PROFILING OF A1 MES-C-MYC MOUSE EMBRYONIC MESENCEPHALON CELLS BY DATA INDEPENDENT LC-MS

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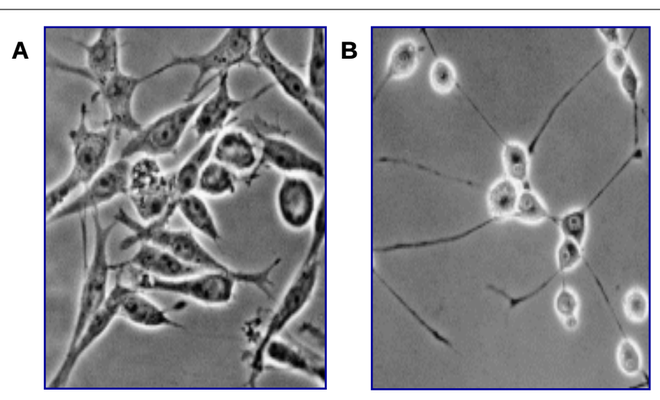
INTRODUCTION

Insights into the mechanisms underlying CNS differentiation and functions can be gained by studying the properties of its cell types during neural differentiation. Although *in vitro* studies using primary cultures obtained directly from tissues have provided useful information, cellular heterogeneity makes it difficult to dissect molecular events at a single cellular phenotype. The variety and the extreme precision of the functions over which CNS presides are assured by the fine-tuning of the communications between different neural cells types.

Mes-c-myc A1, a cell line generated from mouse embryonic mesencephalic CNS, can be cultured under undifferentiated/proliferative or differentiated/non-proliferative conditions. In the presence of serum these cells appear undifferentiated and proliferate whereas serum withdrawal and cAMP stimulation cause cell cycle arrest and neuronal differentiation with ensuing neurite outgrowth, neuronal electrophysiological properties and expression of neuronal markers. In such a cellular model it is possible to distinguish molecules and mechanisms related to different status of proliferation and differentiation.

To address the issue of molecules involved in neural differentiation, we performed the qualitative and quantitative proteomic profiling of mes-c-myc A1 cell line by data independent LC-MS^E analysis, with the aim to survey the molecular changes following neural cell differentiation.



C	A1P	A1D
Cell proliferation	+	-
Neurite outgrowth	-	+
Electrophysiological properties	-	+
Neuronal gene expression	+	++

Figure 1. Experimental system. Phase-contrast photomicrographs showing A1 cells before (A1P) and after (A1D) differentiation. Morphological changes are paralleled by the appearance of functional and molecular features of differentiated neurons as summarized in C.

METHODS

Cell cultures and sample preparation

Mouse CNS immortalized mes-c-myc A1 (A1) cell line was obtained from mouse embryonic mesencephalon cells as previously described[1]. Both undifferentiated A1P and differentiated A1D cells were cultured as previously reported[1].

Monolayer cultures of cell lines were harvested, after three washes in ice-cold PBS, by incubating with a solution containing trypsin and EDTA. After centrifugation, the cell pellets were washed three times with PBS and resuspended in 25 mM NH_4HCO_3/0.5% RapiGest for cell lysis and protein extraction. Samples were sonicated and centrifuged to eliminate cellular debris. The supernatants were collected and protein concentration determined by the Bradford method.

Total protein extracts were subsequently reduced (10 mM DTT), alkylated (10 mM IAA) and enzymatically digested by two subsequent additions [1:100 (w/w) enzyme:protein ratio for 16 hours at 37°C, then 1:50 for 4 hours at 37°C] of TPCK-treated trypsin.

LC-MS conditions

A single dimension LC-MS approach has been used for the separation and analysis of the embryonic mesencephalon cell line samples. The combined qualitative and quantitative experiments were conducted using a 90 min gradient from 5 to 40% acetonitrile (0.1% formic acid) at 250 nL/min using a nanoACQUITY system. An Atlantis 3 µm C18 reversed phase 75 µm x 15 cm nanoscale LC column was used and estimated on-column sample loads were 0.5 µg protein digest for all studies. The data independent, alternate scanning LC-MS^E experiments were performed with a Q-Tof Premier mass spectrometer.

