

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/46094248>

# Proteomic Profile Regulated by the Anticancer Peptide CIGB-300 in Non-Small Cell Lung Cancer (NSCLC) Cells

ARTICLE in JOURNAL OF PROTEOME RESEARCH · OCTOBER 2010

Impact Factor: 4.25 · DOI: 10.1021/pr100728v · Source: PubMed

CITATIONS

9

READS

113

18 AUTHORS, INCLUDING:



**Yasser Perera**

Center for Genetic Engineering and Biotech...

30 PUBLICATIONS 280 CITATIONS

SEE PROFILE



**Lazaro Betancourt**

Center for Genetic Engineering and Biotech...

55 PUBLICATIONS 774 CITATIONS

SEE PROFILE



**Vladimir Besada**

Center for Genetic Engineering and Biotech...

102 PUBLICATIONS 1,048 CITATIONS

SEE PROFILE



**Gabriel Padron**

University of Barcelona

103 PUBLICATIONS 1,350 CITATIONS

SEE PROFILE

## Proteomic Profile Regulated by the Anticancer Peptide CIGB-300 in Non-Small Cell Lung Cancer (NSCLC) Cells

Arielis Rodríguez-Ulloa,<sup>\*,†</sup> Yassel Ramos,<sup>‡</sup> Jeovanis Gil,<sup>‡</sup> Yasser Perera,<sup>§</sup> Lila Castellanos-Serra,<sup>‡</sup> Yairret García,<sup>‡</sup> Lázaro Betancourt,<sup>‡</sup> Vladimir Besada,<sup>‡</sup> Luis J. González,<sup>‡</sup> Jorge Fernández-de-Cossio,<sup>†</sup> Aniel Sanchez,<sup>‡</sup> Joem M. Serrano,<sup>§</sup> Hernán Farina,<sup>||</sup> Daniel F. Alonso,<sup>||</sup> Boris E. Acevedo,<sup>§</sup> Gabriel Padrón,<sup>‡</sup> Alexis Musacchio,<sup>†</sup> and Silvio E. Perea<sup>§</sup>

Department of Bioinformatics, Department of Proteomics, and Laboratory of Molecular Oncology, Center for Genetic Engineering and Biotechnology, Havana, Cuba, and Laboratory of Molecular Oncology, National University of Quilmes, Buenos Aires, Argentina

Received July 14, 2010

CIGB-300 is a proapoptotic peptide-based drug that abrogates the CK2-mediated phosphorylation. This peptide has antineoplastic effect on lung cancer cells *in vitro* and *in vivo*. To understand the mechanisms involved on such anticancer activity, the NCI-H125 cell line proteomic profile after short-term incubation (45 min) with CIGB-300 was investigated. As determined by 2-DE or 2D-LC-MS/MS, 137 proteins changed their abundances more than 2-fold in response to the CIGB-300 treatment. The expression levels of proteins related to ribosome biogenesis, metastasis, cell survival and proliferation, apoptosis, and drug resistance were significantly modulated by the presence of CIGB-300. The protein translation process was the most affected (23% of the identified proteins). From the proteome analysis of the NCI-H125 cell line, novel potentialities for CIGB-300 as anticancer agent were evidenced.

**Keywords:** Comparative proteomic analysis • CIGB-300 • ribosome biogenesis • apoptosis • casein kinase 2 • nucleophosmin • lung cancer

### Introduction

Casein Kinase 2 (CK2) is a serine/threonine protein kinase overexpressed in a variety of solid and hematopoietic tumors.<sup>1–3</sup> In particular, lung cancer has been shown to exhibit high levels of CK2 activity and mRNA expression.<sup>4,5</sup> This kinase phosphorylates a wide range of substrates related to gene expression, cell survival, and apoptotic pathways; its increased expression in tumor cells promotes cellular proliferation and suppresses apoptosis.<sup>6</sup> Previous findings have suggested the potential of CK2 as an attractive anticancer target.<sup>7</sup> Therefore, to inhibit the CK2 activity, different emergent approaches have been assayed so far: (a) down-regulation of CK2 gene expression by antisense oligonucleotide,<sup>8</sup> (b) allosteric inhibition of the enzyme,<sup>9</sup> and (c) blocking of ATP binding site by small compounds.<sup>10</sup> CIGB-300 (formerly P15-Tat) is a novel hypothesis-driven peptide that abrogates the CK2-mediated phosphorylation by its ability to bind the substrate's phosphoacceptor domain instead of the enzyme per se.<sup>11</sup>

The antineoplastic effect of CIGB-300 has been well documented both *in vitro* and *in vivo* by using different cancer cell lines and mouse models.<sup>11–13</sup> In particular, lung cancer cells have been shown to be sensitive to CIGB-300 *in vitro* and *in vivo*. For instance, the cell viability on human non-small cell lung cancer (NSCLC) NCI-H125 and on murine lung transformed epithelial TC-1 cells were similarly reduced (IC<sub>50</sub> = 70  $\mu$ M),<sup>11,12</sup> and the caspase activation was detected after 30 min of CIGB-300 addition.<sup>11</sup> Likewise, CIGB-300 induced massive apoptosis after 10 min upon addition of 100  $\mu$ M CIGB-300 to small cell lung cancer (SCLC) NCI-H82 cells.<sup>13</sup> *In vivo*, CIGB-300 was able to elicit significant antitumor activity both in murine TC-1 and human NCI-H125 implanted tumors.<sup>11,13</sup> CIGB-300 has been already studied in the clinical ground, demonstrating safety, tolerability, and efficacy signs in women with cervical malignancies.<sup>14</sup>

Significant advances have been made in proving the CIGB-300 mechanism of action in lung cancer cells. Recently, the nucleolar chaperone nucleophosmin (B23/NPM) has been described as the major CK2-substrate targeted by CIGB-300.<sup>15</sup> Such interaction inhibited the CK2-mediated phosphorylation on B23/NPM in a dose-dependent manner and led to nucleolar disassemble with subsequent apoptosis.<sup>15</sup> Accumulated evidence suggests that CIGB-300 could ultimately induce apoptosis by impairing the ribosome biogenesis.<sup>15</sup> A detailed description of differential protein expression of cells treated with CIGB-300 is needed to support the proposed mechanism of action for such peptide.

\* To whom correspondence should be addressed. Department of Bioinformatics, Center for Genetic Engineering and Biotechnology, P.O. Box 6162, Havana CP10600, Cuba. Phone: 53-7271-6221. Fax: 53-7271-8070. E-mail: arielis.rodriguez@biocomp.cigb.edu.cu.

<sup>†</sup> Department of Bioinformatics, Center for Genetic Engineering and Biotechnology.

<sup>‡</sup> Department of Proteomics, Center for Genetic Engineering and Biotechnology.

<sup>§</sup> Laboratory of Molecular Oncology, Center for Genetic Engineering and Biotechnology.

<sup>||</sup> National University of Quilmes.

In this work, the proteome of the nuclear fraction from NCI-H125 cells modulated by CIGB-300 was described. Two complementary proteomic techniques, two-dimensional electrophoresis (2-DE) and two-dimensional liquid chromatography coupled to tandem mass spectrometry (2D-LC-MS/MS), were used. The analysis of differentially expressed proteins suggested that CIGB-300 impair the ribosome biogenesis on these cells. Data provided here not only indicate that this peptide regulates different relevant biological processes in cancer but also set the molecular basis for a synergistic interaction with anticancer drugs.

## Materials and Methods

**Peptide.** CIGB-300 is a cyclic peptide (CWMSPRHLGTC) fused to the cell penetrating peptide Tat (<sup>48</sup>GRKKRRQRRPPQ<sup>68</sup>).<sup>11</sup> The CIGB-300 peptide was synthesized on solid phase and purified by reverse phase high performance liquid chromatography (RP-HPLC) to >95% purity on an acetonitrile/H<sub>2</sub>O–trifluoroacetic acid gradient<sup>16</sup> and confirmed by electrospray mass spectrometry (Micromass, U.K.).

**Cell Culture and Treatments.** The non-small cell lung cancer (NSCLC) cell line NCI-H125, kindly provided by Dr. Eduardo Suarez (Center for Molecular Immunology, Havana, Cuba), was originally obtained from ATCC (Rockville, MD). The NCI-H125 cells were cultured in RPMI 1640 (Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS; PAA, Canada) and 100 µg/mL gentamicin (Sigma, USA), at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>.

For proteomic analysis, 20 × 10<sup>6</sup> tumor cells were seeded in appropriate vessels and incubated for 24 h. The cells were incubated with 200 µmol/L of CIGB-300 during 45 min, taking into account that CIGB-300 induces cell death features after 60 min of incubation. For Western blot analysis, cell cultures were treated with 40, 80, or 200 µmol/L of CIGB-300 for 45 min.

**Isolation of Nuclear Proteins.** NCI-H125 cells were collected by trypsinization. After washing with phosphate buffered saline (PBS), the cells were suspended in 500 µL of the isotonic buffer containing 10 mM HEPES-NaOH adjusted to pH 7.5, 0.25 M sucrose, 1 mM EGTA, and protease inhibitors.<sup>17</sup> For plasma membrane solubilization Triton X-100 at final concentration of 0.25% was added. After 15 min at 4 °C, the cell lysate was centrifuged for 15 min at 12,000 × g and 4 °C. Nuclear pellet was washed with 500 µL of a solution containing 10 mM HEPES-NaOH, pH 7.5, 0.34 M sucrose, 0.75 mM spermidine, 0.15 mM spermine, 1 mM EDTA, 0.1% Triton X-100, and protease inhibitors, centrifuged at 1000 × g for 5 min at 4 °C, and decanted. Nuclear proteins were then solubilized in a lysis solution containing 7 M urea, 2 M thiourea, 2% CHAPS, 0.5% ASB-14, 15% glycerol, 2% DTT, and protease inhibitors. The suspension was sonicated (2 × 1 min) and incubated 1 h at room temperature. Free cysteines were blocked with 4% acrylamide during 1 h at room temperature. Anfolytes range 3–10 were added at 2% final concentration and DNA was removed by centrifugation at 60,000 × g during 3 h. The supernatant containing solubilized nuclear proteins was conserved at –70 °C for 2-DE and 2D-LC-MS/MS analysis.

