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Proteome changes induced by overexpression of the p75 neurotrophin receptor ($p75^{NTR}$) in breast cancer cells

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ABSTRACT In breast cancer cells, the neurotrophin receptor $p75^{NTR}$ acts as a prosurvival factor able to stimulate resistance to apoptosis, but its mechanism of action remains incompletely defined. In this study, we investigated the global proteome modification induced by $p75^{NTR}$ overexpression in breast cancer cells treated by the pro-apoptotic agent tumor necrosis factor (TNF)-related-apoptosis-inducing-ligand (TRAIL). $p75^{NTR}$ was stably overexpressed in the MCF-7 breast cancer cells and the impact of a treatment by TRAIL was investigated in wild type *vs.* $p75^{NTR}$ overexpressing cells. Proteins were separated in two-dimensional electrophoresis, and regulated spots were detected by computer assisted analysis before identification by MALDI-TOF/TOF mass spectrometry. In the absence of TRAIL treatment, $p75^{NTR}$ did not induce any change in the proteome of breast cancer cells. In contrast, after treatment with TRAIL, fragments of cytokeratin-8, -18 and -19, as well as full length cytokeratin-18, were up-regulated by $p75^{NTR}$ overexpression. Of note, spectrin alpha-chain and the ribosomal protein RPLP0 were induced by TRAIL, independently of $p75^{NTR}$ level. Interestingly, the well known stress-induced protein HSP-27 was less abundant when $p75^{NTR}$ was overexpressed, indicating that $p75^{NTR}$ overexpression reduced TRAIL induced cell stress. These data indicate that overexpression of $p75^{NTR}$ induces proteome modifications in breast cancer cells and provide information on how this receptor contributes in tumor cell resistance to apoptosis.

KEY WORDS: *breast cancer, neurotrophin receptor $p75^{NTR}$, TRAIL, proteomics*

Introduction

Aside from being a powerful tool to identify new potential biomarkers in oncology, proteomics is increasingly recognized as a method to decipher the molecular mechanisms underlying cancer cell growth and metastasis (Collins *et al.*, 2009). In this context, a promising area of investigation is the analysis of growth factor receptor impact in cancer cells (Kolch and Pitt, 2010), with a double objective: to understand the basic mechanisms of cancer initiation and progression, and to subsequently delineate new therapeutic targets. In breast cancer, some information about the activity of oncogenic receptor proteins has been obtained through proteomics (Hondermarck *et al.*, 2008). This was illustrated for instance with the tyrosine kinase receptors FGFR and TrkA (Com *et al.*, 2007, Vercoutter-Edouart *et al.*, 2000), and mass spectrometry profiling can provide useful data for patient stratification based on expression/activation of specific receptors and regulated proteins (Hochgrafe *et al.*, 2010). A particularly relevant aspect for clinical applications is the definition of cellular modifications induced by growth factor receptor overexpression in breast cancer. As shown in the case of the tyrosine kinase receptor HER2/neu, which is overexpressed in about 10-20% of breast tumors, a better understanding of the modifications induced by the overexpression is a prerequisite for the development of targeted therapies such as herceptin/trastuzumab (Brufsky, 2010). Interestingly, proteomics can be used to gain information, at the global proteome level, on the molecular

Abbreviations used in this paper: 5-FU, 5-Fluorouracil; BEX2, brain-expressed X-2; CK, cytokeratin; DEDD, death effector domain containing DNA bonding; HSP27, heat shock protein 27; MALDI-TOF/TOF, matrix-assisted laser desorption/ionisation-time of flight/time of flight; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NGF, nerve growth factor; NRAGE, neurotrophin receptor-interacting MAGE homolog; $p75^{NTR}$, p75 neurotrophin receptor; RPLP0, 60S acidic ribosomal protein P0; TNF, tumor necrosis factor; TRADD, TNF receptor-associated death domain; TRAIL, TNF-related-apoptosis-inducing-ligand; TRAF, TNF receptor-associated factor.

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and functional modifications accompanying growth factor receptor overexpression in breast cancer cells.

The p75 neurotrophin receptor ($p75^{NTR}$) is a transmembrane receptor and a member of the tumor necrosis factor (TNF) receptor superfamily which exerts diverse functions such as the stimulation of cell survival and differentiation during neuronal development (Schor, 2005). $p75^{NTR}$ has no catalytic activity but recruits specific protein partners that bind intracellular Chopper and death domains (Blochl and Blochl, 2007). For instance, it has been reported that the neurotrophin receptor-interacting MAGE homolog (NRAGE) binds the Chopper domain of $p75^{NTR}$ and the TNF receptor-associated factor 2 and 6 (TRAF2 and TRAF6) bind to the death domain (Blochl and Blochl, 2007). $p75^{NTR}$ is not only expressed in nervous tissues, but also in non-neuronal tissues and in several cancers such as thyroid carcinoma (Rocha *et al.*, 2006), melanoma (Truzzi *et al.*, 2008), bladder (Tabassum *et al.*, 2003), prostate (Quann *et al.*, 2007), stomach (Jin *et al.*, 2007) and liver (Passino *et al.*, 2007) cancers. $p75^{NTR}$ may have opposite functions according to tumor types. Hence, it has been described to exert a tumor-promoting activity by favoring survival and metastasis in brain, prostate cancer and melanomas (Marchetti *et al.*, 2004, Menter *et al.*, 1994, Quann *et al.*, 2007), while it has been proposed as a potential tumor suppressor in bladder (Tabassum *et al.*, 2003), stomach (Jin *et al.*, 2007) and liver (Passino *et al.*, 2007) cancers.

In breast cancer, we have previously shown that $p75^{NTR}$ is expressed in the majority of breast tumors (Descamps *et al.*, 2001a), and preferentially in basal-like breast carcinomas (Reis-Filho *et al.*, 2006). The stimulation of $p75^{NTR}$ leads to increased breast cancer cell survival and resistance to apoptosis, by promoting the activation of the transcriptional factors NF-κB (Descamps *et al.*, 2001b) and $p21^{waf1}$ (Verbeke *et al.*, 2010). Furthermore, the activation of NF-κB requires the binding of TNF receptor-associated death domain protein (TRADD) on the death domain of $p75^{NTR}$ (El Yazidi-Belkoura *et al.*, 2003) and the brain-expressed X-2 protein (BEX2), which binds the death domain of $p75^{NTR}$, plays a key role in the downstream NF-κB activation (Naderi *et al.*, 2007). Despite these data on initial signaling activated by $p75^{NTR}$ in breast cancer cells, there is no information on potential changes induced by $p75^{NTR}$ at the proteome level.

