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A multidimensional approach to an in-depth proteomics analysis of transcriptional regulators in neuroblastoma cells

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HIGHLIGHTS

- An optimised neuroproteomic method for the analysis of transcriptional regulators.
- This method detected more than 1800 nuclear proteins, which constitutes one of the largest datasets reported for a neuronal cell.
- This method will allow in-depth analysis of transcriptional regulators for the study of neurological diseases.

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ABSTRACT

The dynamic regulation of transcriptional events is fundamental to many aspects of neuronal cell functions. However, proteomics methods have not been routinely used in global neuroproteomics analyses of transcriptional regulators because they are much less abundant than the "house-keeping" proteins in cells and tissues. Recent improvements in both biochemical preparations of nuclear proteins and detection sensitivities of proteomics technologies have made the global analysis of nuclear transcriptional regulators possible. We report here an optimised neuroproteomic method for the analysis of transcriptional regulators in the nuclear extracts of SHSY-5Y neuroblastoma cells by combining an improved nuclear protein extraction procedure with multidimensional peptide separation approaches. We found that rigorous removal of cytoplasmic proteins and solubilisation of DNA-associated proteins improved the number of nuclear proteins identified. Furthermore, we discovered that multidimensional peptide separations by either strong cation exchange (SCX) chromatography or electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) analysis detected more than 1800 nuclear proteins, which constitutes one of the largest datasets of nuclear proteins reported for a neuronal cell. Thus, in-depth analysis of transcriptional regulators for studying neurological diseases are increasingly feasible.

1. Introduction

Proteomics approaches have been successfully used for the large-scale analysis of protein expression patterns, post-translational modifications and protein–protein interactions (Cahill, 2001; Pandey and Mann, 2000). The rapid evolution of quantitative proteomics technologies has enabled routine analysis of global proteomic changes among diverse tissues and cells (Gauss et al., 1999; Shevchenko et al., 1996; Yan et al., 2001). More recently, specialised proteomic studies such as those on neuroproteomics have become increasingly useful for understanding the dynamic regulatory protein networks that underlie neuronal development and neurological diseases (Lin et al., 2009; Liu et al., 2006; Tyler et al., 2011). In addition, neuroproteomics has branched into more in-depth studies of the sub-proteomes, including synaptoproteomics and neural plasma membrane proteomics (Zhang, 2010). However, compared with other high-throughput tools for the system-wide analyses of genes and proteins, the sensitivities of proteomics technologies for the characterisation of less abundant signalling molecules and transcriptional regulators have remained low for routine biochemical studies.

The eukaryotic nucleus is an important organelle for regulating gene expression and other diverse functions (Trinkle-Mulcahy

Abbreviations: 2DE, 2D gel electrophoresis; ACN, acetonitrile; ATN1, atrophin-1; C/EBP, CCAAT enhancer binding protein; CID, collision induced dissociation; DMEM, Dulbecco's modified eagle medium; DRPLA, dentatorubral-pallidoluysian atrophy; ERLIC, electrostatic repulsion-hydrophilic interaction chromatographic; FDR, false discovery rate; HD, Huntington's disease; ICAT, isotope-coded affinity tag; IPA, ingenuity pathway analysis; SCX, strong cation exchange; TEAB, triethylammonium bicarbonate; TF, transcription factor.

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and Lamond, 2008). Within the nuclear proteomes, many cellular signals, including signals for the stress response, growth and differentiation, ultimately target specific gene promoters to induce alterations in gene expression or DNA replication. The ability to comprehensively identify and quantify transcriptional regulators is important for understanding their functions under different physiological and diseased conditions. Unfortunately, transcription factors are often underrepresented in global proteomic studies due to their relatively low abundance in comparison with the "house-keeping" proteins, such as metabolic enzymes, cytoskeletal proteins and heat shock proteins. To address this limitation, subcellular fractionation approaches for organelle-specific proteomic analyses have been attempted for more sensitive examinations of low-abundance proteins (Andersen et al., 2002; Boisvert et al., 2010; Dreger et al., 2001; Trinkle-Mulcahy and Lamond, 2008). For example, several groups have investigated the nuclear or chromatin proteomes, using a variety of biochemical approaches for the enrichment of nuclear proteins from diverse cell lines and primary cells (reviewed by Albrethsen et al., 2009). In one study, a 2D gel electrophoresis (2DE) reference map of total nuclear proteins isolated from human liver was established (Jung et al., 2000); however, both heat shock proteins and cytoskeletal proteins were still abundantly represented. Additional subnuclear fractionation can further improve the depth of the proteome coverage. For example, the nuclear proteome of human HeLa cells was extensively analysed by Andersen et al., which resulted in the identification of 271 nucleolar proteins (Andersen et al., 2002). Similarly, Tchapyjnikov et al. (2010) used a nanospray LC/MS/MS-based approach to analyse cell nuclei extracted with a commercially available nuclear extraction kit and identified 154 transcription factors and many other transcriptional co-regulators, kinases and phosphatases. Shakib et al. (2005) analysed the nuclear proteins from NRK49F rat kidney fibroblasts after prolonged hypoxia by 2DE. Among the 791 proteins identified, 17 transcription factors or cofactors were found to be possibly regulated by hypoxia. In addition to 2DE, LC-based shotgun proteomics methods have also been used effectively for the analysis of nuclear proteomes. Shiio and Eisenman used the isotope-coded affinity tag (ICAT) approach to identify Myc-induced changes in the nuclear proteome (Shiio et al., 2003). After chromatin enrichment, they applied ICAT in combination with LC/MS/MS and identified 282 proteins, including 64 known nuclear proteins. Among the 18 transcription factors identified, ATF-3 reduction and NIFK induction were found to be Myc-modulated. Recently, several advanced mass spectrometry-based studies have made notable progress in characterising human chromatin. Garcia's group used three different chromatin extraction methods and identified over 1900 proteins, 40% of which were classified as nuclear proteins by independent bioinformatics analyses (Torrente et al., 2011). Overall, it appears that a balance must be reached between nuclear protein specificity and the depths of the nuclear proteome coverage.

