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Differential protein expression in normal and trisomic cell lines from murine cerebral cortex measured by itraq and Mass Spectrometry

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Abstract: Down syndrome (DS), the most common cause of genetic mental dysfunction, is caused by aberrant protein expression due to a trisomy of chromosome 21 (Ts21). A mouse model of Ts21, based on synteny of human chromosome 21 and murine chromosome 16, is used for studying the mechanisms involved in DS. In this study we measured protein expression in immortalized cell lines derived from normal and trisomy 16 (Ts16) fetal mouse cerebral cortex (CNh and CTb respectively). iTRAQ labeling and mass spectrometry were used to examine each cell line for significant up- or down-regulation of proteins. CTb cells showed increased expression of 71 proteins and decreased expression of 56 proteins when compared to the normal CNh control cells. Several of these differentially expressed proteins have previously been reported to show aberrant expression in DS and other neurological disorders such as Alzheimer's disease, a condition closely associated with Down syndrome. Our results clearly demonstrate the aberrant expression of proteins with various cellular functions within the trisomy model and may lead to a deeper understanding of the proteins and mechanisms involved in the Down syndrome phenotype.

Key words: Trisomy 16/21; Down syndrome; differential protein expression; iTRAQ, mass spectrometry.

Introduction

Down syndrome (DS) is the most complicated genetic anomaly compatible with life and is the most common genetic condition associated with mental disability. Approximately 95% of DS individuals have an additional complete copy of chromosome 21, a condition known as full trisomy 21 (Ts21), while the remainder present with partial Ts21, or Ts21 mosaicism[1]. DS occurs in 1 in 700-800 live births, and becomes more common with increasing maternal

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age[2,3]. DS presents with various complex phenotypes and affects many organs, including the brain, which gives rise to dysmorphic features such as impaired cognitive abilities and early onset Alzheimer's disease. While various other inconsistently occurring abnormalities exist in the heart (congenital disease), muscle (hypotonia) and skeleton (brachycephaly), only impaired cognitive function is common to all individuals with DS[4].

Within the DS brain, the cerebral cortex is strongly affected by the Ts21 genotype, showing an abnormal reduction in size[5], aberrant stratification[6], lamination[7], myelination[8,9] and shallow or missing sulci (fissures/folds of the cerebral cortex)[10]. In addition, there appears to be reductions in the number of neurons present[8,11] as well as impaired synaptogenesis and dendritic deterioration[12]. Because the cerebral cortex is responsible for complex functions such as sensory perception, thought, language, attention, memory and consciousness, alterations to this part of the brain, resulting from the Ts21 condition, likely contribute to the cognitive impairment associated with DS.

A useful animal model for the study of human Ts21 is murine trisomy 16 (Ts16). This is due to a high degree of synteny between the long arm of human autosome 21 and the distal segment of mouse chromosome 16[13] and murine trisomy 16 is considered a model for both DS and AD[14]. Although Ts16 mice die *in utero*, the phenotypic similarities to DS are present in the embryonic and fetal periods of development. This constraint has restricted the use of these animals to studies of early developmental stages and justifies the use of primary cell cultures[14]. Therefore in the present work we have employed an immortalized cell line (CTb) derived from the cerebral cortex of a trisomy 16 mouse fetus[15,16]. This cellular model has been well accepted for the study of DS and AD pathologies[17]. The CTb model has recently been used to study amyloid aggregation[18], endosomal abnormalities related to amyloid precursor protein[19] and apoptosis due to amyloid accumulation[20]. Similar to DS individuals, Ts16 mice exhibit congenital heart defects[21,22], craniofacial and skeletal abnormalities, growth retardation[23] and immunological irregularities[24,25]. In addition, brain abnormalities similar to those in observed in DS occur. These include reduced size, delayed thickening, and other abnormal aspects of cerebral cortex development[26]. Ts16 mice also exhibit a decrease in cholinergic neuron generation[23] and altered electrophysiological properties manifested throughout the central nervous system[27].

Although it has been well established that the Ts21 genotype produces the various DS phenotypes, the exact molecular mechanisms and specific proteins involved are still largely unknown. Here we report on the use of a murine model system for human trisomy 21 that may aid in the identification of relevant proteins involved in DS phenotypes, especially those relevant to the early development. We determined the differential protein expression profiles of cell lines derived from the cerebral cortex of normal and Ts16 fetal mice by the differential peptide labeling method known as "isobaric tags for relative and absolute quantitation" (iTRAQ) coupled with mass spectrometry. A variety of proteins, both up regulated and down regulated, were identified that may play a role in DS pathology.