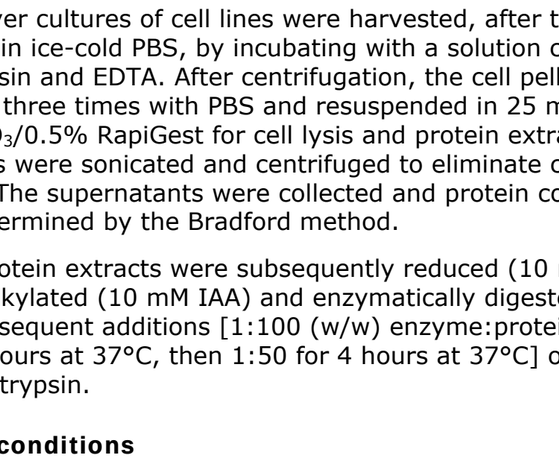


Figure 2. Log-log peptide intensity distribution for A1D vs. A1P (grey = non-regulated peptides; blue = regulated peptides (both replicating and high probability of regulation)).

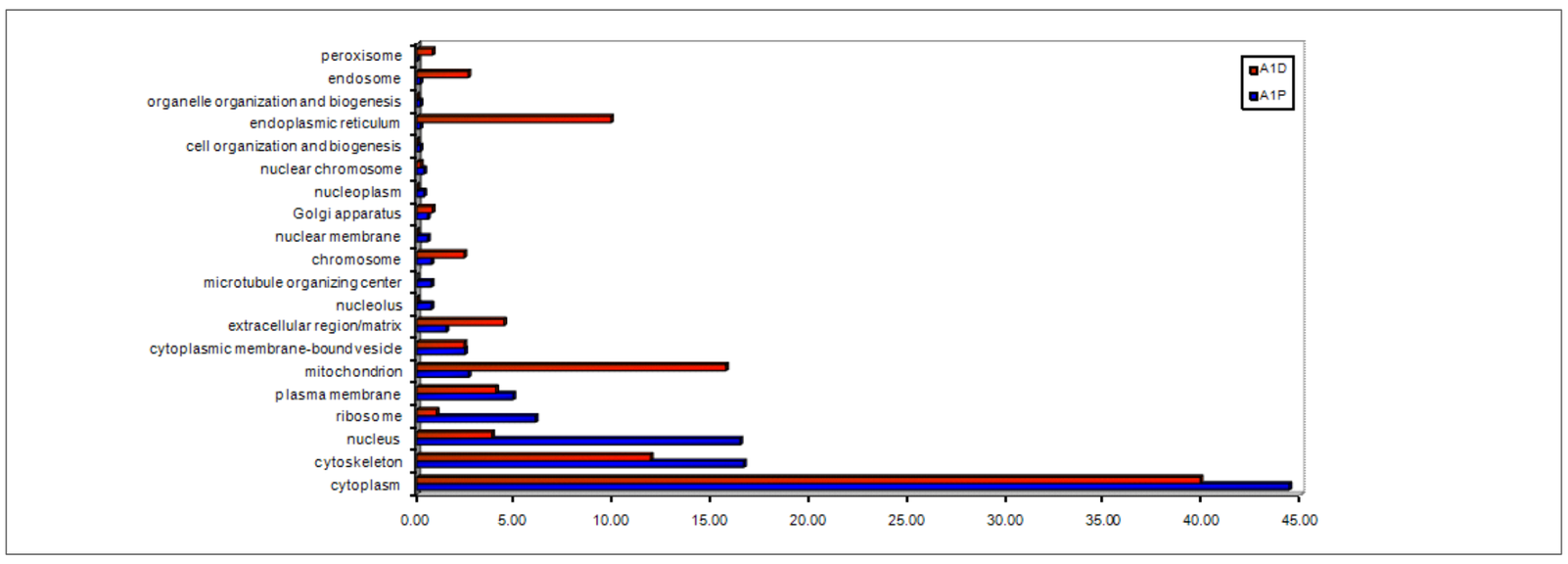


Figure 3. Bar chart of the gene ontology cellular localization categories. Comparison of the GO cellular localization categories obtained for the up- and down-regulated proteins in the A1P (blue bars) vs. A1D (red bars) condition.

