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Profilin-1 Overexpression in MDA-MB-231 Breast Cancer Cells Is Associated with Alterations in Proteomics Biomarkers of Cell Proliferation, Survival, and Motility as Revealed by Global Proteomics Analyses

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Abstract

Despite early screening programs and new therapeutic strategies, metastatic breast cancer is still the leading cause of cancer death in women in industrialized countries and regions. There is a need for novel biomarkers of susceptibility, progression, and therapeutic response. Global analyses or systems science approaches with omics technologies offer concrete ways forward in biomarker discovery for breast cancer. Previous studies have shown that expression of profilin-1 (PFN1), a ubiquitously expressed actin-binding protein, is downregulated in invasive and metastatic breast cancer. It has also been reported that PFN1 overexpression can suppress tumorigenic ability and motility/invasiveness of breast cancer cells. To obtain insights into the underlying molecular mechanisms of how elevating PFN1 level induces these phenotypic changes in breast cancer cells, we investigated the alteration in global protein expression profiles of breast cancer cells upon stable overexpression of PFN1 by a combination of three different proteome analysis methods (2-DE, iTRAQ, label-free). Using MDA-MB-231 as a model breast cancer cell line, we provide evidence that PFN1 overexpression is associated with alterations in the expression of proteins that have been functionally linked to *cell proliferation* (FKPB1A, HDGF, MIF, PRDX1, TXNRD1, LGALS1, STMN1, LASP1, S100A11, S100A6), *survival* (HSPE1, HSPB1, HSPD1, HSPA5 and PPIA, YWHAZ, CFL1, NME1) and *motility* (CFL1, CORO1B, PFN2, PLS3, FLNA, FLNB, NME2, ARHGDIB). In view of the pleotropic effects of PFN1 overexpression in breast cancer cells as suggested by these new findings, we propose that PFN1-induced phenotypic changes in cancer cells involve multiple mechanisms. Our data reported here might also offer innovative strategies for identification and validation of novel therapeutic targets and companion diagnostics for persons with, or susceptibility to, breast cancer.

Introduction

Despite early screening programs and new therapeutic strategies, breast cancer is still the leading cause of cancer death in women in industrialized countries and regions (Ferlay et al., 2010). Mortality of breast cancer patients results primarily from distant metastasis of tumor cells to the vital organs. Global analyses or systems science approaches with omics technologies offer concrete ways forward in biomarker discovery for breast cancer (Korwar et al., 2013). Deregulation of the actin cytoskeleton is a hallmark of both oncogenic transformation and deregulated motility of cancer cells leading to metastatic dissemination. Dynamic remodeling of the actin cytoskeleton involves concerted actions of a large number of actin-binding proteins (ABPs) (Nurnberg et al., 2011). It is therefore conceivable that some ABPs could be targeted as therapeutic molecules in combating tumor development and metastasis.

Profilins (PFNs) belong to a family of small proteins (12–14 kDa) that bind globular (G-) actin and are thought to regulate actin polymerization at the leading edge of migrating cells. Four members have been identified to date, including the ubiquitously expressed PFN1, PFN2 (mainly expressed in neurons), and PFN3 and 4 whose expression is restricted to kidney and testis. Beside binding to actin, PFNs interact with a number of proline-rich-motif (PRM) proteins (Witke,

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2004), which are implicated in a wide range of cellular activities (proliferation, migration, endocytosis, mRNA splicing, and gene transcription) and to the plasma membrane through its interactions with phosphoinositides (Hartwig et al., 1989; Moens et al., 2007).

Recent work has highlighted alteration in PFN1 expression in different cancer types, including breast (Janke et al., 2000), pancreatic (Gronborg et al., 2006), hepatic (Wu et al., 2006), gastric (Oien et al., 2003), and renal (Minamida et al., 2011). Among the different types of cancer, PFN1 has been most widely studied in the context of breast cancer. PFN1 suppresses proliferation of breast cancer cells *in vitro* and inhibits tumorigenic ability of xenografted breast cancer cells *in vivo* (Janke et al., 2000, Zou et al., 2007). PFN1 overexpression elevates the level of $p27^{kip1}$, a major cell-cycle inhibitor, and cause cell-cycle arrest of breast cancer cells at G1 phase; however, silencing $p27^{kip1}$ only partially restores the proliferation defect induced by PFN1 overexpression (Zou et al., 2010), suggesting that it is not the only possible mechanism underlying PFN1's tumor-suppressive effect. Elevating PFN1 expression also promotes apoptosis of breast cancer cells in response to various cytotoxic agents, suggesting that PFN1 plays an important role in regulating cell survival (Yao et al., 2012, 2013; Zou et al., 2010). Finally, PFN1 has been implicated as a negative regulator of mammary carcinoma aggressiveness as it has been shown that lower PFN1 expression correlates with metastatic potential of tumor cells in human breast cancer (Zou et al., 2007).

Consistent with these clinical findings, loss of PFN1 expression promotes migration, stromal invasion, transendothelial migration of breast cancer cells *in vitro*, and vascular dissemination of tumor cells from xenografts *in vivo*. Conversely, overexpression of PFN1 suppresses migration and invasion of breast cancer cells (Bae et al., 2009, 2010; Ding et al., 2014; Zou et al., 2007). Multiple mechanisms including increased sustenance of PI3K signaling and PI3K-dependent membrane recruitment of pro-migratory complexes, and elevated MMP9 and VEGF secretion have been proposed to account for the increased disseminative act of breast cancer cells induced by loss of PFN1 expression (Ding et al., 2014). The above findings clearly demonstrate that PFN1 can orchestrate a number of cellular processes. Therefore, modulating PFN1 expression induces multiple phenotypic changes in breast cancer cells, and little is known about the biological networks regulated by PFN1 that can explain its pleotropic actions on cancer cells *in vivo*.

