

NIH Public Access

Author Manuscript

Exp Cell Res. Author manuscript; available in PMC 2015 January 01.

Published in final edited form as:

Exp Cell Res. 2014 January 1; 320(1): . doi:10.1016/j.yexcr.2013.07.019.

Green fluorescent protein expression triggers proteome changes in breast cancer cells

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Abstract

Green fluorescent protein (GFP) is the most commonly used reporter of expression in cell biology despite evidence that it affects the cell physiology. The molecular mechanism of GFP-associated modifications has been largely unexplored. In this paper we investigated the proteome modifications following stable expression of GFP in breast cancer cells (MDA-MB-231). A combination of three different proteome analysis methods (2-DE, iTRAQ, label-free) was used to maximise proteome coverage. We found that GFP expression induces changes in expression of proteins that are associated with protein folding, cytoskeletal organisation and cellular immune response. In view of these findings, the use of GFP as a cell reporter should be carefully monitored.

Keywords

GFP expression; Proteomic change; Unfolded protein response; Cytoskeleton modifications; Cellular immune response

Introduction

Green fluorescent protein (GFP) is responsible for the green bioluminescence of the jellyfish *Aequorea victoria*. This single-chain 238 amino acid polypeptide emits green fluorescence under 488 nm light. Because of this property GFP has been increasingly used to facilitate experiments in cell biology. It is commonly used as a reporter gene or a fusion tag with a particular protein (for review, see [1]). However, a number of cellular side effects resulting from GFP expression have been reported. For example, apoptosis has been reported in several cell lines when stable expression of GFP was attempted [2]. In muscle cells, overexpression of GFP has been linked to impairment of contraction in experimental models

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2013.07.019.

both *in vitro* and *in vivo* [3-6]. In neurons, expression of fluorescent proteins (eGFP and YFP) appears to alter cellular mechanisms and molecular characteristics [7,8].

In light of these findings, it is surprising that relatively few studies report on the molecular consequences of GFP expression. In endothelial cells, GFP has been shown to induce a marked increase of HSP70 at both mRNA and protein levels in a dose dependent manner. This upregulation leads to an increased expression of cyclooxygenase-2 (COX-2) followed by increased prostaglandin E_2 (PGE₂) production [9]. In 2006, Baens et al. [10] reported a defect in polyubiquitination, a post-translational modification that affects a wide range of cellular processes. Finally, in an attempt to provide a more comprehensive view of the influence of GFP expression in cardiac myocytes, Badrian and Bogoyevitch [11], conducted a DNA microarray analysis which revealed changes in the expression of 212 genes and therefore suggested caution in the interpretation of experimental results where GFP-expressing cells are used as controls. In this study, we used a breast cancer cell line as a model system to analyse proteome changes associated with stable GFP expression.

Materials and methods

Cell culture

Parental MDA-MB-231 breast cancer cells (referred to as wild-type (WT) cells form hereon) were cultured in DMEM supplemented with 10% fetal bovine serum, sodium pyruvate and antibiotics at 37 °C and 5% CO₂. Stable MDA-MB-231 clones expressing GFP were generated using the plasmid pEGFP-C3 and the X-tremeGENE HP DNA transfection reagent (Roche Applied Science, Australia) according to the manufacturer's instructions. Selection of the stable clones (expression of GFP was confirmed by fluorescence microscopy) and subsequent subcultures of one stable clone (used in the following experiments) were performed in the above conditions and with regular growth media supplemented with 1 mg mL⁻¹ G418.

Protein extraction and quantification

For protein extraction, cells grown to 90% confluence in 175 cm² culture flasks were washed twice with PBS and detached from the flask by 5 min incubation at 37 °C in citric saline solution (1.35 M KCl, 0.15 M sodium citrate).

For protein analysis by two-dimensional electrophoresis (2-DE), cells were pelleted by centrifugation, resuspended in IEF buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% IPG buffer (pH 4–7) (GE Healthcare), 1% DTT, 0.001% bromophenol blue) and proteins were extracted with glass beads using a mini bead beater (4×20 s interspersed with 5 min cooling on ice). Samples were then centrifuged at 15,000g (15 min, 20 °C).

For the iTRAQ and MS-label free experiments, a total cell lysate was obtained by extracting the cell pellet in 10 volumes of ice-cold RIPA Buffer (Pierce, Sydney, Australia) using the mini bead beater as described above. Samples were then centrifuged at 15,000g (1 h, 4 °C).

Total protein concentration in the supernatant was determined using the 2-D Quant kit (GE Healthcare), using BSA as the reference standard.

Two-dimensional electrophoresis, protein visualization and image analysis

2-DE was performed essentially as previously described by Coumans et al. [12]. In summary, rehydrated 18 cm IPG strips pH 4–7 (GE Healthcare) were loaded with protein (300 μ g for analytical gels or 400 μ g for preparative gels) and IEF was carried out on the IPGphor II (GE Healthcare Life Science) at 20 °C with a current limit of 50 μ A/strip to a

total volt–hour-product of 30 kVh (analytical gels) or 45 kVh (preparative gels). Second dimension separation was achieved on Protean II XL Cell (Bio-Rad) with home cast 1.5 mm SDS polyacrylamide gels (12%) at 8 mA/gel until the bromophenol blue dye front reached the anodic end of the SDS-gel. Staining of the gels was performed using colloidal Coomassie Brilliant Blue G-250 [13] for analytical study and by Coomassie blue staining (50% methanol, 0.15% Coomassie blue R-250, 0.75% acetic acid) for preparative 2-DE. Image capture was performed as previously described by Coumans et al. [14]. Three biological samples and two technical replicates were grouped and analysed with PDQuest advanced 2-D analysis software (Bio-Rad). For comparison of expression data, the Student's *t*-test function in the PDQuest software was used and a *p*-value 0.05 was considered to be statistically significant.

Sample preparation and iTRAQ labeling

100 μ g of whole cell protein extract from either WT or GFP-expressing MDA-MB-231 cells was precipitated with 9 volumes of ice-cold acetone for 1 h at -20 °C. The protein precipitate was recovered by centrifugation, air dried (*ca*. 2–3 min) and dissolved in 50 mM sodium bicarbonate containing 0.1% SDS. Probe sonication was used to assist dissolution. Protein samples were then reduced using tris(2-carboxyethyl)phosphine (2.5 mM, 60 °C, 60 min) and alkylated with iodoacetamide (2 mM, 20 °C, 10 min), digested with trypsin (0.5 μ g/50 μ g protein) (Promega, Madison, WI) for 16 h at 37 °C and then labeled with the iTRAQ reagents according to the manufacturer's instructions [15].