Nuclear proteomics analyses of neuronal cells have not been widely reported, in part because of the difficulties associated with the unusual morphologies and processes of cells of the central and peripheral nervous systems. In this study, we have developed a comprehensive approach for the characterisation of the nuclear proteome from a SHSY-5Y neuroblastoma cell line. We found that, by both rigorous removal of cytoplasmic proteins and extensive extraction of chromatin-associated proteins, we could dramatically improve the nuclear proteome coverage in this cell line. Furthermore, by adopting multidimensional chromatographic approaches, including ERLIC and SCX fractionations, to further expand the nuclear proteome coverage, we could achieve one of the most in-depth identifications of transcription regulators in SHSY-5Y cells.

2. Materials and methods

2.1. Materials

HPLC-grade solvents and water were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Triethylammonium bicarbonate (TEAB), protease inhibitors cocktail and phosphatase inhibitors cocktail were purchased from Sigma (St. Louis, MO). Sequencing-grade modified trypsin was purchased from Promega Corp. (Madison, WI). PepClean C_{18} spin columns were purchased from Pierce (Rockford, IL). Western blot reagents were obtained from BioRad (Redmond, WA). The antibody against actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the antibody against histone H1 (Clone AE-4) was purchased from Millipore (Billerica, MA).

2.2. Cell lines and cell culture

Human neuroblastoma cell line SHSY-5Y was obtained from ATCC (Manassas, VA). Cells were propagated as monolayers in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and F12 medium supplemented with 0.1 mmol/L nonessential amino acids, 1% penicillin/streptomycin and 10% foetal bovine serum at 37 °C in 5% CO_2. Exponentially growing and nearly confluent (90%) cells were harvested after several passages and were washed twice with PBS.

2.3. Nuclear protein extraction and analysis

2.3.1. Basic extraction method (Method 1)

Nuclear extracts were prepared from the SHSY-5Y cells using a cell lysis and salt extraction procedure described by Dignam et al. (1983). Briefly, the PBS-washed cell pellets were gently resuspended in a hypotonic lysis buffer comprising 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, protease inhibitors and phosphatase inhibitors. After incubation of the re-suspended cells on ice for 15 min, 0.5% NP-40 was added, and the extracts were vigorously vortexed for 10 s to disrupt the cell membranes. The cellular extracts were then centrifuged at 800 × *g* for 10 min at 4 °C to separate the cytoplasmic components (supernatants) from the nuclei-enriched fractions (pellets). The cytoplasmic fractions (supernatants) were stored at −80 °C until subsequent analyses. The nuclear pellets were resuspended in a hypertonic buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 25% (v/v) glycerol, 0.5 mM DTT, 0.2 mM EDTA and cocktails of protease and phosphatase inhibitors). Nuclear proteins were extracted via vigorous agitation for 15 min on ice. The solutions were further sonicated 3 times at 10 s intervals on ice. The resulting solutions were centrifuged at 16,000 × *g* and 4 °C for 15 min. The supernatants containing solubilised nuclear proteins were stored at −80 °C until further analyses. Protein extracts were further concentrated by the addition of 5 volumes of ethanol for precipitation. After centrifugation at 16,000 × *g* for 15 min, the protein pellets were re-suspended in 500 μL of a buffer containing 8 M urea, 50 mM TEAB (pH 8.0) and protease and phosphatase inhibitor cocktails. Protein concentrations were determined using the Bradford assay according to the manufacturer's instructions (Bio-Rad). The proteins were reduced with 10 mM DTT at RT for 1 h and alkylated with 50 mM iodoacetamide for 30 min in the dark. To maintain, trypsin digestion efficiency, the urea concentration was diluted to 1 M with the addition of 50 mM TEAB. For in-solution proteolytic digestion, trypsin was added into the protein solutions at a ratio of 1:25 (trypsin/protein by weight), and the solutions were incubated overnight at 37 °C. The resulting tryptic peptides were desalted using C_{18} spin columns (Pierce) and stored at −80 °C until LC/MS/MS analysis.

2.3.2. Rigorous nuclear protein enrichment method (Method 2)

Similar to the basic method, cytoplasmic extracts and nuclear pellets were first separated by low-speed centrifugation after treating the cells with the hypotonic buffer and NP-40. For more rigorous enrichment of the nuclear proteins, we washed the nuclear pellets an additional 2–3 times with the freshly made hypotonic buffer and NP-40 to more thoroughly remove the cytoplasmic proteins. The proteins in the resulting nuclear pellets were extracted with the hypertonic buffer followed by the sonication and centrifugation steps, as described above. The nuclear proteins in the supernatant were concentrated by ethanol precipitation and resuspended in the urea buffer. Tryptic digestion and LC/MS/MS analysis were performed as described in Method 1.

2.3.3. Solubilisation of chromatin-associated proteins with nuclease treatment (Method 3)

After hypertonic buffer extraction of proteins from the nuclear pellets, as described in Method 2, the remaining nuclear pellets after centrifugation might still contain proteins that were trapped within the DNA and chromatin. These proteins were extracted from the pellets by the addition of ≥250 units of Benzonase® nuclease (Sigma, St. Louis, MO) into the nuclease buffer (20 mM Tris HCl, pH 8.0, 2 mM $MgCl_2$, and 20 mM NaCl) and incubated at 37 °C for 1 h. The resulting proteins were resuspended in the buffer with 8 M urea, digested with trypsin and analysed by LC/MS/MS, as described for Method 1.