In this study, we utilized three quantitative proteomics approaches, including two-dimensional electrophoresis (2-DE) coupled with mass spectrometry (LC-MS/MS), 8-plex isobaric tag for relative and absolute quantification (iTRAQ) 2D-LC–MS/MS and label-free LC-MS/MS quantification, to determine PFN1-induced proteomics changes in MDA-MB-231 cells.

Materials and Methods

Cell culture

Parental MDA-MB-231 breast cancer cells (referred to as wild-type (WT) cells from here on) and stable MDA-MB-231 clones expressing green fluorescent protein (GFP) and GFP-PFN1 were generated as previously described (Coumans et al., 2014; Zou et al., 2007).

Protein extraction and quantification

Protein extraction and quantification were performed as previously described (Coumans et al., 2014). Briefly, cells at 90% of confluence, in 175 cm^2 flasks, were washed twice with PBS, detached from the culture flask by 5 min incubation at 37°C in citric saline solution (1.35 M KCl, 0.15 M sodium citrate), and the cell pellet was used for protein extraction.

For protein analysis by 2-DE, the cell pellet was 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 extracted. Protein samples were then centrifuged at 15,000 *g* (15 min, 20°C) and supernatant retained for further analysis.

In all cases, protein concentration in the supernatant was determined using the 2-D Quant kit (GE Healthcare).

Two-dimensional electrophoresis, protein visualization, and image analysis

2-DE was performed essentially as previously described (Coumans et al., 2011, 2014). In summary, rehydrated 18 cm IPG strips pH 4–7 (GE Healthcare) were loaded with protein (300 μg for analytical gels or 400 μg 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 μA/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 a colloidal Coomassie Brillant Blue (Neuhoff et al., 1988) 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 (Coumans et al., 2009). Three biological samples and two technical replicates were grouped and analysed with PDQuest advanced 2-D analysis software (Bio-Rad).

Sample preparation for iTRAQ labeling

Samples were prepared as previously described (Coumans et al., 2014): 100 μg of whole cell protein extract from WT, GFP, and GFP-PFN1 expressing cells were precipitated with 9X volume of ice-cold acetone for 1 hour at −20°C. Protein precipitates were recovered by centrifugation, air dried, and dissolved in 50 mM sodium bicarbonate, 0.1% SDS. Protein samples were then reduced, alkylated (iodoacetamide), and digested with trypsin. Samples were then labeled with the iTRAQ reagents according to the manufacturer's instructions (Ross et al., 2004). The 8-plex iTRAQ system allows eight samples to be labeled per 2D-LC–MS/MS run. Therefore, the reporter labels were applied to the samples as follows: three biological replicates of GFP expressing cells with reporter labels 113, 114, 115, respectively; three biological replicates of GFP-PFN1 expressing cells with reporter labels 116, 117, 118, respectively, and two biological replicates of WT with reporter labels 119 and 121 respectively were included per run.

Excess unbound iTRAQ labels, trypsin, SDS, and solvents were removed using strong cation exchange (SCX) cartridge.

Eluted peptides were vacuum-dried, and resuspended in 500 μL of 0.2% heptafluorobutyric acid (HFBA) prior to solid phase extraction on a C18 RP peptide macrotrap (Microm Bioresources, Auburn, CA). The eluents were then pooled, vacuum-dried, and the pellet dissolved in 25 μL 1% formic acid, 0.05% HFBA. All labeling experiments were run twice (two separate injections) using 2D-LC–MS/MS, to provide the technical replicates.

Sample preparation for label-free LC-MS quantification

As previously described (Coumans et al., 2014), 100 μg of whole cell protein extract from three biological replicates of each WT, GFP, and GFP-PFN1 expressing cells were treated and trypsin digested as described in the sample preparation for iTRAQ labeling.

After trypsin digestion, the peptide samples were purified using SCX StageTips and C18 StageTips (Thermo Scientific, Australia) following the manufacturer's instructions. Technical replicates were obtained by three separate injections of individual samples.

Mass spectrometry and database search

For protein identification from 2-DE, protein spots from preparative gels were manually excised, destained, digested, and analyzed by LC-MS/MS exactly as previously described (Coumans et al., 2009). Peak lists were generated by MassLynx (version 4.0 SP1, Micromass) using the Mass Measure program and submitted to the database search program Mascot (Matrix Science, London, UK, version 2.3.03). Search parameters were: precursor and product ion tolerances $\pm$ 50 ppm and 0.2 Da respectively; acrylamide (C), carbamidomethyl (C), and oxidation (M) were specified as variable modifications, enzyme specificity was trypsin, one missed cleavage was possible, and the NCBInr 19_8_11 (14987464 sequences; 5132678026 residues) searched.

iTRAQ analysis of peptides (*ca* 5–10 μg total load, 10 μL) was performed as previously described (Coumans et al., 2014), using a 2D-LC–MS/MS (LC Packings autosampler and switchos valves with Dionex Ultimate 3000 pumps, in line to a QStar *Elite* mass spectrometer, ABSciex, Framingham, MA). The WIFF data 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 cut-off for accepting statistically significant changes in the protein expression level.

Label-free quantification was performed as previously described (Coumans et al., 2014). Briefly, solubilized peptides (*ca* 2 μg total load, 2 μL) were separated by nano-LC using an Ultimate 3000 HPLC and autosampler (Dionex, Amsterdam, Netherlands) and analyzed using a linear ion trap-Orbitrap (LTQ-Orbitrap). MS peak intensities were analyzed using Progenesis LC-MS data analysis software v4 (Nonlinear Dynamics, Newcastle upon Tyne, UK). MS/MS spectra were searched against the Swiss-Prot database 24_8_12 (536789 sequences, 190518892 residues) using the database search program Mascot. Parent and fragment ions were searched with tolerances of $\pm$ 6 ppm and $\pm$ 0.6 Da, respectively. Searched peptide charge states were limited to +2 to +4. 'No enzyme' and *Homo sapiens* for the taxonomy were specified. Peptides with an ion score >25 and above were considered for protein identification.