Excess unbound iTRAQ labels, trypsin, SDS and solvents were removed by strong cation exchange (ICAT SCX cartridge, ABSciex, MA), solid phase extraction (SPE) using an Applied Biosystems Opti-Lynx cartridge holder and a syringe pump (KD Scientific, Holliston, MA) at a flow rate of 9.5 mL/h. The eluted peptide solutions were vacuum-dried, and resuspended in 500 μ L of 0.2% heptafluorobutyric acid (HFBA) prior to a further SPE step using reverse phase (RP) chromatography on a C18 RP peptide macrotrap cartridge (Microm Bioresources, Auburn, CA). Briefly, the macrotrap was primed with 1 mL CH₃CN, then 1 mL 50% CH₃CN/0.1% formic acid, and equilibrated using 1 mL of 0.2% HFBA. The resuspended peptides were loaded, the macrotrap washed with 1 mL of 0.2% HFBA and the peptides eluted with 500 μ L 50% CH₃CN/0.1% formic acid, followed by 200 μ L neat CH₃CN. The eluants were then pooled and vacuum-dried. The pellet was dissolved in 25 μ L 1% formic acid, 0.05% HFBA and analysed using 2D-LC–MS/MS. All labeling experiments were run twice (2 separate injections), to provide the two technical replicates and statistical significance was determined using the Student's *t*-test (Microsoft Exel).

Sample preparation for label-free LC–MS quantification

Whole cell protein extracts (100 μ g) from WT and GFP-expressing MDA-MB-231 cells were treated and trypsin digested as described in the sample preparation for iTRAQ labeling.

Following trypsin digestion, the peptide samples were purified using SCX StageTips and C18 StageTips (Thermo Scientific) following the manufacturer's instructions.

Mass spectrometry and database search

For protein identification from 2-DE, relevant protein spots from preparative gels were manually excised, destained and digested as previously described [14]. Peptides were separated using a nanoLC 1D plus system (Eksigent). Samples $(2-7 \,\mu\text{L})$ were concentrated and desalted on a ChromXPC18 pre-column (350 $\mu\text{m} \times 0.5 \,\text{mm}$, Eksigent) with H₂O:CH₃CN (98:2, 0.1% formic acid). After a 4 min wash the pre-column was automatically switched into line with a 3C18-CL-120 nano-column (75 $\mu\text{m} \times -15 \,\text{cm}$), Eksigent. Peptides were eluted using a linear gradient of H₂O:CH₃CN (95:5, 0.1% formic

acid) to H₂O:CH₃CN (60:40, 0.1% formic acid) at ~300 nL/min over 30 min. The precolumn was connected via a fused silica capillary (10 cm, 25 μ m) to a PicoTip emitter (New Objective) where HV (2300 V) was applied and the column tip positioned ~1 cm from the Z-spray inlet of an TripleTOF 5600 mass spectrometer (ABSciex). Positive ions were generated by electrospray and the TripleTof operated in information dependent acquisition mode (IDA). A Tof MS survey scan was acquired (*m*/*z* 350–1500, 0.25 s) and the multiply charged ions (2+ to 5+) in the 400–1250 *m*/*z* range were sequentially selected by Q1 for MS–MS analysis. Nitrogen was used as collision gas and optimum collision energy was chosen (based on charge state and mass). Tandem mass spectra were accumulated for up to 0.05 s (*m*/*z* 100–1800). Peak lists were submitted to the database search program Mascot (Matrix Science). Search parameters were precursor and product ion tolerances ±100 ppm and 0.05 Da respectively; Carbamidomethyl (C) and Oxidation (M) were specified as variable modifications, enzyme specificity was trypsin, one missed cleavage was allowed and the SwissProt 2012_10 (538,259 sequences; 191,113,170 residues), taxonomy *Homo sapiens* searched.

For iTRAQ analysis, solubilized peptides were analysed by 2D-LC-MS/MS (LC Packings autosampler and switchos valves with Dionex Ultimate 3000 pumps, in line to a QStar Elite, ABSciex, Framingham, MA). The procedure was adapted from a previously published approach [16]. Peptides (ca. 5–10 μ g total load, 10 μ L) were initially captured onto a SCX micro column ($0.75 \times \sim 20 \text{ mm}^2$, Poros S10, Applied Biosystems) and the eluant from multiple salt elution steps (5, 10, 15, 20, 25, 30, 40, 50, 100, 250, 500 and 1000 mM ammonium acetate) captured and desalted on a C18 precolumn cartridge (300 μ m id \times 5 mm, packed with 5 μ m C18, 100 Å PepMap, Dionex, Sunnyvale, CA). After a 10 min wash the pre-column was switched (Switchos) into line with a fritless analytical column (75 μ m \times ~12 cm) containing C18 reverse phase packing material (Magic, 5 μ , 200 Å) [17] Peptides were eluted using a linear gradient of buffer A (0.1% formic acid) to buffer B (20:80, H₂O:CH₃CN containing 0.1% formic acid) at ~300 nL/min over 90 min. High voltage (2300 V) was applied through a low volume tee (Upchurch Scientific) at the column inlet and the outlet positioned ~1 cm from the orifice of a QStar Elite mass spectrometer (ABSciex, Framingham, MA). Positive ions were generated by electrospray and the QStar operated in information dependent acquisition mode (IDA). A Tof MS survey scan was acquired (m/z350-1700, 0.75 s) and the 3 largest multiply charged ions (count > 20, charge state 2 and 4) sequentially selected by Q1 for MS-MS analysis. Nitrogen was used as collision gas and an optimum collision energy automatically chosen (based on charge state and mass). Tandem mass spectra were accumulated for up to 2.5 s (m/z 65–2000) with 2 repeats. The WIFF files were submitted to ProteinPilot v4.0 and both ipi.Human v3.58 and NCBInr 24/8/12 databases used for data processing. ProteinPilot v4 search parameters were thorough ID search effort, biological modifications focus, quantification and bias correction used for data processing; cys-carboxyamidomethylation and iTRAQ 8-plex reagents used, enzyme specificity was trypsin. A minimum protein unused score of 1.3 was accepted for all reported proteins (minimum 95% confidence in correct sequence assignment) and a p value of 0.05 was used as the cutoff for accepting statistically significant changes in protein expression level.