2.4. Peptide fractionation and LC/MS/MS

2.4.1. Strong cation exchange chromatography (SCX)

For each SCX separation of the tryptic digests, peptides derived from 300 μg of nuclear proteins were separated on a BioCAD™ Perfusion Chromatography System (ABI) equipped with a polysulfoethyl A column (4.6 × 200 mm, 5 μm, 300 Å, Poly LC, Columbia, MD) plus an upstream guard column (4 × 10 mm). The column was first washed isocratically with the mobile phase A (10 mM KH_2PO_4, 20% acetonitrile (ACN), pH 2.7) for 10 min to remove the unbound materials. The retained peptides were then eluted with a 30 min linear gradient from 0% to 15% mobile phase B (600 mM KCl, 10 mM KH_2PO_4 and 20% ACN, pH 2.7) and were then eluted with another 20 min gradient from 15% to 50% mobile phase B, followed by a final 10 min linear gradient from 50% to 100% mobile phase B, at a flow rate of 1 mL/min. The peptides in each 2-min fraction were desalted via PepClean C_{18} spin columns, dried in a speedvac and then combined into 12 fractions with comparable complexities according to the MS signals observed by MALDI-TOF/TOF MS on a 4800 Protein Analyser (AB Sciex).

2.4.2. Electrostatic repulsion hydrophilic interaction chromatography (ERLIC)

For each ERLIC analysis of the tryptic digests, peptides derived from 300 μg of nuclear proteins were fractionated in ERLIC mode, using a Poly-WAX LP column (4.6 × 200 mm, 5 μm, 300 Å, Poly LC, Columbia, MD) on the BioCAD HPLC. A gradient composed of a mobile phase A (10 mM ammonium acetate in 85% ACN/1% formic acid (FA)) and a mobile phase B (30% ACN/0.1% FA) was used for the separation. It was conducted at an initial gradient from 0% to 15% B for 10 min, then from 15% to 30% B for 25 min, followed by a gradient from 30% to 100% B for 5 min and finally at 100% B for 10 min, at a flow rate of 1 mL/min. After 2-min fractions were collected, C_{18} spin columns were used to desalt the peptides; select neighbouring fractions were combined into 12 fractions based on the MALDI MS signals.

2.4.3. Reversed-phase liquid chromatography and tandem mass spectrometry (RPLC/MS/MS)

Tryptic peptides obtained from total nuclear extracts were either analysed by LC/MS/MS directly or subjected to further SCX or ERLIC fractionations prior to LC/MS/MS. RPLC/MS/MS was performed on an Chromatography System that was equipped with an Ultimate™ 3000 autosampler (Dionex, Sunnyvale, CA) and coupled with an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). Desalted peptides were first trapped on a cartridge (Pepmap C_{18}, 0.5 cm × 300 μm, Dionex) at 2% mobile phase B (mobile phase A: 2% ACN and 0.1% FA; mobile phase B: 85% ACN and 0.1% FA) with a flow rate of 30 μL/min; then, they were transferred onto a 75 μm × 150 mm capillary Acclaim® PepMap100 column (C_{18}, 3 μm, 100 Å, Dionex) and fractionated using a 75-min gradient (3–45% B) at a flow rate of 250 nL/min. After each gradient cycle, the column was washed isocratically at 95% mobile phase B to clean the column and minimise carry-overs, followed by a re-equilibration step with mobile phase A. The eluted peptides were ionised at 2.0 kV via a Proxeon nano electrospray ion source and introduced into the mass spectrometer. The capillary temperature was 275 °C. The MS was operated in a data-dependent mode. Full scan MS spectra (from m/z 300–2000) were acquired in the Orbitrap analyser operating at a resolution of 60,000 at a m/z of 400. The lock mass option was enabled to achieve high mass accuracy. The 10 most intense peptide ions with charge states ≥2 were sequentially isolated to a target value of 10,000 and fragmented in the linear ion trap by low-energy collision-induced dissociation (CID), with a normalised collision energy of 35%. The ion selection threshold was set at 5000 counts for MS/MS. The maximum allowed ion accumulation times were 500 ms for full scans in the Orbitrap and 100 ms for CID measurements in the LTQ.

2.4.4. Protein database search

All of the raw LC/MS/MS spectra were analysed by the Proteome Discoverer (version 1.3.0.339) software suite, using both the SEQUEST (Thermo, CA, USA) and Mascot (version 2.3, Matrix Science, London, UK) search engines. Carbamidomethyl cysteine (+57 Da) was set as a fixed modification, and methionine oxidation (+16 Da) was set as an optional modification. Up to two missed internal tryptic cleavage sites were allowed. Parameters common to all database searches included the use of monoisotopic masses and a mass error tolerance of 10 ppm for the precursor ions and 0.5 Da for the CID fragment ions. The database search was performed against all human proteins (20,233 sequence entries) annotated in the SwissProt protein database (released in October, 2012).

The database search results (.msf files) were further filtered and compiled into a list of nonredundant proteins with Scaffold (version Scaffold_3_3_1; Proteome Software, Inc., Portland, OR). Peptide identifications were accepted if they could be established at ≥95% probability, as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at ≥95.0% probability with a false discovery rate (FDR) of no more than 1% based on the forward and reverse database search approach and if they contained at least one uniquely identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped together to satisfy the principles of parsimony. The accession numbers of the identified proteins were uploaded into the Ingenuity Pathway Analysis (IPA) software (http://www.ingenuity.com) to retrieve the putative cellular localisation and functional information for the respective protein.

3. Results and discussion

3.1. Effective nuclear proteome analysis with the basic method

SHSY-5Y is a well-characterised human neuroblastoma cell line that has been widely used as a model for investigating neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases (Molina-Holgado et al., 2008). In this study, we aimed to optimise a neuroproteomics method for the identification of low-abundance transcriptional regulators in SHSY-5Y cells. We isolated cell nuclei based on a procedure described by Dignam et al. (1983) that utilises a hypotonic procedure for cell lysis and a high-salt extraction to isolate proteins from nuclear pellets (Method 1, Section 2).