**Two-Dimensional Gel Electrophoresis (2-DE).** For both nuclear fractions, treated and nontreated with CIGB-300, three replicate analytical gels and two preparative gels were run. 2-DE was performed by combining IEF in IPG for the first dimension and SDS-PAGE for the second dimension. IPG strips (pI range 4–7, 18 cm) (Amersham Pharmacia, Sweden) were rehydrated overnight in 350 µL of lysis solution containing 2% of anfolytes

range 4–7 and approximately 400 µg of nuclear proteins for analytical gels or 700 µg for preparative gels. Electrofocusing was done at 20 °C in a Multiphor II equipment (Amersham Pharmacia, Sweden) at 50 kV-h under silicone oil. For the second dimension, IPG strips were incubated for 15 min in 3 mL of equilibration solution (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl, pH 8.8). The focalized proteins were transferred to 16.8% polyacrylamide-SDS gels (prepared 24 h before use) and were run at 16 mA for 30 min and then at 30 mA for 6 h. For analytical gels, silver staining was used for detection, while preparative gels were silver stained with a MS compatible procedure.<sup>18,19</sup>

**2-D Image Analysis.** Images from 2-D gels were processed and analyzed using the Melanie 5 software (GeneBio, Switzerland). Spots were automatically detected without previous contrast enhancement, followed by manual editing when necessary. For each class, gel reproducibility was evaluated by using the available tools (pair reports and scatter plot analysis). Heuristic clustering was used for automatic classification of the set of gels. The detection of changes in protein maps between the two classes was first done by automatic analysis and confirmed by visual inspection of the series of gels. Histograms for groups based on percent volume of the spots were recorded, using the central tendency as the mean value and the mean square deviation as the dispersion value. The spots displaying a change in their expression between the classes greater than the 2-fold factor (determined from the mean) were considered for protein identification by MS. For molecular mass and pI estimation according to the protein migration in 2D gel, several intense and well-resolved spots corresponding to different gel zones were selected as landmarks. Theoretical mass and pI were calculated from the sequence of the identified proteins using the Compute pI/M<sub>w</sub> ExPASy tool ([http://ca.expasy.org/tools/pi\\_tool.html](http://ca.expasy.org/tools/pi_tool.html)).

## In-Gel Trypsin Digestion and Protein Identification.

**In-Gel Digestion.** Replicate spots from two preparative gels were pooled and silver staining was removed with Farmer's reagent.<sup>20</sup> Spots were cut in 1–3 mm<sup>3</sup> pieces, dried with acetonitrile, rehydrated in a minimum volume of ammonium bicarbonate (25 mM) containing trypsin (12.5 ng/µL, Promega, USA), and digested at 37 °C for 18 h.

**MS Analysis.** Tryptic peptides from selected spots were obtained and processed as described by González et al.<sup>21</sup> ESI–MS spectra were acquired using a QToF-2 hybrid type orthogonal tandem mass spectrometer (Micromass, U.K.) fitted with a Z-spray nanoflow electrospray ion source operated at 80 °C with a drying gas flow at 50 L/h. Peptides eluted with 60% ACN in 1% formic acid from ZipTips (Millipore, USA) were loaded into the borosilicate nanoflow tips. Capillary and cone voltages of 900 and 35 V were applied, respectively. To acquire the ESI–MS/MS spectra, the first quadrupole was used to select the precursor ion within a window of approximately 2 Th. Argon was used in the collision chamber at 3 × 10<sup>–2</sup> Pa pressure and collision energies between 25 and 50 eV were set to fragment precursor ions. Data acquisition and processing were performed using MassLynx v3.5 (Micromass, U.K.).

**Protein Identification.** Queries were done to sequence databases (Swiss-Prot and NCBI) by MASCOT search engine software.<sup>22</sup> Monoisotopic masses of tryptic peptides were used to identify the proteins by peptide mass fingerprinting (PMF), and additionally the most intense signals observed in every spectrum were sequenced by collision induced dissociation and ESI–MS/MS. The MS/MS ion search option of the MASCOT

program was used for protein identification; no taxonomic restrictions were made. Cysteine propionamide was considered as fixed modification. Variable modifications including deamidation of glutamine and asparagine, and methionine sulfoxide were taken into account. Other restrictions to MASCOT were precursor and fragment ions  $m/z$  tolerance of 0.2 Da, enzyme digestion with trypsin, and up to one missed cleavage. Additionally, MS/MS spectra of proteins identified on the basis of sequencing of one or two peptides were manually interpreted, looking for four or more intense consecutive C-terminal  $y_n$  fragment ions and the complementary b fragment series.

**Proteomic Analysis by Two-Dimensional Liquid Chromatography Coupled to Tandem Mass Spectrometry (2D-LC-MS/MS). Trypsin Digestion.** The nuclear protein extracts, previously reduced and alkylated as described above, of control (400  $\mu$ g) and CIGB-300-treated NCI-H125 cells (400  $\mu$ g) were precipitated with acetone/TCA and independently dissolved in 300  $\mu$ L of buffer containing 50 mM ammonium bicarbonate, 2 M urea, pH 8.0. In the case of the treated sample, the buffer was prepared in  $^{18}\text{O}$ -labeled water. Proteins were digested by two additions of trypsin at an enzyme-to-substrate mass ratio of 1:100, for 16 h for each one, at 37 °C. To inhibit trypsin activity and to stop the proteolytic digestion, samples were reduced and alkylated using the previously described protocol. Both digests were mixed and desalted before cation exchange chromatography.

**2D-LC-MS/MS Analysis.** A combination of strong cation exchange and reversed phase chromatography steps were used to separate the complex mixture of peptides prior to tandem mass spectrometry analysis (MS/MS). Desalted peptides were separated into 14 fractions using a 0.8 mm  $\times$  50 mm SCX II column (Agilent, USA) and NaCl gradient (from 0 to 500 mM in 100 min). Collected fractions were lyophilized and further dissolved in 130  $\mu$ L of 1% formic acid. For each fraction three replicate aliquots of 40  $\mu$ L were separated in independent LC-MS/MS experiments by reverse phase chromatography. Data dependent acquisition MS/MS spectra of the eluted peptides were acquired in three  $m/z$  ranges (400–600, 590–900, or 890–2000). Peptides were separated and identified using an Agilent 1100 series nano LC system (Agilent, USA) coupled online to a QToF-2 hybrid mass spectrometer (Micromass, U.K.). The capillary and cone voltages of the electrospray ionization source were operated with 2.4 kV and 45 V, respectively. Samples were applied at 20  $\mu$ L/min to an LC-Packings PepMap C18 Precolumn Cartridge (5 mm  $\times$  300  $\mu$ m i.d.) for 10 min and then switched onto a C18 capillary column (15 cm  $\times$  75  $\mu$ m i.d., packed with 5  $\mu$ m, Zorbax 300 SB) using a mobile phase containing 0.1% formic acid, 5–45% acetonitrile gradient over 90 min at 300 nL/min flow rate. For protein identification, 1 s survey scans were acquired over the selected mass range and a maximum of 4 concurrent MS/MS acquisitions were triggered for 2+, 3+ charged precursors ions detected at an intensity above a 15 counts/s threshold. Each MS/MS acquisition was completed and switched back to MS mode when the total ion current fell below a 2 counts/s threshold or after a maximum of 6 s of continuous acquisition.

**Data Analysis.** Raw files were processed using MASCOT Distiller 2.3 software. Peptides were assigned to MS/MS spectra using MASCOT search engine against the IPI human database (version 3.61). The following search parameters were selected: 2 Da precursor mass tolerance, 0.8 Da MS/MS ions mass tolerance, tryptic search with up to one missed cleavage, oxidized methionine as variable modification, and cysteine

propionamide as fixed modification. To accept a peptide hit as positive we considered a false discovery rate (FDR) of 3% based on the target-decoy strategy.<sup>23</sup>

Quantification was achieved using ISOTOPICA software.<sup>24,25</sup> The software enables the relative peptide quantification based on the detailed analysis of the observed isotopic ion distribution. The software calculates the best ratio of the nonlabeled ( $^{16}\text{O}_2$ , control condition) and labeled ( $^{18}\text{O}_1 + ^{18}\text{O}_2$ , treated with CIGB-300) to obtain an *in silico* isotopic ion distribution that best match with the observed experimentally. To evaluate the quality of this adjustment, the software calculates the area difference between both isotopic ion distributions, and it is expressed as a coefficient named GOF (goodness of fitting). The relative quantifications of peptides with GOF below 0.6 were not considered for further analysis. All relative quantifications were additionally manually inspected.

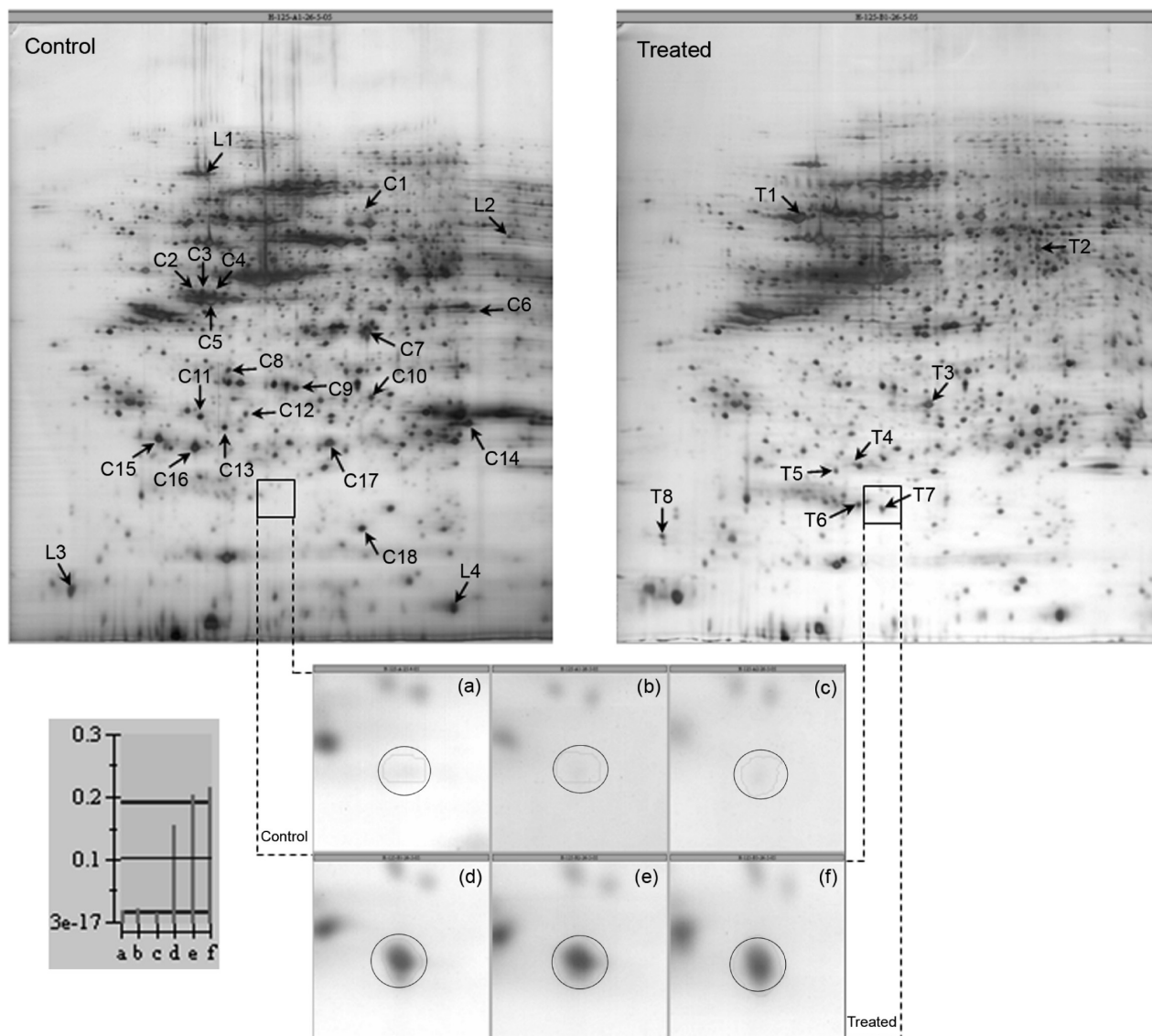
**Western Blot Analysis.** Treated cells were collected by standard trypsinization and subsequently lysed using RIPA buffer (BDH, Germany) according the supplier's recommendations. Then, the cellular lysate was cleared by centrifugation at 12,000 rpm during 15 min at 4 °C and quantified by the Bradford assay (Bio-Rad, USA). Protein samples were loaded into individual SDS-PAGE wells (5  $\mu$ g of total protein/well), electrophoretically resolved, and transferred to a nitrocellulose membrane. The membrane was blocked 4% skimmed milk in PBS, for 18–20 h at 4 °C. Then, 1  $\mu$ g/mL of either antinucleophosmin (B23/NPM) monoclonal antibody (Zymed, USA) or antiprolibitin (PHB) rabbit polyclonal antibody (Sigma, USA) was added and incubated for 1 h at 37 °C. After washing with PBS, the membrane was incubated with antimouse IgG (1:1000) or antirabbit IgG polyclonal (1:1000) secondary antibodies conjugated to horseradish peroxidase (Sigma, USA). Finally, the antigen–antibody reaction was developed using enhanced chemiluminescence (Amersham Life Science, USA), according to the instructions of the manufacturer.

