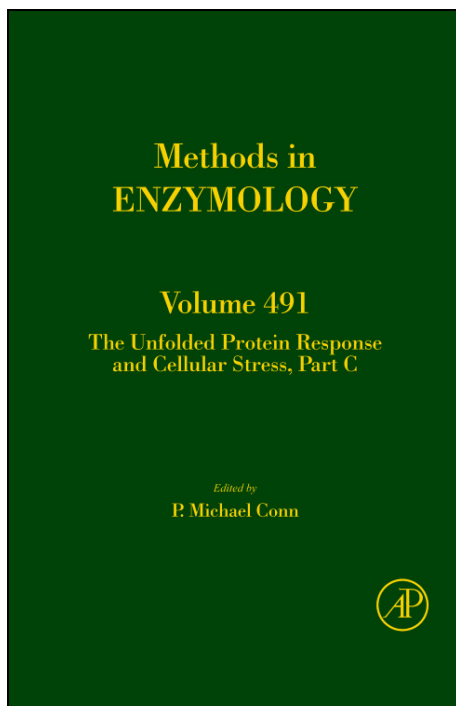


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# SIGNALING PATHWAYS OF PROTEOSTASIS NETWORK UNRAVELED BY PROTEOMIC APPROACHES ON THE UNDERSTANDING OF MISFOLDED PROTEIN RESCUE

Patrícia Gomes-Alves, Sofia Neves, *and* Deborah Penque

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## Abstract

Attempts to promote normal processing and function of F508del-CFTR, the most common mutant in cystic fibrosis (CF), have been described as potential therapeutic strategies in the management of this disease.

Here we described the proteomic approaches, namely two-dimensional electrophoresis (2DE), mass spectrometry (MS), and bioinformatics tools used in our recent studies to gain insight into the proteins potentially involved in low-temperature or mutagenic treatment-induced rescue process of F508del-CFTR. The proteins identified are part of the proteostasis network, such as the unfolded protein response (UPR) signaling pathways that may regulate the processing of CF transmembrane conductance regulator (CFTR) through the folding and trafficking progression. The complete characterization of these signaling pathways and their regulators in CF will certainly contribute to the development of novel therapeutic strategies against CF.

## 1. INTRODUCTION

The unfolded protein response (UPR) is one of the signaling pathways composing the proteostasis network, which regulates and maintains the protein folding and function in the face of many cellular challenges during cell lifetime (Powers *et al.*, 2009). The UPR is a complex molecular cascade activated in response to cellular stressors that induce the endoplasmic reticulum (ER) overloading with unfolded proteins. The UPR is mainly characterized by upregulation of ER chaperones to improve the folding capacity of the ER and reestablish ER homeostasis, by facilitating proteins correct folding or assembly and preventing protein aggregation in the ER lumen and consequently improving cell survival (Citterio *et al.*, 2008; Kelsen *et al.*, 2008). Activation of ER-associated degradation (ERAD) pathways, another signaling pathways composing proteostasis network responsible for the degradation of the excess of misfolded proteins in the ER (van Anken and Braakman, 2005), is also a characteristic feature of UPR.

Dysregulation of cellular proteostasis by a constant flux of misfolded and mistrafficking proteins contributes to a broad range of human diseases such as cystic fibrosis (CF).

CF is mostly caused by the inherited F508del mutation that leads to CF transmembrane conductance regulator (CFTR) misfolding and excessive degradation, ultimately resulting in reduced availability of functional protein.

Under F508del-CFTR overexpression, cells induce ER stress and/or the UPR signaling pathways as a response to a loss of proteostatic control (Bartoszewski *et al.*, 2008; Gomes-Alves and Penque, 2010; Gomes-Alves

*et al.*, 2009, 2010a; Kerbirou *et al.*, 2007). We have shown by proteomics that treatments aimed at rescuing F508del-CFTR, such as low-temperature incubation or “RXR” mutagenic repair (by inactivation of four RXR motifs of F508del-CFTR-4RK mutant), modulate/readjust the proteome components of pathways composing proteostasis network, including the UPR and ERAD (Gomes-Alves and Penque, 2010; Gomes-Alves *et al.*, 2009, 2010a).

Most therapies designed to rescue the trafficking defect of F508del-CFTR cause the UPR/ER stress induction (Gomes-Alves *et al.*, 2010b; Loo *et al.*, 2005; Singh *et al.*, 2008) involving, however, a larger number or higher expression of proteins than those induced by the misfolded protein itself (Gomes-Alves and Penque, 2010; Gomes-Alves *et al.*, 2010b; Loo *et al.*, 2005; Singh *et al.*, 2008). This treatment-induced UPR/ER stress stimulus seems also to readjust some proteostasis imbalance associated with F508del-CFTR expression, which probably endows the cell with the ability to rescue at least partially the mutant CFTR.