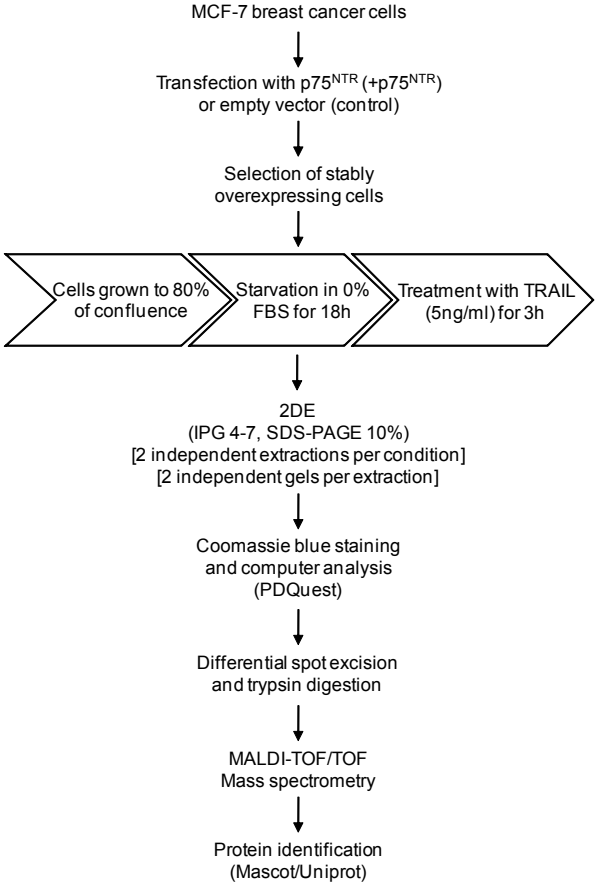


Fig. 1 (Left). Work-flow used *for studying the proteome changes induced by $p75^{NTR}$ overexpression in breast cancer cells.*

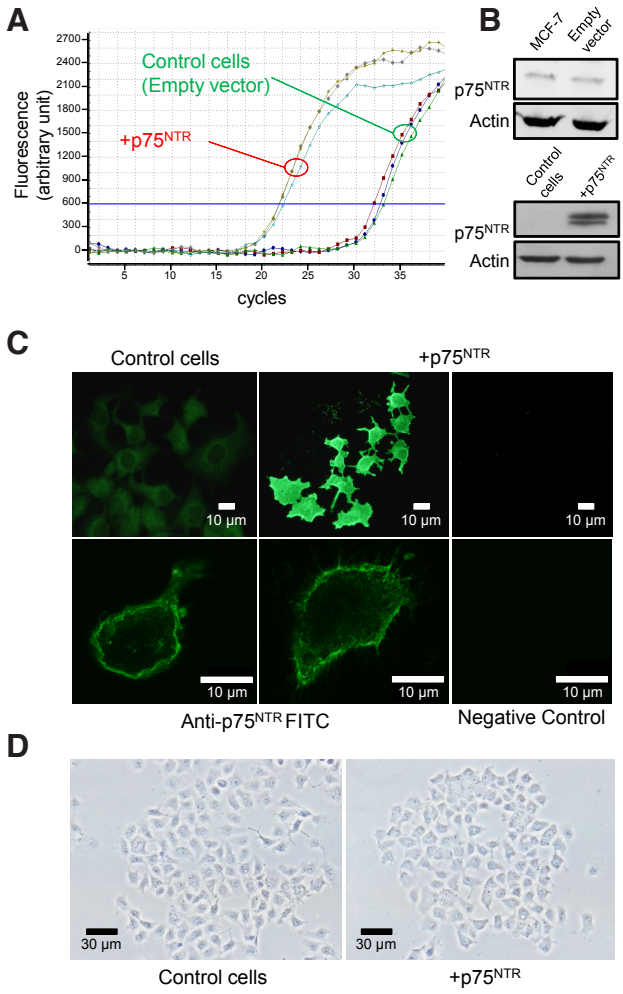


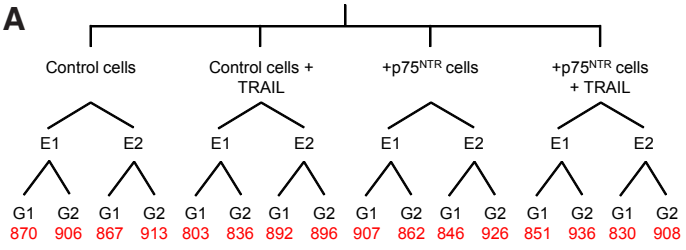
Fig. 2 (Right). Validation of $p75^{NTR}$ overexpression in MCF-7 breast cancer cells. (A) *Total RNA from MCF-7 breast cancer cells was isolated, reverse-transcribed and analyzed by Real Time-PCR. The amplification curves are shown for cells overexpressing $p75^{NTR}$ (+$p75^{NTR}$) and the empty vector transfected cells as control. For +$p75^{NTR}$ and control cells, the 3 different colors of the amplification curves represent triplicates.* **(B)** *Protein extracts of "empty vector" and +$p75^{NTR}$ were also analyzed by Western blot.* **(C)** *Immunocytochemistry (upper lane) and confocal microscopy (lower lane) analysis of $p75^{NTR}$ expression. For negative control, primary antibody was omitted.* **(D)** *Microscopy analysis of +$p75^{NTR}$ versus control cells.*