**Bioinformatics Analysis.** Functional classification of identified proteins was based on the information annotated in the Gene Ontology (GO) database (<http://www.geneontology.org>). The analysis was performed using the DAVID functional annotation tool (<http://david.abcc.ncifcrf.gov/>).<sup>26,27</sup> Biological pathways associated with differentially expressed proteins were identified by using the functional enrichment tool ToppFun.<sup>28</sup> During this analysis, ToppFun retrieves information from databases such as BioCyc (<http://biocyc.org/>), Panther (<http://www.pantherdb.org/>), Reactome ([http://www.reactome.org/cgi-bin/frontpage?DB=gk\\_current](http://www.reactome.org/cgi-bin/frontpage?DB=gk_current)), Kyoto Encyclopedia of Genes and Genome (KEGG, <http://www.genome.jp/kegg/pathway.html>) and MSigDB (<http://www.broadinstitute.org/gsea/msigdb/>). The software computes Bonferroni correction to identified significant enriched pathways ( $p$ -values lower than 0.05), as statistical analysis. Proteins associated with the enriched pathways were visualized in a network context using the Cytoscape software (version 2.6.0).<sup>29</sup>

## Results and Discussion

CIGB-300 is a peptide-based drug that has just started to be explored at the clinical level. The first data concerning safety and efficacy signs in women with cervical malignancies have been already reported.<sup>14</sup> As lung cancer cells have shown to be highly sensitive toward CIGB-300 anticancer effect either *in vitro*<sup>11,12,15</sup> or *in vivo*,<sup>13</sup> these tumors become an attractive target for clinical testing of CIGB-300. The nuclear proteome regulated in the presence of 200  $\mu$ M of CIGB-300 was then





**Figure 1.** Representative bidimensional electrophoresis gels derived from control and CIGB-300 treated cells. Proteins were separated by combining IEF in IPG (pI range from 4 to 7) and SDS-PAGE (mass range from 12 to 120 kDa). Arrows indicate 26 spots significantly modulated (fold change >2) by CIGB-300 treatment. The magnified regions (a–c, control condition; d–f, treated cells) show the effect of CIGB-300 on the spot coded as T7 corresponding to nucleophosmin (B23/NPM) fragment. The histogram of three replicates for each conditions (a–f) is shown at the left of the magnified regions.

studied using the NCI-H125 lung cancer cell line as target cells. Studies on the permeability of CIGB-300 have indicated that such peptide dose represents the inhibitory concentration 90 ( $IC_{90}$ ) for CIGB-300 in NCI-H125 cells, which guarantees the full biological activity and better intracellular uptake in more than 90% of the cells (unpublished observations).

In a first approach, 2-DE in conjunction with quantitative image analysis and sequencing by mass spectrometry was used to investigate the CIGB-300 modulated protein profile. The Figure 1 shows the 2-D pattern for nuclear proteins of NCI-H125 cells cultured with CIGB-300 or medium alone during 45 min. Between 1600 and 2000 spots were detected by image analysis in the three replicate analytical gels of each condition. A total of 26 differentially expressed spots were subjected to trypsin digestion and mass spectrometry analysis. The 22 identified proteins that were up- or down-regulated more than 2-fold after treatment with CIGB-300 are shown in Table 1. The apparent relative molecular weight ( $M_r$ ) and the isoelectric

point (pI) determined from 2-D calibrated gels for the identified proteins were compared with their corresponding theoretical values. With the exception of LIM and SH3 domain protein 1 (LASP1) and B23/NPM protein, all of the identified proteins revealed good correlation between both values (Table 1).

In the case of LASP-1 protein, the  $M_r$  of 36.4 kDa and pI of 6.2 were detected in 2-D gels, similar to 34.5 kDa and pI 6.3 values, previously reported.<sup>30</sup> Two spots (T8 and T7), with  $M_r$  of 16.0 and 17.7 kDa, corresponding to B23/NPM fragments were identified on CIGB-300-treated cells (Figure 1). This finding suggested that CIGB-300 could promote partial B23/NPM degradation in the experimental condition, a relevant observation, as such a protein was reported to be a major target for CIGB-300.<sup>15</sup> Given the fact that B23/NPM stability is increased by its CK2-mediated phosphorylation<sup>31</sup> along with the ability of CIGB-300 to abrogate such a process,<sup>15</sup> B23/NPM degradation is expected to occur, and it represents a subrogated marker of CIGB-300 functionality. However, a decreased ex-

**Table 1.** Differentially Expressed Proteins in NCI-H125 Cells Treated with CIGB-300, Identified Using 2-DE and Mass Spectrometry Approach

spot no.	UniProt_ACC <sup>a</sup>	description	gene symbol <sup>b</sup>	% coverage (matching peptides) <sup>c</sup>	M <sub>r</sub> (kDa) <sup>d</sup>	pI <sup>d</sup>	FC <sup>e</sup>
Apoptosis							
T3	P35232	Prohibitin	PHB	51 (13)	29.8– <b>26.1</b>	5.6– <b>5.5</b>	2.5
T7	P06748	Nucleophosmin (fragment)	NPM1	36 (6)	32.6– <b>17.7</b>	4.6– <b>5.3</b>	11.0
T8	P06748	Nucleophosmin (fragment)	NPM1	23 (6)	32.6– <b>16.0</b>	4.6– <b>4.2</b>	8.0
C11	P52565	Rho GDP-dissociation inhibitor 1	ARHGDIA	30 (3)	23.1– <b>24.6</b>	5.0– <b>4.9</b>	–5.3
C15	P13693	Translationally controlled tumor protein	TCTP	65 (6)	19.6– <b>22.6</b>	4.8– <b>4.7</b>	–5.2
C16	Q04760	Lactoylglutathione lyase	GLO1	84 (17)	20.6– <b>21.9</b>	5.1– <b>4.9</b>	–4.5
C17	P09211	Glutathione S-transferase P	GSTP1	66 (14)	23.2– <b>22.3</b>	5.4– <b>5.5</b>	–3.1
C18	P00441	Superoxide dismutase	SOD1	44 (5)	15.8– <b>16.2</b>	5.7– <b>5.7</b>	–6.8
Cell proliferation							
T2	O60884	DnaJ homologue subfamily A member 2	DNAJA2	50 (12)	45.7– <b>44.5</b>	6.1– <b>6.0</b>	3.0
Glucose catabolic process							
C7	P07195	L-lactate dehydrogenase B chain	LDHB	35 (10)	36.5– <b>33.7</b>	5.7– <b>5.7</b>	–3.8
C14	P60174	Triosephosphate isomerase	TPI1	54 (11)	26.5– <b>23.9</b>	6.5– <b>6.2</b>	–2.9
RNA processing							
C1	P61978	Heterogeneous nuclear ribonucleoprotein K	HNRNPK	20 (6)	51.0– <b>53.0</b>	5.4– <b>5.7</b>	–3.2
C2	P07910	Heterogeneous nuclear ribonucleoproteins C1/C2	HNRNPC	23 (7)	33.5– <b>39.2</b>	4.9– <b>4.8</b>	–16.9
C3	P07910	Heterogeneous nuclear ribonucleoproteins C1/C2	HNRNPC	39 (10)	33.5– <b>39.2</b>	4.9– <b>4.9</b>	–13.9
C4	P07910	Heterogeneous nuclear ribonucleoproteins C1/C2	HNRNPC	18 (5)	33.5– <b>39.0</b>	4.9– <b>5.0</b>	–11.7
C5	P07910	Heterogeneous nuclear ribonucleoproteins C1/C2	HNRNPC	32 (12)	33.5– <b>37.8</b>	4.9– <b>5.0</b>	–3.0
Regulation of transcription							
T4	P45973	Chromobox protein homologue 5	CBX5	24 (4)	22.2– <b>20.7</b>	5.7– <b>5.1</b>	5.4
T6	Q13185	Chromobox protein homologue 3	CBX3	44 (5)	20.8– <b>18.2</b>	5.2– <b>5.1</b>	3.5
Protein ubiquitination							
C9	Q9UL46	Proteasome activator complex subunit 2	PSME2		27.2– <b>27.3</b>	5.4– <b>5.4</b>	–3.2
C10	Q06323	Proteasome activator complex subunit 1	PSME1	38 (9)	28.7– <b>26.4</b>	5.8– <b>5.8</b>	–2.3
Intracellular transport							
T5	Q9NP72	Ras-related protein Rab-18	RAB18	61 (10)	22.7– <b>20.3</b>	5.1– <b>5.0</b>	4.4
C6	Q14847	LIM and SH3 domain protein 1	LASP1		29.7– <b>36.4</b>	6.6– <b>6.2</b>	–3.0
Cytoskeleton organization							
T1	Q13509	Tubulin beta-3 chain	TUBB3	34 (10)	50.4– <b>50.9</b>	4.8– <b>4.9</b>	4.2
C8	Q15691	Microtubule-associated protein RP/EB family member 1	MAPRE1	37 (6)	29.9– <b>29.2</b>	5.0– <b>5.0</b>	–5.6
C12	P43487	Ran-specific GTPase-activating protein	RANBP1	11 (2)	23.2– <b>24.8</b>	5.2– <b>5.1</b>	–3.6
C13	P52566	Rho GDP-dissociation inhibitor 2	ARHGDIB	26 (3)	22.8– <b>23.6</b>	5.1– <b>5.0</b>	–5.1

<sup>a</sup> UniProtKB/Swiss-Prot entry accession number (<http://au.expasy.org>). <sup>b</sup> Recommended gene name (official gene symbol) as provided by UniProtKB/Swiss-Prot. <sup>c</sup> Protein sequence coverage and number of signals matching the theoretical mass of tryptic peptides (C6 and C9 spot were identified only by MS/MS sequencing). <sup>d</sup> Theoretical values were calculated using ExPASy Compute pI/M<sub>r</sub> tool ([http://ca.expasy.org/tools/pi\\_tool.html](http://ca.expasy.org/tools/pi_tool.html)) (signal peptides were not considered for M<sub>r</sub> and pI calculations). Bold-faced numbers indicates M<sub>r</sub> and pI experimental values. <sup>e</sup> Fold change (FC) of differentially regulated proteins [(–) down-regulated].

pression of intact B23/NPM protein (32.6 kDa) could not be determined by 2D gel image analysis; probably this protein spot was oversaturated, avoiding the detection of differential expression levels.