We showed that overexpression in BHK cell lines of “repaired” form of F508del-CFTR, F508del-4RK-CFTR, or low-temperature treatment on BHK cell lines overexpressing the mutant F508del-CFTR induce upregulation of GRP78/BiP (the UPR hallmarker), several ER stress (e.g., GRP94 and GRP75), and several foldases (e.g., PDI) and readjust (to wt type level) some proteome imbalance associated with the proteasome degradation (e.g., Psme2 and Cops5) and folding and transport (e.g., RACK1) of CFTR (Gomes-Alves and Penque, 2010; Gomes-Alves *et al.*, 2009, 2010a).

We are convinced that the complete characterization of the proteostasis signaling pathways and their regulators in CF by high-throughput proteomics will confidently culminate in the development of novel therapeutic strategies against CF.

## 2. 2DE-BASED PROTEOMICS APPROACH ANALYSIS TO STUDY PROTEIN EXPRESSION PROFILE

Proteome profiling of cells/tissues under different conditions (e.g., health vs. disease, treated vs. nontreated) by two-dimensional gel electrophoresis (2DE) and mass spectrometry (MS)-based proteomic approach has contributed to the elucidation of the basic cellular mechanisms of disease by discovering candidate biomarkers and disease targets for new drug development (Penque, 2009).

The main advantage of 2DE technology is its capacity to provide a global view of a sample proteome at a given time by resolving hundreds to thousands of proteins simultaneously on a single gel. In spite of the inability

of 2DE to resolve all proteins present in a sample (e.g., highly hydrophobic proteins, low abundance proteins, extremely basic or acidic pI values, molecular size out of gel limits) or the growing developments in gel-free MS-based approaches, 2DE in combination with MS still remains the most popular technology in proteomics (Penque, 2009).

In the next sections we provide detailed information on the optimal conditions and procedures for 2DE/MS-based proteomics analysis used in our previous publications (Gomes-Alves and Penque, 2010; Gomes-Alves *et al.*, 2009), as an approach able to uncover cellular mechanisms involved on treatments-induced mistrafficking protein rescue.

### 3. EXPERIMENTAL DESIGN

#### 3.1. Cell lines

The importance of using recombinant CFTR-expressing cell lines as heterologous model systems is recognized from previous studies (Chang *et al.*, 1999; Mendes *et al.*, 2003; Roxo-Rosa *et al.*, 2006), since endogenous CFTR-expressing models are limited and the expression level is usually too low to study CFTR biogenesis, trafficking and function.

Adherent cell lines, such as BHK cells, not expressing (BHK-L) or stably expressing wild type (wt)-, F508del-CFTR (mutant), or F508del/4RK-CFTR ("repaired") are cultivated in DMEM/F12 media containing 5% fetal calf serum (Gibco, Invitrogen) and 500  $\mu$ M methotrexate (MTX—Teva Pharma) in culture flasks (Nunc Prand Products) until 80% of confluence (Roxo-Rosa *et al.*, 2006). Cells are then trypsinized and washed once in PBS (Gibco, Invitrogen) and pellets with  $5 \times 10^6$  cells should be quickly frozen on dry ice and stored at  $-80^\circ\text{C}$  until analysis. All BHK cell lines were provided by Roxo-Rosa *et al.* (2006).

#### 3.2. Low-temperature treatment and *in vivo* labeling

Low temperature (26–30  $^\circ\text{C}$ ) apparently stabilizes the mutant protein during folding in the ER, allowing some CFTR molecules to escape ER quality control and traffic through the Golgi apparatus to the plasma membrane (Denning *et al.*, 1992; Mendes *et al.*, 2003; Sharma *et al.*, 2001). Alternatively, Rennolds *et al.* (2008) have suggested that at low-temperature, F508del-CFTR may use a nonconventional trafficking pathway and bypasses the Golgi apparatus where CFTR becomes Endo-H resistant.

To investigate the effect of low temperature in the proteostasis environment involved in F508del-CFTR rescue,  $6 \times 10^5$  BHK cells stably expressing wild type (wt)- or F508del-CFTR should be seeded per 35 mm culture

dishes (Nunc Prand Products), grown to about 80% of confluence and then incubated at 37 °C (control) or 26 °C for 24 or 48 h in the same media without MTX. Cells are incubated 30 min in methionine-free  $\alpha$ -minimal essential medium (MEM, Gibco, Invitrogen) prior to the metabolic labeling in the same medium with 140 mCi/ml of [<sup>35</sup>S]-methionine (MP Biomedicals), in the last 3 h of incubation at 37 or 26 °C, respectively.