To evaluate the nuclear protein isolation efficiency of the basic method, Western blotting was performed on the nuclear and cytoplasmic extracts (Fig. 1A). A histone H1 band (32–33 kDa) was detected specifically in the nuclear fraction but not in the cytoplasmic extracts. In contrast, actin was detected only in the cytoplasmic fraction but not in the nuclear extracts. These data suggested that the basic nuclear protein enrichment method was effective. To further evaluate the quality of the nuclear preparations and to assess the degree of cytoplasmic protein contamination of the nuclear extracts, we examined both cytoplasmic and nuclear extracts by MS (Fig. 1B–E and Supplemental Tables 1 and 2). Among the approximately 1400 proteins that were identified from the cytoplasmic extracts, approximately two thirds were annotated primarily as cytoplasmic proteins according to the bioinformatics analysis by IPA, whose contents are curated mainly from the scientific literature (Fig. 1D–E). In comparison, among the >600 proteins found in the nuclear extract, ~72% were annotated as nuclear proteins by IPA, which suggests that the basic method was highly effective and produced a level of nuclear extract purity that was comparable to that from many published studies on nuclear proteomes (Abdolzade-Bavil et al., 2004; Dreger, 2003; Escobar et al., 2005; Salzano et al., 2006). For example, among the 1900 proteins identified from the chromatin preparations by Garcia's group, ~40% were classified as nuclear proteins by DAVID Bioinformatic Resources (http://david.abcc.ncifcrf.gov/) (Torrente et al., 2011). On the other hand, Henrich et al. identified 124 unique proteins from the human Burkitt's lymphoma B-cell line, using a sucrose density gradient centrifugation followed by 2DE (Henrich et al., 2007). Using PSORT, a protein subcellular localisation prediction algorithm, they determined that over 90% of the identified proteins were predicted to be nuclear. Of note, the different approaches to protein localisation using PSORT, DAVID, IPA and other methods do not enable a direct comparison of these nuclear protein preparations.

Using the basic approach, we found 332 overlapping proteins that were common to both nuclear and cytoplasmic extracts in one of the representative experiments (Fig. 1B). The overlap between the two extracts could have been caused by either inefficiencies associated with the extraction methods or intracellular translocations of certain proteins. Overall, the basic method was effective at producing enriched nuclear proteins.

3.2. Improvement of nuclear protein enrichment efficiencies

Because of the presence of ~20% of the cytoplasmic proteins in the nuclear extracts (Fig. 1D), we further evaluated whether repeated washing of the nuclear pellets could improve the removal of cytoplasmic contaminants (Method 2, Section 2, Fig. 2A). With a two-step wash procedure, we found 15.1% ± 0.3% more proteins, with around 70% being nuclear proteins (data not shown). However, there was no further gain with a three-step wash procedure, either with regard to the protein numbers or the percentages of nuclear proteins found (Supplemental Fig. 1 and Supplemental Tables 3 and 4), which suggests that the presence of cytoplasmic proteins in the nuclear extracts was unlikely to be the result of casual "contamination".

Cell nuclei contain large amounts of DNAs and RNAs, which might be associated with transcription factors and other regulators of gene expressions. To release the proteins that might be trapped within the nucleic acids and chromatin, we added Benzonase, a commercially available nuclease, to the pellets after hypertonic extraction, to digest the nucleic acids and possibly release more chromatin-associated proteins (Method 3, Section 2, Fig. 2A). The addition of Benzonase markedly reduced the cell lysate viscosity during the sample processing (data not shown). Indeed, approximately 82 additional unique proteins and an average of 1082 additional peptides were identified after Benzonase treatment, compared with the yield from Method 2 alone (Fig. 2B and C and Supplemental Tables 5 and 6). Methods 2 and 3 enabled the identifications of similar numbers of "transcriptional regulators" classified according to IPA (highlighted in Supplemental Tables 5 and 6), with 18 more unique transcriptional regulators identified only after the Benzonase treatment. Surprisingly, the IPA bioinformatics predictions of proteins identified following the Benzonase treatment did not show a higher percentage of nuclear proteins; the nuclear protein purity obtained from this method was ~68%, which is comparable to ~66% from Method 2 (Fig. 2D and E). This finding suggests that some "cytoplasmic proteins" could indeed be associated with nucleic acids (or chromatin on occasion) and could possibly perform 'moonlighting' functions in the nucleus.

3.3. Expanding the nuclear proteome coverage by multidimensional fractionations

The nuclear proteomes have been studied in a variety of human organs and cells (Albrethsen et al., 2009; Andersen et al., 2002; Dreger et al., 2001; Henrich et al., 2007; Schenk et al., 2012; Tchapyjnikov et al., 2010; Torrente et al., 2011; Wilkie and Schirmer, 2006). Most of the studies used crude nuclear pellets without further purification or fractionation. It is well established that orthogonal protein or peptide fractionation prior to LC/MS/MS is a highly effective approach for identifying low-abundance proteins. We therefore evaluated the effectiveness of SCX- and ERLIC-based multidimensional peptide separation methods for expanding the nuclear proteome coverage relative to the coverage from the analysis of unfractionated nuclear preparations. From 300 μg nuclear proteins prepared by combining Methods 2 and 3 (see Section 2, Fig. 2A), an average of 2497 non-redundant proteins and approximately 12,603 unique peptides were identified via ERLIC separation, whereas an average of 2623 non-redundant proteins and over 9800 unique peptides were identified via SCX separation (Fig. 3A and B and Supplemental Tables 7–9). Both separation methods were equally effective at dramatically improving the coverage of the nuclear proteomes, with each approach more than doubling the number of proteins identified over the unfractionated approach (Fig. 3A). Furthermore, a combined analysis that involved both SCX and ERLIC fractionations almost tripled the number of proteins identified in comparison with the unfractionated analysis, bringing the total number of proteins to >3000 (Fig. 3A and B). After multidimensional peptide separations, the predicted percentage of nuclear proteins decreased from ~64% to ~54–61% (Fig. 3C and D), which suggests that some low-abundance proteins identified only after SCX and ERLIC fractionations were mainly localised in the cytoplasm in SHSY-5Y cells. Even at a low nuclear protein purity of 50%, our combined SCX/ERLIC analysis detected >1800 nuclear proteins (Among the 3422 of total proteins identified in Fig. 3A, around 55% are classified as nuclear proteins, see Fig. 3D, resulted in 1882 estimate nuclear proteins), which constitute one of the largest datasets reported for a neuronal cell. To understand the details of the improved proteome coverage due