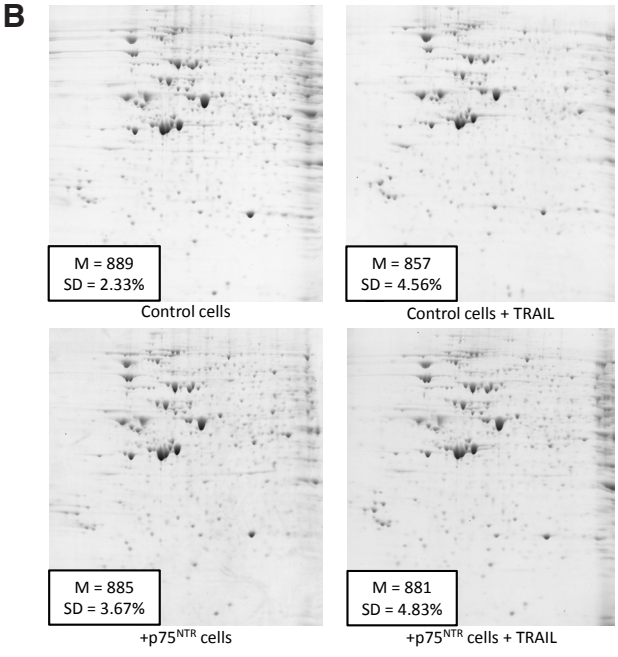
In the present study, we have used a proteomic approach to investigate proteome modifications induced by p75^{NTR} in breast cancer cells. Breast cancer cell lines have been described to express lower levels of p75^{NTR} than breast tumor biopsies (Descamps *et al.,* 2001a) and therefore we first stably overexpressed p75^{NTR} in the MCF-7 cell line to restore a p75^{NTR} level similar to *in vivo* expression. Overexpressing *versus* control cells were treated or not with the well known pro-apoptotic agent tumor necrosis factor (TNF)-related-apoptosis-inducing-ligand (TRAIL). We then separated proteins of total cell lysate using two-dimensional electrophoresis and regulated proteins were identified by MALDI-TOF/TOF analysis. Several proteins that exhibited a different expression level when p75^{NTR} was overexpressed were identified, pointing to potential mechanisms used by this receptor to promote cell survival and resistance to apoptosis.

Results

Establishment and validation of p75^{NTR} overexpressing cells

In order to investigate the impact of p75^{NTR} on the proteome of breast cancer cells, we overexpressed it in the MCF-7 cells. After cell transfection and selection, we first validated the p75^{NTR} overexpression at the mRNA level by Q-PCR (Fig. 2A). The amplification curves show an increase of 10 cycles between the +p75^{NTR} cells and control cells, indicating a 1000 fold increase of p75^{NTR} mRNA. The reference gene was found at the same level of expression between +p75^{NTR} and control cells (data not shown).





The overexpression of p75^{NTR} was also observed at the protein level by Western blot (Fig. 2B). A well defined classical double band (Pincheira *et al.*, 2009, Unsain *et al.*, 2008) was observed around 70-75 kDa in the lane corresponding to +p75^{NTR} cells. We also observed here that the insertion of the empty vector did not affect the amount of endogenous p75^{NTR}. Furthermore, immunocytochemistry confirmed a higher level of p75^{NTR} in the +p75^{NTR} cells (Fig. 2C) as indicated by comparison of green intensity. Confocal analysis indicated that the overexpressed p75^{NTR} was correctly addressed to the cell membrane. Of note, green intensity observed between control cells and +p75^{NTR} cells is not proportionate because of different exposure times. Finally, the overexpression of p75^{NTR} did not modify the morphological characteristics of MCF-7 cells (Fig. 2D).

Proteome changes in p75^{NTR} overexpressing cells

Proteins from both +p75^{NTR} and control cells, treated or not with the pro-apoptotic agent TRAIL, were separated by 2-DE before analysis with PDQuest software. As indicated, two different extractions were performed in order to avoid the biological bias, and two different gels were run for each extraction to eliminate the technical bias. So we obtained and analyzed by PDQuest a total of 16 gels perfectly reproducible (Fig. 3). A representative example of these gels is shown (Fig. 4). The spot distribution observed here is consistent and very similar to what was described for the MDA-MB-231 cells (Lottin *et al.*, 2002). Nearly 800 spots were obtained in the range of molecular weight 15-250 kDa and pI 4-7. Software analysis allowed the detection of 9 differential spots between the

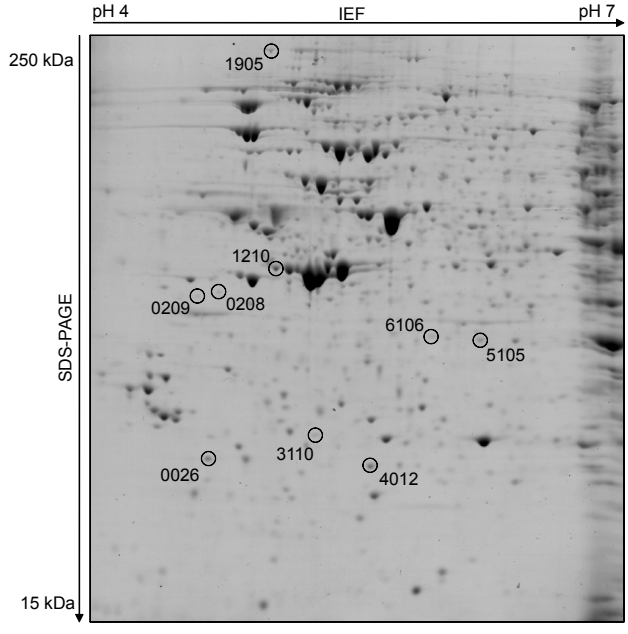


Fig. 3 (Left). Reproducibility of the 2DE strategy. (A) *The different technical replicates are listed with, for each of them, the number of detected spots by PDQuest (in red). E, extraction; G, gel.* **(B)** *Representative gel of each condition with, in the lower left corner, the mean spot number (M) for each condition and the standard deviation (SD) in percentage.*

Fig. 4 (Right). Representative 2DE gel obtained for +p75^{NTR} breast cancer cells. *This gel was obtained as described in Materials and Methods for +p75^{NTR} cells treated with TRAIL (5 ng/ml until 3 h), after a colloidal Coomassie Blue staining.*

different experimental conditions (Fig. 4). These variations were induced either by TRAIL treatment or by the p75^{NTR} overexpression under TRAIL treatment. Fig. 5 displays these variations with gel magnification for spots of interest. For each spot, there are four pictures corresponding to the four conditions: "empty vector" cells, +p75^{NTR} cells, "empty vector" cells treated with TRAIL and +p75^{NTR} cells treated with TRAIL. On the PDQuest generated histograms, we can see four columns per condition, corresponding to the four technical replicates of each culture condition (Fig. 5). Mass spectrometry analysis, performed on a MALDI-TOF/TOF apparatus, allowed the identification of the 9 spots (Table 1). Representative mass spectra obtained for heat shock protein 27 are shown in Fig. 6 and all spectra and information used for protein identification are in supplemental data. We observed three different cases: proteins induced by TRAIL treatment with no influence of p75^{NTR} (spectrin alpha-chain, the ribosomal protein RPLP0), proteins increased by p75^{NTR} under TRAIL stimulation (cytokeratin-8, -18, -19) and one protein less expressed with overexpression of p75^{NTR} under TRAIL treatment (HSP27).