To increase the coverage of the proteome modulated by CIGB-300 in the nuclear fraction of NCI-H125 cells, a second comparative proteomic study using 2D-LC–MS/MS was performed. A total of 509 protein hits were identified (Supporting Information), of which 118 hits, corresponding to 127 proteins, were differentially expressed in CIGB-300-treated cells (Table 2). Interestingly, through this proteomic approach 12 of the 22 proteins identified by 2-DE analysis were also identified; the expression levels of these proteins were similarly modulated by CIGB-300 in both experiments (Table 2). Thus, such a finding serves as an internal cross-validation between both experimental approaches.

Changes in two differentially expressed proteins were validated through Western blot analysis of total cell extracts from NCI-H125 cells. CIGB-300 down-regulated the B23/NPM ex-

pression in a dose-dependent manner with the highest rate at 200  $\mu$ M, the selected dose for proteomic studies (Figure 2). Although fragments of lower apparent molecular weight from B23/NPM protein were not detected, the decreased expression of B23/NPM protein supported the detrimental effect of CIGB-300 on its stability. The increased expression of prohibitin (PHB) was also validated using the same experimental conditions. Similar to proteomic data, CIGB-300 up-regulated the PHB protein at 200  $\mu$ M of CIGB-300; no significant effect was observed at lower peptide concentrations (Figure 2).

For better understanding of putative cellular processes affected by CIGB-300 on this lung cancer cell line, the differentially expressed proteins were classified in terms of their biological functions using the information from the Gene Ontology database. A considerable number of the identified proteins were involved in translation (23%), cytoskeleton organization (18%), RNA processing (11%), and apoptosis (7%) (Figure 3). Pathway analysis demonstrated the enrichment of metabolic, cytoskeletal signaling, translation, and gene expres-

**Table 2.** Differentially Expressed Proteins in NCI-H125 Cells Treated with CIGB-300, Identified Using 2D-LC–MS/MS

protein hit	IPI_ACC <sup>a</sup>	UniProt_ACC <sup>a</sup>	description	gene symbol <sup>b</sup>	% coverage (peptides) <sup>c</sup>	FC <sup>d</sup>
Translation						
8	IPI00008529	P05387	60S acidic ribosomal protein P2	RPLP2	63.48 (5)	2
11	IPI00181728	Q8TDN6	Brix domain-containing protein 2	BXDC2	21.81 (3)	2.7
12	IPI00554723	P27635	60S ribosomal protein L10	RPL10	13.55 (2)	2.7
14	IPI00550021	P39023	60S ribosomal protein L3	RPL3	10.42 (3)	7.2
15	IPI00008530	P05388	60S acidic ribosomal protein P0	RPLP0	17.35 (2)	2.7
17	IPI00012772	P62917	60S ribosomal protein L8	RPL8	12.06 (2)	9.6
19	IPI00413324	P18621	60S ribosomal protein L17	RPL17	15.76 (3)	2.9
21	IPI00003918	P36578	60S ribosomal protein L4	RPL4	15.93 (4)	2.6
22	IPI00012795	Q13347	Eukaryotic translation initiation factor 3 subunit I	EIF3I	24 (3)	2
25	IPI00745266	Q9Y262	Eukaryotic translation initiation factor 3 subunit L	EIF3L	9.93 (3)	2.2
29	IPI00216587	P62241	40S ribosomal protein S8	RPS8	6.25 (1)	12.1
30	IPI00456758	P46776	60S ribosomal protein L27a	RPL27A	8.78 (1)	3.1
31	IPI00555744	P50914	60S ribosomal protein L14	RPL14	5.45 (1)	3.7
34	IPI00329389	Q02878	60S ribosomal protein L6	RPL6	5.21 (1)	9.2
43	IPI00215719	Q07020	60S ribosomal protein L18	RPL18	6.91 (1)	2.9
45	IPI00221093	P08708	40S ribosomal protein S17	RPS17	7.41 (1)	2.4
46	IPI00008708	O76021	Ribosomal L1 domain-containing protein 1	RSL1D1	4.08 (1)	5
4	IPI00025447	Q61Q15	Elongation factor 1-alpha	EEF1A1	33.33 (9)	-2
6	IPI00014424	Q05639	Elongation factor 1-alpha 2	EEF1A2	34.34 (8)	-2
15	IPI00008438	P46783	40S ribosomal protein S10	RPS10	32.73 (5)	-2.5
25	IPI00013415	P62081	40S ribosomal protein S7	RPS7	27.32 (3)	-2.5
36	IPI00783097	P41250	Glycyl-tRNA synthetase	GARS	8.12 (3)	-3.3
38	IPI00007074	P54577	Tyrosyl-tRNA synthetase, cytoplasmic	YARS	10.61 (3)	-2.5
41	IPI00816063	P30050	Isoform 2 of 60S ribosomal protein L12	RPL12	14.39 (1)	OFF
	IPI00024933		Isoform 1 of 60S ribosomal protein L12		11.52 (1)	
46	IPI00295400	P23381	Tryptophanyl-tRNA synthetase, cytoplasmic	WARS	4.03 (1)	-5
	IPI00412737		Tryptophanyl-tRNA synthetase isoform b		4.42 (1)	
50	IPI00329633	P26639	Threonyl-tRNA synthetase, cytoplasmic	TARS	8.85 (3)	-3.3
52	IPI00300074	Q9NSD9	Phenylalanyl-tRNA synthetase beta chain	FARSB	5.43 (2)	-2
53	IPI00031820	Q9Y285	Phenylalanyl-tRNA synthetase alpha chain	FARSA	4.72 (1)	-2
55	IPI00215780	P39019	40S ribosomal protein S19	RPS19	10.34 (2)	-3.3
69	IPI00032635	Q9BX40	Isoform 1 of Protein LSM14 homologue B	LSM14B	6.75 (1)	-3.3
	IPI00471933		Isoform 3 of Protein LSM14 homologue B		10.88 (1)	
71	IPI00375127		Similar to mKIAA0038 protein	EIF4H	7.82 (2)	-3.3
	IPI00014263	Q15056	Isoform Long of Eukaryotic translation initiation factor 4H		9.27 (2)	
Apoptosis						
35	IPI00000816	P62258	14-3-3 protein epsilon	YWHAE	16.08 (2)	2.6
17	IPI00025512	P04792	Heat shock protein beta-1	HSPB1	33.66 (3)	-3.3
21	IPI00218733	<b>P00441</b>	Superoxide dismutase [Cu-Zn]	SOD1	44.81 (2)	OFF
26	IPI00003815	<b>P52565</b>	Rho GDP-dissociation inhibitor 1	ARHGDI1A	24.02 (2)	-10
37	IPI00550900	<b>P13693</b>	Translationally controlled tumor protein	TCTP	15.12 (2)	-3.3
40	IPI00220766	<b>Q04760</b>	Lactoylglutathione lyase	GLO1	20.65 (2)	-5
64	IPI00026260	P22392	Isoform 1 of Nucleoside diphosphate kinase B	NME2	9.87 (1)	-2.5
	IPI00795292		Isoform 3 of Nucleoside diphosphate kinase B		5.62 (1)	
72	IPI00219757	<b>P09211</b>	Glutathione S-transferase P	GSTP1	9.52 (2)	-10
Cell proliferation						
16	IPI00441473	O14744	Protein arginine N-methyltransferase 5	PRMT5	15.70 (6)	2
32	IPI00032406	<b>O60884</b>	DnaJ homologue subfamily A member 2	DNAJA2	7.04 (1)	2.8
33	IPI00395865	Q16576	Histone-binding protein RBBP7	RBBP7	12 (2)	2.3
44	IPI00013953	Q9NNW5	WD repeat-containing protein 6	WDR6	1.25 (1)	4.2
20	IPI00020956	P51858	Hepatoma-derived growth factor	HDGF	18.33 (2)	-2
22	IPI00014197		Isoform 1 of Protein CDV3 homologue	CDV3	31.40 (2)	OFF
	IPI00798278	Q9UKY7	Isoform 2 of Protein CDV3 homologue		38.03 (2)	
30	IPI00307226	P01588	Erythropoietin	EPO	12.44 (2)	OFF
Glucose catabolic process						
1	IPI00219018	P04406	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	71.94 (16)	-10
2	IPI00479186	P14618	Isoform M2 of Pyruvate kinase isozymes M1/M2	PKM2	54.24 (17)	-10
	IPI00220644		Isoform M1 of Pyruvate kinase isozymes M1/M2		51.04 (15)	
3	IPI00465439	P04075	Fructose-bisphosphate aldolase A	ALDOA	43.96 (8)	-10
5	IPI00169383	P00558	Phosphoglycerate kinase 1	PGK1	35.49 (9)	-5
7	IPI00027497	P06744	Glucose-6-phosphate isomerase	GPI	29.03 (8)	-10
8	IPI00451401		Isoform 2 of Triosephosphate isomerase	TPI1	35.74 (6)	-10
	IPI00797270	<b>P60174</b>	Isoform 1 of Triosephosphate isomerase		47.39 (7)	
	IPI00607708		Isoform 2 of L-lactate dehydrogenase A chain	LDHA	33.43 (6)	-10
9	IPI00217966	P00338	L-lactate dehydrogenase		30.75 (6)	
16	IPI00219217	<b>P07195</b>	L-lactate dehydrogenase B chain	LDHB	14.37 (4)	-5
44	IPI00916111	P40925	Malate dehydrogenase	MDH1	9.38 (2)	-2.5
65	IPI00219525	P52209	6-phosphogluconate dehydrogenase, decarboxylating	PGD	3.93 (1)	-5
RNA processing						
23	IPI00026089	O75533	Splicing factor 3B subunit 1	SF3B1	3.53 (3)	2
24	IPI00217686	Q8IY81	Putative rRNA methyltransferase 3	FTSJ3	5.43 (2)	6.8
	IPI00218592		Isoform ASF-3 of Splicing factor, arginine/serine-rich 1	SFRS1	21.39 (2)	2
27	IPI00215884	Q07955	Isoform ASF-1 of Splicing factor, arginine/serine-rich 1		17.34 (2)	
	IPI00218591		Isoform ASF-2 of Splicing factor, arginine/serine-rich 1		14.73 (2)	

