidative stress) were included in the experimental design, which allowed us to draw relevant biological conclusions. The challenge was to perform single 2-way ANOVA of multivariate response data. Some approaches have been developed with multifactorial design, such as comparison of

Student's test results by Venn diagram (42) or 2-way ANOVA (38), but are debatable because their robustness is very dependent on biological replication included in the study and present the problem of correction for multiple testing. To resolve this issue, we adopted a robust method of 2-way ANOVA for multivariate response data, as proposed by Legendre and Anderson (30), which uses RDA for variance partitioning. The method is largely applied in ecological research (31). Its robustness derives from permutation testing for the effects of main factors and the interaction between them; permutation tests do not require the normality of residuals.

This approach combines multiple regression and principal component analysis to establish relationships between response variables (expression level) and explicative variables (experimental conditions). The method highlights trends in the scatter of data points that are maximally and linearly related to a set of constraining (explanatory) variables. In other words, RDA determines the proportion of expression level variance that is accounted for by experimental conditions. Hence, for a specific probe, the expression levels measured in several experimental conditions could be summarized by the proportion of explained variance of each condition. For example, the RDA of an experimental design with 2 factors (A and B) at 2 levels (absence, presence), in triplicate conditions, allows the representation of a list of 12 expression level values associated with a specific probe (genes) by only 2 values, each being the proportion of the explained variance of each factor. This representation of data is realized without loss of generality. This approach, based on the ordination method, proposes a different analytical view for multifactorial design, compared to classical multivariate approaches, which could be powerless for the analysis of design with few biological replicates. Its advantage is that it

quantifies the effects associated with each experimental condition, and establishes a threshold for the selection of candidate transcripts that is more realistic than the classical statistical threshold (i.e. the percentage of variance explained by an experimental condition is more interpretable of a P-value in biological problems). Nevertheless, RDA assumes that the relationships between explanatory and response variables are linear and may underestimate the percentage of variance that could be explained by nonlinear models (true for any form of linear ANOVA).

In the present study, based on such representation, we selected probes which had at least 20% of variance explained by each experimental condition to investigate transcripts modulated by  $\Delta F508$  mutation and oxidative conditions. Using this analytical technique we were able to demonstrate that pathways involved in cell death and apoptosis were overrepresented in the biological processes modulated by oxidative stress in CF cells (Figure 5). The overrepresentation of these biological processes adds to the body of knowledge regarding the link between CFTR defects, inflammation and apoptosis (43).

#### ***Anti-apoptotic profile of CF cell lines under oxidative stress***

To study the transcriptomic response of CF cells in the presence of oxidative stress, we decided to work with DMNQ, which elicited intracellular oxidative stress and oxidative DNA damage. With this oxidative stress, we were able to evoke a chronic stress response, as demonstrated by increased GSH after 24-h exposure (Figure 4A), without significant biological perturbation of resistance integrity and cell function (transepithelial transport, Figure 4B and 4C). The DMNQ concentration used in our protocol induced intracellular stress that mimicked the oxidative stress present in bronchial epithelial cells



of CF patients. DMNQ was also chosen because it produces apoptosis via mitochondrial release of cytochrome c and caspase-9 and -3 activation in C17.2 cells (45) and that intracellular oxidative stress is associated with enhanced apoptosis in pancreatic (CFPAC1) and tracheal CF (CFT-2) cells (41).

In our study, after chronic oxidative stress induction, the amount of oligonucleosomes (Figure 8) released by the *CuFi-1* cell line after exposure to DMNQ was lower in *CuFi-1* cells compared to their controls (*NuLi-1*), suggesting resistance of the CF cell lines to apoptosis under oxidative stress. This intriguing result was confirmed by the same technique in the *CFBE41o-* cell line. Furthermore, we saw no activation of the Caspase 3/7 in *CuFi-1* cells compared to their controls (*NuLi-1*) following DMNQ exposure (Figure 9).

These results add to the controversy regarding the susceptibility of CFTR-deficient epithelial cells to apoptosis (43). Rottner et al (41) has shown that CF cells are more sensitive to apoptosis induced by actinomycin D or staurosporine. However, Yalcin et al. (54) have reported that increased apoptosis in the epithelial cells may be related with the presence of chronic infections rather than CFTR dysfunction. Anti-apoptotic profiles have also been described in CFTR<sup>(-/-)</sup> mice (13). Although these results highlight the potential role of CFTR in cell survival, the impact on apoptosis might be cell specific and depend on the environmental condition (intrinsic or extrinsic stimulus) surrounding the CFTR deficient cells.

In order to identify potential molecular pathways that could be involved in the modulation of cell survival by CFTR, we performed supervised classification based on the Mfuzz c-means algorithm (Figure 6). This analysis demonstrates that there are

transcripts that are upregulated in presence of CFTR defect and further upregulated in presence of DMNQ. There are also transcripts that are downregulated in presence of CFTR defect and further downregulated in presence of DMNQ. Furthermore, the level of expression of each of these transcripts is dependent, in our experimental model, on at least two factors (oxidative stress and CFTR mutation). The variance of some of the transcripts is mainly dependent on the presence of oxidative stress whereas other transcripts depend more on the CFTR mutation (Figure 6). From these results, we can hypothesize that depending on the type and level of stress applied to CF cells that the molecular survival or death pathways activated might be different.

To identify potential pathways that could be implicated in the resistance of CF cells to oxidative stress induced by DMNQ we have confirmed by RT-qPCR the expression level of 3 molecular targets well studied in cell survival in another cell line (*CFBE41o-corrCFBE41o-*) as well as in a primary culture of bronchial epithelial cells. We demonstrated that oxidative stress was associated with increased VEGF in both cellular models (Figure 7). This observation is particularly interesting since it has been shown that VEGF protein level is elevated in CF patients (34, 35). VEGF's protective activity in the oxidative condition is dependent on BCL2A1 (24), the other molecular pathway potentially involved in resistance to apoptosis and potentially involved in CF resistance to apoptosis (27). BCL2A1 protein also reduces the release of pro-apoptotic cytochrome c from mitochondria and blocks caspase activation. It is the only member of its family to be associated with the inflammatory response (51). Our microarray and RT-qPCR analyses revealed upregulation of BCL2A1 gene after DMNQ-induced stress (Figure 7), and this upregulation in CF cells indicates its potential participation in anti-apoptosis action.

According to our results, VEGFA and BCL2A1 are upregulated in both cell lines but also in primary bronchial cells of CF patients arguing in favor of an increase antiapoptotic activity in CF.

Finally, we also obtained potential evidence of MAPK signaling pathway participation in this process. MAPK genes comprise a large family of protein kinases that enable cells to respond to exogenous and endogenous stimuli (39). They build up an intracellular network that translates and integrates signals into cytoplasmic and nuclear processes. The phosphorylation states of MAPK signaling pathways modulate multiple cellular functions, such as proliferation, differentiation and apoptosis (44). We have observed modulation of mRNA expression of several molecules in the MAPK pathway. MAP3K5 (also named ASK1) is downregulated in *CuFi-1* and *CFBE41o-* exposed to DMNQ. However, we could not identify a significant decrease of MAP3K5 in the primary culture of epithelial cells. This lack of decrease in the primary culture could be secondary to a lack of power of our sample size or to the complexity and variability of the signaling pathways involved in apoptotic regulation. Taken together, these elements suggest that deficient apoptotic signaling in CF cell lines, either *CuFi-1* or *CFBE41o-*, under oxidative conditions, could derive from a complex regulatory system implicating VEGFA, BCL2A1 and the MAPK signaling pathway.

In conclusion, the new, permutational, multivariate, 2-way ANOVA used in our study identified interesting transcripts by computing the proportion of their variance explained by experimental conditions. The approach could be relevant in biomedical problems by integrating more than one experimental factor. Based on this analysis, we demonstrated the modulation of biological processes associated with survival functions in

574 CF cell lines under oxidative stress. Modulation could potentially be explained by  
575 dysregulation of the MAPK signaling pathway and the potential role of BCL2A1 and  
576 VEGFA, pro- and anti-apoptotic genes, respectively, in the apoptotic response.