For comparison of expression data, Student's t-test was used and a p value of ≤ 0.05 was considered to be statistically significant.

Data analysis

To determine the possible biological consequences following stable GFP-PFN1 expression in MDA cells, protein classification analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009a, 2009b). 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 (0.6) and Kmeans clustering (Franceschini et al., 2013).

Results

To assess the influence of PFN1 overexpression on the proteome of breast cancer cells, stable sublines of GFP and GFP-PFN1 expressers were generated and GFP expression in both expressing cells was confirmed by fluorescence microscopy.

Determination of false protein quantification rates using replicates within WT and GFP/GFP-PFN1 expressers

Because analytical variability is inherent in quantitative proteomics studies, we determined the false protein quantification rates as previously described (Coumans et al., 2014). Considering replicates and assuming equal protein loading, the expected ratio for all proteins identified should be equal to 1. The ratio between our technical averages for WT and GFP or GFP-PFN1 expressers, reveals that significant differences ($p \leq 0.05$) were observed from one biological replicate to another. Using a minimum of three peptides per protein for quantification (iTRAQ and label-free analyses), combined distribution analysis of these protein quantification ratios (up- and downregulated proteins) (Fig. 1), reveals that with a p value of ≤ 0.05, in iTRAQ analysis an arbitrary cut-off of 1.3 will lead to only 2.12% of false positive quantified proteins (FPP) between biological replicate, in 2-DE analysis an arbitrary cut-off value of 1.5 will lead to less than 2% of false positive quantified proteins, while in label-free analysis a 1.4% of FPP is obtained with an arbitrary cut-off value of 1.6.

2-DE analysis of WT, GFP, and GFP-PFN1 expressers

Two technical replicates of three biological replicates of WT, GFP, and GFP-PFN1 expressers were analyzed by 2-DE. On average, 715 protein spots (MW 15 to 150 kDa, pH 4–7) were detected and further quantified using the PDQuest software. Gels were of high quality with reproducible protein patterns between sample replicates and between independent experiments (average inter-assay CV = 22%).

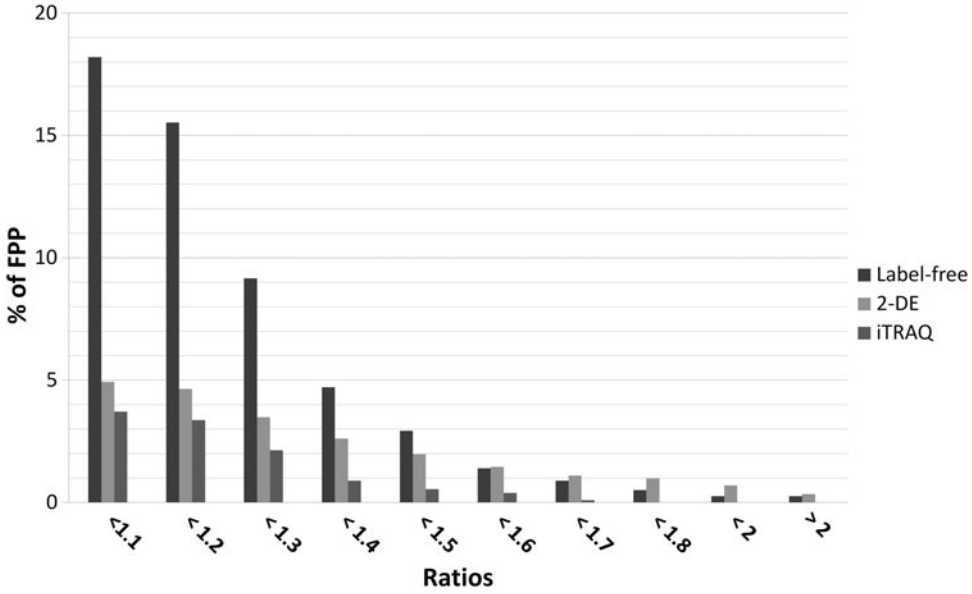

FIG. 1. Distribution of the combined protein ratios with significant changes ($p \leq 0.05$) within the biological replicates of WT, GFP, and GFP-PFN1 expressing MDA-MB-231 cells for 2-DE, iTRAQ, and label-free proteomics approaches. The % of false positive proteins (FPP) with ratios ranging from 1 to >2 is reported.

Differential expression analysis revealed three tendencies. First, we noticed that GFP expression in MDA-MB-231 cells induced proteome expression changes (Coumans et al., 2014) and that some of these modifications are compensated by GFP-PFN1 expression (Table 1A, Fig. 2, Supplementary Table S1; See Supplementary material at www.liebertpub.com/omi). This is the case for seven protein spots that had a fold change >3 between the calculated MDA-GFP/WT and MDA-GFP-PFN1/MDA-GFP ratios. For instance, LASP1 is upregulated in MDA-GFP cell as the MDA-GFP/WT ratio is equal to 1.56, but downregulated by the expression of GFP-PFN1 with a ratio of −1.77, giving a total fold change >3. Among these protein spots, two were upregulated in MDA-GFP-PFN1, while five were downregulated. Second, expression of the combination of both GFP and PFN1 seems to increase to some extent the differential expression of some protein spots (Table 1C, Fig. 2, Supplementary Table S1) as the MDA-GFP-PFN1/WT ratio is higher than the MDA-GFP-PFN1/MDA-GFP ratio. This is the case for three protein spots, two being downregulated and one upregulated by the expression of the combination of both proteins (GFP, PFN1) in MDA cells. Finally, GFP-PFN1 expression also induces proteome modifications that exclusively depend on PFN1 overexpression in MDA cells as the ratio MDA-GFP/MDA is not significantly different. This is the case for 10 protein spots, nine being upregulated and one being downregulated (Table 1B, Fig. 2, Supplementary Table S1). Identification by LC-MS/MS (Table 1A, B, C and Supplementary Table S1, 2) reported multiple possible identifications for five of the differentially expressed protein spots. Protein spots 4 and 13, both reported PLS3 as a possible identification, suggesting that this protein is post-translationally modified as many modifications such as phosphorylation, lysine acylation, and Asn-Gln deamidation alter the pI. Moreover, it has been estimated that about 20% of the protein spots in a pH 4–7 IEF gel will contain multiple proteins (Lim et al., 2003).