The 3-h cell metabolic labeling allows the visualization of proteins that are synthesized and further processed during this time of incubation with the [<sup>35</sup>S]-methionine (*de novo* synthesis—visualization of treatment effects on protein synthesis), while Coomassie staining allows the visualization of all proteins at steady state present in the sample.

## 4. 2D GEL ELECTROPHORESIS

### 4.1. Sample preparation

Pellets of  $5 \times 10^6$  (or  $0.8 \times 10^6$  for radiolabeled cells) cells are lysed directly in 450  $\mu$ l of lysis buffer [7 M urea (Sigma-Aldrich), 2 M thiourea (Sigma-Aldrich), 4% (w/v) CHAPS (Sigma-Aldrich), 60 mM DTE (Sigma-Aldrich), 0.75% (v/v) ampholine 3.5–10.0 (GE Healthcare), 0.25% (v/v) ampholine 4.0–6.0 (GE Healthcare)], and 0.12  $\mu$ l of protease inhibitor mixture (leupeptin 1 mg/ml; aprotinin 2 mg/ml; pepabloc 50 mg/ml; E64 3.5 mg/ml; benzamidin 50 mg/ml). After 30 min at 37 °C rocking, samples are sonicated three times 15 s in a waterbath sonifier (Selecta) and then clarified by centrifugation at 14,000 $\times g$  for 30 min. Supernatants are collected for a new microtube.

### 4.2. 2D-PAGE

In the first dimension of 2DE (isoelectric focusing, IEF), proteins are separated on the basis of their isoelectric point in an immobilized pH gradient gel (IPG) and in the second-dimension (SDS-PAGE) proteins undergo an additional separation according to their molecular weight under denaturing conditions. There are different 2DE methodologies or possibilities and all require the use of biological and technical replicates to produce meaningful results (Penque, 2009). A guidelines called MIAPE (Minimum Information About Proteomics Experiments) gel electrophoresis that act as checklists of the essential information that should be reported about particular 2DE experimental techniques has been also recently created (<http://psidev.sourceforge.net>) (see below).

In our 2DE procedure, total extracted protein samples (supernatants) are loaded onto 18 cm Immobiline DryStrips (GE Healthcare) with a nonlinear wide range pH gradient (pH 3–10) with a top overlay of 3 ml of mineral oil

(Amersham Bioscience) to prevent evaporation and urea crystallization during next steps of IEF on an IPGphor IEF system (Amersham Bioscience). For IEF, an active rehydration at 50 V for at least 12 h at 20 °C is performed before the focusing program of a total of about 60 kVh as follows: 200 V for 1 h, 500 V for 1 h, 1000 V for 2 h, 2000 V for 2 h, 3000 V for 2 h, increased gradient voltage to 8000 V for 1 h, and 8000 V for 5 h. After IEF is run, IPG strips were equilibrated in IEF equilibration solution (50 mM Tris–HCl, pH 8.8 (Merck), 6 M urea (Sigma–Aldrich), 30% (v/v) glycerol (Sigma–Aldrich), 2% (w/v) sodium dodecyl sulfate (SDS) (GE Healthcare), and traces of bromophenol blue (Merck) with 2% (w/v) DTE (Sigma–Aldrich) for 15 min and with 2.8% (w/v) iodoacetamide (Sigma–Aldrich) for another 15 min, to, respectively, reduce and alkylate proteins before application onto the top of a SDS–PAGE for molecular weight separation (second dimension), constituted by 8–16% (w/v) gradient polyacrylamide and run overnight at 3 W/gel (Ettan DALT twelve System, GE Healthcare). The strip gels are “glued” onto the top of a SDS–PAGE with boiled 0.5% (w/v) agarose in running buffer. If the second dimension is not carried out immediately after IEF, strips can be stored for prolonged times at –80 °C.

### 4.3. Coomassie and fluorography

Proteins once displayed on the 2D gel must be visualized in a valuable form to enable their accurate qualitative/quantitative analysis by dedicated software. The critical factors of staining methods are their sensitivity, reproducibility and the linear range of detection. The most used standard procedures of protein staining include Coomassie brilliant blue (CBB), and variants such as colloidal CBB (C–CBB) or blue silver (C–CBB modified), and MS-compatible silver stain (Gorg *et al.*, 2004). Other choices include DIGE (difference gel electrophoresis), SYPRO<sup>®</sup> Ruby (fluorescent dyes), or radiolabeling (e.g., <sup>35</sup>S–methionine or <sup>32</sup>P–phosphorous) (Minden, 2007; Roxo-Rosa *et al.*, 2004).