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## Legends and figures

### **Figure 1. Volcano plot showing distribution of the expression ratio in *CuFi-1* compared to *NuLi-1* cells**

In the figure, upregulated, downregulated are illustrated in grey and immune or inflammatory transcripts are illustrated in black squares.  $P < 0.05$  values are considered significant, and the vertical lines indicate a 2-fold difference between the 2 cell lines.

### **Figure 2. GO term analysis of genes expressed differentially in *CuFi-1* and *NuLi-1* cells**

These bar plots summarize the results of GO term analysis for 3 selections of transcripts: (A) modulated transcripts, (B) upmodulated transcripts, (C) downmodulated transcripts. In this representation, biological functions fitting 4 criteria were showed: significantly over-represented ( $P < 0.05$ ), composed of at least  $>30$  transcripts (for (A) a minimum of 50 transcripts and for (B) and (C) a minimum of 30 transcripts), odds-ratio  $< 1.5$ , associated with 1 of 4 biological processes: cellular survival, inflammation, ribonucleotide metabolism, transduction signal. The observed (in light grey) and expected (dark grey) number of transcripts for each GO term are illustrated in the bar plot. The GO terms that were overrepresented in the *CuFi* but not associated with the 4 selected biological processes were not included in the figure.

### **Figure 3. Comparison of expression log-ratios between microarray and RT-qPCR experiments**

The expression log-ratio microarray results of 19 transcripts were compared to their expression log-ratio by RT-qPCR. We also report the observed modulation or detection of these transcripts in other experiments. All the expression data were done in triplicate, starting with three distinct cultures of each cell lines (with or without treatment). For each gene, error bars on the histogram for the relative expression are indicated.

**Figure 4. Dose-response study evaluating the effect of DMNQ on *CuFi-1* and *NuLi-1* cell lines**

(A) Total GSH was measured to evaluate the oxidative stress generated by different DMNQ concentrations (0, 10, 15, 20  $\mu$ M) on *CuFi-1* and *NuLi-1* cell lines. The impact of DMNQ (B) on total current ( $\mu$ A) was also recorded. (C) The physiological impact of DMNQ was also estimated by measuring the transepithelial resistance (Ohms). The data are shown as means $\pm$ SD. Significant differences were revealed by parametric 2-way ANOVA (\* $P \leq 0.05$  of difference from the controls). The gene expression study was done with a concentration of 15 $\mu$ M.

**Figure 5. Comparative GO term analysis**

Enrichment of GO term following the selection of transcripts based on IQR and taking into account the % of explained variance. (A) Based on the IQR filters, selection of 687 transcripts having an IQR value equal or superior to 0.5 which have % of explained variance superior to 40 % (decomposed in at least 20 % explained by the mutation and at least 20 % explained by the oxidant). (B) A Gene ontology analysis was performed on the selected gene to test the overrepresentation of biological processes for this selection. Among the most represented pathways, oxidation/reduction, cell death and apoptosis were significantly overrepresented.

**Figure 6. Downmodulated and upmodulated trends of gene expression for *CuFi-1* vs *NuLi-1* cells in the absence/presence of DMNQ**

Expression profiles were clustered according to the Mfuzz algorithm from standardized expression data on 96 probes (corresponding to 59 distinct transcripts) with IQR  $> 0.5$  associated with cell death and apoptotic processes. Expression profiles of (A) upregulated transcripts dependent on DMNQ and  $\Delta$ F508 mutation, and (B) downregulated transcripts dependent on DMNQ and  $\Delta$ F508 for *CuFi-1* vs *NuLi-1*. To the right of each cluster, we

illustrate for each transcript the repartition of the total variance between the factors mutation and oxidant. The central black square highlights those transcripts where the total variance is explained by at least 40% but not more than 60% for one factor.

**Figure 7. Evaluation of MAP3K5, VEGFA and BCL2A1 mRNA expression by RT-qPCR on CF and non-CF cell lines after oxidative stress and in CF and non-CF primary bronchial cells**

(A) The HPRT1 gene was used to normalize MAP3K5, VEGFA and BCL2A1 expression. VEGFA and BCL2A1 expression in CF and non-CF cells was significantly upregulated (\*\* $P < 0.001$ ) after DMNQ treatment, as described previously in *CuFi/NuLi* cell lines. MAP3K5 was significantly decreased (\*\* $P < 0.01$ ) in CF and non-CF cell lines after DMNQ treatment. (B) The HPRT1 gene was used to normalize MAP3K5, VEGFA and BCL2A1 expression. VEGFA and BCL2A1 expression was significantly upregulated (\*  $P < 0.05$ ) in bronchial CF cells. There was no significant increase of the MAP3K5 gene in the bronchial CF cells. The data are reported as fold expression obtained by standard curve analysis.

**Figure 8. Oligonucleosomes release after DMNQ treatment in CF and non-CF cell lines**

As a specific marker of apoptosis induction, histone-associated DNA fragments (mono- and oligonucleosomes) were quantified by photometric enzyme immunoassay in CF and non-CF cell lines. 2-way ANOVA was performed on background-normalized absorbance. There is a significant increase in the oligonucleosome release in the cytosol of the *NuLi-1* cells compared to the *CuFi-1* cells in presence of DMNQ. In the *corrCFBE41o-* (non-CF) and *CFBE41o-* (CF) cells, in presence of DMNQ, There is an increase of oligonucleosomes in the CF and non CF cells, but the increase was more important in *corrCFBE41o-* (non-CF) compared to *CFBE41o-* (CF) cells.

**Figure 9. Caspase 3/7 after DMNQ treatment in CF and non-CF cell lines**

As a specific marker of apoptosis induction, Caspase 3/7 activity was quantified by liminometric assay in CF (CuFi-1) and non-CF (NuLi-1) cell lines. Following DMNQ treatement, there is a significant increase (\* $P < 0.05$ ) in the caspase activity in the *NuLi* cells but not in the *CuFi* cells.



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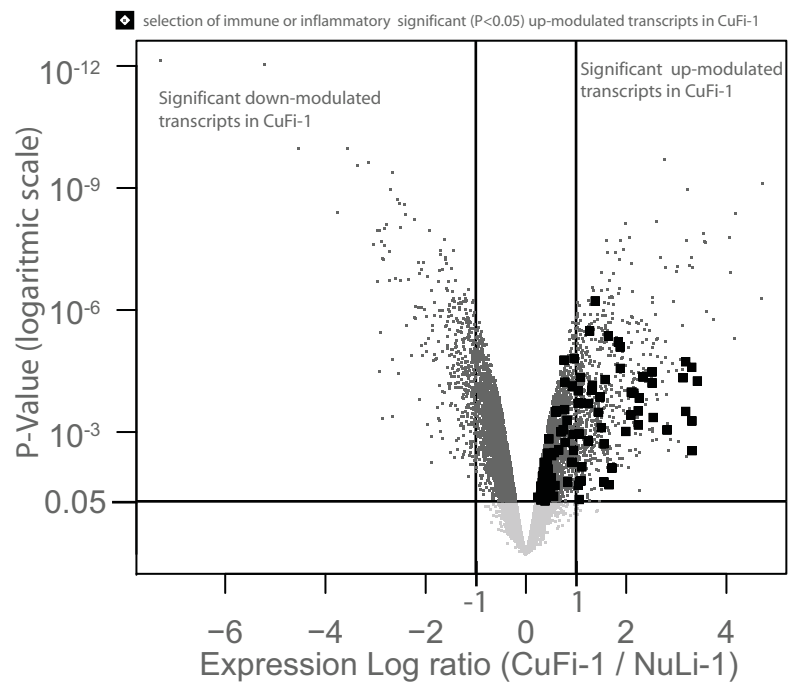
## References