Protein abundance change in iTRAQ and label-free analysis of WT, GFP, and GFP-PFN1 expressers

We used two additional distinct mass spectrometry experimental approaches to profile and quantify the proteome changes occurring following PFN1 overexpression in cells. From our iTRAQ experiments (IPI human database, unused score >1.3 (≥95% confidence)), ProteinPilot identified 6281 proteins before grouping, representing 11471 distinct peptides (≥95% confidence) and 39608 spectra. These data collapsed into 1264 proteins after grouping. Combination of the technical replicates brought a common result of 874 proteins. In our label-free experiments, 130163 MS/MS spectra were identified and searched against the Swiss-Prot database 24_8_12. A total of 4359 peptides were identified, but to increase confidence in protein identification, only peptides with an ion score >25 were selected. After conflict resolution, the combination of the technical replicates allowed the identification of 828 proteins (ANOVA $p \leq 0.05$). For quantification purpose, we chose to consider only proteins identified with at least three unique peptides in both iTRAQ and label-free analysis.

By comparing reporter ion ratios, we find that expression of PFN1 in WT cells significantly downregulates (fold change >1.3, $p \leq 0.05$) six proteins (Table 1A, B and Supplementary Table S1). Two of these proteins (AKR1B1, LGALS3) compensate the effect of GFP expression in MDA cells (Table 1A, Supplementary Table S1) as these proteins are also significantly differentially expressed (fold change >1.3, $p \leq 0.05$) in GFP-expressing cells. One of these proteins (AKR1B1) was previously identified in our 2-DE analysis, confirming its differential expression. A total of seven proteins (Table 1B, Supplementary Table S1) were found to be upregulated, one of these proteins (PLS3) having previously been identified in our 2-DE analysis (Spots 4, 13).

Our label-free experiments identified 20 significantly (fold change >1.6, $p \leq 0.05$) downregulated proteins and seven

upregulated proteins (Table 1A, B, C, and Supplementary Table S1). Few of these proteins were previously identified either in our 2-DE and/or iTRAQ analyses. A number of these proteins (nine downregulated, four upregulated) compensate the influence of GFP expression in MDA cells (Table 1A, Supplementary Table S1) while differential expression of the others is uniquely due to PFN1 overexpression in MDA cells (Table 1B, Supplementary Table S1). Finally, the differential expression of S100A11 (Table 1C, Supplementary Table S1) is further increased by the GFP-PFN1 expression.

For several of the proteins identified (CFL1, HDGF, TXNDC5, CORO1B, HSPA1A/B, and PGAM1) by 2-DE and iTRAQ and/or label-free, the significant fold change observed by 2-DE is not always found in our MS base analysis methods. Several factors can explain this discrepancy. First, protein physicochemical properties and variation in sample preparation between 2-DE and MS analysis may play an important role in their quantitative extraction. Second, comigration of several proteins as observed for spot 13 is another challenge in 2-DE analysis, as discussed previously. Fold change difference between iTRAQ and label-free could be explained by the use of only reverse phase separation and less starting material in label-free. Finally, in MS analysis, even if stringent ion score criteria are used, as it is the case in this study, correct assignment of the proteolytic peptides to the parent protein, as well as assignment of the MS/MS spectra to the precursor, could lead to significantly different quantifications for a particular protein. Moreover, post-translational modifications can also often lead to challenges in the assignment of MS/MS spectra to sequence databases, as many more MS/MS spectra are collected than can be assigned to existing databases.

Data analysis of the differentially expressed proteins in GFP-PFN1 expresser

Using DAVID (Huang et al., 2009a, 2009b), we identified four major biological process clusters: (1) regulation of cell death (21.7% of the proteins identified, (*p*-value 8.1E-5) including YWHAZ, PRDX1, TXNDC5, HSPB1, NPM1, HSPA1A/B, LGALS1, HSPD1, HSPA5, MIF, NME2, CFL1, HSPE1), (2) cytoskeleton organization (16.7%, (*p*-value: 5.9E-5) including FLNA, FLNB, NPM1, PLS3, STMN1, CFL1, PFN2, CAP2, ARHGDIB, LASP1), (3) cell proliferation (11.7%, (*p*-value: 8,3E-3), including HSPD1, PCNA, MIF, HDGF, FKBP1A, PRDX1, TXNRD1), and (4) cell proliferation regulation (6.7%, (*p*-value: 6.3E-1) including S100A6, S100A11, NPM1, NME2).

In cancer, there is compelling evidence that tumor growth results from uncontrolled cell proliferation but also of reduced cell death (Evan et al., 2001). The cell cycle is driven forward by cell cycle associated (CDKN1A (p21), CDKN1B (p27), TP53 (p53), CCDN1), and regulatory (EGFR) proteins that were not identified in this study but were added to strengthen the STRING network analysis of the predicted protein–protein interactions performed to identify functionally linked proteins (Fig. 3).

Discussion

Upregulation of PFN1 expression level has been shown to strongly inhibit the motility/invasiveness and/or proliferation of several breast cancer cell lines (MDA-MB-231, MDA-MB468, CAL51, BT474) (Ding et al., 2014; Janke et al., 2000; Roy et al., 2004). However, molecular mechanisms by which PFN1 regulates the aggressiveness of mammary carcinoma are mostly unknown. This is the first study aimed at determining the proteomics changes upon GFP-PFN1 overexpression in MDA-MB-231 breast cancer cells.