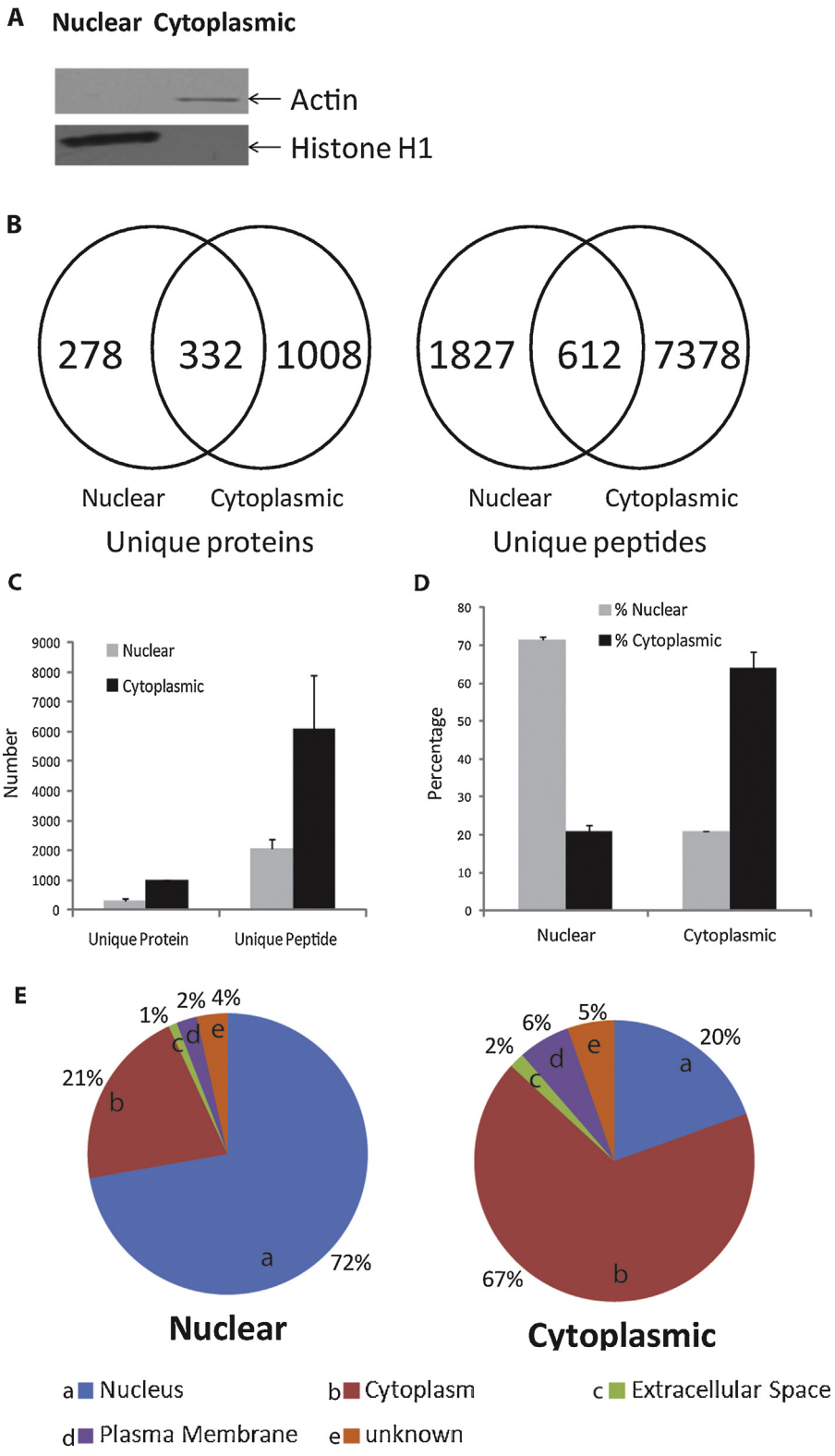

Fig. 1. Comparison of nuclear and cytoplasmic extract isolated with the basic method. (A) Western blot detection of cellular compartment-specific proteins in SHSY-5Y cell nuclear and cytoplasmic extracts. Histone H1 (32–33 kD) was found mainly in the nuclear extracts, while actin (42 kD) was enriched mainly in the cytoplasmic extracts. (B and C) Comparison of unique proteins and peptides identified from the nuclear and cytoplasmic extracts. Nuclear (grey bar); cytoplasmic (black bar), (B) shows data from one of the representative experiments with Method 1. Peptide and protein identification criteria are specified in Section 2. Scaffold was used to filter for and compare unique proteins and peptides identified from the analysis of 50 μg of each cytoplasmic or nuclear extract. As expected, more proteins and peptides were discovered in the cytoplasmic extracts. However, 278 unique proteins and 1827 unique peptides were found in only the nuclear extracts in Fig. 1B (Supplemental Tables 1 and 2). (D and E) Cellular localisation of the proteins identified from the cytoplasmic and nuclear extracts. The predictions were made by the IPA software. (D) Subcellular localisation annotation: nucleus (grey bar); cytoplasm (black bar). (E) Shows analysis results from one of the representative experiments. Subcellular localisation annotation: nucleus (a, blue); cytoplasm (b, red); extracellular space (c, green); plasma membrane (d, purple); unknown (e, orange). (For interpretation of the references to colour in the text, the reader is referred to the web version of the article.)