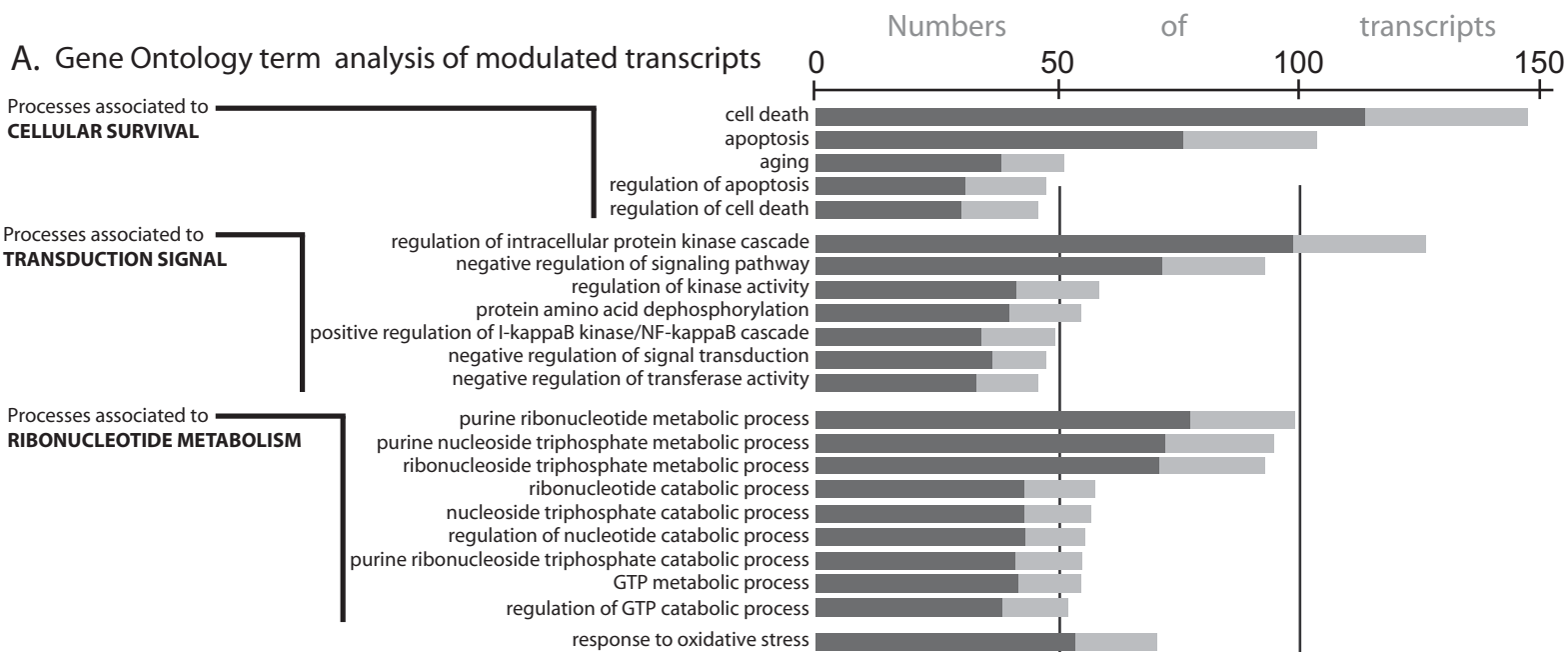
1. **Aldallal N, McNaughton EE, Manzel LJ, Richards AM, Zabner J, Ferkol TW, and Look DC.** Inflammatory response in airway epithelial cells isolated from patients with cystic fibrosis. *Am J Respir Crit Care Med* 166: 1248-1256, 2002.
2. **Arsalane K, Dubois CM, Muanza T, Begin R, Boudreau F, Asselin C, and Cantin AM.** Transforming growth factor-beta1 is a potent inhibitor of glutathione synthesis in the lung epithelial cell line A549: transcriptional effect on the GSH rate-limiting enzyme gamma-glutamylcysteine synthetase. *Am J Respir Cell Mol Biol* 17: 599-607, 1997.
3. **Barrett T, Troup DB, Wilhite SE, Ledoux P, Rudnev D, Evangelista C, Kim IF, Soboleva A, Tomashevsky M, Marshall KA, Phillippy KH, Sherman PM, Muertter RN, and Edgar R.** NCBI GEO: archive for high-throughput functional genomic data. *Nucleic Acids Res* 37: D885-D890, 2009.
4. **Becker MN, Sauer MS, Muhlebach MS, Hirsh AJ, Wu Q, Verghese MW, and Randell SH.** Cytokine secretion by cystic fibrosis airway epithelial cells. *Am J Respir Crit Care Med* 169: 645-653, 2004.
5. **Bensalem N, Ventura AP, Vallee B, Lipecka J, Tondelier D, Davezac N, Santos AD, Perretti M, Fajac A, Sermet-Gaudelus I, Renouil M, Lesure JF, Halgand F, Laprevote O, and Edelman A.** Down-regulation of the Anti-inflammatory Protein Annexin A1 in Cystic Fibrosis Knock-out Mice and Patients. *Mol Cell Proteomics* 4: 1591-1601, 2005.
6. **Bezzetti V, Borgatti M, Nicolis E, Lampronti I, Dehecchi MC, Mancini I, Rizzotti P, Gambari R, and Cabrini G.** Transcription factor oligodeoxynucleotides to NF-kappaB inhibit transcription of IL-8 in bronchial cells. *Am J Respir Cell Mol Biol* 39: 86-96, 2008.
7. **Bianchi ME.** DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol* 81: 1-5, 2007.
8. **Bouvet GF, Voisin G, and Berthiaume Y.** Exploring epigenetic modulation in Cystic Fibrosis: relationship between DNA methylation and inflammatory gene expression in epithelial cells. *Pediatr Pulmonol* 278: A159, 2012.
9. **Cantin AM, White TB, Cross CE, Forman HJ, Sokol RJ, and Borowitz D.** Antioxidants in cystic fibrosis. Conclusions from the CF antioxidant workshop, Bethesda, Maryland, November 11-12, 2003. *Free Radic Biol Med* 42: 15-31, 2007.
10. **Coakley RD, Grubb BR, Paradiso AM, Gatzky JT, Johnson LG, Kreda SM, O'Neal WK, and Boucher RC.** Abnormal surface liquid pH regulation by cultured cystic fibrosis bronchial epithelium. *Proc Natl Acad Sci USA* 100: 16083-16088, 2003.
11. **de la Rosa G, Yang D, Tewary P, Varadhachary A, and Oppenheim JJ.** Lactoferrin acts as an alarmin to promote the recruitment and activation of APCs and antigen-specific immune responses. *Journal of immunology (Baltimore, Md : 1950)* 180: 6868-6876, 2008.
12. **Dehecchi MC, Nicolis E, Norez C, Bezzetti V, Borgatti M, Mancini I, Rizzotti P, Ribeiro CM, Gambari R, Becq F, and Cabrini G.** Anti-inflammatory effect of miglustat in bronchial epithelial cells. *J Cyst Fibros* 7: 555-565, 2008.
13. **Dimagno MJ, Lee S-H, Hao Y, Zhou S-Y, McKenna BJ, and Owyang C.** A proinflammatory, antiapoptotic phenotype underlies the susceptibility to acute