Discussion

The effects of p75^{NTR} on both apoptosis and cell survival was classically described in neuronal-like models such as PC12 cells, but proteome changes induced by p75^{NTR} have never been reported and our present study provides a pioneer investigation in this area. The first point that we have observed is that the sole overexpression of p75^{NTR} did not induce any proteome modification by itself, whereas in case of a stress produced by the pro-apoptotic agent TRAIL, this overexpression induced several modifications. The requirement of an apoptotic stress for the induction of anti-apoptotic signaling from growth factor receptors has already been reported, and in breast cancer cells it has been shown with the activation of the serine/threonine kinase Akt following fibroblast growth factor receptor activation (Vandermoere *et al.*, 2005). Together, there are two different types of proteome changes that have been detected in this study: protein induced by TRAIL with no impact of p75^{NTR} overexpression and proteins regulated by p75^{NTR} overexpression under TRAIL treatment.

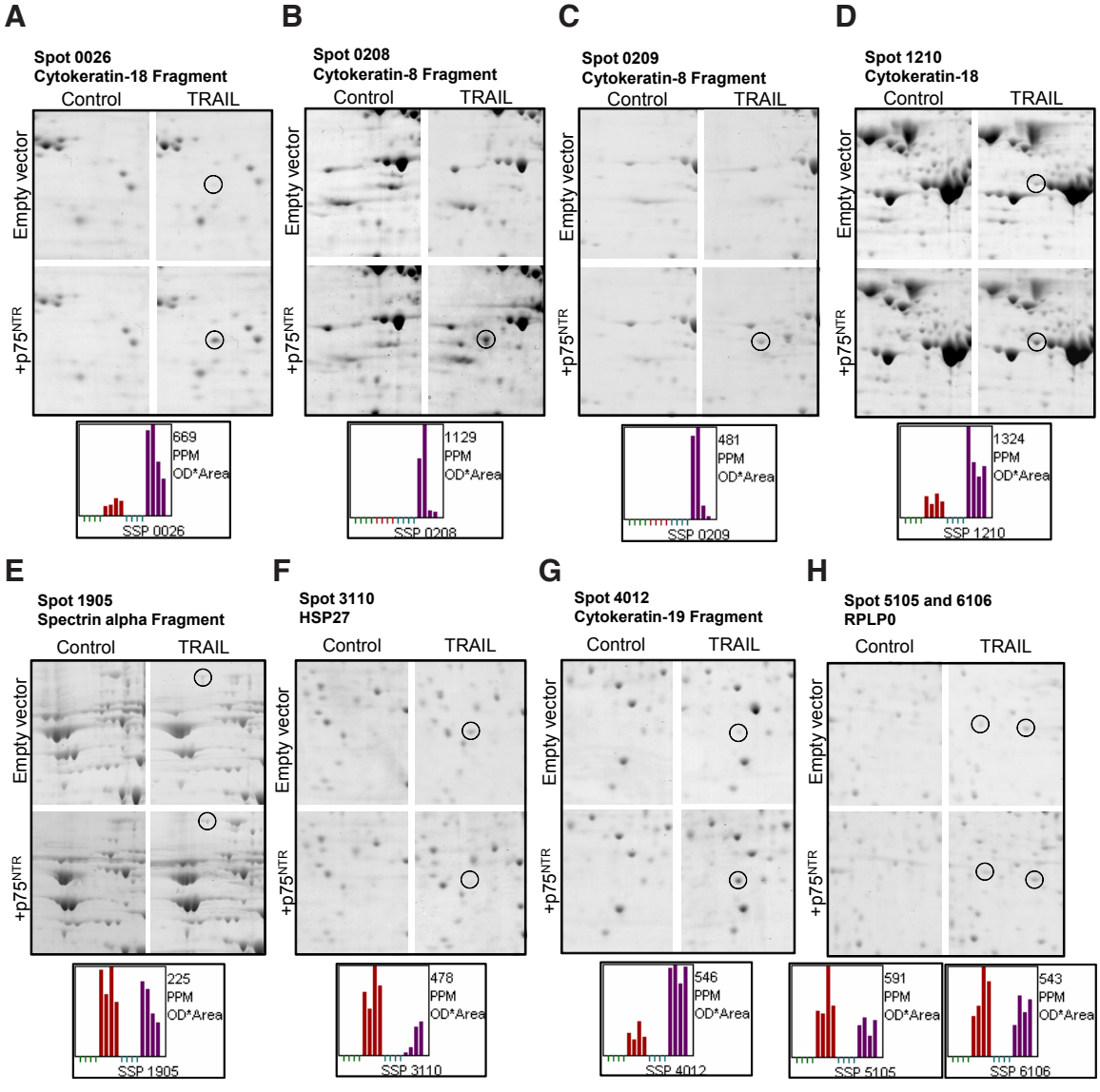


Fig. 5. Differential proteins in +p75^{NTR} breast cancer cells and relative abundance. *Gel quantitative analysis was performed with the PDQuest software.* **(A,D)** *cytokeratin-18.* **(B,C)** *cytokeratin-8.* **(E)** *spectrin alpha-chain 2.* **(F)** *heat shock protein 27.* **(G)** *cytokeratin-19.* **(H)** *60S acidic ribosomal protein P0. Histograms were generated automatically by the PDQuest software for each spot of the analysis. Green columns are for "empty vector" conditions, red ones correspond to "empty vector" cells treated by TRAIL, blue columns are for +p75^{NTR} conditions and finally purple columns represents +p75^{NTR} breast cancer cells treated by TRAIL. The bigger column represents the maximum spot intensity observed between the four replicates gels, and the other are expressed in function of this reference.*

Proteins induced by TRAIL with no impact of p75^{NTR} overexpression

Spectrin is a cytoskeletal protein that lines the intracellular side of the plasma membrane of many cell types, forming a scaffolding and playing an important role in maintenance of plasma membrane integrity and cytoskeletal structure by binding short actin filament. It has been demonstrated that it can be cleaved by caspase-3 to give rise to a 150 kDa fragment (Williams *et al.*, 2003). Our study shows that a TRAIL treatment is able to induce the cleavage of spectrin in MCF-7 cells. It is interesting to note that this breast cancer cell line lacks caspase-3; the spectrin cleavage can nevertheless be performed by caspase-7, which is structurally and functionally close to caspase 3 (Liang *et al.*, 2001). Furthermore, it is well described that both intrinsic and extrinsic pathways of apoptosis can lead to the cleavage of spectrin under a stress treatment (Kulkarni *et al.*, 2006). Spectrin fragment, which acts in tetramer complexes, also regulates the balance between calpains- and caspases-mediated cell death (Bignone and Baines, 2003). High concentration of alpha II spectrin fragment has been described to inhibit calpains but not caspases. However, the physiological significance of this calpain inhibition by spectrin fragment remains unclear. In our study, TRAIL induced spectrin cleavage, and this cleavage was not influenced by p75^{NTR} level. Spectrin cleavage is considered as an early marker of apoptosis and the fact that we observed the fragment under TRAIL treatment illustrates the initiation of apoptosis in breast cancer cells. Given that p75^{NTR} overexpression had no impact on spectrin cleavage in our experiments, we can postulate that the anti-apoptotic activity of p75^{NTR} involves other intermediates.