PFN1 overexpression alters the expression of proteins involved in cell proliferation and apoptosis

Overexpression of PPIA in several cancers, including breast, has been reported (for review: see Lee, 2010). Under cancer stressful conditions, this protein might contribute to cell survival through its chaperone activity and its antioxidant effect establishing a stable cancer growth environment. In cancer, it has been suggested that PPIA regulation is controlled by p53 or by the hypoxia inducible factor (HIF)-1α. A causative relationship resulting from the direct interaction between PPIA and p53, has been shown during apoptosis and cell cycle regulation (Baum et al., 2009). P53 is known to interact with several proteins involved in cell proliferation; cell proliferation regulation and cell death regulation, some of which have been identified in this study (see Table 2 for reported evidence of their roles). Consequently the reported interaction between PFN1 and PPIA might be of fundamental importance (Fig. 3).

Other molecular chaperones identified included heat shock proteins (HSPs). Their expression is induced by various stressors and their function to restore protein homeostasis. This might explain why GFP expression induced their expression in MDA cells (Table 1, Sacchetti et al., 2001, and Coumans et al., 2014). In the current study, we showed that GFP-PFN1 expression repressed HSPE1, HSPB1, HSPD1, and HSPA5 expression in MDA cells. In cancer, it has been shown that HSP expression and/or activity is abnormally high. A good correlation between HSP expression and resistance to chemotherapy has been established. Moreover, their depletion or inhibition frequently reduces tumor size, eventually inducing involution (for reviews, see (Calderwood et al., 2006; Garrido et al., 2006; Jego et al., 2013). Apoptosis occurs via two pathways mediated by caspases: (1) the intrinsic/mitochondrial pathway and (2) the extrinsic/death receptors pathway as well as via caspase-independent pathways. HSPs have been shown to interfere with these pathways either directly or indirectly (Lanneau et al., 2008). Recently, it has been reported that PFN1 overexpression sensitizes breast cancer cells to staurosporine-induced apoptosis through the typical intrinsic apoptotic pathway (Yao et al., 2013). In addition, they correlated this with an activation of $p53^{R273H}$ (a common tumor-associated hotspot mutation of p53) pathway in the cytoplasm that might be mediated by HSPs.

PFN1 overexpression alters the expression of proteins involved in cell motility

Cell migration is fundamental to tumor cell invasion and metastasis and involves the dynamic remodeling of the cytoskeleton. Zou et al. (2007) have previously reported that PFN1 overexpression induces actin stress-fibers, upregulates focal adhesion, and dramatically inhibits motility in MDA-MB-231 cells. In agreement with these findings, we find that several cytoskeletal associated proteins known to be involved

TABLE 1. LIST OF THE DIFFERENTIALLY EXPRESSED PROTEINS

	Underexpressed protein in GFP-PFN1 expressers					*Overexpressed protein in GFP-PFN1 expressers*				
	Spot nbr in Fig 3	*Protein name*	*Gene symbol*	*Uniprot acc. Nbr*	*E.C. Number*	*Spot nbr in Fig 3*	*Protein name*	*Gene symbol*	*Uniprot acc. Nbr*	*E.C. Number*
A	Spot 15	Spliceosome RNA helicase DDX39B	DDX39B	Q13838	3.6.4.13.	Spot 7	Phosphoglucomutase 2	PGM2	Q96G03	5.4.2.2., 5.4.2.7.
	Spot 15	4-trimethylaminobutyraldehyde dehydrogenase	ALDH9A1	B9EKV4	1.2.1.47., 1.2.1.3., 1.2.1.19.	Spot 21	Cofilin-1	CFL1	P23528	
	Spot 17	Heat shock protein beta-1	HSPB1	P04792			Proliferating cell nuclear antigen	PCNA	P12004	
	Spot 18	Protein NDRG1	NDRG1	Q92597						
	Spot 19	Aldose reductase	AKR1B1	P15121	1.1.1.21.		Eukaryotic initiation factor 4A-III	EIF4A3	P38919	3.6.4.13.
	Spot 19	LIM and SH3 domain protein 1	LASP1	Q14847			Glutathione S-transferase omega-1	GSTO1	P78417	2.5.1.18., 1.8.5.1., 1.20.4.2.
	Spot 9	Prolyl 4-hydroxylase subunit alpha-1	P4HA1	P13674	1.14.11.2.		Eukaryotic initiation factor 4A-I	EIF4A1	P60842	3.6.4.13.
	Spot 9	Copine-2	CPNE2	Q96FN4						
		Galectin-3 isoform 1	LGALS3	P17931						
		Peptidyl-prolyl cis-trans isomerase	FKBP1A	P62942	5.2.1.8.					
		Galectin-1	LGALS1	P09382						
		Stathmin	STMN1	P16949						
		Cathepsin D	CTSD	P07339	3.4.23.5.					
		Macrophage migration inhibitory factor	MIF	P14174	5.3.2.1.					
		10 kDa heat shock protein, mitochondrial	HSPE1	P61604						
		60 kDa heat shock protein, mitochondrial	HSPD1	P10809						
		Malate dehydrogenase, mitochondrial	MDH2	P40926	1.1.1.37					
		Peroxiredoxin-1	PRDX1	Q06830	1.11.1.15.					
		78 kDa glucose-regulated protein	HSPA5	P11021						
B	Spot 10	Hepatoma-derived growth factor	HDGF	P51858		Spot 3	TXNDC5 protein	TXNDC5	Q8NBS9	
		Major vault protein	MVP	Q14764		Spot 4	Plastin-3	PLS3	P13797	
		Histone H1.0	H1F0	P07305		Spot 5	Coronin-1B	CORO1B	Q9BR76	
		Leukocyte elastase inhibitor	SERPINB1	P30740		Spot 6	Perilipin-3	PLIN3	O60664	
		Acidic leucine-rich nuclear phosphoprotein 32 family member A	ANP32A	P39687		Spot 11	Growth factor receptor-bound protein 2	GRB2	P62993	
		Peptidyl-prolyl cis-trans isomerase A	PPIA	P62937	5.2.1.8.	Spot 12	Adenylyl cyclase-associated protein 2	CAP2	P40123	
		Thioredoxin reductase 1, cytoplasmic	TXNRD1	Q16881	1.8.1.9.	Spot 12	UDP-N-acetylhexosamine pyrophosphorylase	UAP1	Q16222	2.2.7.83., 2.2.7.23.
		Protein S100-A6	S100A6	P06703		Spot 13	Heat shock 70 kDa protein 1A/1B	HSPA1A/B	P08107	