- pancreatitis in cystic fibrosis transmembrane regulator (-/-) mice. *Gastroenterology* 129: 665-681, 2005.
14. **Donaldson SH, and Boucher RC.** Update on pathogenesis of cystic fibrosis lung disease. *Curr Opin PulmMed* 9: 486-491, 2003.
  15. **Falcon S, and Gentleman R.** Using GStats to test gene lists for GO term association. *Bioinformatics (Oxford, England)* 23: 257-258, 2007.
  16. **Futschik ME, and Carlisle B.** Noise-robust soft clustering of gene expression time-course data. *J Bioinform Comput Biol* 3: 965-988, 2005.
  17. **Gambardella S, Biancolella M, D'Apice MR, Amati F, Sangiuolo F, Farcomeni A, Chillemi G, Bueno S, Desideri A, and Novelli G.** Gene expression profile study in CFTR mutated bronchial cell lines. *Clin Exp Med* 6: 157-165, 2006.
  18. **Gruenert DC, Willems M, Cassiman JJ, and Frizzell RA.** Established cell lines used in cystic fibrosis research. *Journal of Cystic Fibrosis* 3: 191-196, 2004.
  19. **Guilbault C, Novak JP, Martin P, Boghdady ML, Saeed Z, Guiot MC, Hudson TJ, and Radzioch D.** Distinct pattern of lung gene expression in the Cftr-KO mice developing spontaneous lung disease compared with their littermate controls. *Physiol Genomics* 25: 179-193, 2006.
  20. **Hampton TH, Ballok AE, Bomberger JM, Rutkowski MR, Barnaby R, Coutermarsh B, Conejo-Garcia JR, O'Toole GA, and Stanton BA.** Does the F508-CFTR mutation induce a proinflammatory response in human airway epithelial cells? *AJP: Lung Cellular and Molecular Physiology* 303: L509-L518, 2012.
  21. **Hampton TH, and Stanton BA.** A novel approach to analyze gene expression data demonstrates that the DeltaF508 mutation in CFTR downregulates the antigen presentation pathway. *American journal of physiology Lung cellular and molecular physiology* 298: L473-482, 2010.
  22. **Haraldsen G, Balogh J, Pollheimer J, Sponheim J, and K  chler AM.** Interleukin-33 - cytokine of dual function or novel alarmin? *Trends in immunology* 30: 227-233, 2009.
  23. **Haston CK, Cory S, Lafontaine L, Dorion G, and Hallett MT.** Strain-dependent pulmonary gene expression profiles of a cystic fibrosis mouse model. *Physiol Genomics* 25: 336-345, 2006.
  24. **He C-H, Waxman AB, Lee CG, Link H, Rabach ME, Ma B, Chen Q, Zhu Z, Zhong M, Nakayama K, Nakayama KI, Homer R, and Elias JA.** Bcl-2-related protein A1 is an endogenous and cytokine-stimulated mediator of cytoprotection in hyperoxic acute lung injury. *The Journal of clinical investigation* 115: 1039-1048, 2005.
  25. **Irizarry RA.** Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res* 31: 15e-15, 2003.
  26. **Jacquot J, Tabary O, Le RP, and Clement A.** Airway epithelial cell inflammatory signalling in cystic fibrosis. *Int J BiochemCell Biol* 40: 1703-1715, 2008.
  27. **Jungas T, Motta I, Duffieux F, Fanen P, Stoven V, and Ojcius DM.** Glutathione levels and BAX activation during apoptosis due to oxidative stress in cells expressing wild-type and mutant cystic fibrosis transmembrane conductance regulator. *JBiolChem* 277: 27912-27918, 2002.

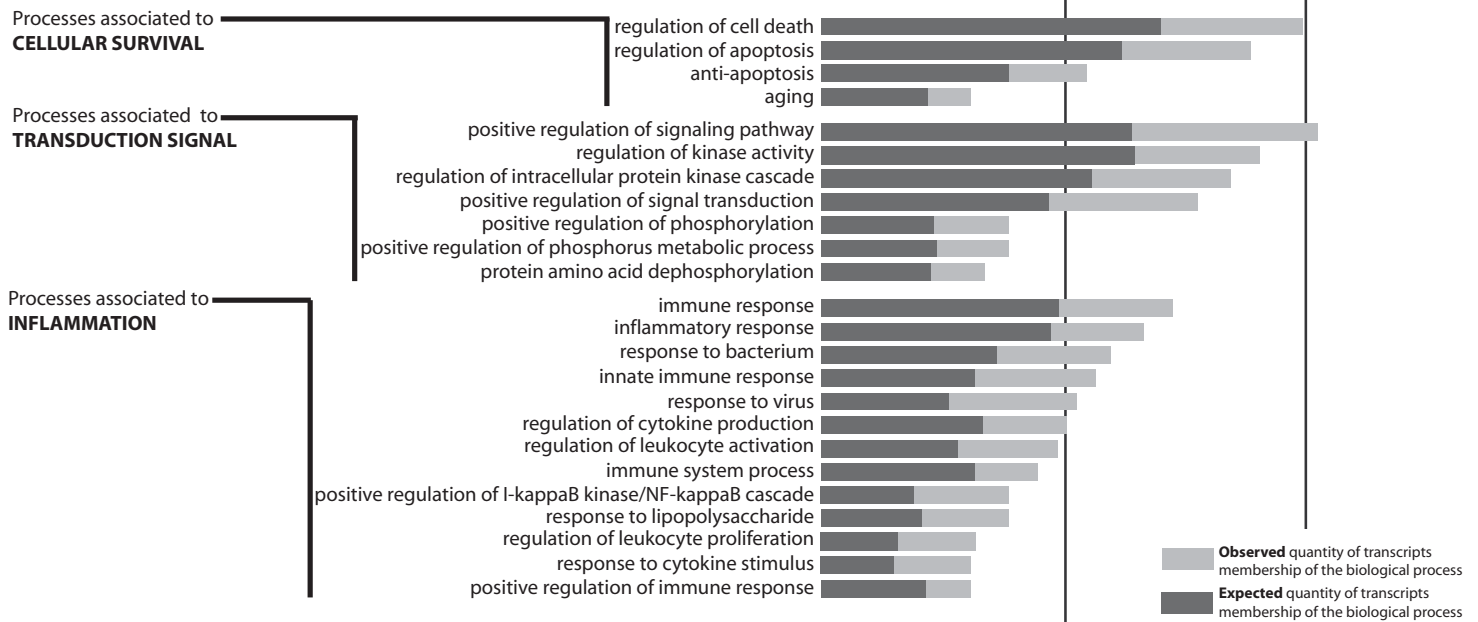
28. **Lagrange-Puget M, Durieu I, Ecochard R, Abbas-Chorfa F, Draï J, Steghens JP, Pacheco Y, Vital-Durand D, and Bellon G.** Longitudinal study of oxidative status in 312 cystic fibrosis patients in stable state and during bronchial exacerbation. *Pediatr Pulmonol* 38: 43-49, 2004.
29. **Lee CG, Da Silva CA, Dela Cruz CS, Ahangari F, Ma B, Kang M-J, He C-H, Takyar S, and Elias JA.** Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. *Annu Rev Physiol* 73: 479-501, 2011.
30. **Legendre P, and Anderson MJ.** Distance-based redundancy analysis: Testing multispecies responses in multifactorial ecological experiments. *Ecological Monographs* 69: 1-24, 1999.
31. **Legendre P, and Legendre L.** *Numerical Ecology*. Elsevier, 2012, p. 1006.
32. **Luciani A, Villella VR, Esposito S, Brunetti-Pierri N, Medina D, Settembre C, Gavina M, Pulze L, Giardino I, Pettoello-Mantovani M, D'Apolito M, Guido S, Masliah E, Spencer B, Quarantino S, Raia V, Ballabio A, and Maiuri L.** Defective CFTR induces aggresome formation and lung inflammation in cystic fibrosis through ROS-mediated autophagy inhibition. *Nature cell biology* 2010.
33. **Machen TE.** Innate immune response in CF airway epithelia: hyperinflammatory? *Am J Physiol Cell Physiol* 291: C218-C230, 2006.
34. **Martin C, Coolen N, Wu Y, Thevenot G, Touqui L, Prulière-Escabasse V, Papon JF, Coste A, Escudier E, Dusser DJ, Fajac I, and Burgel PR.** CFTR dysfunction induces vascular endothelial growth factor synthesis in airway epithelium. *Eur Respir J* 42: 1553-1562, 2013.
35. **McColley SA, Stellmach V, Boas SR, Jain M, and Crawford SE.** Serum vascular endothelial growth factor is elevated in cystic fibrosis and decreases with treatment of acute pulmonary exacerbation. *Am J Respir Crit Care Med* 161: 1877-1880, 2000.
36. **Norkina O, Kaur S, Ziemer D, and De Lisle RC.** Inflammation of the cystic fibrosis mouse small intestine. *Am J Physiol Gastrointest Liver Physiol* 286: G1032-G1041, 2004.
37. **Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, Ohara RB, Simpson GL, Solymos P, Stevens HH, and Wagner H.** vegan: Community Ecology Package. In: *veganr-forger-project* 2013.
38. **Pavlidis P.** Using ANOVA for gene selection from microarray studies of the nervous system. *Methods* 31: 282-289, 2003.
39. **Raman M, Chen W, and Cobb MH.** Differential regulation and properties of MAPKs. *Oncogene* 26: 3100-3112, 2007.
40. **Ratjen F, and Doring G.** Cystic fibrosis. *Lancet* 361: 681-689, 2003.
41. **Rottner M, Tual-Chalot S, Mostefai HA, Andriantsitohaina R, Freyssinet J-M, and Martínez MC.** Increased Oxidative Stress Induces Apoptosis in Human Cystic Fibrosis Cells. *PLoS ONE* 6: e24880, 2011.
42. **Smyth GK.** Limma: linear models for microarray data. In: *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*, edited by Gentleman R, Carey V, Huber W, Irizarry R, and Dudoit S. New York: Springer, 2005, p. 397-420.
43. **Soleti R, Porro C, and Martínez MC.** Apoptotic process in cystic fibrosis cells. *Apoptosis* 18: 1029-1038, 2013.