As referred before in Section 3.2, the proteins labeled *in vivo* with [<sup>35</sup>S]–methionine are visualized by autoradiography as follows: the gel is fixed in 30% (v/v) methanol and 10% (v/v) acetic acid for 30 min, washed four times 15 min in water, and treated for 1 h with 1 M Na salicylate, pH 7. The gels are then dried in a vacuum system for 2 h at 80 °C and film exposed (Fuji Film) with intensifying screen. “Cold” proteins (without radiolabeling) are visualized by Coomassie “blue silver” staining exactly as described (Candiano *et al.*, 2004).

Metabolic labeling is much more sensitive [ $<0.1$ – $0.01$  ng (Penque, 2009)] than the “blue silver” Coomassie (detection limit 1–100 ng/spot) (Candiano *et al.*, 2004). Moreover, [<sup>35</sup>S]–methionine labeling detects the presence of a number of proteins that are undetectable by silver staining or Western blotting (Chen and Casadevall, 1999). The digitalized images from

both Coomassie stained and fluorographic 2DE-maps are obtained using a proper scanner (obtained image quality attributes must be compatible with the requests of the analysis software), as for example ImageScanner (GE Healthcare).

#### 4.4. 2D maps image analysis

Subsequent analysis of the spot pattern on a gel by specialized software enables multiple-gel comparison, whereby quantitative and qualitative changes are detected by comparison with control gels. The critical parameter in computer-based image analysis is the quality of the images. This requires high quality and reproducible 2D gels and also high quality acquisition of the image with image capturing devices, as mentioned above. Image analysis software is designed for automated spot detection, matching, normalization and quantification. However corrections by user intervention are usually necessary.

In our studies the dedicated software used for gel image analysis was Progenesis PG200v2006-Nonlinear. An average gel image is created for each group of gels and a maximum of absences allowed must be defined (according to the analysis objectives and depending on the gels replica number). In order to standardize the intensities of the labeling among all spots present in the several 2DE-maps, analysis is carried out by taking into account the normalized volume of spots (or %vol., i.e., the volume of each spot over the volume of all spots in the gel).

#### 4.5. Statistical analysis

The difference in expression levels between groups (control, disease, and treatment) for a given protein is statistically assessed by using the ANOVA test, for  $n$  observations, where  $n$  is the number conditions (groups) analyzed. Differences over 1.5-fold are considered statistically significant when  $p < 0.05$ . Proteins of interest are then selected and excised from gels for subsequently MS analysis (see below).

### 5. PROTEIN IDENTIFICATION BY MS

Two specific types of MS analysis can be used for protein identification: the peptide-mass fingerprint (PMF), which involves determination of the masses of the peptides in the digest and is normally performed in a MALDI-TOF instrument, and the amino acid sequencing of the peptides (MS/MS), normally obtained by ESI tandem MS or MALDI-TOF/TOF (Penque, 2009).



MS or MS/MS spectra data are normally used to search a predicted mass map or protein sequence within a database to identify the protein of interest using software such as Sequest (Thermo) or Mascot (MatrixScience) (Aebersold and Mann, 2003; Steen and Mann, 2004). Using a database search, candidate proteins are ranked from a list of most closely matched candidates using various scoring algorithms.

### 5.1. Protein gel cut and digestion

After spot excision from gels, protein spots are destained as follows: wash spots with type I (Millipore) water for 20 min and then incubate with 50% (v/v) acetonitrile (ACN) for 30 min, at 37 °C with shaking. After destaining, spots are dehydrated by incubation with ACN first for 5 min and further 30 min with fresh ACN, at RT, with shaking. The gel pieces are lyophilized by a Speedvac evaporator (Savant, Farmingdale, USA) for 30–60 min, followed by trypsin digestion. For trypsin digestion spots are rehydrated with digestion buffer containing 6.7 ng/l of trypsin (Promega, Madison, WI, USA) in 50 mM  $\text{NH}_4\text{HCO}_3$  and incubated for 30 min at RT. Digestion buffer is removed and 50 mM  $\text{NH}_4\text{HCO}_3$  is added to the spots, which are incubated overnight (12–16 h) at 37 °C.

After overnight digestion with trypsin, supernatants are collected for new tubes and extraction of tryptic peptides is performed by successive incubation of the gel piece with 1% (v/v) TFA/50% (v/v) ACN and with 100% (v/v) ACN. For each step samples are sonified for 10 min in a waterbath sonifier (Selecta). The respective supernatants are pooled and dehydrated with a speed vacuum rotor (Savant). The pellet is resuspended in 5  $\mu\text{l}$  of 0.1% (v/v) TFA/50% (v/v) ACN and finally sonified for 15 min in the waterbath sonifier. The sample (0.5  $\mu\text{l}$ ) is spotted on the MALDI plate and 0.5  $\mu\text{l}$  of  $\alpha$ -CHCA are added to the sample, the mixture is allowed to dry at RT (Bensalem *et al.*, 2007).