(*continued*)

TABLE 1. (CONTINUED)

	Underexpressed protein in GFP-PFN1 expressers					*Overexpressed protein in GFP-PFN1 expressers*				
	Spot nbr in Fig 3	*Protein name*	*Gene symbol*	*Uniprot acc. Nbr*	*E.C. Number*	*Spot nbr in Fig 3*	*Protein name*	*Gene symbol*	*Uniprot acc. Nbr*	*E.C. Number*
		14-3-3 protein eta	YWHAH	Q04917		Spot 13	Plastin-3	PLS3	P13797	
		14-3-3 protein zeta/delta	YWHAZ	P63104		Spot 13	Annexin A6	ANXA6	P08133	
		Kinectin	KTN1	Q86UP2		Spot 14	Rho GDP-dissociation inhibitor 2	ARHGDIB	P52566	
		Calumenin	CALU	O43852		Spot 2	Vacuolar protein sorting-associated protein 28 homolog	VPS28	Q9UK41	
		Transketolase	TKT	P29401	2.2.1.1.		26S protease regulatory subunit 4	PSMC1	P62191	
							Filamin-B	FLNB	O75369	
							Filamin-A	FLNA	P21333	
							NME1-NME2 protein	NME2	P22392	2.7.4.6., 2.7.13.3
							Small nuclear ribonucleoprotein Sm D2	SNRPD2	P62316	
							Nucleophosmin	NPM1	P06748	
							Ras-related protein Rab-7a	RAB7A	P51149	
C	Spot 20	Phosphoglycerate mutase 1	PGAM1	P18669	3.3.3.13., 5.4.2.1., 5.4.2.4.	Spot 1	Profilin-2	PFN2	P35080	
	Spot 8	ATP synthase subunit d, mitochondrial	ATP5H	O75947						
		Protein S100-A11	S100A11	P31949						

(A) Proteome expression changes induced by GFP expression in MDA-MB-231 cells and compensated by GFP-PFN1 expression, **(B)** Proteome expression changes induced by combined expression of both GFP and PFN1, **(C)** Proteome modifications depending exclusively of PFN1 overexpression.

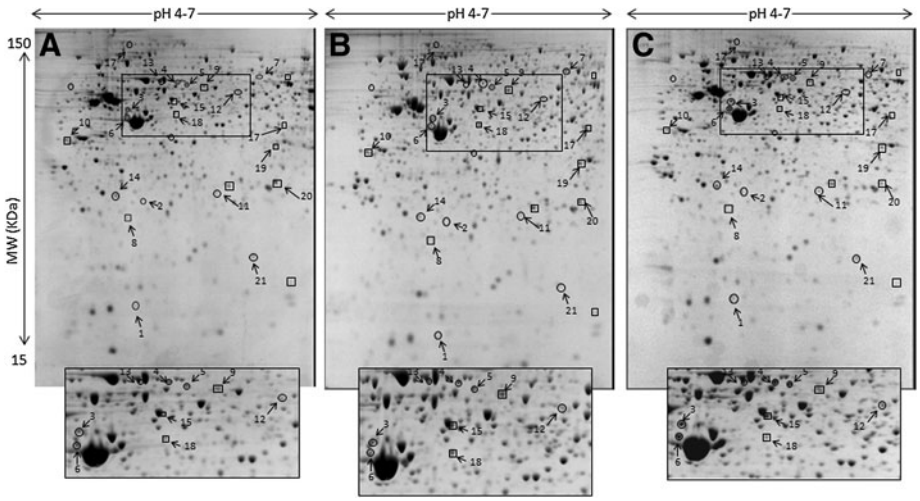

FIG. 2. 2-DE protein profiles of WT **(A)**, MDA-MB-231 stably expressing GFP **(B)**, and GFP-PFN1 **(C)** Spots highlighted had at least a 1.5-fold expression change in MDA-GFP-PFN1 when compare to MDA and MDA-GFP protein spots. Spots highlighted with ○ are overexpressed and the spot highlighted with □ is under-expressed. Spots that are numbered are identified in Table 1 and in the supplemental data.

in protrusion at the leading edge (CFL1, CORO1B, PFN2), cell adhesion (FLNA, FLNB, LASP1), and metastasis (NME2, ARHGDIB, STMN1) have modified expression (see Table 2 for literature evidence).

The cofilin pathway regulates the dynamics of actin-based structures (stress-fibrers, lamellipodia) that are involved in the early steps of motility initiation (for review: (Wang et al., 2007)). This pathway is composed of a group of kinases and phosphatases that both activate/dephosphorylate CFL1 (phospholipase Cγ (PLCγ, slingshot, and chronophin) and inhibit/phosphorylate CFL1 (LIM kinase (LIMK)). High CFL1 expression has been reported in a range of cancer cells including breast cancer cells (Wang et al., 2007), however it is not exclusively the expression level that determines the invasive phenotype of the cells but rather the balance between the expression of proteins regulating CFL1 activity. Indeed, it has been demonstrated that it is the disruption of the balance between CFL1 and LIMK1, and therefore CFL1 phosphorylation alteration, that leads to cell protrusion inhibition and chemotaxis to EGF (Mouneimne et al., 2006). Moreover, in *Dictyostelium* (Aizawa et al., 1996) and human glioblastoma cells (Yap et al., 2005) moderate CFL1 overexpression increases cell migration velocity, but higher levels of expression are reported to inhibit cell motility in the human lung cancer cell line H1299 (Lee et al., 2005). In our study, LIMK1 was not detected by any of the methods used,