- 817 44. **Takeda K, Noguchi T, Naguro I, and Ichijo H.** Apoptosis Signal-Regulating  
818 Kinase 1 in Stress and Immune Response. *Annu Rev Pharmacol Toxicol* 48: 199-225,  
819 2008.
- 820 45. **Tamm C, Zhivotovsky B, and Ceccatelli S.** Caspase-2 activation in neural  
821 stem cells undergoing oxidative stress-induced apoptosis. *Apoptosis* 13: 354-363,  
822 2008.
- 823 46. **Tarran R, Button B, and Boucher RC.** Regulation of normal and cystic  
824 fibrosis airway surface liquid volume by phasic shear stress. *AnnuRev Physiol* 68:  
825 543-561, 2006.
- 826 47. **Trinh NT, Prive A, Maille E, Noel J, and Brochiero E.** EGF and K<sup>+</sup> channel  
827 activity control normal and cystic fibrosis bronchial epithelia repair. *Am J Physiol*  
828 *Lung Cell Mol Physiol* 2008.
- 829 48. **Valdivieso AG, and Santa-Coloma TA.** CFTR activity and mitochondrial  
830 function. *Redox Biology* 1: 190-202, 2013.
- 831 49. **Vankeerberghen A, Cuppens H, and Cassiman J-J.** The cystic fibrosis  
832 transmembrane conductance regulator: an intriguing protein with pleiotropic  
833 functions. *Journal of Cystic Fibrosis* 1: 13-29, 2002.
- 834 50. **Verhaeghe C, Remouchamps C, Hennuy B, Vanderplasschen A, Chariot A,**  
835 **Tabruyn SP, Oury C, and Bours V.** Role of IKK and ERK pathways in intrinsic  
836 inflammation of cystic fibrosis airways. *Biochem Pharmacol* 73: 1982-1994, 2007.
- 837 51. **Vogler M.** BCL2A1: the underdog in the BCL2 family. 19: 67-74, 2011.
- 838 52. **Wright JM, Merlo CA, Reynolds JB, Zeitlin PL, Garcia JGN, Guggino WB,**  
839 **and Boyle MP.** Respiratory epithelial gene expression in patients with mild and  
840 severe cystic fibrosis lung disease. *Am J Respir Cell Mol Biol* 35: 327-336, 2006.
- 841 53. **Xu Y, Clark JC, Aronow BJ, Dey CR, Liu C, Wooldridge JL, and Whitsett JA.**  
842 Transcriptional adaptation to cystic fibrosis transmembrane conductance regulator  
843 deficiency. *JBiolChem* 278: 7674-7682, 2003.
- 844 54. **Yalçın E, Talim B, Özçelik U, Doğru D, Cobanoğlu N, Pekcan S, and Kiper**  
845 **N.** Does defective apoptosis play a role in cystic fibrosis lung disease? *Arch Med Res*  
846 40: 561-564, 2009.
- 847 55. **Zabner J, Karp P, Seiler M, Phillips SL, Mitchell CJ, Saavedra M, Welsh M,**  
848 **and Klingelhutz AJ.** Development of cystic fibrosis and noncystic fibrosis airway  
849 cell lines. *AmJPhysiol Lung Cell MolPhysiol* 284: L844-L854, 2003.
- 850 56. **Zabner J, Scheetz TE, Almabrazi HG, Casavant TL, Huang J, Keshavjee S,**  
851 **and McCray PB, Jr.** CFTR DeltaF508 mutation has minimal effect on the gene  
852 expression profile of differentiated human airway epithelia. *Am J Physiol Lung Cell*  
853 *MolPhysiol* 289: L545-L553, 2005.
- 854 57. **Ziady AG, and Hansen J.** Redox balance in Cystic Fibrosis. *The International*  
855 *Journal of Biochemistry & Cell Biology* 2014.