Table 2. Continued

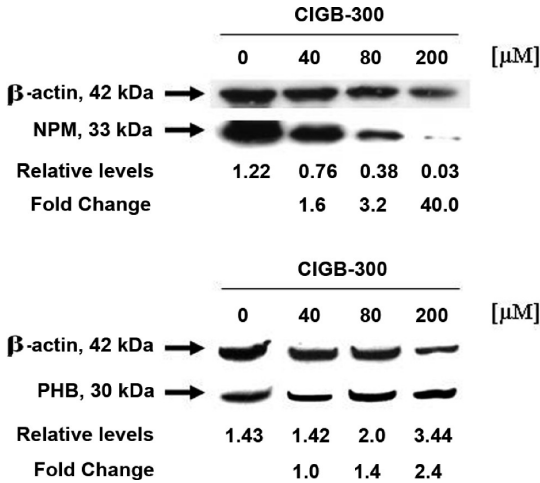
protein hit	IPI_ACC <sup>a</sup>	UniProt_ACC <sup>a</sup>	description	gene symbol <sup>b</sup>	% coverage (peptides) <sup>c</sup>	FC <sup>d</sup>
36	IPI00003377	Q16629	Isoform 1 of Splicing factor, arginine/serine-rich 7	SFRS7	8.82 (1)	3.1
	IPI00790699		Isoform 3 of U2-associated protein SR140		2.91 (2)	
40	IPI00143753	O15042	Isoform 1 of U2-associated protein SR140	SR140	2.82 (2)	2.3
	IPI00829908		Isoform 2 of U2-associated protein SR140		2.82 (2)	
	IPI00215965		Isoform A1-B of Heterogeneous nuclear ribonucleoprotein A1		24.73 (7)	
10	IPI00797148	P09651	Isoform 2 of Heterogeneous nuclear ribonucleoprotein A1	HNRNPA1	34.46 (7)	−3.3
	IPI00465365		Isoform A1-A of Heterogeneous nuclear ribonucleoprotein A1		28.75 (7)	
	IPI00644968	P0C7M2	Putative heterogeneous nuclear ribonucleoprotein A1-like protein 3	HNRPA1L3	28.75 (7)	
	IPI00414696		Isoform A2 of Heterogeneous nuclear ribonucleoproteins A2/B1		33.14 (7)	
11	IPI00396378	P22626	Isoform B1 of Heterogeneous nuclear ribonucleoproteins A2/B1	HNRNPA2B1	32.01 (7)	−2
	IPI00179964		Isoform 1 of Polypyrimidine tract-binding protein 1		15.82 (4)	
24	IPI00334175	P26599	Isoform 2 of Polypyrimidine tract-binding protein 1	PTBP1	15.27 (4)	−2.5
	IPI00412714		Isoform 4 of Plasminogen activator inhibitor 1 RNA-binding protein		11.89 (2)	
27	IPI00470497	Q8NC51	Isoform 2 of Plasminogen activator inhibitor 1 RNA-binding protein	SERBP1	3.98 (1)	−5
	IPI00470498		Isoform 3 of Plasminogen activator inhibitor 1 RNA-binding protein		11.70 (2)	
	IPI00410693		Isoform 1 of Plasminogen activator inhibitor 1 RNA-binding protein		3.92 (1)	
54	IPI00328840	Q86V81	THO complex 4	THOC4	9.85 (1)	−3.3
58	IPI00003438	O75937	DnaJ homologue subfamily C member 8	DNAJC8	11.46 (1)	−5
59	IPI00299000	Q9UQ80	Proliferation-associated protein 2G4	PA2G4	8.12 (2)	−2
Regulation of transcription						
20	IPI00513773	Q6NZI2	Isoform 2 of Polymerase I and transcript release factor	PTRF	14.33 (2)	2.4
	IPI00176903		Isoform 1 of Polymerase I and transcript release factor		11.03 (2)	
31	IPI00872855	Q01844	RNA-binding protein EWS	EWSR1	6.40 (2)	−2.5
	IPI00853059		Isoform 2 of Far upstream element-binding protein 1		9.17 (3)	
34	IPI00375441	Q96AE4	Isoform 1 of Far upstream element-binding protein 1	FUBP1	6.06 (2)	−5
42	IPI00234252	Q92922	SWI/SNF complex subunit SMARCC1	SMARCC1	3.17 (2)	−10
45	IPI00031812	P67809	Nuclease-sensitive element-binding protein 1	YBX1	9.26 (1)	−3.3
67	IPI00171798	O94776	Metastasis-associated protein MTA2	MTA2	4.64 (1)	OFF
DNA replication						
60	IPI00413611	P11387	DNA topoisomerase 1	TOP1	3.66 (2)	−2
70	IPI00292858	P19971	Thymidine phosphorylase	TYMP	3.11 (1)	−3.3
Protein ubiquitination						
39	IPI00384051	<b>Q9UL46</b>	Proteasome activator complex subunit 2	PSME2	12.85 (2)	−2.5
48	IPI00645078	P22314	Ubiquitin-like modifier-activating enzyme 1	UBA1	2.36 (2)	−2.5
51	IPI00395627	Q9HB71	Isoform 1 of Calcyclin-binding protein	CACYBP	9.21 (1)	−5
62	IPI00479722	<b>Q06323</b>	Proteasome activator complex subunit 1	PSME1	9.24 (1)	−3.3
63	IPI00029623	P60900	Proteasome subunit alpha type-6	PSMA6	7.32 (1)	−5
68	IPI00219913	P54578	Ubiquitin carboxyl-terminal hydrolase 14	USP14	5.87 (1)	−3.3
Proteolysis						
32	IPI00011229	P07339	Cathepsin D	CTSD	10.19 (3)	−3.3
61	IPI00026216	P55786	Puromycin-sensitive aminopeptidase	NPEPPS	3.16 (2)	−2
Intracellular transport						
26	IPI00001639	Q14974	Importin subunit beta-1	KPNB1	5.37 (3)	2
39	IPI00017376	Q15437	Protein transport protein Sec23B	SEC23B	3 (1)	2.2
	IPI00010154	P31150	Rab GDP dissociation inhibitor alpha	GDI1	4.47 (2)	
12	IPI00645255	Q5SX87	GDP dissociation inhibitor 2	GDI2	25.10 (5)	−5
	IPI00303882		Isoform B of Mannose-6-phosphate receptor-binding protein 1		20.74 (4)	−5
23	IPI00106668	O60664	Isoform A of Mannose-6-phosphate receptor-binding protein 1	M6PRBP1	29.88 (3)	−10
33	IPI00643041	P62826	GTP-binding nuclear protein Ran	RAN	14.81 (2)	−3.3
Cytoskeleton organization						
1	IPI00556012	Q5BKV1	Myosin, heavy chain 9, nonmuscle	MYH9	43.58 (9)	2
2	IPI00007752	P68371	Tubulin beta-2C chain	TUBB2C	55.06 (16)	2.4
3	IPI00909140	P07437	Tubulin beta chain	TUBB	54.96 (10)	2.8
	IPI00013475	Q13885	Tubulin beta-2A chain	TUBB2A		
4	IPI00031370	Q9BVA1	Tubulin beta-2B chain	TUBB2B	36.18 (11)	2.5
	IPI00013683	<b>Q13509</b>	Tubulin beta-3 chain	TUBB3	44.89 (12)	2.7
5	IPI00180675	Q71U36	Tubulin alpha-1A chain	TUBA1A	39.25 (13)	
	IPI00218343	Q9BQE3	Tubulin alpha-1C chain	TUBA1C	33.41 (12)	2.3
	IPI00930688	P68363	Tubulin alpha-1B chain	TUBA1B	42.35 (14)	
7	IPI00646779	Q9BUF5	Tubulin beta-6 chain	TUBB6	19.69 (6)	2.8
9	IPI00292496	Q3ZCM7	Tubulin beta-8 chain	TUBB8	10.59 (3)	2.3
	IPI00397526		Isoform 1 of Myosin-10		6.02 (8)	
10	IPI00790503	P35580	Isoform 3 of Myosin-10	MYH10	5.96 (8)	2.1
	IPI00479307		Isoform 2 of Myosin-10		5.97 (8)	
18	IPI00017454	Q9H853	Putative tubulin-like protein alpha-4B	TUBA4B	10.79 (2)	2
	IPI00220573	P19105	Myosin regulatory light chain 12A	MYL12A	10.53 (1)	
28	IPI00033494	O14950	Myosin regulatory light chain 12B	MYL12B	10.47 (1)	2
	IPI00220278	P24844	Myosin regulatory light polypeptide 9	MYL9	10.47 (1)	
41	IPI00335168	P60660	Myosin light polypeptide 6	MYL6	10.60 (1)	2.3
18	IPI00216691	P07737	Profilin-1	PFN1	55.71 (4)	−3.3
28	IPI00843975	P15311	Ezrin	EZR	7.17 (3)	−5



**Table 2.** Continued

protein hit	IPI_ACC <sup>a</sup>	UniProt_ACC <sup>a</sup>	description	gene symbol <sup>b</sup>	% coverage (peptides) <sup>c</sup>	FC <sup>d</sup>
35	IPI00419979	Q13177	Serine/threonine-protein kinase PAK 2	PAK2	5.90 (1)	-10
43	IPI00012011	P23528	Cofilin-1	CFL1	25.30 (2)	-10
49	IPI00028091	P61158	Actin-related protein 3	ACTR3	12.20 (2)	-3.3
57	IPI00003817	<b>P52566</b>	Rho GDP-dissociation inhibitor 2	ARHGDI2	12.44 (1)	OFF
Cell adhesion						
42	IPI00025861	P12830	Cadherin-1	CDH1	2.27 (1)	2.1
Others						
13	IPI00215637	O00571	ATP-dependent RNA helicase DDX3X	DDX3X	13.60 (5)	2.0
	IPI00293616	O15523	ATP-dependent RNA helicase DDX3Y	DDX3Y	10.30 (4)	
37	IPI00376379	Q7Z794	Keratin, type II cytoskeletal 1b	KRT77	4.67 (2)	2.4
38	IPI00021304	P35908	Keratin, type II cytoskeletal 2 epidermal	KRT2	4.19 (2)	2.6
13	IPI00003865	P11142	Isoform 1 of Heat shock cognate 71 kDa protein	HSPA8	14.86 (8)	-2
	IPI00792641	B3KSI4	Transketolase isoform 2	TKT	18.70 (5)	
14	IPI00643920	B4DE31	cDNA FLJ54957, highly similar to Transketolase		16.01 (5)	-5
19	IPI00746165	O75083	Isoform 1 of WD repeat-containing protein 1	WDR1	16.17 (4)	-5
29	IPI00289499	P31939	Bifunctional purine biosynthesis protein PURH	ATIC	10.30 (3)	-2.5
47	IPI00465431	P17931	Galectin-3	LGALS3	11.60 (2)	-2.5
56	IPI00299977	Q9NRX4	14 kDa phosphohistidine phosphatase	PHPT1	16.80 (1)	-2.0
	IPI00644196	P36952	Isoform 2 of Serpin B5		7.36 (1)	
66	IPI00783625		Isoform 1 of Serpin B5	SERPINB5	4.53 (1)	-2.5

<sup>a</sup> Accession numbers in IPI (International Protein Index) (<http://www.ebi.ac.uk/IPI>) and UniProtKB/Swiss-Prot databases (<http://au.expasy.org>) The UniProt accession numbers highlighted in bold indicate those proteins (12) that were also identified by 2-DE analysis. <sup>b</sup> Recommended gene name (official gene symbol) as provided by UniProtKB/Swiss-Prot. <sup>c</sup> Protein sequence coverage and number of identified peptides. <sup>d</sup> Fold change (FC) of differentially regulated proteins [(-) down-regulated].