For label-free quantification, solubilised peptides were analysed using a linear ion trap-Orbitrap (LTQ-Orbitrap). Peptides were reconstituted in 10 μ L of 0.1% formic acid and were separated by nano-LC using an Ultimate 3000 HPLC and autosampler (Dionex, Amsterdam, Netherlands). 0.2 μ L (*ca.* 2 μ g total load) was loaded onto a micro C18 precolumn (500 μ m × 2 mm, Michrom Bioresources, Auburn, CA, USA) with Buffer C (98% H₂O, 2% CH₃CN, 0.1% TFA) at 10 μ L min⁻¹. After a 4-min wash the pre-column was switched (Valco 10 port valve, Dionex) into line with a fritless nano column (75 μ m id × 10 cm) containing reverse phase C18 media (5 μ m, 200 Å Magic, Michrom Bioresources).

Peptides were eluted using a linear gradient of Buffer C (98% H₂O, 0.1% TFA) to Buffer D (98% CH₃CN, 2% H₂O, 0.1% formic acid) at 250 nL min⁻¹ over 60 min. High voltage (2000 V) was applied to a low volume tee (Upchurch Scientific, Oak Harbor, WA, USA) and the column tip positioned ~0.5 cm from the heated capillary (*T*=280 °C) of an Orbitrap Velos (Thermo Electron, Bremen, Germany) mass spectrometer. Positive ions were generated by electrospray and the Orbitrap operated in data-dependent acquisition mode. A survey scan *m*/*z* 350–1750 was acquired in the Orbitrap (Resolution=30,000 at *m*/*z* 400, with an accumulation target value of 1,000,000 ions) with lockmass enabled. Up to the 10 most abundant ions (>5000 counts) with charge states +2 to +4 were sequentially isolated and fragmented within the linear ion trap using collisionally induced dissociation with an activation *q*=0.25 and activation time of 30 ms at a target value of 30,000 ions. *m*/*z* ratios selected for MS/MS were dynamically excluded for 30 s.

MS peak intensities were analysed using Progenesis LC–MS data analysis software v4 (Nonlinear Dynamics, Newcastle upon Tyne, UK). Ion intensity maps from each run were aligned to a reference sample and ion feature matching was achieved by aligning consistent ion m/z and retention times. The peptide intensities were normalized against total intensity (sample specific log-scale abundance ratio scaling factor) and compared between groups by oneway analysis of variance (ANOVA, p 0.05 for statistical significance) and *post-hoc* multiple comparison procedures. Type I errors were controlled for by False Discovery Rate (FDR) with q value significance set at 0.01 [18,19]. MS/MS spectra were searched against the Swiss-Prot database 24_8_12 (536,789 sequences, 190,518,892 residues) using database search program MASCOT (Matrix Science, London, UK, \langle www.matrixscience.com \rangle). Parent and fragment ions were searched with tolerances of ±6 ppm and ±0.6 Da, respectively. Searched peptide charge states were limited to +2 to +4. 'No enzyme' and *H. sapiens* for the taxonomy were specified. Only peptides with a high ion score >25 and above were considered for protein identification. Statistical proteome modifications between WT and GFP-expressing cells were assessed by Student's *t*-test analysis (Microsoft Exel).

Data analysis

To determine the possible biological consequences following stable GFP expression in MDA cells, protein classification analysis of the down- and up-regulated proteins was performed using the PANTHER classification system ((http://www.pantherdb.org)) [20,21] which allows sorting of protein lists into molecular function, biological process, cellular component, protein class and pathway. Moreover, an analysis of the predicted protein–protein interactions was performed using STRINGv9.05 analysis tool ((http://string-db.org)) using a high confidence score (0.7) and Kmeans clustering [22].

Results

The objective of this study was to characterise the proteome changes occurring following stable expression of GFP in human breast cancer cells. Following fluorescence microscopy to confirm GFP expression (data not shown), proteins were extracted and analysed using a combination of three different proteome analysis methods (2-DE, iTRAQ, label-free).

Determination of false protein quantification rates using replicates within MDA and MDA-GFP expressing cells

Analytical variability is inherent in quantitative proteomic studies. 2-DE analysis requires a lot of manual work and expertise, and while careful optimization of sample processing reduces experimental variability and increases 2-D gel reproducibility, experimental and software-related post-experimental variability still occurs (for review, see [23]). Discovery based proteomics approaches provide a fast method of screening complex protein sample

mixtures for relative expression changes. Several studies have recently reported detailed comparisons between different types of MS-based quantification approaches [24-26]. Labelfree quantification provides identification and quantification of proteins with minimal manipulation and avoids the costly process of introducing stable isotopes into samples. However, because samples are prepared and measured separately, so quantification performance in terms of accuracy, precision, and reproducibility is limited [27]. The iTRAQ approach generally offers better precision but underestimation or "ratio compression" arising from contamination during precursor ion selection specific to MS/MS quantification in complex samples occurs [28]. Therefore, to reduce false positive protein identification and quantification, Wang et al. [26] have proposed a strategy to determine experimentally the arbitrary fold change cutoff to apply. We applied a similar strategy to determine the fold change cutoff to consider for the three proteomic methods that we used. Briefly, because the expected ratio for all proteins identified should be equal to 1 (between replicates and assuming equal protein loading), we calculated the ratio between our technical average for WT and GFP expressers of MDA-MB-231. We found that significant differences $(p \ 0.05)$ occur from one biological replicate to another. In 2-DE analysis, a total of 30 (5%) and 29 (5.7%) proteins for WT and GFP expressers of MDA-MB-231 respectively showed a significant change. Using a minimum of 3 peptides per protein for quantification, our iTRAO and label-free analyses showed, a total of 26 (4%) and 67 (25%), respectively for WT cells and a total of 18 (2.6%) and 64 (24.5%) proteins respectively, for GFP-expressers which showed a significant fold-change. We then analysed the distribution of the protein quantification ratios and determined the percentage of proteins below these ratios. The combined ratios for MDA and MDA-GFP and up- and down-regulated proteins are presented in Fig. 1. With a p value of 0.05, in iTRAQ analysis an arbitrary cutoff of 1.2 will lead to only 2.8% of false positive quantified proteins between biological replicates, in 2-DE analysis an arbitrary cutoff value of 1.5 will lead to less than 2% of false positive quantified proteins while in label-free analysis this percentage is obtained with an arbitrary cutoff value of 1.6. These ratio cutoffs which are in agreement with the recommendation of Millioni et al. [23] for 2-DE analysis and within the range of previous published data for iTRAO and label-free analysis [26,29] were used in further analyses.