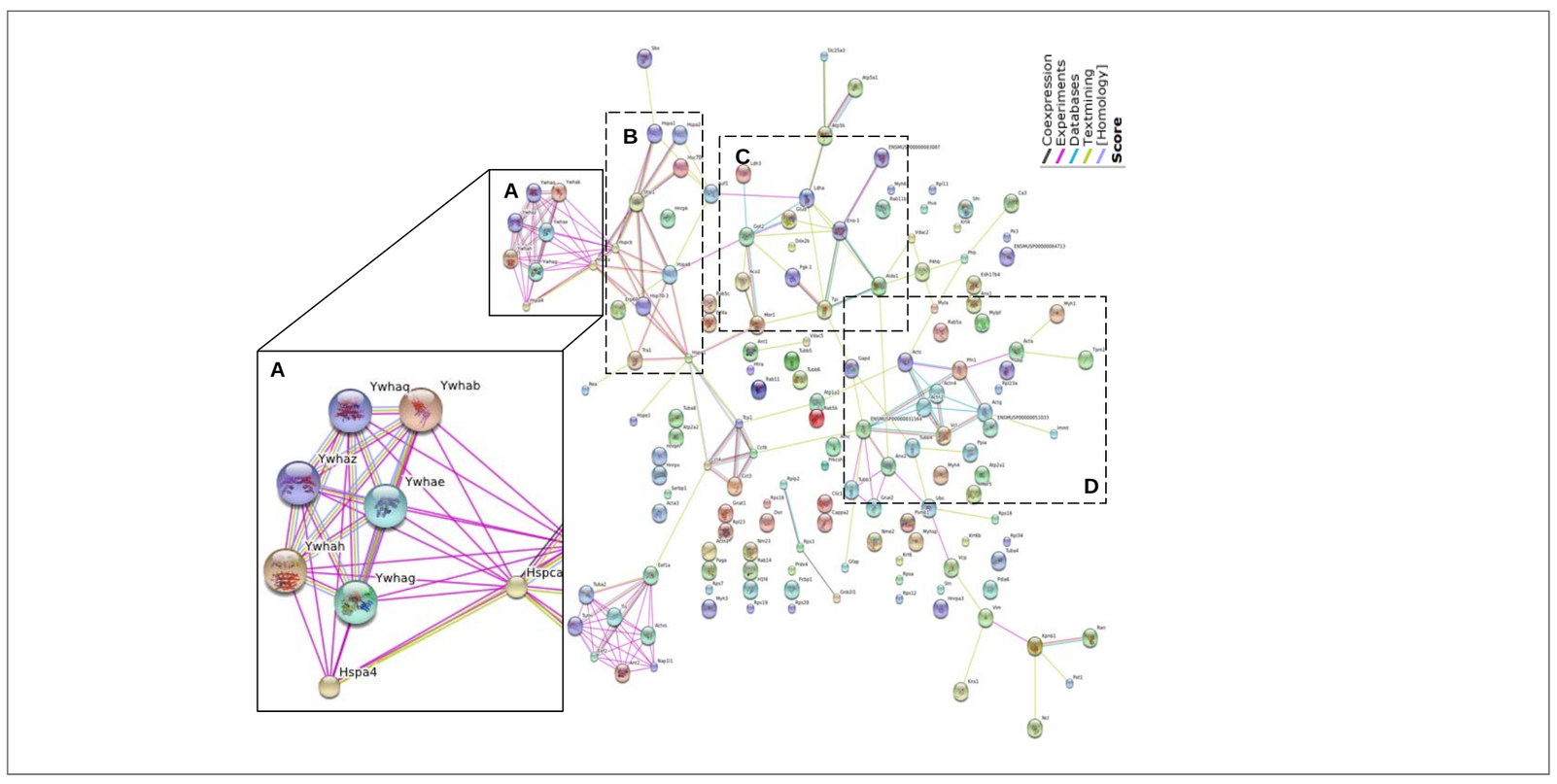


Figure 4. Graphical view of protein interaction network obtained for differentially expressed proteins in A1 cell line under proliferative/differentiated status. The correspondence of accession number (Table 1)/gene name for the 14-3-3 cluster (panel A) is the following: Ywhaq, P68254; Ywhaz, P63101; Ywhab, Q9CQV8; Ywhae, P62259; Ywhah, P68510; Ywhag, P61982; Hspa4, Q61316; Hspca, P07901.

RESULTS

Qualitative Profiling

With a mere estimated amount of 0.5 µg of protein digest loaded per injection, stringent database search criteria (10 ppm precursor/20 ppm product ion tolerances and at least 2 peptides identified to a protein) and identification of the protein in at least 2 out of 3 replicate injections, a total of 245 proteins was identified across both conditions. Homologues identifications were not considered.

The qualitative profiles of A1 cells allowed the identification of 169 proteins common to both conditions. Furthermore, 32 and 44 proteins were uniquely identified, *i.e.* not detected in the other condition of interest, to the A1D and A1P conditions, respectively.