RPLP0 is a structural subunit of the ribosomal complex, localized in both cytoplasm and nucleus, which is involved in mRNA translation. In our study, we found that RPLP0 is increased upon TRAIL treatment in both control and p75^{NTR} overexpressing cells. The RPLP0 mRNA is usually used as a reference gene in PCR or RT-PCR and has been widely used in mRNA quantification of cancer samples, including breast cancer cells (Lyng *et al.*, 2008). However, it has been shown that RPLP0 is not the more stable reference gene in breast cancer cells (McNeill *et al.*, 2007). Analysis by RT-PCR of a set of breast tumor mRNA extracts has shown that other genes are statistically more stable than RPLP0. Also, RPLP0 mRNA was found to be up-regulated in ovarian and endometrial tumors compared to control tissues (Artero-Castro *et al.*, 2011). To our knowledge, we are the first to show an upregulation of RPLP0 under stress induced by a pro-apoptotic agent, reinforcing the idea that RPLP0 should be used with caution as a reference for gene expression in cancers.

Proteins regulated by p75^{NTR} overexpression under TRAIL treatment

In this study, we identified the three well described cytokeratin (-8, -18 and -19) that are known to be involved in cell resistance to apoptosis and survival pathways. Cytokeratin-8 fragment and cytokeratin-18 fragment are known to form an heterodimere complex under stress condition (Waseem *et al.*, 2004). Cytokeratin-8, -18 and -19 and their fragments were found co-localized in cytoplasmic inclusion in MCF-7 cells under TRAIL-induced apoptosis (MacFarlane *et al.*, 2000). This may be a component of the cell death program required to prevent the spread of cell damage before removal of the apoptotic cell by phagocytosis. Death effector domain containing DNA binding protein (DEDD), a highly conserved and ubiquitous

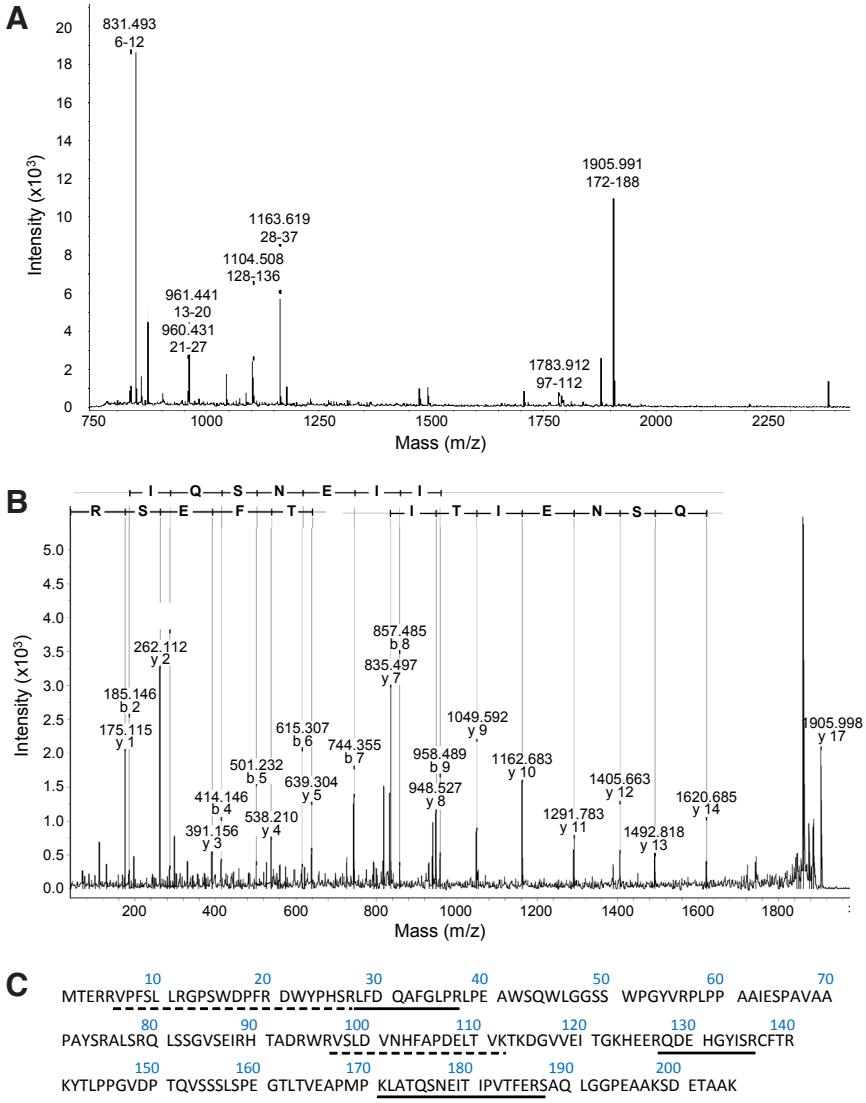


Fig. 6. MALDI-TOF and MS-MS spectra corresponding to HSP27. *Shown are MALDI-TOF* **(A)** *and MS-MS* **(B)** *spectra of spot number 3110 presented in Fig. 4. The spot was excised from the gel and trypsin-digested. After MALDI-TOF and database searching, 6 trypsic peptides matched with theoretical masses, leading to sequence coverage of 36%, provided a clear identification of HSP27 that was confirmed by sequencing of three peptides by MS-MS. The amino-acid sequence underlined once corresponds to the 6 trypsic peptides obtained in MS and the sequences underlined twice corresponded to the 3 peptides obtained in MS-MS* **(C)**.