2-DE analysis of MDA-MB-231 expressing GFP

By 2-DE, an average of 705 protein spots (MW 15–150 kDa, pH 4–7) was detected and further quantified using the PDQuest software. To reduce operator-related variability, we used only high quality gels with reproducible protein patterns between sample replicates and between independent experiments (average inter-assay CV=22%). In total, 45 protein spots were found to be differentially expressed (fold change > 1.5, p 0.05). Two protein spots could only be detected in MDA control cells and one in MDA cells expressing GFP. 19 protein spots were down-regulated while 24 were up-regulated in MDA-GFP cells when compared to MDA control cells. LC–MS/MS analysis of these protein spots returned 23 identifications (Fig. 2, Table 1 and Supplemental data). The remaining spots were either not excised confidently or invisible after Coomassie blue R-250 staining.

Protein identification and quantification in iTRAQ and label-free analysis

We used two additional distinct mass spectrometry experimental approaches to profile and quantify the proteome changes occurring following GFP expression in cells. From our iTRAQ experiments (IPI human database, unused score > 1.3 (95% confidence)), ProteinPilot identified 6281 proteins before grouping, representing 11,471 distinct peptides (95% confidence) and 39,608 spectra. These data collapsed into 1264 proteins after grouping. Combination of the technical replicates brought a common result of 874 proteins. Our label-free experiments identified 130,163 MS/MS spectra. These were searched against the Swiss-Prot database 24_8_12 and 4359 peptides were identified. To increase confidence

in protein identification, only peptides with an ion score >25 were selected. After conflict resolution 828 proteins across all replicates (ANOVAs p 0.05) were identified. When looking at the number of peptides per protein identification, notable differences arise between the iTRAQ and label-free analysis. For quantification purposes, we chose to consider only proteins identified with at least 3 peptides in both iTRAQ and label-free analysis. Number of proteins identified and quantified with these criteria in both experimental set up as well as the overlap of protein identification is shown in Fig. 3.

Protein abundance change in iTRAQ and label-free analysis of MDA-expressing GFP

In iTRAQ analysis, a total of 104 differentially expressed proteins were identified (fold change > 1.2 with p 0.05), of which 62 were down-regulated and 42 were up-regulated in MDA expressing GFP (Table 1). Five of these proteins were previously identified as differentially expressed using 2-DE. However, between these common identifications conflicting results arise (as for example, HDGF, MAT2A). This might be explained by the fact that LC–MS/MS analysis of individual spots from our 2-DE analysis sometimes revealed several protein identifications which is not uncommon with these types of approaches [30].

In our label-free analysis, a combined analysis of 3 biological replicates and 3 technical replicates of MDA-GFP and MDA cells respectively identified 30 differentially expressed proteins (fold change > 1.6, p 0.05), 5 being down-regulated and 24 up-regulated in MDA-GFP cells when compared to MDA cells (Table 1). Within these proteins, 7 were also identified as differentially expressed in our 2-DE/iTRAQ experiments.

Protein classification analysis of the differentially expressed proteins following stable GFP expression in MDA cells

Using the PANTHER classification system, we determined the percentage of proteins found in each of the categories identified within molecular function and biological process (Fig. 4). It is also important to mention that many proteins were associated with several Gene Ontology (GO) annotations, for example, the 73 up-regulated proteins were associated with 93 and 167 GO terms in molecular function and biological process respectively.

The major categories within molecular function were binding, catalytic activity and structural molecule activity encompassing 76.8% and 79.6% function hits for the down- and up-regulated proteins respectively. Within biological process, the three major categories were metabolic process, cellular process and transport (63.1%) for the down-regulated proteins and metabolic process, cellular process and immune response (52.6%) for the up-regulated proteins identified in our combine analysis (Fig. 4).

STRING network analysis of the predicted protein–protein interactions was performed to identify functionally linked proteins. The network is presented under the evidence view, whereby lines represent the existence of evidence (neighborhood, gene fusion, co-occurrence, co-expression, experiments, databases, textmining) used in predicting the association. The gene symbols used in this network are listed in Table 1. Several well defined clusters (Fig. 5) are revealed. The green cluster groups are mostly down-regulated proteins that are constituents of the ribosome, the pink cluster has both down- and up-regulated proteins that correspond to some elongation factors and proteasome subunit, a third cluster shown in red, represents down-regulated proteins involved in the DNA metabolic process while the last major cluster shown in yellow represents up-regulated proteins that bind to unfolded proteins and down-regulated proteins that bind to nucleotides. This suggests the occurrence of some feedback mechanisms.

Discussion

GFP is widely used in cell biology as a reporter of expression. However, GFP expression has been shown to induce molecular changes within the cell and sometimes even toxicity [2,10,11]. Nonetheless, only a few investigations of the molecular changes induced by GFP expression have been published.

Metabolic process changes following stable GFP expression in MDA cells

In both, down- and up-regulated protein groups, the largest category within biological process was metabolic process comprising 40.6% and 27% of the processes respectively (Fig. 4).

Changes in protein expression occur principally within primary metabolic process (94.9% of the down-regulated and 82% of the up-regulated proteins). The majority of these modifications relate to protein metabolism (59.7%, 59.6% respectively), this is followed by the nucleobase, nucleoside, nucleotide and nucleic acid metabolic process (35.8%, 17% respectively), carbohydrate metabolism (3%, 14.9% respectively), and lipid metabolism (1.5%, 8.5% respectively).