### 5.2. MALDI analysis—Equipment and specifications

Peptides are analyzed on an Applied Biosystems 4700 ProteomicsAnalyzer with TOF/TOF ion optics. Data are acquired in positive MS reflector mode with six spots of protein standards (Calibration Mixture 2, Applied Biosystem) used for calibration (4000 Series Explorer Software v3.0 RC1). Three or five S/N best precursors of each spectrum can be selected for MS/MS analysis. The interpretation of the MS+MS/MS data is carried out by the GPS Explorer software (Version 3.5, Applied Biosystems) and a local copy of the MASCOT search engine (Version 2.0). Monoisotopic peptide-mass values are considered, a MS mass tolerance is set at 50 ppm and a MS/MS fragment tolerance set at 0.25 Da. Identification restrictions can be applied. Trypsin is normally the digestion enzyme used and one missed

cleavage site is usually allowed. Cys carbamidomethylation and Met oxidation are set as fixed and variable amino acid modifications, respectively. A taxonomic restriction to specific species protein sequences may be included. For MS all peaks with S/N greater than 5 and for MS/MS all peaks with S/N greater than 3 are searched against the database (e.g., Swissprot, NCBItr, etc.). The criteria to accept the identification are significant homology scores achieved in Mascot ( $p < 0.05$ ), but depending on the goals of the study or on the standards of the journal where the author wishes to publish, more stringent criteria may be used (e.g., minimum of matched peptides, minimum of sequence coverage, etc.).

## 6. BIOCHEMICAL VALIDATION

The generated data from a quantitative proteomics analysis has required validation/verification that can be performed by traditional biochemical assays (e.g., Western blotting, immunocyto/histochemistry, or other) or more recently by MS-based approaches (e.g., selected/multiple reaction monitoring (SRM/MRM)-based workflows) (Kitteringham *et al.*, 2009; Picotti *et al.*, 2010)

In our study, Western blot assay using antibodies specific to relevant proteins is used to validate/verify the proteomic data obtained. Briefly, protein extracts from cell lines are quantified (Bradford; Bio-Rad protein assay) and 15  $\mu$ g of each cell sample (triplicates) are separated on NuPAGE<sup>®</sup> Novex 4–12% (w/v) Bis-Tris gel 1.0 mm, 15 well (Invitrogen), transferred to nitrocellulose membranes (Schleicher & Schuell). After transference, the membranes are blocked with PBS plus 5% (w/v) fat free milk for 2 h and probed in the same solution for 2 h at RT with: 1:300 rabbit anti-GRP78/BiP Ab (Sigma-Aldrich), 1:500 mouse anti-PDI (Santa Cruz Biotechnology), 1:2000 rabbit anti-GRP94 (Abcam), 1:500 mouse anti-GRP75 (Abcam), 1:1000 rabbit anti-S100A6 (Abcam), 1:1000 mouse anti-HSP72 (Stressgen); 1:1000 goat anti-Psme2 (Abcam), 1:10000 mouse anti-COPS5 (Abcam) or 1:1000 rabbit anti-RACK1 (Sigma-Aldrich). After three washes of 15 min each with PBS–0.5% (v/v) Tween 20, membranes were probed with appropriated secondary antibody, 1:5000 anti-mouse IgG HRP (from sheep) or 1:5000 anti-rabbit IgG HRP (from donkey) (both from GE Healthcare). The immunoreactions are developed on washed membranes as before using enhanced chemiluminescence-ECL (GE Healthcare).

For Western blots normalization, all membranes are washed with the stripping buffer [1.5% (w/v) glycine, 0.1% (w/v) SDS; 1% (v/v) Tween 20; pH 2.2] five times for 10 min, washed with PBS and one last time with PBS-T, and reprobed as above with 1:1000 mouse anti- $\alpha$  tubulin (Sigma-Aldrich) and developed using ECL. The abundance of proteins is calculated

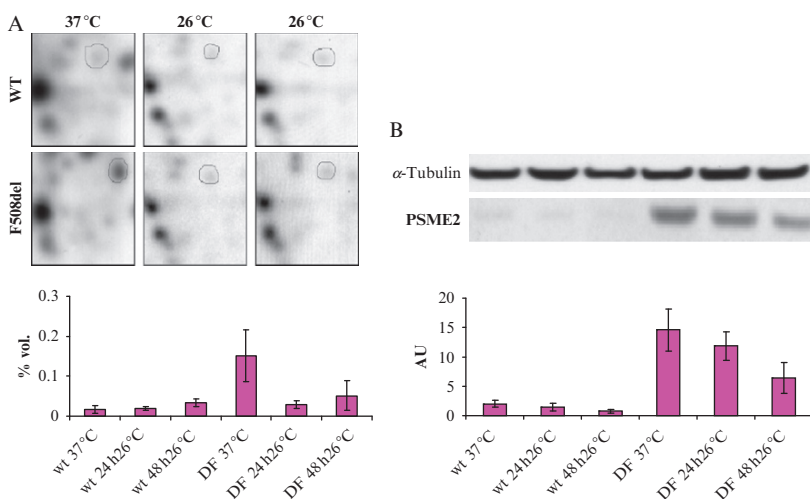
from densitometry of immunoblots ( $n = 3$  replicates) using the Progenesis PG200v2006. Figures 13.1 and 13.2 are illustrated examples of 2DE-maps and validation data obtained for PSME and GRP78/BiP, respectively, under the different conditions studied.