**Figure 2.** Validation of nucleophosmin (B23/NPM) and prohibitin (PHB) expression levels by Western blot analysis. NCI-H125 cells were treated with different concentrations of CIGB-300 during 40 min. Total cell lysates were separated on a SDS-PAGE gel and immunoblotting was performed with anti-B23/NPM and anti-PHB antibodies.  $\beta$ -Actin expression was used as control.

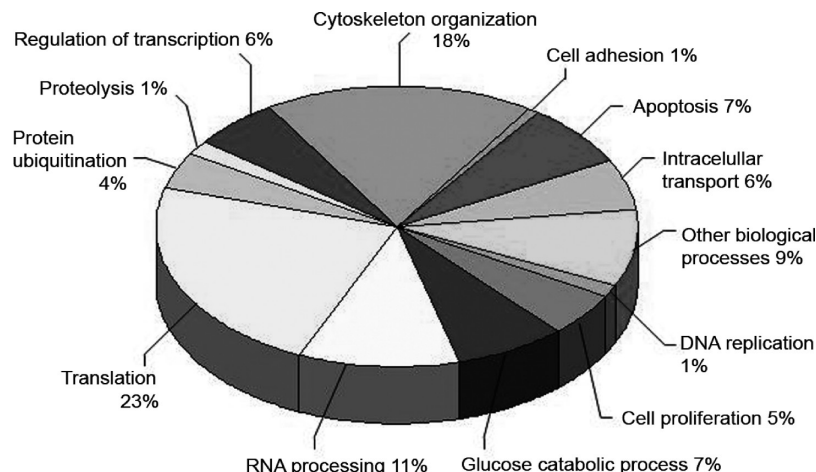
sion processes in the identified data set (Figure 4). Pathways are referred to according to annotations derived from different databases (Figure 4). Particularly, metabolic pathways are related to glycolysis and gluconeogenesis, whereas cytoskeletal proteins are mainly related to Gap junction and Rho GTPase signaling pathways.

Differentially expressed proteins related to the apoptosis process support the pro-apoptotic function of CIGB-300. Among these proteins, PHB is a negative regulator of cell proliferation that represses the E2F-mediated transcription pathway and activates the p53 transcriptional activity.<sup>32,33</sup> Thereby PHB has been described as a potential tumor suppressor protein. In this study, PHB expression was increased by CIGB-300 treatment (Table 1), such up-regulation was validated using Western blot analysis as referred above (Figure 2). Furthermore, a decrease of the translationally controlled

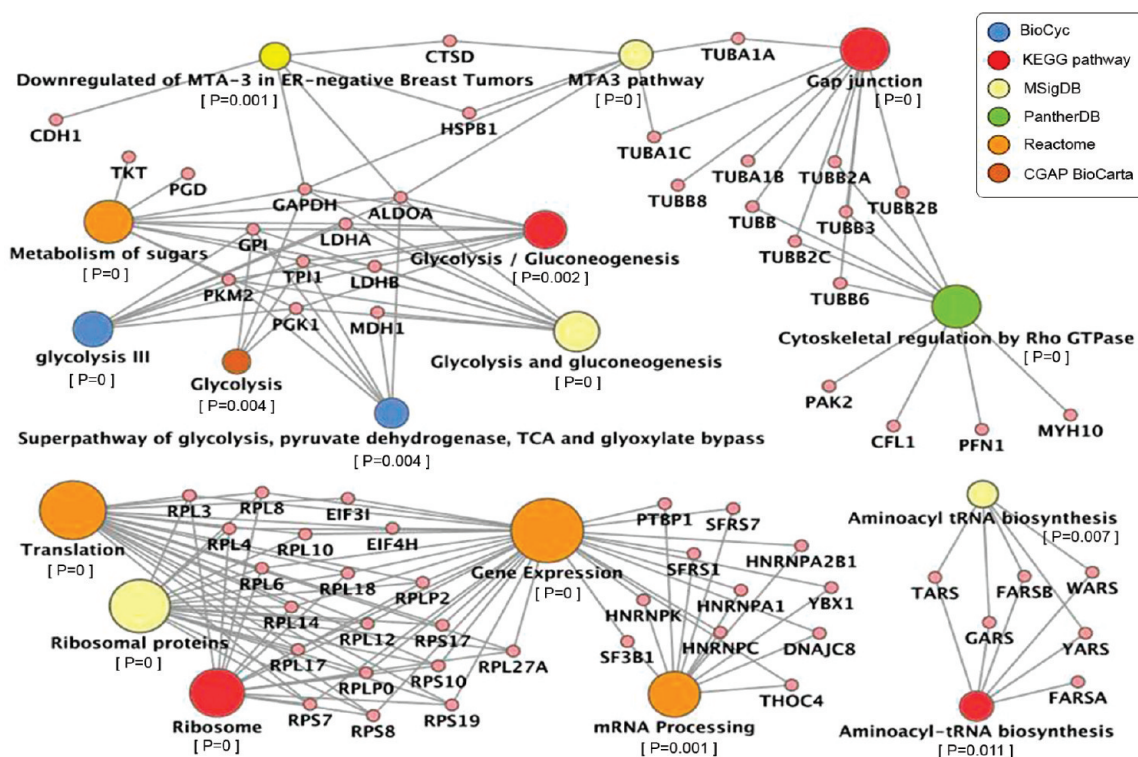
tumor protein (TCTP) level was observed on CIGB-300 treated cells as confirmed through the two proteomic approaches used in this study (Table 1 and Table 2). TCTP is an antiapoptotic protein;<sup>34</sup> previous studies have demonstrated that down-regulation of TCTP is related with suppression of tumor malignant phenotype.<sup>35</sup>

From a mechanistic view, the down-regulation of B23/NPM protein induced by CIGB-300 is of great relevance (Figure 2). This nucleolar protein promotes cell survival through the inhibition of distinct pro-apoptotic pathways including the inactivation of the tumors suppressors IRF1 and p53.<sup>36,37</sup> Besides, B23/NPM regulates the ribosome biogenesis pathway by several mechanisms including the processing of pre-rRNA molecule in the internal transcribed spacer (ITS2) sequence to form the 5.8S and 28S rRNA.<sup>38</sup> In addition, B23/NPM elicits chaperone activity preventing protein aggregation at the nucleolus during ribosome assembly,<sup>39</sup> and mediating the nucleocytoplasmic transport of both ribosomal subunits.<sup>40</sup>

Besides B23/NPM, two other proteins related to ribosome biogenesis were down-regulated 2-fold in the presence of CIGB-300: DNA topoisomerase I (TOP1) and proliferation-associated protein 2G4 (PA2G4) (Table 2). TOP1 regulates rRNA transcription.<sup>41</sup> This protein catalyzes the transient breaking and rejoining of a single strand of DNA; such enzymatic function of TOP1 has been demonstrated to be activated by CK2 phosphorylation.<sup>42</sup> PA2G4 regulates the intermediate and late steps of rRNA processing and also contributes to maturation of the 60S ribosomal subunit.<sup>43</sup> Furthermore, PA2G4 represses the inhibitory phosphorylation of eukaryotic initiation factor 2  $\alpha$  (eIF2 $\alpha$ ), exerting as a consequence a positive regulation on protein translation.<sup>44</sup> In line with these findings, ribosomal proteins as well as different aminoacyl-tRNA synthetases were modulated by CIGB-300 treatment (Table 2, Figure 4). Ribosomal proteins failing to assemble into ribosome subunits undergo proteosomal degradation.<sup>45</sup> However, a set of proteins related to ubiquitin dependent degradation was down-regulated on CIGB-300-treated cells (Table 2). Therefore, the expected decrease of proteasome activity would explain the nuclear



**Figure 3.** Distribution of the 137 proteins differentially expressed according to their biological functions.



**Figure 4.** Pathways related to identified proteins. Nodes represent both pathways and proteins, and edges represent functional associations between them. The color of pathway nodes indicates the original data source, and the size is proportional to the number of identified proteins in each pathway according to ToppFun results. P-value of each pathway is given in brackets.

accumulation of ribosomal proteins observed in the presence of CIGB-300. Altogether, the inhibitory effect of CIGB-300 on B23/NPM stability, along with modulation of TOP1, PA2G4 and ribosomal proteins constitute experimental evidence supporting that CIGB-300 indeed impairs the ribosome biogenesis process.

Proteins that regulate cytoskeleton assembly and stability were identified in this study (Table 2, Figure 4). Particularly, ezrin, a protein important for cellular adhesion and migration<sup>46</sup> that has been implicated in growth and metastasis of several human cancers,<sup>47–51</sup> was down-regulated on CIGB-300-treated cells. In addition, the 14 kDa phosphohistidine phosphatase protein (PHPT1) that regulates cell migration and invasion of lung cancer cells through the cytoskeletal reorganization pathway<sup>52</sup> was also down-regulated by CIGB-300 (Table 2). The

expression of Cadherin-1 (CDH1), a cell–cell adhesion glycoprotein considered as a negative regulator of cancer invasion,<sup>53,54</sup> was increased on CIGB-300-treated cells (Table 2). Therefore, the concomitant regulation of ezrin, PHPT1, and CDH1 suggests the inhibition of cellular motility in CIGB-300-treated cells.

In addition, other proteins related to metastasis were modulated on NCI-H125 cells by CIGB-300. Thymidine phosphorylase (TP) protein, which is an angiogenic factor that stimulates the chemotaxis of endothelial cells and also confers resistance to the hypoxia-induced apoptosis,<sup>55,56</sup> was down-regulated by CIGB-300 (Table 2). TP has been related to tumor invasion and metastasis in several cancers, such as cervical cancer and NSCLC.<sup>57–60</sup> The expression of Hepatoma-derived growth factor, a protein involved in anchorage-independent growth

and cell invasion of NSCLC,<sup>61</sup> was also decreased on CIGB-300-treated cells (Table 2). Furthermore, Glucose-6-phosphate isomerase, which functions as an extracellular cytokine stimulating cell motility,<sup>62</sup> was down-regulated by CIGB-300 (Table 2), while the Chromobox protein homologue 5, implicated in silencing metastasis-related genes,<sup>63</sup> was up-regulated on CIGB-300-treated cells (Table 1). Finally, the expression of Cathepsin D was decreased by CIGB-300 (Table 2). This protein regulates cell proliferation, invasion and metastasis.<sup>64,65</sup> Thus, the modulation of these proteins supports a potential antime-tastatic effect of CIGB-300. Experiments in our lab are evaluating this hypothesis.