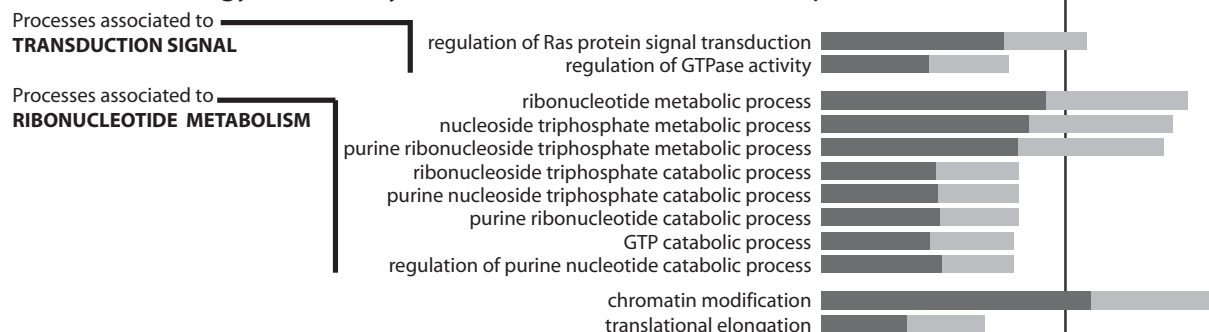




### B. Gene Ontology term analysis of up-modulated transcripts

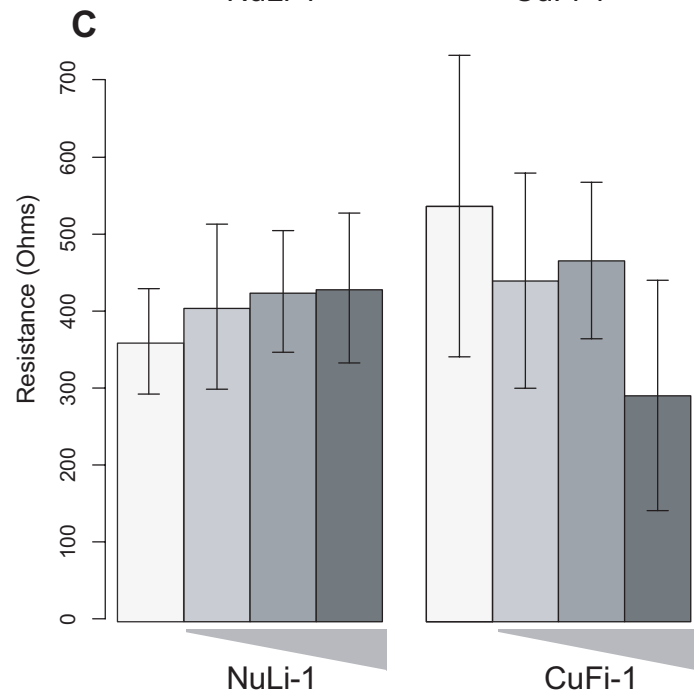
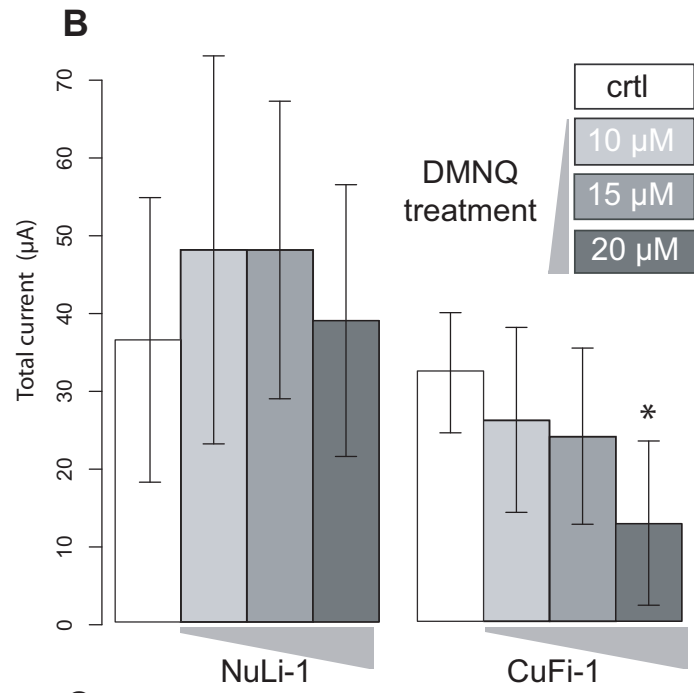
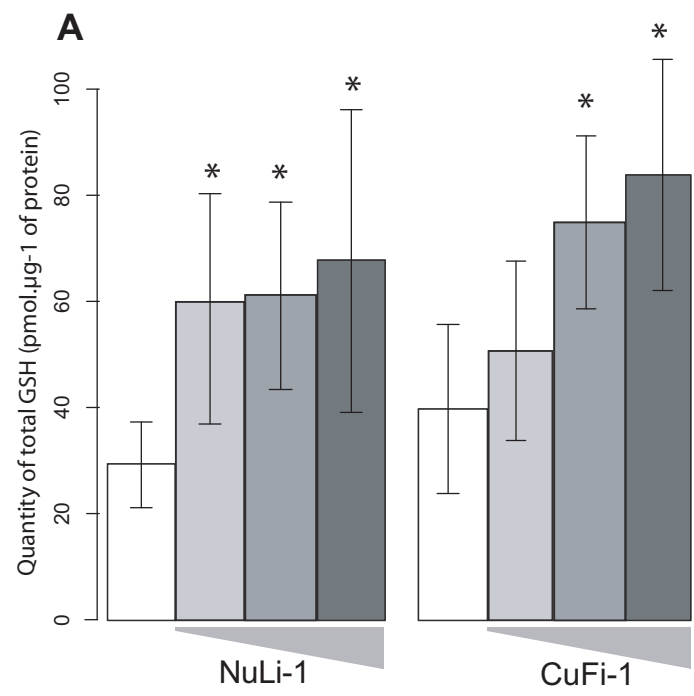


### C. Gene Ontology term analysis of down-modulated transcripts

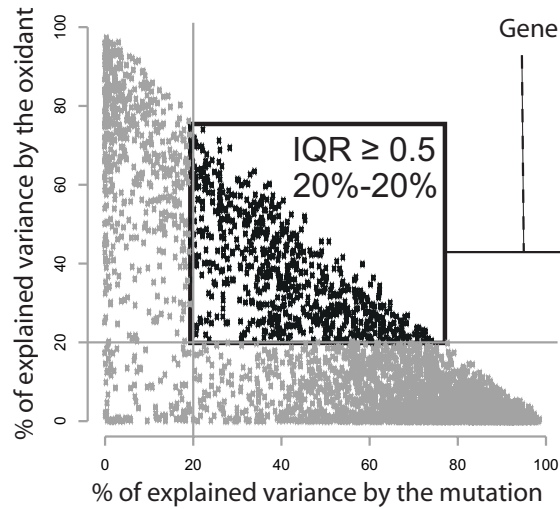


Detected in CF patients (except microarray experlments)	Observed modulations in microarray experiments				Observed modulations in PCR experiments	
						<div>NuLi-1</div> <div>CuFi-1</div>
Bonfield, Panuska et al. 1995 (2)		▲		4.15	18.72	IL6
Schuster, Haarmann et al. 1995 (3)				1.88	1.92	IL8
Bonfield, Panuska et al. 1995 (2)						
Dean, Dai et al. 1993 (2) (3)						
Schuster, Haarmann et al. 1995 (3)		▲	▲	1.17	0.87	IL1B
Greally, Hussain et al. 1993 (4)				1.58	1.02	IL1A
Schuster, Haarmann et al. 1995 (3)						
Adib-Conquy, Pedron et al. 2008 (1)				1.32	0.40	IL1RN
Kronborg, Hansen et al. 1993 (3) (4)						
Schwiebert, Estell et al. 1999 (5)			▲	1.09	2.09	CCL5
Adib-Conquy, Pedron et al. 2008 (1)				3.18	2.86	CXCL10
				3.20	2.73	CXCL11
		▲		2.83	5.86	TNFAIP6
Kelley and Elmer 2000 (5)			▲	0.97	1.04	STAT1
Adib-Conquy, Pedron et al. 2008 (1)				0.8	0.16	CFOS
Adib-Conquy, Pedron et al. 2008 (1)			▲	3.18	1.16	THBS1
MacGregor, Gray et al. 2008 (2)						
Henke, Renner et al. 2006 (5)		▲	▲	0.59	2.09	S100A8
Henke, Renner et al. 2006 (5)		▲	▲	0.89	1.45	S100A9
Foell, Seeliger et al. 2003 (4)				2.28	2.85	S100A12
				-1.52	4.29	HLADQB1
				-2.13	4.19	HLADRB1
				2.19	4.61	C15
Owens, Shroyer et al. 2008 (6)				-1.62	-1.05	ALOX5
(1)polymorphonuclear leukocytes (2)bronchoalveolar lavage (3)sputum (4)serum (5)epithelial cells (6)sinonasal mucosa	Verhaagehe et al	Kaur et al	Xu et al	Voisin et al log-Ratio (cuFi-1/NuLi-1)	RT-PCR log-Ratio	





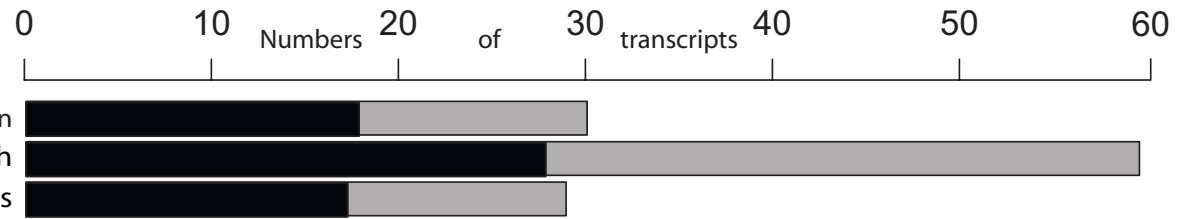
(A)