protein, exists predominantly as mono- or diubiquitinated. Ubiquitinated DEDD interacts with the K8/18 intermediate filament, in order to facilitate their degradation during apoptosis (Lee *et al.*, 2002). It has also been demonstrated that the complexes K8/K18 can sequestrate caspases and so decrease apoptosis (Lin *et al.*, 2008); this complex also acts as a modulator of apoptotic pathways by controlling the trafficking of some receptors like FasR at the cell surface (Gilbert *et al.*, 2001). For cytokeratin-18, our study evidenced an increase of both, the full length protein and a fragment, when $p75^{NTR}$ was overexpressed. That could therefore participate in the anti-apoptotic function of this receptor. A comparative study demonstrated that cytokeratin-8 could be involved in drug resistance mechanisms, and more precisely to the 5-FU resistance of MCF-7 cells, with a potential relation with 14-3-3sigma (Zheng *et al.*, 2010). Concerning cytokeratin-19 it has been reported that this protein can be secreted in the extracellular matrix and have a pro-metastatic effect (Alix-Panabieres *et al.*, 2009). The fragment of cytokeratin-19 identified in this study is described as Cyfra21-1, which is used as a biomarker of carcinomas that can be assayed in blood and urine (Dittadi *et al.*, 1996). This cytokeratin fragment, induced by caspases, can be found in some inclusion in the MCF-7 cells in TRAIL-induced apoptosis as reported for cytokeratin-8 and -18 (MacFarlane *et al.*, 2000). Together, our study confirms that cytokeratin regulations are induced during breast cancer cell apoptosis and that $p75^{NTR}$ clearly interferes with this process. Precise analysis of cytokeratin distribution in breast epithelial cells was published in the early 90's with the demonstration that normal breast epithelial cells produce cytokeratins -5, -6, -7 and -17, whereas tumor cells produce mainly keratins -8, -18 and -19 (Trask *et al.*, 1990). This distribution was secondarily confirmed in tumor samples (Page *et al.*, 1999) and cytokeratin immunodetection is now eventually used at the clinical level to help discriminate benign from malignant cells on histopathological slides. It is interesting to note that $p75^{NTR}$ overexpression upregulates cytokeratins -8, -18 and -19 that are overexpressed in tumor cells, and although there are various potential regulators of cytokeratin expression, our data reveal that $p75^{NTR}$ can influence cytokeratin levels in breast cancer.

HSP27 is a cytoplasmic protein known to be involved in stress resistance and actin organization. Heat shock proteins such as HSP90, HSP70 and HSP27 are induced in response to a wide variety of physiological and environmental stress, including heat, reactive oxygen species or anticancer drugs, such as TRAIL, in order to protect cells from apoptosis (Zhuang *et al.*, 2010). HSP27 can activate protective pathways such as NF-κB in hepatocellular carcinoma cells (Guo *et al.*, 2009), and it is interesting to note that $p75^{NTR}$ upregulates NF-κB in breast cancer (El Yazidi-Belkoura *et al.*, 2003, Naderi *et al.*, 2007). In our case, $p75^{NTR}$ is overexpressed and so NF-κB might be activated without the necessity of HSP27 implication. HSP27 upregulation decreases the efficiency of Herceptin treatment by increasing Her2 protein stability in SK-BR-3 breast cancer cells (Kang *et al.*, 2008). It has also been reported that HSP27 can differentiate tolerogenic macrophages and may therefore support human breast cancer progression (Banerjee *et al.*, 2011). These macrophages lose the capacity to kill cancer cells but become extremely proangiogenic, inducing significant neovascularization, a process that is critically important for tumor development. In lung cancer, a down-regulation by siRNA of HSP27 sensitizes the cell to a TRAIL-induced apoptosis (Zhuang *et al.*, 2010). It has been shown that the expression of HSP27 is under the influence of the transcription factor Brn-3b in breast cancer cells (Fujita *et al.*, 2011). Interestingly, a recent report described that the transcriptional factor Brn-3b is regulated by the estrogen

TABLE 1

MASS SPECTROMETRY IDENTIFICATION OF THE PROTEINS REGULATED BY TRAIL AND $P75^{NTR}$ OVEREXPRESSION

Spot Number	Protein Name	Accession Number	MW (kDa)	PI	Score Mascot	Peptide Sequence (MS2)	Score MS2	Percentage of Coverage
0026	**CK18 Fragment**	P05783	20.2	5.0	241.8	KVKLEAEIATYRR KLEAEIATYRR RTVQSLEIDLDSMRN RAQYDELARK	70.3 62.7 61.1 47.6	19.5
0208	**CK8 Fragment**	P05787	45	5.0	316.7	RASLEAAIADAEQRG RLEGLTDEINFLRQ KLSELEAALQRA	121.5 110.7 86.3	49.9
0209	**CK8 Fragment**	P05787	45	5.0	291.8	RASLEAAIADAEQRG RLEGLTDEINFLRQ KLSELEAALQRA	105.7 99.9 87.9	54.9
1210	**CK18**	P05783	47.9	5.2	212.7	RAQIFANTVDNARI RIVLQIDNARL KVKLEAEIATYRR	100.7 76.9 35.0	45
1905	**Spectrin Fragment**	Q13813	150	5.2	400.7	KSADESGQALLAAGHYASDEVRE KALINADELASDVAGAEALLDRH KHQAFEAELSANQSRI KHQALQAEIAGHEPRI	153.1 134.6 71.3 43.3	15.9
3110	**HSP27**	P04792	22.8	5.0	224.1	KLATQSNEITIPVTFESRA RQDEHGYISRC RLFDQAFGLPRL	116.0 54.3 53.9	36.1
4012	**CK19 Fragment**	P08727	21.1	4.9	296.4	RQSSATSSFGGLGGGSVRF RIVLQIDNARL RVLDELTLART	164.1 74.8 57.6	36.8
5105	**RPLP0**	P05388	34.3	5.6	306.8	KTSFFQALGITTKI RAGAIAPCEVTVPAQNTGLGPEKT RVLALSVETDYTFPLAEKV	115.8 113.7 77.3	49.5
6106	**RPLP0**	P05388	34.3	5.6	348.0	RAGAIAPCEVTVPAQNTGLGPEKT RVLALSVETDYTFPLAEKV KTSFFQALGITTKI KEDLTEIRDMLLANKV	123.6 106.9 89.1 28.4	65.9

This table sums up the principal information obtained by mass spectrometry to identify the proteins. Spot number, protein name, accession number, molecular weight, PI, mascot score, peptide sequence, MS2 score for each peptide and the global percentage of coverage sequence are indicated for each protein.

receptor-α and nerve growth factor receptors, through the activation of the MAPK pathway (Ounzain *et al.*, 2011). In our case, the down-regulation of HSP27 protein is correlated with the overexpression of p75^{NTR} under a TRAIL treatment and therefore our results suggest that p75^{NTR} overexpression decreases cellular stress, subsequently diminishing the necessity for HSP27 synthesis.