Neuronal and glial markers (*i.e.* vimentin, nestin, neuron specific enolase, peripherin and glial fibrillary acidic protein), previously reported in A1 cells, were detected, attesting their bipotent neuronal and glial fingerprint. In addition, in both A1P and A1D samples, two neuronal specific intermediate filaments (NF-M and NF-66) were also identified.

Quantitative Profiling

The normalized intensities of A1D *vs.* A19P accurate mass-retention time clusters were plotted on a natural log scale (Figure 2). Quantitative expression results were obtained by comparing the peak area/intensity of each peptide in the two conditions2,3. A deviation from the main diagonal reflects fold changes of peptide intensities or, in other words, protein regulation.

	description	*ratio**	*variance*	*type*
Q64433	10 kDa heat shock protein mitochondrial	-0.85	0.056	down
P63038	60 kDa heat shock protein mitochondrial	-0.58	0.001	down
Q99KI0	Aconitate hydratase mitochondrial	-0.52	0.009	down
P48962	ADP/ATP translocase 1	-0.92	0.094	down
P51881	ADP/ATP translocase 2	-0.89	0.077	down
Q03265	ATP synthase subunit alpha mitochondrial	-0.90	0.082	down
P56480	ATP synthase subunit beta mitochondrialGlial	-0.83	0.047	down
P03995	fibrillary acidic protein astrocyte (GFAP)	-0.31	0.092	down
P26443	Glutamate dehydrogenase 1 mitochondrial	-0.67	0.003	down
P08249	Malate dehydrogenase mitochondrial	-0.73	0.014	down
Q8VDN2	Sodium/potassium-transporting ATPase	-0.64	0.001	down
P20152	Vimentin	-0.32	0.086	down
Q9CQV8	14-3-3 protein beta/alpha	0.96	0.085	up
P62259	14-3-3 protein epsilon	1.06	0.153	up
P68510	14-3-3 protein eta	0.74	0.005	up
P61982	14-3-3 protein gamma	0.73	0.004	up
O70456	14-3-3 protein sigma	0.75	0.007	up
P68254	14-3-3 protein theta	0.81	0.020	up
P63101	14-3-3 protein zeta/delta	0.83	0.026	up
P16045	Galectin-1	1.26	0.349	up
P15532	Nucleoside diphosphate kinase A	1.36	0.477	up
Q01768	Nucleoside diphosphate kinase B	1.17	0.251	up

*Table 1. Excerpt of significantly regulated A1 cell proteins. *A1P vs. A1D ^{e}log ratio.*

The entire differentially expressed protein data set was further filtered (≥ 2 out of 3 replicate injections, 30% fold change) providing a list of significantly regulated proteins, some of them are listed in Table 1, along with their relative fold change and variance.

A comparison of the GO cellular localization categories of regulated proteins (Figure 3), revealed a significant increase of expression of mitochondrial proteins in A1D (15.7%) vs. A1P (2.6%), supporting the recent hypothesis of mitochondrial participation in neuronal differentiation[4].

The interaction network between the differentially expressed proteins shown in Figure 4 reveals that specific populations cluster on the basis of their reciprocal interactions. Several proteins involved in common metabolic pathways (C), remodelling of cytoskeleton (D) or belonging to the same protein family (B) were differentially expressed upon A1 differentiation.

A specific cluster of proteins up-regulated in proliferating A1 cells (A) belongs to the 14-3-3 family. The 14-3-3 proteins are involved in a wide range of cellular functions, including the regulation of tryptophan 5-mono-oxygenase, a key enzyme in serotonin synthesis.

The latter is of particular interest as A1 cells express serotonin under both proliferation and differentiating conditions (Figure 5A and 5B for A1D and A1P, respectively). It is worth noting that the distribution of serotonin of immunofluorescence is highly reminiscent of vesicular compartmentalization as shown in the high magnifications (Figure 5C and 5D).

CONCLUSION

- **Proteomic profiling of undifferentiated (A1P) *vs.* differentiated (A1D) mes-c-myc A1 by qualitative and quantitative LC-MS^E analysis**
- **Approximately 250 proteins identified across both conditions with a single LC-MS experiment with significant fractions univocally expressed in the A1P and/or A1D conditi**
- **Quantitative results in agreement with previously reported neuronal markers**
- **Most differentially expressed proteins are involved in neuronal differentiation, survival, metabolic processes, protein synthesis and remodelling of cytoskeleton**
- **Large fractions detected as unique or significantly up-regulated in A1D cells share a mitochondrial localization with both structural roles or involved in metabolic activities, suggesting a key role of mitochondria in neuronal differentiatio**

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