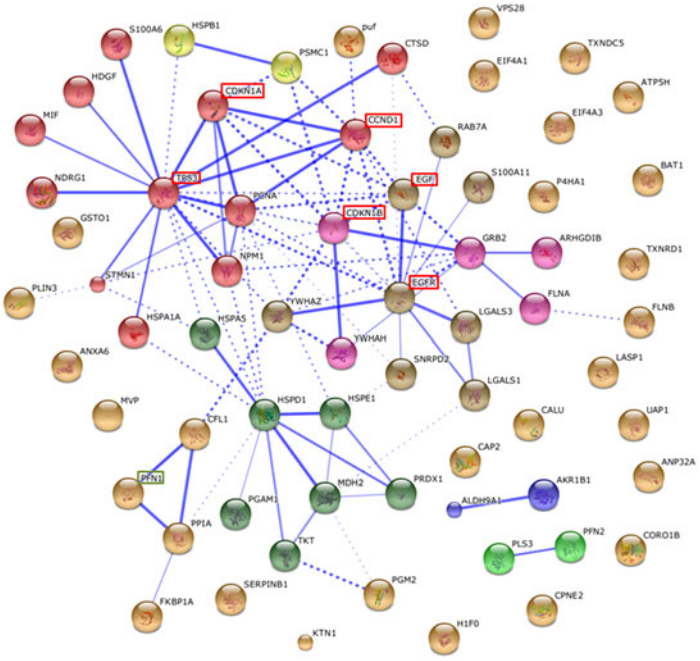

FIG. 3. Confidence view of a protein-protein interactions map generated using STRINGv9.05 analysis tools (http://string-db.org). Settings used to generate this map were: high confidence score (0.6) and Kmeans clustering. PFN1 is highlighted in *green*, while the protein symbols highlighted in *red* were added for analysis purposes.

Table 2. Literature Evidence of Function in Cell Proliferation, Apoptosis, and Cell Motility of Proteins Identified to Be Differentially Expressed in Response to GFP-PFN1 Expression

Protein	*Function*	*Publication*
FKBP1A (also known as FKBP12)	Physiologic regulator of the cell cycle as deficient (FKBP12−/−) mice are arrested in the G1 phase	(Aghdasi et al. 2001)
HDGF	HDGF silencing suppressed proliferation of MCF7 cells, but stimulated their migration and invasion capacity. It has been suggest that this might be due to the induction of a member of the PI3K pathway (PIK3R1)	(Guo et al. 2011)
MIF	Establish molecular links between MIF and p53, apoptosis and cell cycle regulation.	(Hudson et al. 1999, Kleemann et al. 2000, Fingerle-Rowson et al. 2003, Nguyen et al. 2003)
	Provide evidence of a dual role in breast cancer. Exogenous MIF stimulated MDA-MB-231 proliferation and blockade of MIF receptor CD74 blocked MDA-MB-231 proliferation induced by autocrine MIF activity.	(Verjans et al. 2009)
PRDX1	PRDX1 biological function might depends on their interaction with other proteins such as MIF and PPIA. MIF inhibits PRDX1 peroxidase activity while PPIA binds to all PRDX and enhance their thiol-specific antioxidant activity suggesting that they might modulate inflammatory reactions.	(Jung et al. 2001, Lee et al. 2001)
TXNRD1	Thioredoxin system is important in different cellular processes such as proliferation, apoptosis, and gene expression in a thiol-dependent manner.	(for review: (Arner et al. 2006, Arner 2009)
	High expression of PRDX1 and TXNRD1 associated with worse prognosis in breast cancer.	(Cha et al. 2009, Cadenas et al. 2010)
	TXNRD1 knockdown in a mouse lung cancer cell line result in a loss of self-sufficient growth, with a defective progression through the S phase and a decreased expression of DNA polymerase alpha.	(Yoo et al. 2007)
PCNA	In breast tumors, PCNA is a usefulness marker of proliferative activity as its expression appears to correlate poorly with Ki67 and mitotic count.	(Sullivan et al. 1993)
S100	Interact with various protein partners in a calcium-dependent manner influencing many vital cellular processes.	(for review: (Santamaria-Kisiel et al. 2006))
	In breast cancer, high level of S100A11 mRNA has been associated with poor prognosis.	(McKiernan et al. 2011)
	Knocked down of S100A11 expression in lung adenocarcinoma cells A549 and LTEP-a-2, was shows to significantly inhibited cell proliferation *in vitro* and *in vivo.*	(Hao et al. 2012)
	High expression of S100A11 has been demonstrated equally to lead to an increase in cell protrusions and pseudopodia.	(Sakaguchi et al. 2000)
	Depletion of S100A6 level in endothelial cells leads to an arrest in G2/M phase as well a decrease in cyclin-dependent kinase 1 (CDK1), cyclin A1 (CCNA1) and cyclin B1 (CCNB1) which are essential for cell-progression. This might be due be due to their interaction with HSP70/90 and p53.	(Bao et al. 2012)

(*continued*)

TABLE 2. (CONTINUED)