Of the 41 down-regulated proteins within the protein metabolic process category, 51.2% are involved in translation, while the 28 are up-regulated proteins within this subcategory, 88.9% are involved in some kind of protein modification (*i.e.*, folding, modification, complex assembly, proteolysis). Furthermore 24 down-regulated proteins are found within the nucleic acid metabolic process while only 8 within this category are up-regulated. This might indicate that stable expression of GFP in MDA-MB-231 cells have the potential to induce a decrease of pre-translation and translation events while increasing the post-translation molecular events.

The endoplasmic reticulum (ER) provides an optimal and unique environment for protein folding, assembly and modifications [31]. It is well known that if accumulation of unfolded or misfolded proteins occurs in the ER, activation of complex cytoplasmic and nuclear signalling pathways lead to a homeostatic cell response, called the "unfolded protein response" (UPR). This adaptive response coordinates translation suppression, and, an increased production of molecular chaperones that amplify the protein folding capacity of the ER and a promotion of ER-associated degradation (ERAD) that clears misfolded proteins (for review, see [32]). A recent microarray study by Dombroski et al. [33] reported on the variation in the ER-stress response by exposing B cells to two standard drugs known to inhibit protein folding and modification in the ER. Interestingly, the expression of 18 proteins identified in this study was also found to be modified in the Dombroski et al. [33] study suggesting that GFP expression induces ER-stress in the cell and possibly UPR. A few of our differentially expressed proteins are classic UPR products (for example, CCT4, CCT7, DNAJA1, HSPH1, HSPA5, HSPE1, HSPB1, USP14). These findings are consistent with the presence of significant amounts of unfolded GFP proteins after overexpression in mammalian cells [34].

Changes in the expression of proteins involved in cellular process following stable GFP expression in MDA cells

The second largest functional category in which both down- and up-regulated proteins sorted is cellular process 11.6% and 13.8% respectively (Fig. 4). Functions such as cell adhesion, cell motion, cell cycle, cellular component organisation (more specifically morphogenesis) and cell communication (Fig. 4) were identified. Interestingly, several differentially expressed proteins are involved in cell adhesion, cell motion and cellular

morphogenesis (Fig. 4). Among these proteins, 16 were found to be structural constituents of the cytoskeleton (ACTG1, COF1, SEPT6, GSTO1, CNN3, LMNA, MSN, TPM4, CAPZA1, CORO1C, PLS3, PDLIM1, PLSL, ACTR3, EEF1G, MARS). This suggests that expression of GFP could have a subtle influence on cytoskeletal architecture, cell morphology and motility.

In 2011, Comley et al. [7] reported that expression of YFP in neurons induces morphological changes that are similar to early pathological changes in neurons and additionally they suggested that this could be dose-dependent. Other evidences of the effect of GFP expression on cytoskeletal function have been reported. Huang et al. [4] found that transgenic expression of GFP caused a dilated cardiomyopathy in transgenic mouse lines. Krestel et al. [35] showed that co-expression of eGFP and β -galactosidase in the cytoplasm of forebrain neurons of mice induced growth retardation, weakness, and premature lethality and finally that eGFP expression induced a contractile dysfunction disturbing actin–myosin interactions in muscle cells [3].

Stable GFP expression in MDA cells may lead to altered cellular immune response in vivo

Two other categories of interest within biological process are the immune response and the response to stimulus categories. The category is well represented with 18 up-regulated proteins (MIF, EEF1G, PRDX1, HSPA5, CAPNS1, LGALS3BP, PRDX4, LGALS1, CTSD, LGALS3, FKBP3, TXN, HSPB1, TXNRD1, HLA-A, FKBP1A, MARS, LTA4H), compared to only 7 down-regulated proteins (XRCC5, XRCC6, GSTO1, GSTP1, HSPH1, ILF2, DNAJA1). Within this category we found that 9 up-regulated proteins also belong to the response to stimulus category. These are involved in cellular defense response (HLA-A, FKBP3, CTSD, LGALS3, FKBP1A), response to stress (HSPB1, TXN) and response to toxin (EEF1G, MARS) and suggest an activation of a cell stress response in MDA cells.

Badrian and Bogoyevitch [11] reported on the transcriptional changes associated with GFPexpression in cardiac myocytes, observing that a number of the up-regulated genes coded for protein products involved in the response to stimulus pathways of the cells. Moreover, other *in vivo* and *in vitro* experimental data [2,4,7] showed that expression of a fluorescent protein in cells/tissues modifies their molecular composition and that this seems to be independent of the plasmid used. Liu et al. [2] have shown that GFP expression using three different plasmids leads to a similar pro-apoptotic effect.

Influence of the plasmid and selection by the way of the neomycin resistance (neo^r) gene using G418 has been previously studied in two cell lines (BHK, CHO) by Yallop et al. [36,37]. They reported that while growth and metabolism of both cell lines was affected by the presence of G418 in the culture medium in a manner of increased metabolic load this effect was greatly compensated by addition of serum and glutamine to the culture medium [37]. Moreover, they further elucidated the metabolic changes occurring by measuring the activity and relative expression of key enzymes involved in glucose and glutamine metabolism. They reported that while an increase in enzymatic activity was observed, this was not correlated with an increase in the expression levels of these key enzymes [36]. Nevertheless, stable GFP expression can only be achieved through the transfection of a plasmid containing a selectable marker. Therefore, while we cannot absolutely rule out some changes in the proteome induced by G418 or the process of transfection, particularly relevant for metabolic pathway, the influence might still be minimal in serum-based culture as used in our study.

Taken together, our results and those of others suggest that great care should be taken in the analysis of gene and/or protein expression profile modifications which involve co-expression with GFP as it can induce a variety of molecular changes. Future studies will

need to take this into account in regard to experimental design and data interpretation and perform necessary controls to ensure that any results reported are a direct effect of the experimental intervention and not simply a consequence of fluorescent protein expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by Grants from the Collaborative Research Network (CRN) on Mental Health and Wellbeing in Rural and Regional Communities and the University of New England (PM, JC) and National Institute of Health (2R01 CA108607) (PR). Some mass spectrometric results were obtained at the Bioanalytical Mass Spectrometry Facility within the Analytical Centre of the University of New South Wales. Subsidized access to this facility is gratefully acknowledged.

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Abbreviations

GFP	green fluorescence protein
2-DE	two-dimensional electrophoresis
iTRAQ	isobaric tags for relative and absolute quantitation



Fig. 1.