## 7. DATA MINING AND PUBLICATION

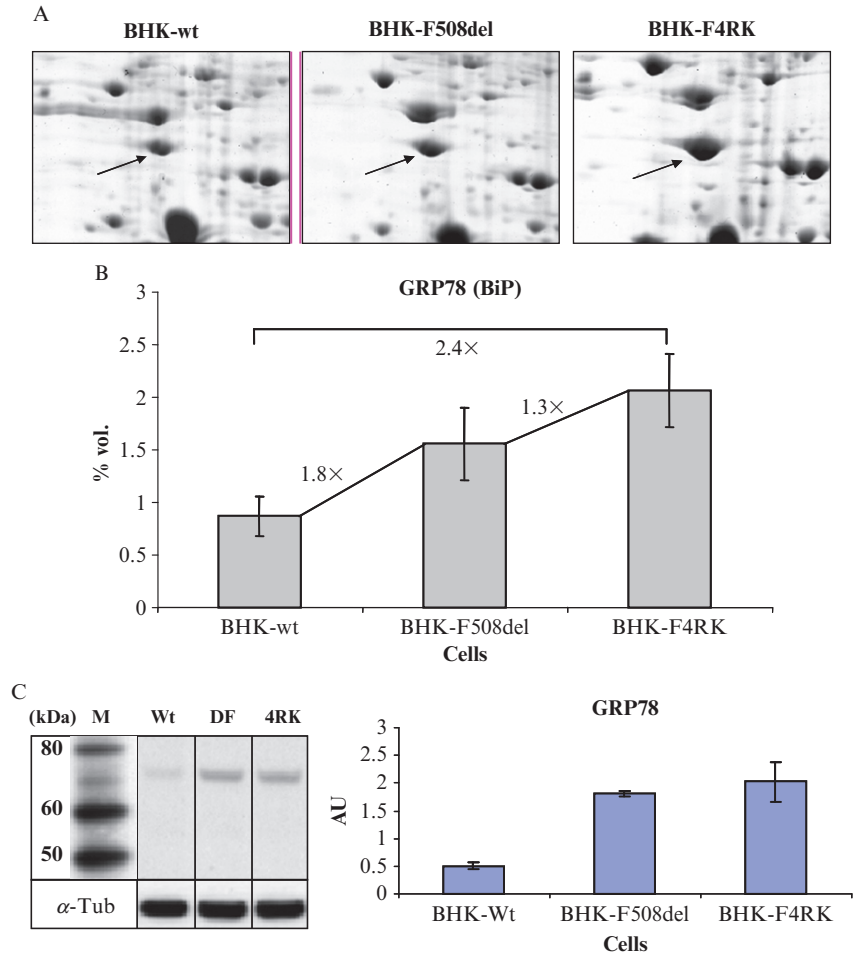
### 7.1. Bioinformatics analysis

Bioinformatics tools that deal with high-throughput data and allow its efficient and accurate analysis, interpretation and correlation, have accompanied the important progresses in the proteomics field as they are fundamental for the continuous development of proteomics technology.

Gene Ontology (GO) ([www.geneontology.org](http://www.geneontology.org)) is used to search for biological processes, cellular components and molecular functions associated to the proteins identified. In order to collect all the available



**Figure 13.1** 2DE analysis (A) and Western blot validation (B) of Psme2 protein expression in BHK-wt 37 °C, BHK-wt 26 °C/24 h, BHK-wt 26 °C/48 h, BHK-F508del 37 °C, BHK-F508del 26 °C/24 h and BHK-26 °C/48 h cells. Close-up views of representative 2DE areas of Psme (A), spots are shown. The 2DE graphic shows the normalized % volume, that is, the volume of each spot over the volume of all spots in the gel (ANOVA test,  $p < 0.05$ ;  $n = 4/5$  gels/group, total  $n = 26$  gels). The Western blot graphic shows the relative normalized abundance of Psme2 (B), in those BHK cell lines calculated from densitometry of immunoblots using  $\alpha$ -tubulin as internal standard ( $n = 3$  independent replicates/each Ab reaction). Progenesis PG200v2006 software (Nonlinear) was used for both Western blot and 2DE densitometry analyses.