Cancer cells use glycolysis, even in aerobic conditions, to produce ATP and intermediaries metabolites for anabolic reactions.<sup>66</sup> Eight glycolytic proteins were down-regulated by CIGB-300 treatment in this study (Tables 1 and 2, Figure 4). Besides, the enzyme 6-phosphogluconate dehydrogenase, which catalyzes the third step of the pentose phosphate pathway oxidative branch, was down-regulated 5-fold by CIGB-300. The antioxidant defense system is essential for cancer cell survival.<sup>67,68</sup> The expression of the enzyme superoxide dismutase, which catalyzes the reduction of superoxide to hydrogen peroxide, was decreased on CIGB-300-treated cells (Tables 1 and 2). These results support the inhibitory effect of CIGB-300 on the proliferation of tumor cells. As the exacerbation of the glycolytic pathway has been considered an alternative way of tumor cells to resist chemotherapy,<sup>69</sup> our findings suggest that CIGB-300 could add a benefit to the standard anticancer therapies. In line with this hypothesis, Glutathione S-transferase P and Lactoylglutathione lyase, two glutathione conjugation proteins related to chemotherapy resistance,<sup>70,71</sup> were down-regulated on CIGB-300-treated NCI-H125 cells (Table 2). Additionally, the down-regulation of the Nuclease-sensitive element-binding protein 1 (YB-1) on CIGB-300-treated cells (Table 2) is of great importance as this factor activates the expression of the P-glycoprotein 1 coding gene related to the multidrug-resistant phenotype.<sup>72,73</sup> Furthermore, YB-1 participates in the nucleotide excision repair pathway and plays a role in the cisplatin-DNA adducts repair.<sup>74</sup> This protein is overexpressed in cisplatin-resistant cells.<sup>75</sup> Besides the role of TP protein in metastasis, this protein, superoxide dismutase, and heat shock protein  $\beta$ -1 have been also related to cisplatin resistant phenotype on tumor cells.<sup>76–79</sup> These three proteins were down-regulated on CIGB-300-treated cells (Table 2). The findings observed in this study suggest that combination of CIGB-300 with some anticancer drugs may produce a synergistic interaction with increased overall antitumor effect.

## Conclusions

This study is the first proteomic analysis on cancer cells treated with the anticancer peptide CIGB-300. A total of 137 differentially expressed proteins were identified by two complementary proteomics approaches. CIGB-300 seems to promote the B23/NPM instability on NCI-H125 cells and the impairment of the ribosomal biogenesis and translation process. Such data are in line with previous findings demonstrating that CIGB-300 leads to apoptosis by blocking the CK2-mediated phosphorylation on B23/NPM with subsequent interference with the nucleolar assembly. Furthermore, other proteins implicated in apoptosis and cell proliferation were found modulated likely as a consequence from the above-mentioned effect of CIGB-300. These proteins could represent surrogate biomarkers of the CIGB-300 effect on tumor cells. Proteins related to glyco-

lysis, antioxidant defense, angiogenesis, and metastasis were modulated on CIGB-300-treated cells, giving important clues about potential anticancer effects of CIGB-300 other than the pro-apoptotic action. CIGB-300 decreases the expression level of proteins implicated in chemotherapy resistance. Thus, such findings could support the combination of CIGB-300 with standard chemotherapeutic drugs to improve the antitumoral response. Overall, the dissection of the CIGB-300 modulated proteome not only documented the pathways supporting the demonstrated anticancer effect of CIGB-300 in lung cancer cells but also uncovered other potentialities of this drug for cancer therapy.

**Acknowledgment.** This work was supported by HeberBiotec SA and Biorec. The Proteomic and Bioinformatics Departments at the Center for Genetic Engineering and Biotechnology would like to thank to INSPUR Company (China) for their kind donation of a computer cluster used for data storage and processing.

**Supporting Information Available:** A complete table of identified proteins. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## References

- (1) Laramas, M.; Pasquier, D.; Filhol, O.; Ringeisen, F.; Descotes, J. L.; Cochet, C. *Eur. J. Cancer* **2007**, *43* (5), 928–934.
- (2) Landesman-Bollag, E.; Romieu-Mourez, R.; Song, D. H.; Sonenshein, G. E.; Cardiff, R. D.; Seldin, D. C. *Oncogene* **2001**, *20* (25), 3247–3257.
- (3) Kim, J. S.; Eom, J. I.; Cheong, J. W.; Choi, A. J.; Lee, J. K.; Yang, W. I.; Min, Y. H. *Clin. Cancer Res.* **2007**, *13* (3), 1019–1028.
- (4) Daya-Makin, M.; Sanghera, J. S.; Mogentale, T. L.; Lipp, M.; Parchomchuk, J.; Hogg, J. C.; Pelech, S. L. *Cancer Res.* **1994**, *54* (8), 2262–2268.
- (5) O-charoenrat, P.; Rusch, V.; Talbot, S. G.; Sarkaria, I.; Viale, A.; Socci, N.; Ngai, I.; Rao, P.; Singh, B. *Clin. Cancer Res.* **2004**, *10* (17), 5792–5803.
- (6) Trembley, J. H.; Wang, G.; Unger, G.; Slaton, J.; Ahmed, K. *Cell. Mol. Life Sci.* **2009**, *66* (11–12), 1858–1867.
- (7) Unger, G. M.; Davis, A. T.; Slaton, J. W.; Ahmed, K. *Curr. Cancer Drug Targets* **2004**, *4* (1), 77–84.
- (8) Wang, G.; Unger, G.; Ahmad, K. A.; Slaton, J. W.; Ahmed, K. *Mol. Cell. Biochem.* **2005**, *274* (1–2), 77–84.
- (9) Hung, M. S.; Xu, Z.; Lin, Y. C.; Mao, J. H.; Yang, C. T.; Chang, P. J.; Jablons, D. M.; You, L. *BMC Cancer* **2009**, *9*, 135.
- (10) Sarno, S.; Reddy, H.; Meggio, F.; Ruzzene, M.; Davies, S. P.; Donella-Deana, A.; Shugar, D.; Pinna, L. A. *FEBS Lett.* **2001**, *496* (1), 44–48.
- (11) Perea, S. E.; Reyes, O.; Puchades, Y.; Mendoza, O.; Vispo, N. S.; Torrens, I.; Santos, A.; Silva, R.; Acevedo, B.; Lopez, E.; Falcon, V.; Alonso, D. F. *Cancer Res.* **2004**, *64* (19), 7127–7129.
- (12) Perea, S. E.; Reyes, O.; Baladron, I.; Perera, Y.; Farina, H.; Gil, J.; Rodriguez, A.; Bacardi, D.; Marcelo, J. L.; Cosme, K.; Cruz, M.; Valenzuela, C.; Lopez-Saura, P. A.; Puchades, Y.; Serrano, J. M.; Mendoza, O.; Castellanos, L.; Sanchez, A.; Betancourt, L.; Besada, V.; Silva, R.; Lopez, E.; Falcon, V.; Hernandez, I.; Solares, M.; Santana, A.; Diaz, A.; Ramos, T.; Lopez, C.; Ariosa, J.; Gonzalez, L. J.; Garay, H.; Gomez, D.; Gomez, R.; Alonso, D. F.; Sigman, H.; Herrera, L.; Acevedo, B. *Mol. Cell. Biochem.* **2008**, *316* (1–2), 163–167.
- (13) Perera, Y.; Farina, H. G.; Hernandez, I.; Mendoza, O.; Serrano, J. M.; Reyes, O.; Gomez, D. E.; Gomez, R. E.; Acevedo, B. E.; Alonso, D. F.; Perea, S. E. *Int. J. Cancer* **2008**, *122* (1), 57–62.
- (14) Solares, A. M.; Santana, A.; Baladron, I.; Valenzuela, C.; Gonzalez, C. A.; Diaz, A.; Castillo, D.; Ramos, T.; Gomez, R.; Alonso, D. F.; Herrera, L.; Sigman, H.; Perea, S. E.; Acevedo, B. E.; Lopez-Saura, P. *BMC Cancer* **2009**, *9*, 146.
- (15) Perera, Y.; Farina, H. G.; Gil, J.; Rodriguez, A.; Benavent, F.; Castellanos, L.; Gomez, R. E.; Acevedo, B. E.; Alonso, D. F.; Perea, S. E. *Mol. Cancer Ther.* **2009**, Epub ahead of print. DOI: 10.1158/1535-7163.MCT-08-1056.
- (16) Fields, G. B.; Noble, R. L. *Int. J. Pept. Protein Res.* **1990**, *35* (3), 161–214.
- (17) Klose, J.; Kobalz, U. *Electrophoresis* **1995**, *16* (6), 1034–1059.