Distribution of the combined protein ratios with significant changes (p 0.05) within the biological replicates of MDA and MDA-GFP for 2-DE, iTRAQ and label-free proteomics approaches. The percent of false positive proteins (FPP) with ratios ranging from 1 to >2 is reported.



Fig. 2.

2-DE protein profiles of WT cells (A) GFP expressers (B) of MDA-231 cells. Spots highlighted presented at least a 1.5-fold expression change in MDA-GFP when compared to MDA protein spots. Spots highlighted with \diamond are only detected in MDA cells, while the spot highlighted with Δ is only detected in GFP expressers of MDA-231 cells. Protein spots labeled with \bigcirc are over-expressed while protein spots labeled with \square are under-expressed in GFP expressers of MDA-231 cells. Spot numbers reflect identifications as reported in Table 1 and in the Supplemental data.

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The number of proteins identified (with one peptide) and quantified (with a minimum of three unique peptides) overlapping between the iTRAQ and label-free approaches after running technical and biological replicates.





Fig. 4.

Distribution of the proteins differentially expressed between MDA and MDA-GFP classified following ontology analysis using the PANTHER classification system ((http://www.pantherdb.org)). The different categories within molecular function and biological process are reported. Black indicates percent of proteins down-regulated in MDA-GFP, while gray indicates those up-regulated.



Fig. 5.

Evidence view of a protein–protein interactions map generated using STRINGv9.05 analysis tools ((http://string-db.org)). Settings used to generate this map were high confidence score (0.7) and Kmeans clustering. The gene symbols used are listed in Table 1 and disconnected nodes are hidden.

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Table 1

List of the identified proteins. Fold change and p value reported in *italics* are given as a supplemental comparison when proteins were quantified but not found differentially expressed according to our selection criteria.

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Expression in	Protein name	Gene symbol	Uniprot acc. no.	E.C. number	Fold change		
MDA-GFF cens compared to MDA cells					2-DE	iTRAQ	Label-free
\rightarrow	Cofilin-1	CFL1	P23528		-2.01 (spot 1)		-1.18 (0.27)
→	Ubiquitin carboxyl-terminal hydrolase 14	USP14	P54578	3.4.19.12	-2.09 (spot 2)		
→	Protein disulfide-isomerase	P4HB	P07237	5.3.4.1	-2.09 (spot 2)		1.28 (0.031)
\rightarrow	Phosphoglycerate mutase 1	PGAMI	P18669	3.3.3.13, 5.4.2.1, 5.4.2.4	-2.05 (spot 3)		
\rightarrow	Proteasome subunit alpha type-6	PSMA6	P60900	3.4.25.1	-1.78 (spot 4)		
\rightarrow	Elongation factor 1-delta	EEF1D	P29692		-1.54 (spot 5)		
→	Nucleophosmin	NPM1	P06748		-1.53 (spot 6)	-1.43 (0.059)	-2.45 (0.101)
Conflicting data	Hepatoma-derived growth factor	HDGF	P51858		-1.53 (spot 6)	1.28	
\rightarrow	V-type proton ATPase subunit d 1	ATP6V0D1	P61421		-1.53 (spot 6)	-1.53	
\rightarrow	Ribosome biogenesis protein WDR12	WDR12	Q9GZL7		-1.63 (spot 7)		
\rightarrow	26S protease regulatory subunit 8	PSMC5	P62195			-1.25	
\rightarrow	40S ribosomal protein S19	RSP19	P39019			-1.20	
\rightarrow	40S ribosomal protein S2	RSP2	P15880			-1.29	-1.26 (0.043)
\rightarrow	40S ribosomal protein S20 isoform 2	RSP20	P60866			-1.23	
\rightarrow	40S ribosomal protein S23	RSP23	P62266			-1.59	
\rightarrow	40S ribosomal protein S3 isoform 2	RSP3	P23396			-1.23	
\rightarrow	40S ribosomal protein S3a	RSPS3A	P61247			-1.28	
\rightarrow	40S ribosomal protein S9	RPS9	P46781			-1.27	
\rightarrow	4F2 cell-surface antigen heavy chain	SLC3A2	P08195			-1.63	
\rightarrow	60S acidic ribosomal protein P0	RPLP0	P05388			-1.29	
\rightarrow	60S ribosomal protein L10a	RPL10A	P62906			-1.27	
\rightarrow	60S ribosomal protein L11	RPL11	P62913			-1.37	
\rightarrow	60S ribosomal protein L17	RPL17	P18621			-1.23	
\rightarrow	60S ribosomal protein L5	RLP5	P46777			-1.20	
\rightarrow	Actin, cytoplasmic 2	ACTG1	P63261			-1.23	
\rightarrow	Actin-related protein 2/3 complex subunit 2	ARPC2	015144			-1.39	