Protein	*Function*	*Publication*
NPM1	In cancer, its role involved the stability and the regulation of p53 and ARF activity.	(for review: (Grisendi et al. 2006, Yung 2007))
	In MDA-MB-231, plakoglobin may regulate the shuttles of NPM1 rapidly between the nucleus and cytoplasm and change its function from oncogenic to tumor suppression as it has been shown that increased of plakoglobin increased the level of nucleolar NPM while decreasing its cytoplasmic pools and that this is concurrent to a decrease in cell proliferation, migration and invasion *in vivo*.	(Lam et al. 2012)
LGALS1	In breast, suggested as a possible diagnostic marker for metastatic breast cancer as greater expression was reported in the highly metastatic cell line MDA-MB-231 compare to less metastatic one.	(Lahm et al. 2001, Imai et al. 2008)
	Well-documented role in cell proliferation and apoptosis in activated human T cells and T leukemia cell lines.	(for review: (Wells et al. 1999, Scott et al. 2004, Hsu et al. 2006)
	A β-galactoside binding protein (β-GBP) (human homologue of LGALS1 gene) induce a promptly arrested of the cell cycle during transition from S phase to G2 leading to apoptotic death in three human mammary cell lines differing in their oncogenic potential, oestrogen receptor expression and expression of the EGF receptor family.	
HSPB1 and HSPA5	Mediated resistance to apoptosis through stabilization of p53.	(Garrido et al. 2006, Li et al. 2012)
YWHAZ	Most significantly increased proteins in breast ductal carcinoma as compared to the corresponding normal tissues.	(Zang et al. 2004)
	Depletion results in an inhibition of the anchorage-independent growth which cause increased apoptosis via the mitochondrial pathway	(Li et al. 2008)
NME1-NME2 (also known as NM23B or NM23-H2)	Overexpression induced apoptosis and depletion led to the increase of a member of the Bcl-2 family (Bcl2L10) a central regulator of the intrinsic apoptosis pathway.	(Kang et al. 2007)
	In MDA-MB-231 cell, its overexpression suppresses motility, invasiveness and anchorage independent growth.	(McDermott et al. 2008)
	Co-expression of the Lbc proto-oncogene product and NM23-H2 inhibit RhoA activation which leads to a decrease in RhoA-GTP and suppression of stress fibers formation.	(Miyamoto et al. 2009)
CFL1	Active dephosphorylated form of cofilin translocate to the mitochondrial membrane inducing the release of cytochrome c and apoptosis.	(Chua et al. 2003, Klamt et al. 2009)
STMN1	Induction of wild type p53 activated p21 and EGR-1 leading to a decreased in stathmin expression.	For review: (Belletti et al. 2011)
	Both stathmin overexpression and downregulation cause abnormalities in progression of M phase.	
	Stathmin depletion results in cell cycle arrest and induction of apoptosis in *in vitro* models including breast cancer cell lines.	
	$p27^{Kip1}$ binding to stathmin inhibits ECM-driven cell migration.	

(*continued*)

Table 2. (Continued)

Protein	*Function*	*Publication*
FLN	Contribute to the initiation of cell migration and possibly compensate for each other in an event of single knockdown.	(Baldassarre et al. 2009)
	Down-regulation of FLNA has been associates with an increase of human breast cancer cell motility and invasion possibly by facilitating focal adhesion and turnover	(Xu et al. 2010)
LAPS1	Repression of LASP1 expression by siRNA arrested cells in G2/M phase of cell cycle and significantly inhibit migration	(Grunewald et al. 2006)

probably because of its relatively lower abundance compared to the other proteins. Further studies will be necessary to investigate the equilibrium between CFL1 and LIMK1.

CORO1B, which is known to regulate the recycling of actin at the leading edge of migrating cells through modification of the level of activated CFL1 has also been identified as differentially expressed. Depleting CORO1B increases the phospho-CFL1 level altering lamellipodial dynamics and actin filament architecture. This effect results from the simultaneous interaction with the Arp2/3 complex, which inhibits actin nucleation, and the recruitment of the phosphatase Slingshot 1L (SSH1L), which in turn enhances cofilin dephosphorylation and its activation (Cai et al., 2007a, 2007b). Therefore, the increased CORO1B expression observed in this study would further increase the levels of activated CFL1 and contribute to the destabilization of the equilibrium between CFL1 and LIMK1 necessary for cell motility.

Furthermore, a new link between the ROCKs signaling pathway, which regulates cell adhesion, migration, invasion, cytokinesis, apoptosis, and oncogenic transformation (Riento et al., 2003), and CORO1B has been identified. Indeed, it has been noted that CORO1B is a negative regulator of ROCK signaling (Rana et al., 2012). Their results established that the breast tumor-promoting factor NRG-1 controlled the interaction between ROCK2 and CORO1B. Additionally, they reported that an increased expression of CORO1B decreases ROCK signaling through the phosphatase SSH1L, reducing MYPT-1 and myosin light chain (MLC) phosphorylation that is important in tumor cell motility.

We also observed an increase in PFN2 and ARHGDIB expression. A recent report (Mouneimne et al., 2012) showed a differential effect of PFN1 and PFN2 on cell protrusion and motility and suggested that PFN2 promotes the activity of the Ena/VASP protein, EVL, which suppresses protrusive activity and cell motility. However, the authors also suggested that correlation between PFN2 expression and patient outcome might be phenotype dependent. A biphasic pattern of expression, along with breast cancer progression, was also observed for ARHGDIB. This recent study reported an increased expression in hyperplasia and benign tissues, followed by a decrease in higher grades of malignant cancers and metastatic lymph nodes (Rivera Rosado et al., 2011). This suggested that ARHGDIB might play dual roles in regulation of breast cancer cell functions depending on the genetic background associated with disease stages such as the status of Rho GTPases and other regulatory proteins.

Conclusion

Our study provides molecular evidence that PFN1 modulates the activity of proteins involved in cell proliferation, cell death, and motility, which are all critical steps in cancer development and progression. Moreover, this study strongly suggests that protein silencing or overexpression might trigger significant molecular changes that cannot be assessed by analyzing only the expression status of a few proteins and extrapolating their influence on the phenotype. It is more likely that the activity and balance between the expressions of many proteins will determine the output of the signaling pathway and therefore the phenotype of the cell. Much is still to be understood regarding the molecular pathways involving PFN1 in cancer development/progression before we can properly and effectively design targeted approaches to cancer therapy.

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Author Disclosure Statement

The authors declare that there are no conflicting financial interests.

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