Conclusion

Our study is the first to identify the global proteome modification induced by the overexpression of p75^{NTR} in breast cancer cells. We found that p75^{NTR} can act on the modulation of several proteins. As p75^{NTR} has been shown to be overexpressed in breast tumors (Descamps *et al.*, 2001a), the proteome changes that we observed in our study could also occur *in vivo* and therefore impact the proteome content of breast tumors. In addition, breast cancer cells produce neurotrophins and the changes in proteome induced by p75^{NTR} overexpression that we report here may provide indications on the mechanisms at play in the neurotrophin-induced tumor cell resistance to apoptosis. Finally, our study emphasizes that proteomics can be used to delineate the impact of membrane receptor overexpression in tumor cells, underlining the broad potential applications of this approach in oncology.

Materials and Methods

The work-flow used in this study is presented in Fig. 1.

Cell culture

MCF-7 breast cancer cells were obtained from the American Type Culture Collection (ATCC) and routinely grown as a monolayer. They were cultured in Minimal Essential Medium (MEM) (Gibco®) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 5 μg/ml insulin, 40 U/ml penicillin-streptomycin, 40 μg/ml gentamycin, at 37°C in a humidified atmosphere of 5% CO_2.

Establishment of p75^{NTR} overexpressing cells

The p75^{NTR} expression vector (pCTAP-p75^{NTR}) was prepared by inserting the human p75^{NTR} cDNA (p75^{NTR}: NM_002507.3) into the pCTAP-A vector (Stratagene) between the restricted enzyme sites EcoRI and XhoI. Cell transfections were done using the Cell Line Nucleofector Kit V (Amaxa) according to the manufacturer's instructions. Cells were selected with 600 μg/ml G418 (Invitrogen), the resulting G418 resistant cell populations were stored as frozen stocks and used for all the experiments within 20 passages. Expression of p75^{NTR} was not modified with passages in culture as revealed by western blot analysis.

RT-PCR

Total RNA from MCF-7 breast cancer cells were isolated with RNeasy kit (Qiagen) followed by a DNAse treatment. Reverse transcription was performed with 1 μg of RNAs, 0.5 μg of random hexamers, 200 units of Moloney murine leukemia virus reverse transcriptase for 10 min at 25°C, 50 min at 37°C and 15 min at 70°C in a final volume of 20 μl. Real time PCR amplifications were performed using a Quantitect SYBR®Green PCR kit with 2 μl of 1/10 cDNA and 500 nM of primers. The primers used were as follows: for p75^{NTR} 5'-ACGGCTACTACCAGGATGAG-3' and 5'-TGGCCTC-GTCGGAATACGTG-3'. The subsequent PCR conditions were carried out in the following manner: 95°C for 30 s, 58°C for 40 s and 72°C for 40 s. Data were analyzed using the MX4000 PCR system software (Stratagene) with the SYBRGreen option (including dissociation curves).

Western blot analysis

Protein extraction of subconfluent MCF-7 breast cancer cells was performed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% NP-40, 1 mM phenylmethanesulfonyl fluoride, 1 mM orthovanadate, 1% SDS, 1% protease inhibitors) 30 min at 4°C. Insoluble material was removed by centrifugation at 4°C for 20 min at 14,800 g after 6 min at 95°C. Total protein concentration was determined using BCA assay (Sigma). 30 μg of lysates were separated on 10% SDS-polyacrylamide gel, transferred onto a nitrocellulose membrane (0.45 μm) in transfer buffer (48 mM Tris-Base, 39 mM Glycine, 0.04% SDS, 20% (v/v) methanol) and blocked for 1h at room temperature in Tris-buffered saline with Tween-20 (TBS-T) (20 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.1% Tween-20) and 5% BSA (for actin) or skimmed milk (for p75^{NTR}). Incubation with primary antibodies (anti-actin: 1/10,000 and anti-p75^{NTR}: 1/2,000) was performed in blocking buffer overnight at 4°C. After washing with TBS-T, membranes were incubated with anti-rabbit IgG peroxidase antibody (Jackson laboratories, 1/10,000) for 1h at room temperature. The reaction was revealed using the kit West Pico chemiluminescent substrate (Pierce) and LAS-4000 mini from Fujifilm Life Science.

Immunofluorescence

p75^{NTR} overexpressing cells (+p75^{NTR}) and control MCF-7 cells were seeded onto type I collagen coated-glass coverslips and fixed with 4% paraformaldehyde solution (20 min, 4°C). Cells were PBS washed and permeabilized 20 min at room temperature with a PBS solution containing 0.05% saponin and 50 mM NH_4Cl. After 2 PBS washes, cells were blocked 30 min in PBS, 0.05% saponin and 2% BSA. Primary rabbit polyclonal antibody against p75^{NTR} (1/500) was incubated with cells overnight at 4°C. After 6 PBS washes, the secondary donkey anti-rabbit AlexaFluor 488 antibody (Invitrogen, 1/4000 in blocking solution) was incubated for 1h at 37°C. In control experiments, cells were not incubated with the primary antibody. After 6 washes, slides were mounted with Mowiol and cells were analyzed by ECLIPSE Ti-U Nikon fluorescent microscope and photographed.

Sample preparation for two-dimensional electrophoresis

Each culture condition had 2 independent extractions. MCF-7 breast cancer cells grown until 80% of confluence, were starved for 18h in MEM without FBS supplemented by 2 μg/ml of fibronectin and 30 μg/ml of transferrin, and then treated or not with the proapototic agent TRAIL for 3h at 5 ng/ml. After 2 washes with an isotonic buffer (250 mM sucrose, 1 mM EDTA, 10 mM Tris HCl pH 7.5), cells were harvested in 1 ml of isotonic buffer per 100 mm Petri dish. Cells were centrifuged 5 min at 1,000 rpm and the volume of cell pellet was determined. Then, 1 volume of isotonic buffer was used to resuspend the cells plus 8 volumes of 1.25X concentrated lysis buffer (final concentration: urea 7 M, thiourea 2 M, CHAPS 4%, DTT 40 mM, spermine base 20 mM). After 30 min of incubation at room temperature, the lysate was centrifuged 15 min at 10,000 rpm to precipitate nucleic acids. The protein supernatant was then carefully collected and the protein concentration estimated using a Bradford-type protein assay (Bio-Rad). Finally, 0.5% by volume of carrier ampholytes 3-10 (Bio-Lyte® 3/10, Bio-Rad) was added, and protein aliquots (450 μg per sample) were stored at -80°C until use.