Expression in	Protein name	Gene symbol	Uniprot acc. no.	E.C. number	Fold change		
MDA-GFF cells compared to MDA cells					2-DE	iTRAQ	Label-free
→	Actin-related protein 3	ACTR3	P61158			-1.22	
\rightarrow	Aspartate-tRNA ligase, cytoplasmic	DARS	P14868	6.1.1.12		-1.32	
\rightarrow	ATP synthase subunit alpha, mitochondrial	ATP5A1	P25705			-1.47	
\rightarrow	ATP synthase subunit beta, mitochondrial	ATP5B	P06576			-1.52	-1.41 (0.008)
\rightarrow	Bifunctional purine biosynthesis protein PURH	ATIC	P31939	2.1.2.3		-1.35	
\rightarrow	Caprin-1	CAPRIN1	Q14444			-1.26	
\rightarrow	Coronin-1C	COROIC	Q9ULV4			-1.29	-
\rightarrow	DNA replication licensing factor MCM2	MCM2	P49736	3.6.4.12		-1.31	
\rightarrow	DNAJ homolog subfamily A member 1	DNAJA1	P31689			-1.30	
\rightarrow	Elongation factor 1-beta	EEF1B2	P24534			-1.79	-
\rightarrow	Eukaryotic initiation factor 4A-I	EIF4A1	P60842	3.6.4.13		-1.21	-1.73
\rightarrow	Eukaryotic initiation factor 4A-III	EIF4A3	P38919	3.6.4.13		-1.25	-2.05
\rightarrow	Eukaryotic translation initiation factor 3 subunit F	EIF3F	O00303	3.4.19.12		-1.49	-1.5(0.018)
\rightarrow	Eukaryotic translation initiation factor 4 gamma 1	EIF4G1	Q04637			-1.31	-1.15 (0.18)
\rightarrow	Glutathione S-transferase omega-1	GST01	P78417	2.5.1.18, 1.8.5.1, 1.20.4.2			-1.99
\rightarrow	Glutathione S-transferase P	GSTP1	P09211	2.5.1.18		-1.26	
\rightarrow	Guanine nucleotide-binding protein subunit beta-2-like 1	GNB2L1	P63244			-1.55	
\rightarrow	Heterogeneous nuclear ribonucleoprotein Q	SYNCRIP	O60506			-1.28	-1.3 (0.031)
\rightarrow	Importin subunit alpha-2	KPNA2	P52292			-1.95	-
\rightarrow	Importin subunit beta-1	KPNB1	Q14974			-1.35	-1.33 (0.28)
\rightarrow	Inosine-5'-monophosphate dehydrogenase 2	IMPDH2	P12268	1.1.1.205		-1.47	
\rightarrow	Interleukin enhancer-binding factor 2	ILF2	Q12905			-1.23	
\rightarrow	Nucleolar and coiled-body phosphoprotein 1	NOLC1	Q14978			-1.25	
\rightarrow	Nucleolar RNA helicase 2 isoform 1	DDX21	Q9NR30	3.6.4.16		-1.33	
\rightarrow	Phosphate carrier protein, mitochondrial	SLC25A3	Q00325			-1.37	
\rightarrow	Polyadenylate-binding protein 1	PABPC1	P11940			-1.30	-1.11 (0.063)
\rightarrow	Probable ATP-dependent RNA helicase DDX5	DDX5	P17844	3.6.4.13		-1.27	
\rightarrow	Proliferating cell nuclear antigen	PCNA	P12004			-1.32	-2.68
\rightarrow	Proteasome subunit alpha type-5	PSMA5	P28066	3.4.25.1		-1.32	
\rightarrow	Protein-L-isoaspartate(D-aspartate) O-methyltransferase	PCMT1	P22061	2.1.1.77		-1.25	

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Expression in	Protein name	Gene symbol	Uniprot acc. no.	E.C. number	Fold change		
MDA-GFP cells compared to MDA cells					2-DE	iTRAQ	Label-free
$\rightarrow$	Putative RNA-binding protein 3	RBM3	P98179			-1.46	
$\rightarrow$	Ras GTPase-activating protein-binding protein 1	G3BP1	Q13283	3.6.4.12		-1.47	
→	Ras-related C3 botulinum toxin substrate 2	RAC2	P15153			-1.23	
$\rightarrow$	Ribosome maturation protein SBDS	SBDS	Q9Y3A5			-1.26	
→	RNA-binding protein FUS	FUS	P35637			-1.29	-1.64 (0.118)
$\rightarrow$	rRNA 2'-O-methyltransferase fibrillarin	FBL	P22087	2.1.1		-1.23	
$\rightarrow$	Scaffold attachment factor B2	SAFB2	Q14151			-1.26	
$\rightarrow$	Signal recognition particle 9 kDa protein	SRP9	P49458			-1.22	
$\rightarrow$	Small nuclear ribonucleoprotein Sm D2	SNRPD2	P62316			-1.26	
$\rightarrow$	SUMO-activating enzyme subunit 1	SAE1	Q9UBE0			-1.29	
$\rightarrow$	T-complex protein 1 subunit alpha	TCP1	P17987			-1.29	
$\rightarrow$	T-complex protein 1 subunit delta	CCT4	P50991			-1.20	
$\rightarrow$	T-complex protein 1 subunit eta	CCT7	Q99832			-1.24	-1.43 (0.082)
→	T-complex protein 1 subunit theta	CCT8	P50990			-1.24	
→	X-ray repair cross-complementing protein 5	XRCC5	P13010	3.6.4			-2.16
$\rightarrow$	X-ray repair cross-complementing protein 6	XRCC6	P12956	3.6.4		-1.41	-1.49 (0.078)
Only detected in GFP cell	RuvB-like 2	RUVBL2	Q9Y230		(spot 22)		
~	Protein-glutamine gamma-glutamyltransferase 2	TGM2	P21980	2.3.2.13	1.51 (spot 8)	1.36	2.23
<i>←</i>	Geranylgeranyl transferase type-2 subunit alpha	RABGGTA	Q92696	2.5.1.60	1.54 (spot 9)		
~	Plastin-2	LCP1	P13796		2.59 (spot 10)	1.77	2.68
~	Heat shock protein 105 kDa	1HASH1	Q92598		1.74 (spot 11)		
<i>←</i>	Methionine-tRNA ligase	MARS	P56192	6.1.1.10	1.75 (spot 12)		
~	Insulin-degrading enzyme	IDE	P14735	3.4.24.56	1.72 (spot 13)		
~	Endoplasmic reticulum aminopeptidase	ERAP1	80ZN60	3.4.11	1.72 (spot 13)		
<i>←</i>	Moesin	MSN	P26038		1.72 (spot 14)	1.11 (0.12)	
~	Prelamin-A/C	LMNA	P02554		1.72 (spot 14)		1.21 (0.026)
~	Succinate dehydrogenase	SDHA	P31040	1.3.5.1	1.93 (spot 15)		
~	Heterogeneous nuclear ribonucleoprotein H	HNRNPH1	P31943		1.61 (spot 16)	1.09~(0.46)	-1.3 (0.039)
←	Sorting nexin-6	SNX6	CHNU60		1.61 (spot 16)		