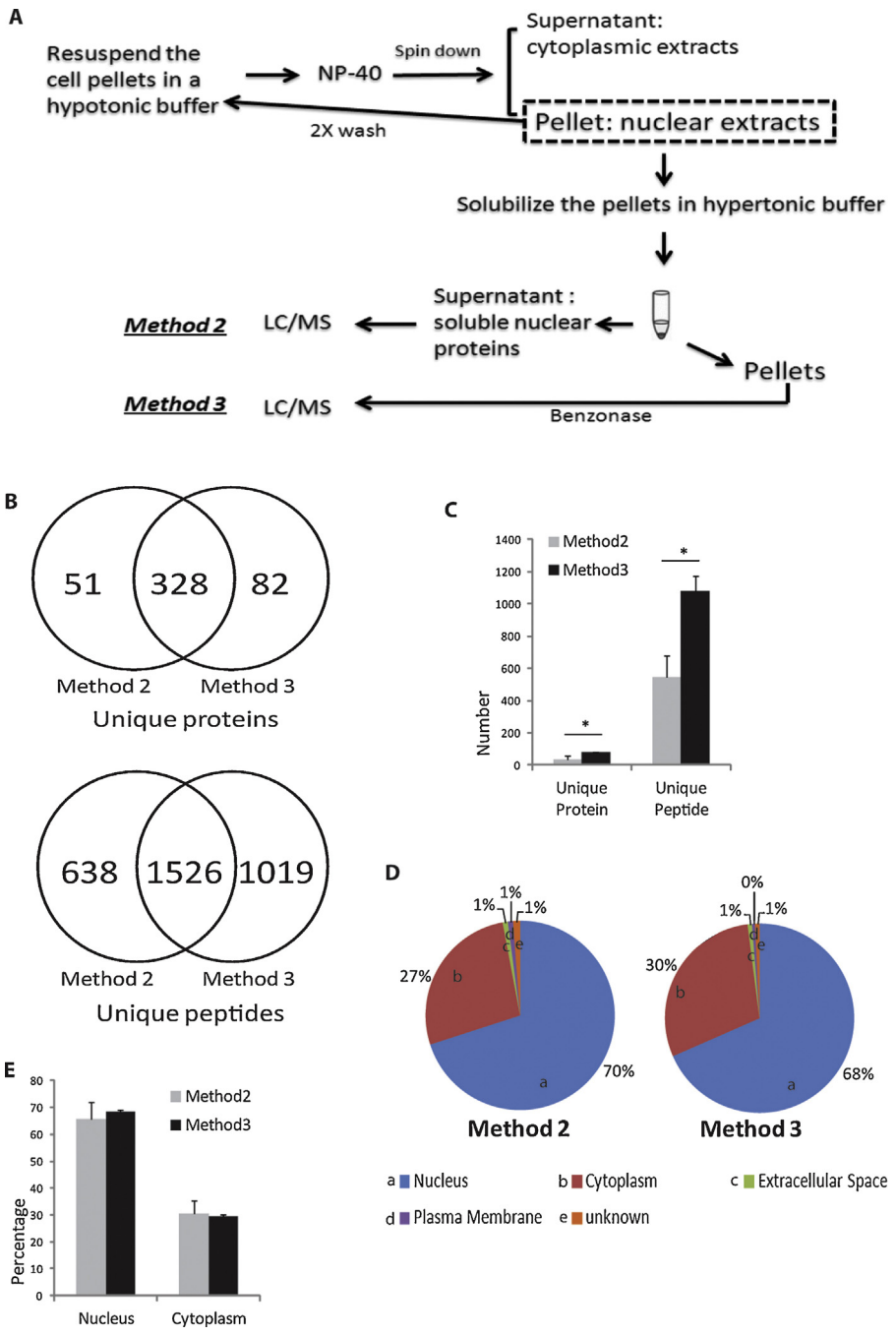

Fig. 2. Effect of nuclease treatment on nuclear protein recovery. (A) After initial isolation of the nuclear pellets from SHSY-5Y cells that included repeated washes to remove loosely associated cytoplasmic proteins, nuclear proteins were extracted with the hypertonic buffer, and the supernatants were digested either immediately (see Section 2: Method 2) or after Benzonase® nuclease digestion of the pellets (see Section 2: Method 3). Free nuclear proteins from Method 2 and DNA-associated proteins recovered from Method 3 were identified by LC/MS/MS analysis. (B and C) Comparison of the unique proteins and peptides identified from either method. (B) Shows data from one of the representative experiments. In addition to the large degree of protein and peptide overlap found between the two methods, nuclease digestion of DNA resulted in the identification of ~40% more peptides and 20% more proteins in Method 3, when compared with Method 2 alone (*$p \leq 0.05$). Method 2 (grey bar); Method 3 (black bar). (D and E) Comparison of IPA-annotated nuclear proteins between the two methods. (D) Shows data from one of the representative experiments. The relative percentages of the nuclear proteins between the two methods were comparable. Subcellular localisation annotation: nucleus (a, blue); cytoplasm (b, red); extracellular space (c, green); plasma membrane (d, purple); unknown (e, orange) (E) Subcellular localisation annotation: method 2 (grey bar); method 3 (black bar). (For interpretation of the references to colour in the text, the reader is referred to the web version of the article.)

to these two fractionation methods, we further analysed the number of unique proteins and peptides identified in each ERLIC and SCX fraction (Fig. 4). Interestingly, the ERLIC method offered less peptide fractionation (Fig. 4A) compared with SCX (Fig. 4B), which suggests that the peptides derived from this nuclear preparation are similar in polarity but more heterogeneous in pI. All of the experiments above were repeated at least twice, and the results were reproducible (data not shown). Further optimisation of these fractionation conditions (e.g., gradients) will likely lead to even deeper coverage of the nuclear proteomes.