Gene Ontology analysis of selected transcripts

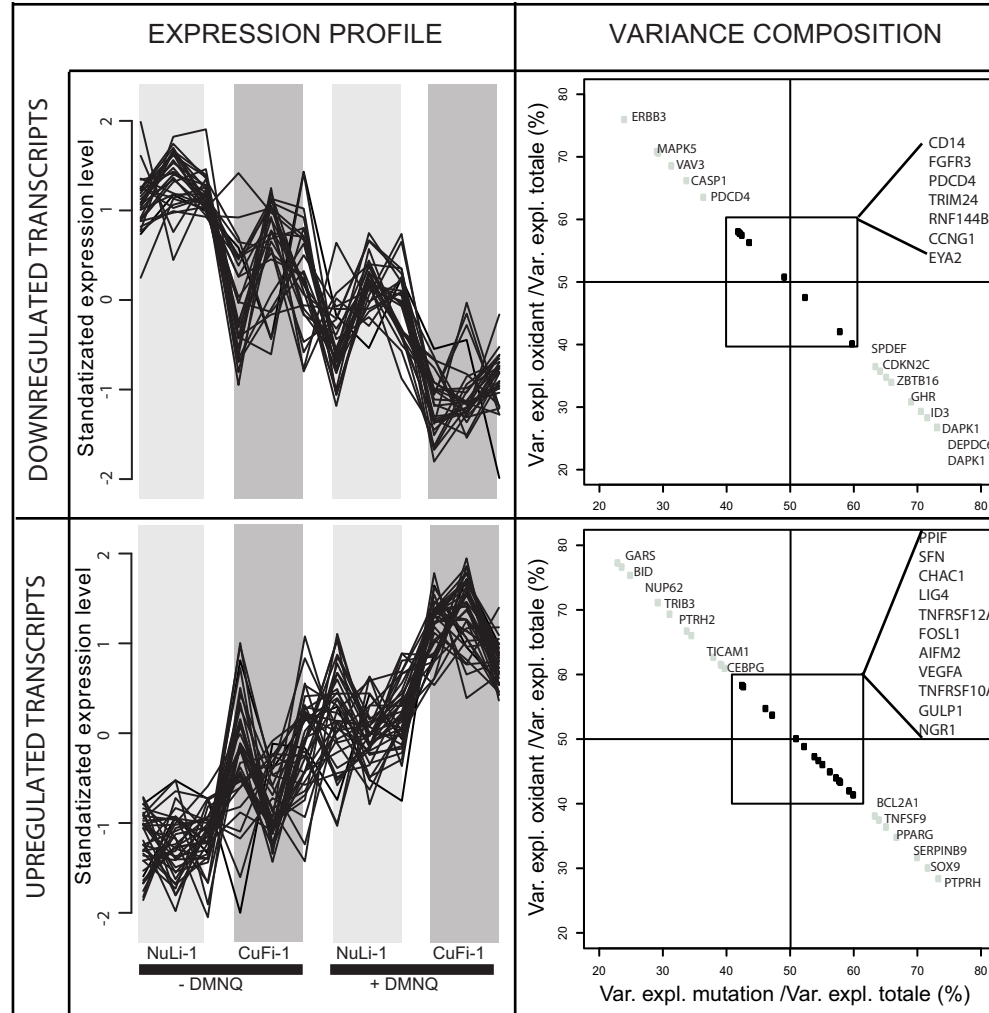
(B)

Oxidation Reduction  
cell death  
apoptosis



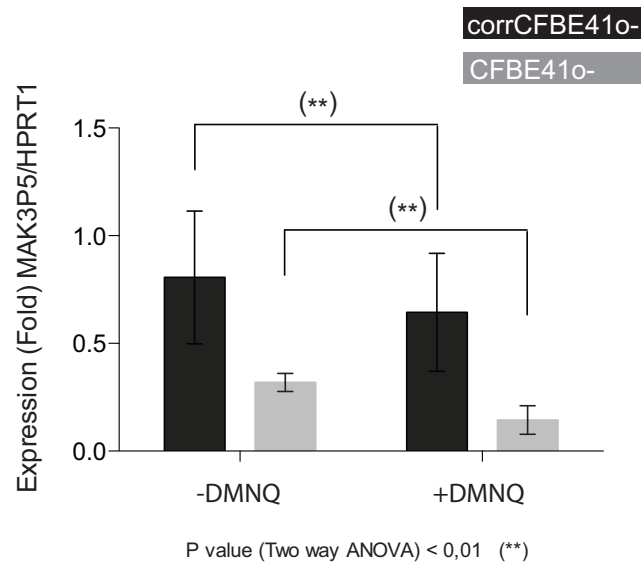
The selected overrepresented biological processes

A)

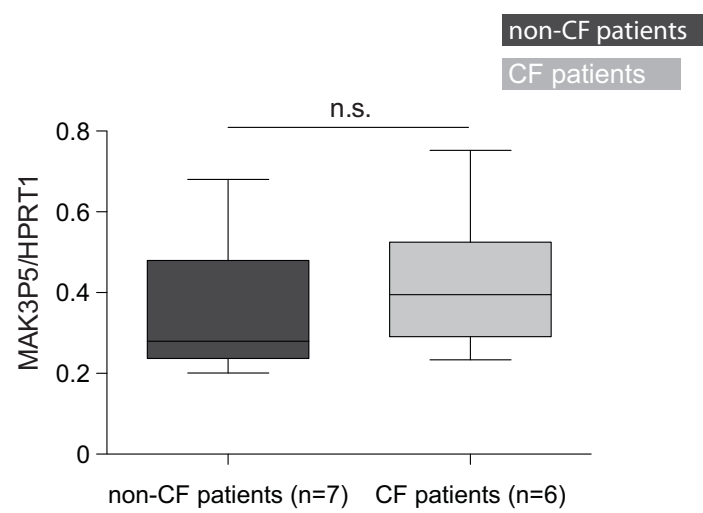


B)

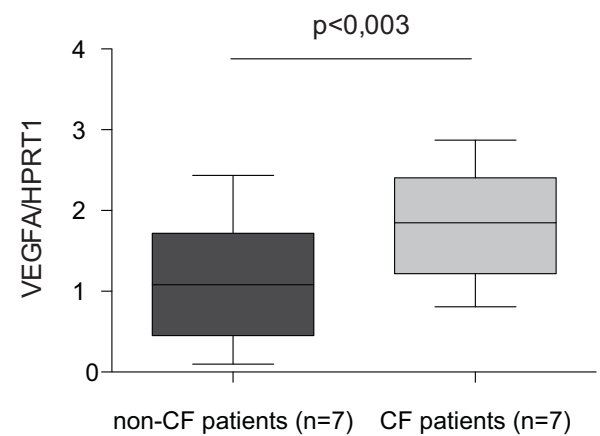
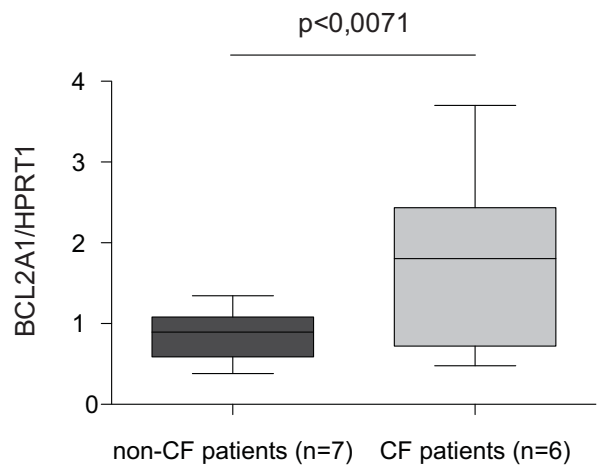
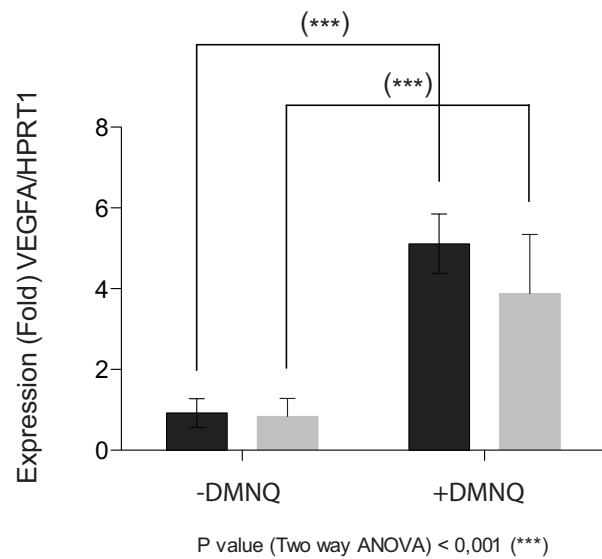
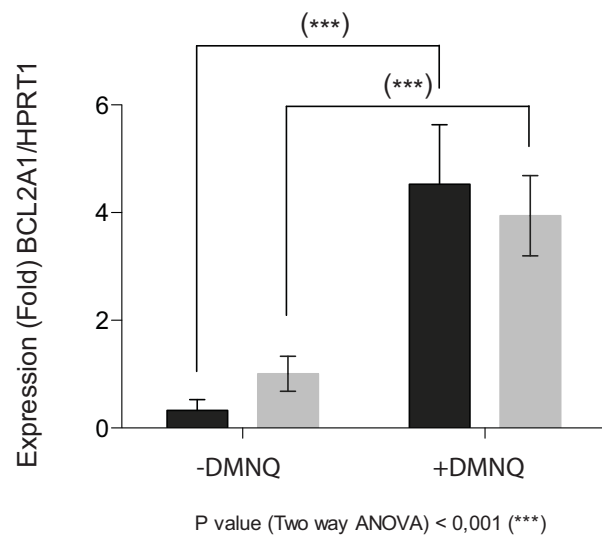
## Downregulation after DMNQ treatment