Expression in	Protein name	Gene symbol	Uniprot acc. no.	E.C. number	Fold change		
MDA-GFP cells compared to MDA cells					2-DE	iTRAQ	Label-free
÷	Synaptic vesicle membrane protein VAT-1 homolog	VAT1	Q99536		2.35 (spot 17)		1.3 (0.25)
~	Thioredoxin reductase 1	<b>TXNRD1</b>	Q16881	1.8.1.9	1.56 (spot 18)		1.44 (0.017)
Conflicting data	S-adenosylmethionine synthase isoform type-2	MAT2A	P31153	2.5.1.6	1.53 (spot 19)	-1.53	
~	Elongation factor 1-gamma	EEF1G	P26641		1.53 (spot 19)	1.13 (0.080)	
~	Leukotriene A-4 hydrolase	LTA4H	P09960	3.3.2.6	1.61 (spot 20)		
~	BAG family molecular chaperone regulator 2	BAG2	095816		2.12 (spot 21)		
~	Aldose reductase	AKRIBI	P15121	1.1.1.21	1.56 (spot 23)	1.36	1.4 (0.181)
~	LIM and SH3 domain protein 1	LASP1	Q14847		1.56 (spot 23)	1.56 (0.051)	1.88 (0.065)
~	Heat shock protein beta-1	HSPB1	P04792		1.73 (spot 24)	1.64	
~	10 kDa Heat shock protein, mitochondrial	HSPE1	P61604				2.11
~	60 kDa Heat shock protein, mitochondrial	HSPD1	P10809			1.02 (0.41)	1.61
~	78 kDa Glucose-regulated protein	HSPA5	P11021			1.17 (0.042)	1.89
$\leftarrow$	Activated RNA polymerase II transcriptional coactivator p15	SUB1	P53999			1.21	
~	Annexin A1	ANXA1	P04083				1.66
$\leftarrow$	AP-2 complex subunit beta	AP2B1	P63010			1.35	
~	ATP-citrate synthase	ACLY	P53396	2.3.3.8		1.09 (0.002)	1.69
~	B-cell receptor-associated protein 31	BCAP31	P51572			1.23	
~	Calmodulin	<b>CALM1</b>	P62158			2.03	
~	Calpain small subunit 1	<b>CAPNS1</b>	P04632			1.18(0.034)	2.06
~	Calpastatin	CAST	P20810			1.55	
~	Calponin-3	CNN3	Q15417			1.46	2.11
~	Calreticulin	CALR	P27797			1.25	1.24 (0.298)
~	Cathepsin D	CTSD	P07339	3.4.23.5		1.80	3.16
~	Clathrin light chain A	CLTA	P09496			1.28	
~	Copine-1	<b>CPNE1</b>	Q99829			1.61	
~	Cystatin-B	CSTB	P04080			1.39	
~	Extended synaptotagmin-1	ESYT1	Q9BSJ8				1.71
~	F-actin-capping protein subunit alpha-1	<b>CAPZA1</b>	P52907				2.03
~	Far upstream element-binding protein 1	FUBP1	Q96AE4			1.42	
~	Galectin-1	LGALS1	P09382			1.29 (0.388)	1.67

Exp Cell Res. Author manuscript; available in PMC 2015 January 01.

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Expression in	Protein name	Gene symbol	Uniprot acc. no.	E.C. number	Fold change		
MDA-GFF cells compared to MDA cells					2-DE	iTRAQ	Label-free
~	Galectin-3	LGALS3	P17931			1.30	
~	Galectin-3-binding protein precursor	<b>LGALS3BP</b>	Q08380			1.21	
~	Gamma-enolase	ENO2	P09104	4.2.1.11		1.32	1.33 (0.13)
~	Glucose-6-phosphate 1-dehydrogenase	G6PD	P11413	1.1.1.49		1.23	
~	Glucosidase 2 subunit beta	PRKCSH	P14314			1.23	1.87 (0.076)
~	HLA class I histocompatibility antigen, A-2 alpha chain	HLA-A	P01892			1.03(0.101)	2.01
~	HLA class I histocompatibility antigen, A-2 alpha chain	HLA-A	P01892				2.01
÷	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	HADH	Q16836	1.1.1.35			1.69
$\leftarrow$	Importin-9	IPO9	Q96P70			1.39	
~	Inhibitor of nuclear factor kappa-B kinase-interacting protein	IKBIP	Q70UQ0			1.85	
<i>←</i>	Leukocyte elastase inhibitor	<b>SERPINB1</b>	P30740			1.29	
<i>←</i>	Macrophage migration inhibitory factor	MIF	P14174	5.3.2.1		1.12 (0.628)	2.49
<i>←</i>	Malate dehydrogenase, mitochondrial	MDH2	P40926	1.1.1.37		1.38	1.91
~	Non-histone chromosomal protein HMG-17	HMGN2	P05204			1.33	
~	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1	NUCKS1	Q9H1E3			1.62	
~	PDZ and LIM domain protein 1	<b>PDLIM1</b>	000151			1.32	
$\leftarrow$	Peptidyl-prolyl cis-trans isomerase FKBP1A	FKBP1A	P62942	5.2.1.8			2.64
÷	Peptidyl-prolyl cis-trans isomerase FKBP3	FKBP3	Q00688	5.2.1.8		1.31	
~	Peroxiredoxin-1	PRDX1	Q06830	1.11.1.15		1.14 (0.221)	1.64
<i>←</i>	Peroxiredoxin-4	PRDX4	Q13162	1.11.1.15		1.24	
~	Phosphatidylethanolamine-binding protein 1	PEBP1	P30086			1.69	
~	Plastin-3	PLS3	P13797			1.22 (0.133)	2.16
~	Proteasome subunit alpha type-7	PSMA7	O14818	3.4.25.1		1.60	
~	Septin-6	SEPT6	Q14141				1.66
<i>←</i>	SH3 domain-binding glutamic acid-rich-like protein 3	SH3BGRL3	Q9H299			1.77	
<i>←</i>	Stathmin	STMN1	P16949			1.46	1.94
~	Superoxide dismutase [Cu-Zn]	SOD1	P00441	1.15.1.1		1.87	
~	Thioredoxin	TXN	P10599			1.55	
~	Thymidylate kinase	DTYMK	P23919	2.7.4.9		1.37	

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<b>Expression in</b>	Protein name	Gene symbol	Uniprot acc. no.	E.C. number	Fold change		
MDA-GFP cells compared to MDA cells					2-DE	iTRAQ	Label-free
~	Thymosin beta-10	TMSB10	P63313			2.26	
~	Thymosin beta-4	TMSB4X	P62328			2.70	
~	Tropomyosin alpha-4 chain	TPM4	P67936			1.28	-
~	Tubulin-specific chaperone A	TBCA	075347			1.61	
~	Ubiquitin carboxyl-terminal hydrolase isozyme L3	UCHL3	P15374	3.4.19.12		1.25 (0.128)	1.60