- (18) Heukeshoven, J.; Dernick, R. *J. Chromatogr.* **1985**, 326, 91–101.
- (19) Jensen, O. N.; Wilm, M.; Shevchenko, A.; Mann, M. Sample preparation methods for mass spectrometric peptide mapping directly from 2-DE gels. In *2-D Proteome Analysis Protocols*; Link, A. J., Ed.; Human Press: Totowa, NJ, 1999; pp 513–530.
- (20) Gharahdaghi, F.; Weinberg, C. R.; Meagher, D. A.; Imai, B. S.; Mische, S. M. *Electrophoresis* **1999**, 20 (3), 601–605.
- (21) Gonzalez, L. J.; Castellanos-Serra, L.; Badock, V.; Diaz, M.; Moro, A.; Perea, S.; Santos, A.; Paz-Lago, D.; Otto, A.; Muller, E. C.; Kostka, S.; Wittmann-Liebold, B.; Padron, G. *Electrophoresis* **2003**, 24 (1–2), 237–252.
- (22) Perkins, D. N.; Pappin, D. J.; Creasy, D. M.; Cottrell, J. S. *Electrophoresis* **1999**, 20 (18), 3551–3567.
- (23) Elias, J. E.; Gygi, S. P. *Nat. Methods* **2007**, 4 (3), 207–214.
- (24) Fernandez-de-Cossio, J.; Gonzalez, L. J.; Satomi, Y.; Betancourt, L.; Ramos, Y.; Huerta, V.; Amaro, A.; Besada, V.; Padron, G.; Minamino, N.; Takao, T. *Nucleic Acids Res.* **2004**, 32 (Web Server issue), W674–W678.
- (25) Fernandez-de-Cossio, J.; Gonzalez, L. J.; Satomi, Y.; Betancourt, L.; Ramos, Y.; Huerta, V.; Besada, V.; Padron, G.; Minamino, N.; Takao, T. *Rapid Commun. Mass Spectrom.* **2004**, 18 (20), 2465–2472.
- (26) Dennis, G., Jr.; Sherman, B. T.; Hosack, D. A.; Yang, J.; Gao, W.; Lane, H. C.; Lempicki, R. A. *Genome Biol.* **2003**, 4 (5), 3.
- (27) Huang, d. W.; Sherman, B. T.; Lempicki, R. A. *Nat. Protoc.* **2009**, 4 (1), 44–57.
- (28) Chen, J.; Bardes, E. E.; Aronow, B. J.; Jegga, A. G. *Nucleic Acids Res.* **2009**, 37 (Web Server issue), W305–W311.
- (29) Shannon, P.; Markiel, A.; Ozier, O.; Baliga, N. S.; Wang, J. T.; Ramage, D.; Amin, N.; Schwikowski, B.; Ideker, T. *Genome Res.* **2003**, 13 (11), 2498–2504.
- (30) Demalte-Annessi, I.; Sanchez, J.-C.; Hoogland, C.; Rouge, V.; Binz, P.-A.; Appel, R. D.; Hochstrasser, D. F. SWISS-2DPAGE database entry. <http://www.expasy.org/swiss-2dpag/protein/ac=Q14847> (accessed Apr, 2010).
- (31) Tawfic, S.; Olson, M. O.; Ahmed, K. *J. Biol. Chem.* **1995**, 270 (36), 21009–21015.
- (32) Wang, S.; Zhang, B.; Faller, D. V. *EMBO J.* **2002**, 21 (12), 3019–3028.
- (33) Fusaro, G.; Dasgupta, P.; Rastogi, S.; Joshi, B.; Chellappan, S. *J. Biol. Chem.* **2003**, 278 (48), 47853–47861.
- (34) Liu, H.; Peng, H. W.; Cheng, Y. S.; Yuan, H. S.; Yang-Yen, H. F. *Mol. Cell. Biol.* **2005**, 25 (8), 3117–3126.
- (35) Tuynder, M.; Susini, L.; Prieur, S.; Besse, S.; Fiucci, G.; Amson, R.; Telerman, A. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, 99 (23), 14976–14981.
- (36) Kondo, T.; Minamino, N.; Nagamura-Inoue, T.; Matsumoto, M.; Taniguchi, T.; Tanaka, N. *Oncogene* **1997**, 15 (11), 1275–1281.
- (37) Li, J.; Zhang, X.; Sejas, D. P.; Bagby, G. C.; Pang, Q. *J. Biol. Chem.* **2004**, 279 (40), 41275–41279.
- (38) Savkur, R. S.; Olson, M. O. *Nucleic Acids Res.* **1998**, 26 (19), 4508–4515.
- (39) Szebeni, A.; Hingorani, K.; Negi, S.; Olson, M. O. *J. Biol. Chem.* **2003**, 278 (11), 9107–9115.
- (40) Ahlemann, M.; Zeidler, R.; Lang, S.; Mack, B.; Munz, M.; Gires, O. *Mol. Carcinog.* **2006**, 45 (12), 957–967.
- (41) Christensen, M. O.; Barthelme, H. U.; Boege, F.; Mielke, C. *J. Biol. Chem.* **2002**, 277 (39), 35932–35938.
- (42) Durban, E.; Goodenough, M.; Mills, J.; Busch, H. *EMBO J.* **1985**, 4 (11), 2921–2926.
- (43) Squatrito, M.; Mancino, M.; Donzelli, M.; Areces, L. B.; Draetta, G. F. *Oncogene* **2004**, 23 (25), 4454–4465.
- (44) Squatrito, M.; Mancino, M.; Sala, L.; Draetta, G. F. *Biochem. Biophys. Res. Commun.* **2006**, 344 (3), 859–868.
- (45) Lam, Y. W.; Lamond, A. I.; Mann, M.; Andersen, J. S. *Curr. Biol.* **2007**, 17 (9), 749–760.
- (46) Pujuguet, P.; Del Maestro, L.; Gautreau, A.; Louvard, D.; Arpin, M. *Mol. Biol. Cell* **2003**, 14 (5), 2181–2191.
- (47) Hunter, K. W. *Trends Mol. Med.* **2004**, 10 (5), 201–204.
- (48) Osawa, H.; Smith, C. A.; Ra, Y. S.; Kongkham, P.; Rutka, J. T. *Neuro-Oncol.* **2009**, 11 (4), 381–393.
- (49) Wang, H. J.; Zhu, J. S.; Zhang, Q.; Sun, Q.; Guo, H. *World J. Gastroenterol.* **2009**, 15 (16), 2016–2019.
- (50) Li, Q.; Wu, M.; Wang, H.; Xu, G.; Zhu, T.; Zhang, Y.; Liu, P.; Song, A.; Gang, C.; Han, Z.; Zhou, J.; Meng, L.; Lu, Y.; Wang, S.; Ma, D. *Cancer Lett.* **2008**, 261 (1), 55–63.
- (51) Xie, J. J.; Xu, L. Y.; Xie, Y. M.; Zhang, H. H.; Cai, W. J.; Zhou, F.; Shen, Z. Y.; Li, E. M. *Int. J. Cancer* **2009**, 124 (11), 2549–2558.
- (52) Xu, A.; Hao, J.; Zhang, Z.; Tian, T.; Jiang, S.; Hao, J.; Liu, C.; Huang, L.; Xiao, X.; He, D. *Lung Cancer* **2010**, 67 (1), 48–56.
- (53) Shibamura, H.; Hirano, T.; Tsuji, K.; Wu, Q.; Shrestha, B.; Konaka, C.; Ebihara, Y.; Kato, H. *Lung Cancer* **1998**, 22 (2), 85–95.
- (54) Kase, S.; Sugio, K.; Yamazaki, K.; Okamoto, T.; Yano, T.; Sugimachi, K. *Clin. Cancer Res.* **2000**, 6 (12), 4789–4796.
- (55) Akiyama, S.; Furukawa, T.; Sumizawa, T.; Takebayashi, Y.; Nakajima, Y.; Shimaoka, S.; Haraguchi, M. *Cancer Sci.* **2004**, 95 (11), 851–857.
- (56) Ikeda, R.; Tajitsu, Y.; Iwashita, K.; Che, X. F.; Yoshida, K.; Ushiyama, M.; Furukawa, T.; Komatsu, M.; Yamaguchi, T.; Shibayama, Y.; Yamamoto, M.; Zhao, H. Y.; Arima, J.; Takeda, Y.; Akiyama, S.; Yamada, K. *Biochem. Biophys. Res. Commun.* **2008**, 370 (2), 220–224.
- (57) Takao, S.; Akiyama, S. I.; Nakajo, A.; Yoh, H.; Kitazono, M.; Natsugoe, S.; Miyadera, K.; Fukushima, M.; Yamada, Y.; Aikou, T. *Cancer Res.* **2000**, 60 (19), 5345–5348.
- (58) Yu, E. J.; Lee, Y.; Rha, S. Y.; Kim, T. S.; Chung, H. C.; Oh, B. K.; Yang, W. I.; Noh, S. H.; Jeung, H. C. *Mol. Cancer Res.* **2008**, 6 (10), 1554–1566.
- (59) Ueda, M.; Terai, Y.; Kumagai, K.; Ueki, K.; Kanemura, M.; Ueki, M. *Int. J. Cancer* **2001**, 91 (6), 778–782.
- (60) Sato, J.; Sata, M.; Nakamura, H.; Inoue, S.; Wada, T.; Takabatake, N.; Otake, K.; Tomoike, H.; Kubota, I. *Int. J. Cancer* **2003**, 106 (6), 863–870.
- (61) Zhang, J.; Ren, H.; Yuan, P.; Lang, W.; Zhang, L.; Mao, L. *Cancer Res.* **2006**, 66 (1), 18–23.
- (62) Funasaka, T.; Haga, A.; Raz, A.; Nagase, H. *Int. J. Cancer* **2002**, 101 (3), 217–223.
- (63) Kirschmann, D. A.; Lininger, R. A.; Gardner, L. M.; Seftor, E. A.; Odero, V. A.; Ainsztein, A. M.; Earnshaw, W. C.; Wallrath, L. L.; Hendrix, M. J. *Cancer Res.* **2000**, 60 (13), 3359–3363.
- (64) Liaudet-Coopman, E.; Beaujoui, M.; Derocq, D.; Garcia, M.; Glondou-Lassis, M.; Laurent-Matha, V.; Prebois, C.; Rochefort, H.; Vignon, F. *Cancer Lett.* **2006**, 237 (2), 167–179.
- (65) Vashishta, A.; Ohri, S. S.; Proctor, M.; Fusek, M.; Vetvicka, V. *Anticancer Res.* **2006**, 26 (6B), 4163–4170.
- (66) Kroemer, G.; Pouyssegur, J. *Cancer Cell* **2008**, 13 (6), 472–482.
- (67) Pelicano, H.; Carney, D.; Huang, P. *Drug Resist. Updates* **2004**, 7 (2), 97–110.
- (68) Hileman, E. O.; Liu, J.; Albitar, M.; Keating, M. J.; Huang, P. *Cancer Chemother. Pharmacol.* **2004**, 53 (3), 209–219.
- (69) Xu, R. H.; Pelicano, H.; Zhou, Y.; Carew, J. S.; Feng, L.; Bhalla, K. N.; Keating, M. J.; Huang, P. *Cancer Res.* **2005**, 65 (2), 613–621.
- (70) Su, F.; Hu, X.; Jia, W.; Gong, C.; Song, E.; Hamar, P. *J. Surg. Res.* **2003**, 113 (1), 102–108.
- (71) Antognelli, C.; Baldracchini, F.; Talesa, V. N.; Costantini, E.; Zucchi, A.; Mearini, E. *Cancer J.* **2006**, 12 (3), 222–228.
- (72) Ohga, T.; Uchiumi, T.; Makino, Y.; Koike, K.; Wada, M.; Kuwano, M.; Kohno, K. *J. Biol. Chem.* **1998**, 273 (11), 5997–6000.
- (73) Saji, H.; Toi, M.; Saji, S.; Koike, M.; Kohno, K.; Kuwano, M. *Cancer Lett.* **2003**, 190 (2), 191–197.
- (74) Gaudreault, I.; Guay, D.; Lebel, M. *Nucleic Acids Res.* **2004**, 32 (1), 316–327.
- (75) Yahata, H.; Kobayashi, H.; Kamura, T.; Amada, S.; Hirakawa, T.; Kohno, K.; Kuwano, M.; Nakano, H. *J. Cancer Res. Clin. Oncol.* **2002**, 128 (11), 621–626.
- (76) Ikeda, R.; Furukawa, T.; Mitsuo, R.; Noguchi, T.; Kitazono, M.; Okumura, H.; Sumizawa, T.; Haraguchi, M.; Che, X. F.; Uchimiya, H.; Nakajima, Y.; Ren, X. Q.; Oiso, S.; Inoue, I.; Yamada, K.; Akiyama, S. *Biochem. Biophys. Res. Commun.* **2003**, 301 (2), 358–363.
- (77) Hisano, T.; Ono, M.; Nakayama, M.; Naito, S.; Kuwano, M.; Wada, M. *FEBS Lett.* **1996**, 397 (1), 101–107.
- (78) Hour, T. C.; Lai, Y. L.; Kuan, C. I.; Chou, C. K.; Wang, J. M.; Tu, H. Y.; Hu, H. T.; Lin, C. S.; Wu, W. J.; Pu, Y. S.; Sternecker, E.; Huang, A. M. *Biochem. Pharmacol.* **2010**, 80 (3), 325–34.
- (79) Lee, J. H.; Sun, D.; Cho, K. J.; Kim, M. S.; Hong, M. H.; Kim, I. K.; Lee, J. S.; Lee, J. H. *J. Cancer Res. Clin. Oncol.* **2007**, 133 (1), 37–46.

PR100728V