3.4. Key neuronal transcription factors identified from in-depth nuclear proteomic analysis

Until recently, conventional neuroproteomics techniques do not have the sensitivities for routine analysis of less abundant neuronal

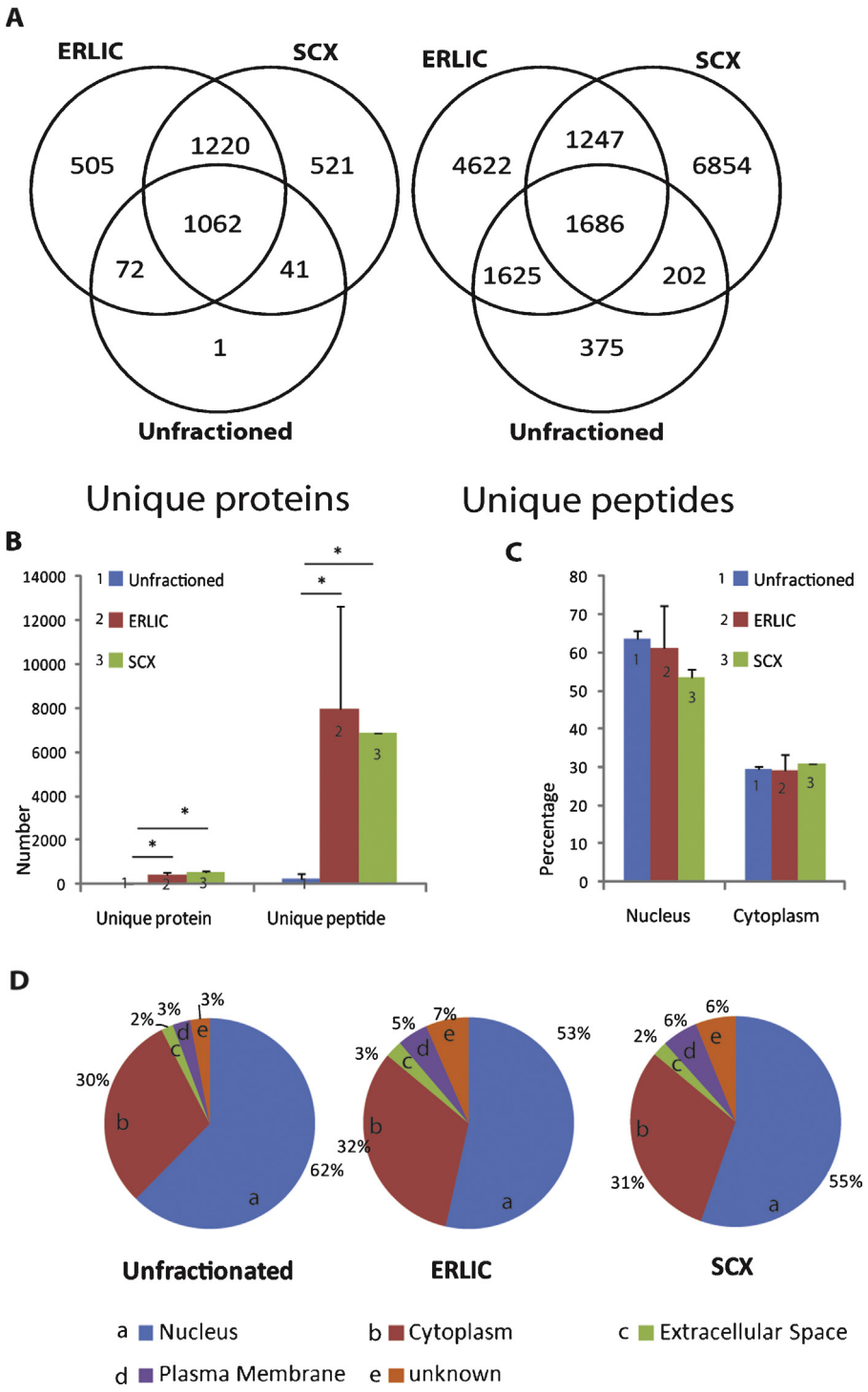

Fig. 3. Comparison of the proteome coverage among the unfractionated preparation and the ERLIC and SCX fractionated preparations. (A and B) Comparison of the numbers of unique proteins and peptides. Unfractionated (1, blue bar), ERLIC (2, red bar) and SCX (3, green bar). (A) Shows data from one of the representative experiments. The ERLIC and SCX fractionations increased the number of unique proteins or peptides that were identified by more than 140% and 150%, respectively ($^*p \leq 0.05$). (C and D) show annotated localisations of the proteins that were identified. Unfractionated (1, blue bar), ERLIC (2, red bar) and SCX (3, green bar). (D) Shows data from one of the representative experiments. Subcellular localisation annotation: nucleus (a, blue); cytoplasm (b, red); extracellular space (c, green); plasma membrane (d, purple); unknown (e, orange). (For interpretation of the references to colour in the text, the reader is referred to the web version of the article.)

transcription factors, which play crucial roles in cells that underlie neurological diseases. The new method described here can dramatically improve the coverage of the nuclear proteomes and enable the analysis of neuronal transcription factors (Supplemental Table 10). Here, we have identified more than 3000 proteins in the nuclear extracts; these proteins comprise one of the largest mammalian nuclear proteomics datasets published to date (Abdolzade-Bavil et al., 2004; Boisvert et al., 2010; Chaerkady et al., 2009; Shakib et al., 2005; Shiio et al., 2003; Tchapyjnikov et al., 2010; Torrente et al., 2011). Using an independent bioinformatics analysis by IPA, 215 proteins from the ERLIC separation and 303 proteins from the SCX separation were classified as "transcriptional regulators" (Supplemental Table 11), which is comparable to the ~300 transcription factors found in human brain tissues by a recent genetic survey