**Figure 13.2** GRP78 (BiP) gel spot representation in BHK-wt, -F508del, and -F4RK 2D-maps (A). %Volume of GRP78 in each cell line is graphically presented. The fold change between the three groups is also given (B). GRP78 Western blot analysis (C). Fifteen micrograms of total protein lysates from BHK-wt (lane 1), BHK-F508del (DF) (lane 2), and BHK-F4RK (lane 3) cells was electrophoretically separated on precasting 4–12% (w/v) gradient polyacrylamide mini gel and immunoblotted for GRP78 using rabbit anti-GRP78 Ab (1:300) and for  $\alpha$ -tubulin using mouse anti- $\alpha$ -tubulin (1:10000) as normalizer standard of the reaction. The antigen–antibody complex was detected by ECL system. The graphic shows the relative normalized abundance GRP78 in those BHK cell lines calculated from densitometry of immunoblots ( $n = 3$  replicates/each Ab reaction) using the Progenesis PG200v2006 software (Nonlinear).

information about each protein, PIKE (Protein Information and Knowledge Extractor) software can also be used, this bioinformatics tool retrieves a set of biological information like: gene name, location, tissue specificity,

function, GO terms and keywords (<http://proteo.cnb.uam.es:8080/pike/>) (see Fig. 13.3A). Protein interactions networks can also be accessed through web-free software, such as Cytoscape, using available protein–protein interaction databases (<http://www.cytoscape.org/>). Other commercial available tools such as Ingenuity Pathway Analysis (Ingenuity Systems Inc.) are able to retrieve functional biological networks where proteins of interest are involved.

## 7.2. Clustering analysis

By hierarchical clustering, a powerful data mining approach for a first exploration of proteomic data, proteins can be group blindly according to their expression profiles facilitating their interpretation. The clustering results depend on parameters such as data preprocessing, between-profile similarity measurement, and the dendrogram construction procedure—methodology reviewed elsewhere (Meunier *et al.*, 2007).

In the “low-temperature” study and after the differential protein expression analysis amongst the six experimental conditions, each spot identified showing statistical significant difference relative to the BHK-wt 37 °C condition are included in the relationship analysis amongst each other (columns) and the six phenotypes (rows), using the normalized volume quantity data.

To avoid distortion in clustering analysis on the account of a small subset of abundant protein spots, standardization of data is performed using the  $z$ -score calculation Eq. (13.1); where  $\bar{x}$  is the arithmetic mean and  $s$  is the standard deviation, calculated from the volume quantity data for a given protein spot in the six phenotypes, and  $x$  is the individual volume quantity to be normalized. Special care is taken relating to the missing values in the six experimental conditions for an identified spot, which are regarded as a missing spot coloration in 2D gel and as so, interpreted as zero volume quantity. For a given spot, the null information are first substituted by the  $\bar{x}$  data in the analyzed phenotypes (column), and then submitted to the standardization process.

$$z = (x - \bar{x}) \frac{1}{s} \quad (13.1)$$

In order to classify the individual protein spots (columns), and the phenotypes(rows), according to the global expression profile, a two way hierarchical clustering analysis can be executed using geWorkbench, version 1.5.1 (<http://www.geworkbench.org/>), a free open source genomic analysis platform developed at Columbia University with funding from the NIH Roadmap Initiative (1U54CA121852-01A1) or the National Cancer Institute. The normalization process is done directly in the platform choosing the option “Mean-variance normalizer,” which was followed by “Fast