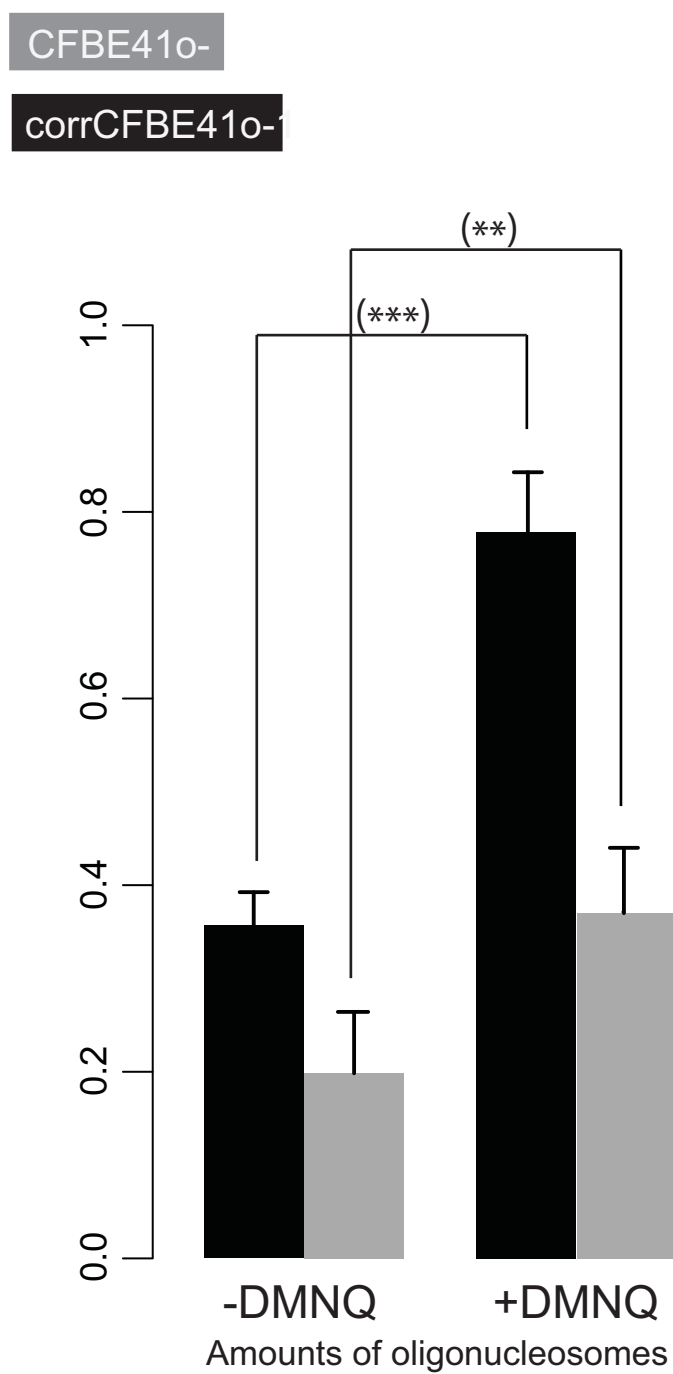
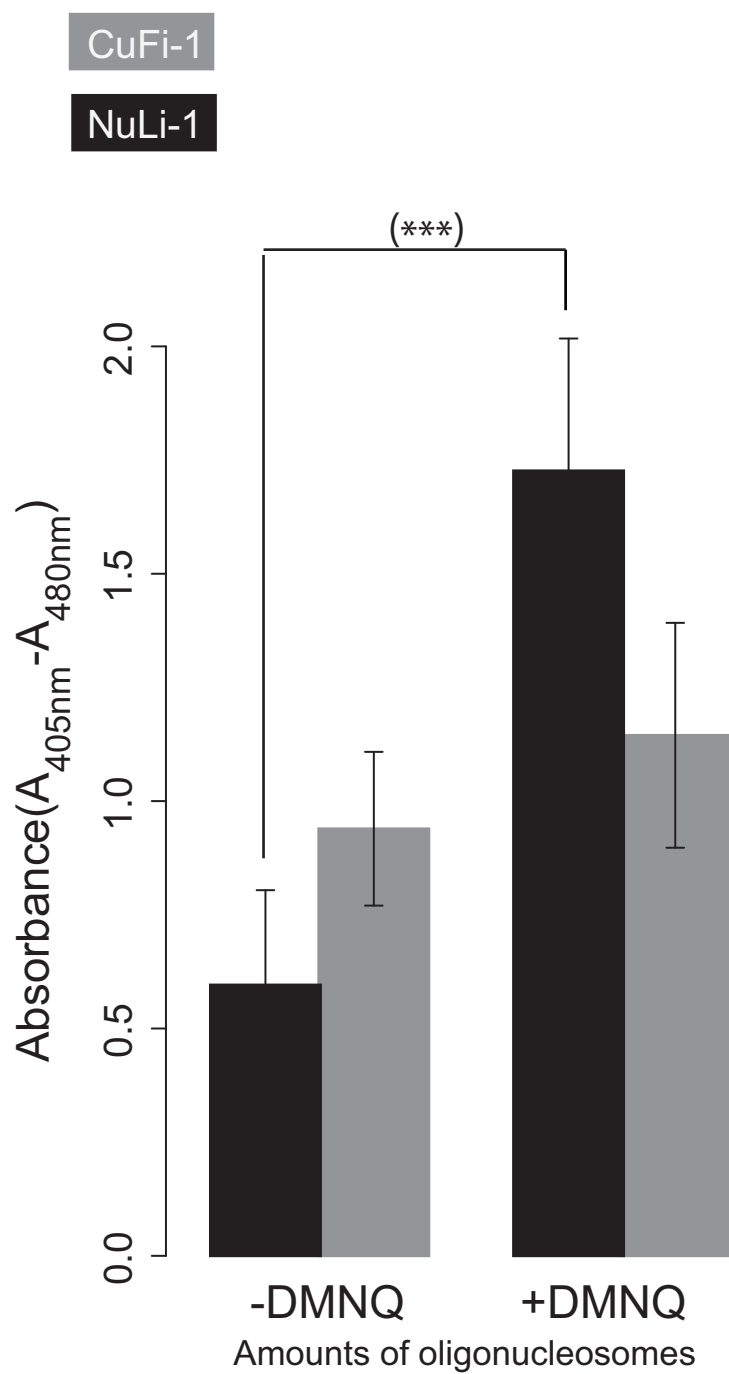


## MAK3P5/HPRT1



## Upregulation after DMNQ treatment





P value (Two way ANOVA) < 0,05 (\*)  
P value (Two way ANOVA) < 0,01 (\*\*)  
P value (Two way ANOVA) < 0,001 (\*\*\*)