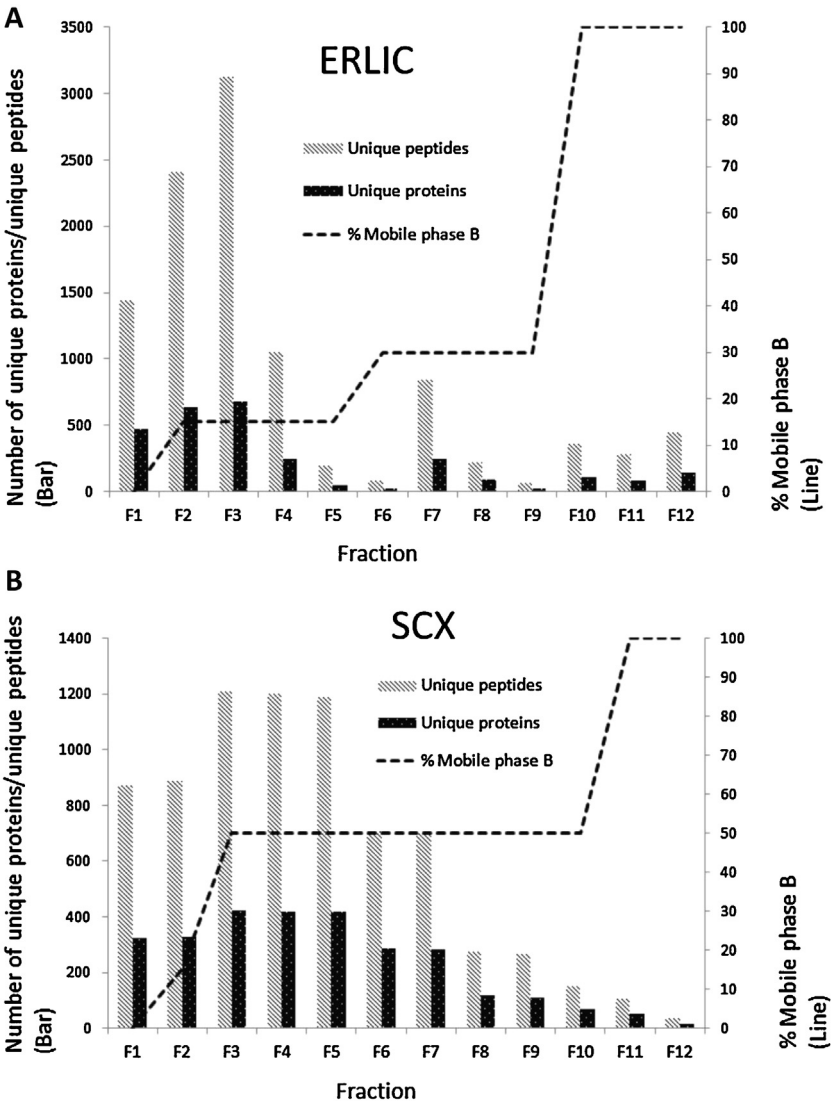

Fig. 4. Number of unique proteins and unique peptides identified in each (A) ERLIC and (B) SCX fraction.

(Vaquerizas et al., 2009). For example, zinc finger proteins are known to mediate specific protein–DNA interactions (Matthews and Sunde, 2002); over 30 zinc finger proteins were found in this study, some of which have been reported to be involved in transcriptional regulation (Blaiseau et al., 1997; Martinez-Pastor et al., 1996). One of the transcription factors detected in this study is the CCAAT enhancer-binding protein (CEBP) (Table 1). As a member of the basic leucine zipper DNA-binding protein family, CEBP family members are enriched in neurons and up-regulated following brain injuries in animal models of neuronal regeneration (Cortes-Canteli et al., 2004; Nadeau et al., 2005). Its key functions include the regulation of neuronal cell growth, differentiation, learning, memory and apoptosis (Cortes-Canteli et al., 2002; Hatakeyama et al., 2006; Marshall et al., 2003; Menard et al., 2002). Additional transcription regulators found in this study included atrophin-1 (ATN1) and huntingtin (Table 1). ATN1 is localised in both the nuclei and cytoplasm of neurons in the human CNS (Wood et al., 2000). Accumulations of ATN1 mutants have been associated with the development of dentatorubral-pallidoluysian atrophy neurodegeneration (Suzuki and Yazawa, 2011) and Huntington's disease (HD) (Costa Mdo et al., 2006; Schilling et al., 2001). Huntingtin protein polymorphism can lead to the incorporation of a different number of glutamines in the protein; HD patients tend to have a large number of glutamines in huntingtin (Perutz, 1996).

Table 1
Select transcriptional regulators identified in this study.

Gene symbol	Protein name	Protein identification probability[a]	Number of unique peptides identified	Percentage sequence coverage	Identification approach
ATN1	Atrophin-1	99.7%	2	5%	SCX
CEBPZ	CCAAT/enhancer-binding protein zeta	99.9%	16	24%	ERLIC
CEBPG	CCAAT/enhancer-binding protein gamma	99.7%	2	48%	SCX
HTT	Huntingtin	99.9%	5	4%	ERLIC

[a] Protein identification probability calculated by Scaffold, using the Protein Prophet method.

Overall, with the optimised multidimensional method, in-depth neuroproteomics analysis of neuronal cells and tissues appears quite feasible.

4. Conclusions

We have optimised a method for identifying transcriptional regulators in a neuroblastoma cell line by a combination of improved nuclear protein isolation and multidimensional peptide fractionations. Using this method, we have identified many transcriptional regulators that were barely detectable in previous neuroproteomics analyses. Therefore, targeted nuclear proteomics analysis could provide an opportunity for a better understanding of neuronal cell functions and diseases. Because the approaches described in this study can be readily combined with different quantitative proteomics methodologies, discovering quantitative changes among the transcription regulators and their post-translational modifications underlying diverse neurological phenomena could soon become a reality.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jneumeth.2013.03.016.

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