Two dimensional electrophoresis

For each condition, 2 independent two dimensional electrophoresis (2DE) gels were performed for each extraction. IEF was carried out using 18 cm IPG Strips pH 4–7 (Bio-Rad). IPG strips were reswollen 2 h in 345 mL DeStreak (Hydroxyethyl disulfide, GE Healthcare Bio-Sciences) rehydration solution and 0.5% v/v carrier ampholytes 3–10 (Bio-Lyte® 3/10, Bio-Rad). The samples (450 μg) were then cup-loaded near the anode of the IPG strips and focused in a Protean IEF cell (Bio-Rad) at a temperature of 20°C. The IPG strips were initially conditioned for 30 min at 250V (rapid voltage ramping), linearly ramped to 1000 V (1 h) and maintained at 1000 V for 1 h more. Then the electric voltage was slowly increased to reach 10 000 V in 1 h and focused at this voltage to give a total of 45 kVh. After focusing, the strips were equilibrated for 2x15 min in 6M Urea, 30% w/v glycerol, 2% w/v SDS, 0.125M Tris, 0.1M HCl, containing either 50mM DTT (first equilibration step) or 150mM iodoacetamide (second equilibration step). The equilibrated IPG strips were sealed on top of 10% polyacrylamide gels (20 cm x 18.3 cm

x 1 mm) and SDS-PAGE was conducted at 25 V for 1 h, followed by 9W/gel until the dye front reached the bottom of the gel. After migration, the gels were washed 3x5 min with ddH_20 and stained using Bio-Safe™ Coomassie (Bio-Rad) according to manufacturer recommendations.

Spot detection and quantification

The gels were first scanned on GS800 scanner (Bio-Rad). Spot detection, quantitation, and analysis were performed using the PDQuest™ 2-D Analysis Software, version 8.0 (Bio-Rad). Gels were grouped by treatment condition and each group processed using the same analysis parameters. Spot detection parameters were selected using the Spot Detection Wizard of PDQuest. Since the different groups of gels varied in degree of streaking, the spot detection parameters were optimized and applied across all gels. Limits were put on the horizontal and vertical radius of a spot to minimize the effect of streaking and maximize the number of spot detected. Other selections used in all analyses included the floating ball method for background subtraction, a pixel size of 5 x 5 for spot detection, and the power-mean for noise filtering. We used the manual matching function instead of automated routine for gel matching to obtain the highest gel matching. Spot detection and quantitation were determined after background subtraction and noise filtering.

In-gel protein digestion

The protein spots were excised manually and washed 5 times for 6 min with 100 μl water. Then the gel spots were soaked in acetonitrile and dried under vacuum before rehydration with a reduction buffer (ammonium bicarbonate 100 mM, DTT 10 mM) 1 h at 56°C and 5 min at room temperature. After removing this buffer, gel spots were incubated with an alkylation buffer (ammonium bicarbonate 100 mM, iodoacetamide 55 mM) 45 min at room temperature and protected from light. Then, protein spots were washed with a 25 mM ammonium bicarbonate buffer followed by acetonitrile and finally dried under vacuum. The gel pieces were re-swollen in 100 μl of 25 mM ammonium bicarbonate with 125 ng of Trypsin Gold (Mass spectrometry grade, Promega) 1 h on ice. The protein spots were incubated 12 h at 37°C after addition of 30 μl of 25 mM ammonium bicarbonate.

Mass spectrometry analysis

The MALDI target plate (AnchorChip™, Bruker Daltonics) was covered with extracted peptides mixed up with α-cyano-4-hydroxy-cinnamic acid matrix (0.3 mg/ml in acetone:ethanol, 3:6 v/v). The molecular mass measurements were performed in automatic mode using FlexControl™ 3.3 software on an Ultraflex™ II TOF/TOF instrument and in the reflectron mode for MALDI-TOF peptide mass fingerprint (PMF) or LIFT mode for MALDI-TOF/TOF peptide fragmentation fingerprint (PFF). External calibration over a 1000-3200 mass range was performed using the $[M+H]^+$ monoisotopic ions of bradikinin 1-7, angiotensin I, angiotensin II, substance P, bombesin, and adrenocorticotropic hormone (clip 1–17 and clip 18–39) from a peptide calibration standard kit (Bruker Daltonics). Briefly, an accelerating voltage of 25 kV, a reflector voltage of 26.3 kV and a pulsed ion extraction of 160 ns were used to obtain the MS spectrum. Each spectrum was produced by accumulating data from 800 laser shots. A maximum of five precursor ions per sample were chosen for LIFT-TOF/TOF MS/MS analysis. Precursor ions were accelerated to 8 kV and selected in a timed ion gate. Metastable ions generated by laser-induced decomposition (LID) were further accelerated by 19 kV in the LIFT cell and their masses measured in reflectron mode. Peak lists were generated from MS and MS/MS spectra using Flexanalysis™ 3.3 software (Bruker Daltonics). Parameters and thresholds used for MS peak picking were: Baseline substraction, algorithm TopHat; smoothing, Savitzky-Golay (width 0.2 m/z & 1 cycle); peak detection algorithm = SNAP; Signal to noise threshold = 6; Quality factor threshold = 50. Parameters and thresholds used for MS/MS peak picking were: Baseline substraction, algorithm TopHat; Smoothing, Savitzky-Golay (width 0.15 m/z & 4 cycles); peak detection algorithm = SNAP; Signal to noise threshold = 3; Quality factor threshold = 30. Database searches, through Mascot 2.2.1 (Matrix Science Ltd, London, UK), using combined PMF and PFF datasets were performed against the UnitProt 2010_10 (number of entries: 20253 in Homo sapiens Taxonomy) database via ProteinScape 2.1 (Bruker Daltonics). A mass tolerance of 75 ppm and 1 missing cleavage site for PMF and MS/MS tolerance of 0.5 Da and 1 missing cleavage site for MS/MS search were allowed. Carbamidomethylation of cysteine and oxidation of methionine residues were also considered. Relevance of protein identities was judged according to the probability based Mowse score calculated with a *P*-value of 0.05 ($p< 0.05$).

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