A

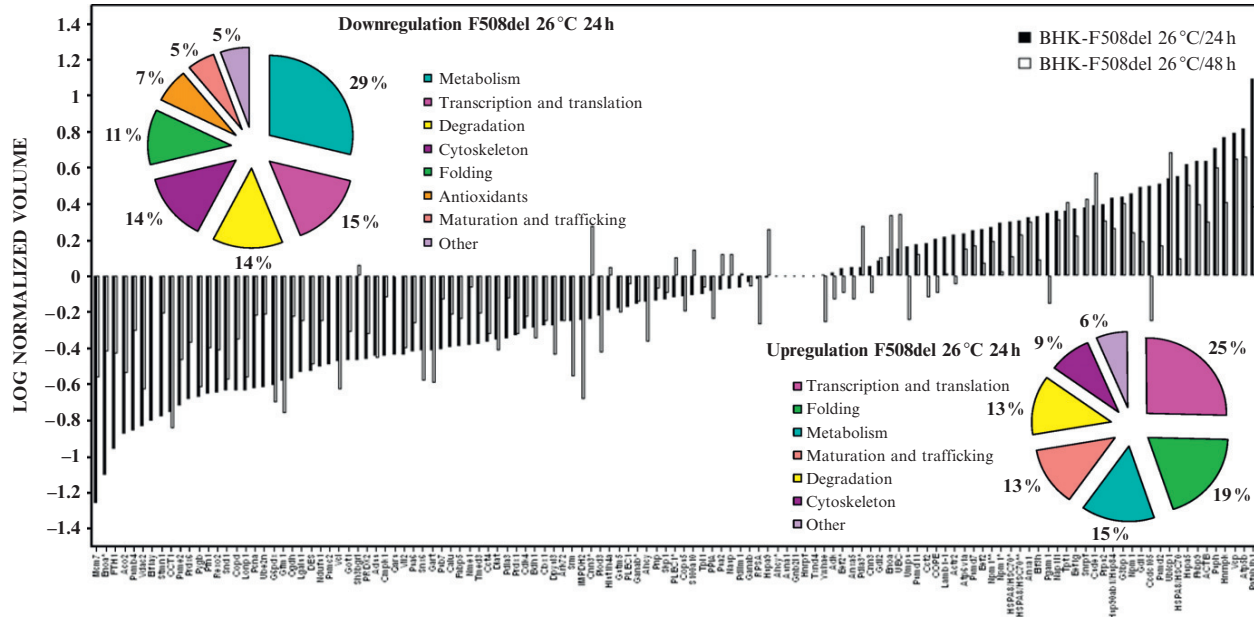
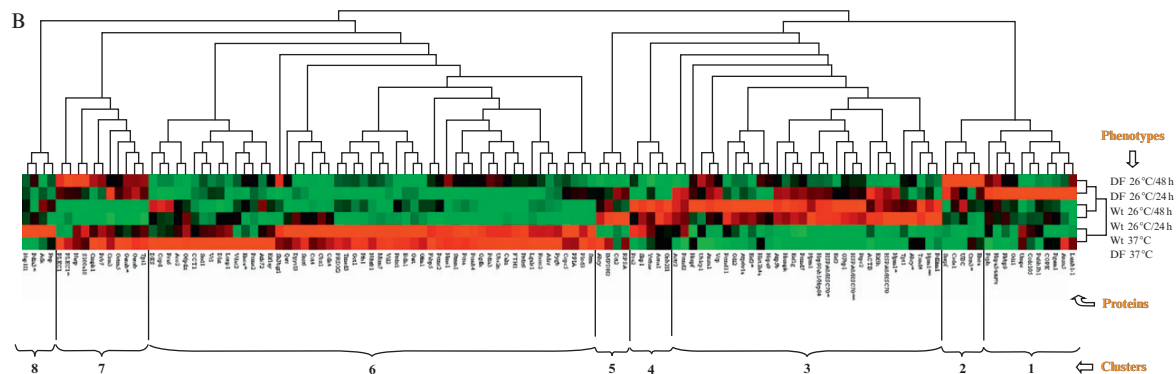


Figure 13.3 (Continued)



**Figure 13.3** Graphical representation of the log normalized volume of proteins in BHK-F508del (A). Expression levels of those proteins in BHK-F508del at 37 °C were considered as reference to determine the up- or downregulation of the correspondent protein in the other phenotype. Proteins up- and downregulated in F508del cells were also classified according to their molecular function. Unsupervised hierarchical clustering (Euclidean distances and average linkage) of the expression profiles of the 139 protein spots differentially expressed in the six phenotypes studied (B). Each horizontal row in the heat map represents the expression level for one protein in the different phenotypes (columns). The relative abundance is displayed by color intensity (light red—more abundant; light green—less abundant).

Hierarchical Clustering Analysis.” The euclidean distance metrics Eq. (13.2) was chosen to measure the dissimilarity between the data and the best agglomerative classification for our data is obtained from “Average Linkage.”

$$d(x, y) = [\sum_i (x_i - y_i)^2]^{1/2} \quad (13.2)$$

Figure 13.3B shows the output of the geWorkbench, where the two generated dendrograms (protein spots and phenotypes) are linked by a heat map graph of the standardized data, allowing a better interpretation of the clustering results.

### 7.3. MIAPE: The Minimum Information About a Proteomics Experiment

MIAPE (<http://www.psdev.info/index.php?q=node/91>) is a project of the HUPO proteomics standards initiative that establishes the minimum information that must be reported on a proteomics experiment (where samples came from, how analyses of them were performed, etc.) so other authors can reproduce the results of the original work. The requirements of the various journals also differ, so important details may be lacking in some cases, or presented in an inappropriate form.

As so, when authors are interested in publish results obtained from proteomics approaches it is practical and useful to consult the MIAPE guidelines already published, most information is available at the webpage <http://www.psdev.info/index.php?q=node/91>. Before submitting a paper for publication it is also essential to consult the requisites of the journal of interest about the data that authors must present for the type of approach they have been using in their investigation.

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