Results and Discussion

Results

Protein identification by iTRAQ

In this study, solubilized proteins were derived from immortalized mouse cell lines of CNh (normal) and CTb (chromosome 16 trisomy: mouse Down syndrome). The pair of cell

lines were each cultured in three separate rounds of production to yield three replicate pairs of normal and trisomic samples and each pair was analyzed in a separate iTRAQ differential expression experiment. After trypsin digestion and iTRAQ labeling, liquid chromatography and MS of three replicates, an average of 12070 spectra were collected, resulting in an average of 4464 peptide sequences per experiment. After assigning each peptide to a parent protein at the appropriate confidence level (95%), 941, 941and 1073 proteins were identified in replicates one, two and three respectively. Five hundred and seventy proteins were present in all 3 replicates, while 289 were present in 2 of the 3 and 505 in a single replicate only. In total, 1364 unique protein sequences were identified.

Identified proteins were considered significantly up- or down-regulated when the protein expression level between trisomic and normal cells was ≥1.3 and ≤0.7 respectively (≥1.5 and ≤0.6 respectively if originating from only a single replicate) and at least two peptides at greater than 95% confidence contributed to the protein identification. Using these cutoff criteria, 71 proteins were up-regulated (Table 1 and Table 3 supplemental) and 56 were down-regulated in (Table 2 and Table 3 supplemental) the trisomic CTb cells compared to the control CNh cells. Based on Gene Ontology groupings of the aberrantly expressed proteins, the functional clustering of aberrantly expressed proteins is shown in Figure 1. The chromosomal location of each aberrantly expressed protein was identified and presented in Tables 1 and 2.

Metabolic enzymes

Carbohydrate metabolism

Almost all enzymes involved in glycolysis were identified in our iTRAQ analysis. Those that were up-regulated in the trisomic cell line CTb included glucose-6-phosphate isomerase, and pyruvate kinase isoform M2, while phosphofructokinase was down-regulated.

Other enzymes involved in carbohydrate metabolism that were up-regulated in the trisomic cell line were transketolase, an enzyme of the pentose phosphate pathway and the reductive metabolic enzyme, fructose-bisphosphate aldose A.

NAD/NADP synthesis

In addition to transketolase, one other protein involved in NAD synthesis was found to be differentially expressed: nicotinamide phosphoribosyltransferase expression was decreased in all three replicates of trisomic samples.

Fatty acid and lipid metabolism

Prosaposin, a precursor protein for saposins involved in glycosphingolipid metabolism, exhibited increased expression in the trisomic cell line.

Nucleotide biosynthesis

Two enzymes, nucleoside diphosphate kinase A and B, both involved in nucleotide biosynthesis, were significantly over-expressed in the CTb cell line when compared to the CNh cell line.

Glutamate metabolism

Glutamate dehydrogenase, a protein involved in glutamate metabolism in the brain was identified by iTRAQ and displayed increased expression in the trisomic cell line compared to the control.

Table 1:

Name	Symbol	UniProt ID	Pep (95%) Mean	114: 115 E1	114: 115 E2	114: 115 E3	114: 115 Mean	GO ID	GO Function	Chr
Heat shock 105kDa/110kDa protein 1	Hsph1	Q61699	3.3	4.3	nq	4.6	4.4	GO:0051085	chaperone mediated protein folding	5
Nucleoside diphosphate kinase B	Nme2	Q5NC82	12.3	3.4	1.9	4.1	3.1	GO:0043066	negative regulation of apoptosis	11
Transketolase	Tkt	P40142	7.3	3.7	3.3	2.0	3.0	GO:0040008	regulation of growth	14
Serine proteinase inhibitor, clade H	Serpinh1	Q8BVU9	9.0	2.8	2.3	3.6	2.9	GO:0051082	unfolded protein binding	7
Aldo-keto reductase family 1, member B8	Akr1b8	P45377	8.0	3.3	3.0	2.3	2.9	GO:0055114	oxidation-reduction process	6
Glycosyltransferase 25 domain cont.	Glt25d1	Q8K297	2.3	3.9	1.4	nq	2.6	GO:0005788	endoplasmic reticulum lumen	8
Plastin 3 (T-isoform)	Pls3	B1AX58	10.0	2.5	3.0	2.4	2.6	GO:0005509	calcium ion binding	X
Catalase	Cat	Q542K4	5.0	2.4	2.7	2.8	2.6	GO:0006979	response to oxidative stress	2
Prosaposin	Psap	Q3TXJ0	2.0	4.2	1.5	2.0	2.6	GO:0048589	developmental growth	10
N-myc downstream regulated gene 1	Ndrg1	Q545R3	1.0	3.0	2.1	nq	2.5	GO:0015630	microtubule cytoskeleton	15
Sorting nexin 9	Snx9	Q4V9K0	1.0	2.9	2.1	nq	2.5	GO:0007154	cell communication	17
Unkempt homolog (Drosophila)	Unk	unk	3.0	2.9	3.1	1.4	2.5	GO:0008150	unk: phosphoribosyl Pi synthesis	11
Von Willebrand factor A domain cont. 5A	Vwa5a	D3Z671	2.7	2.9	1.7	nq	2.3	GO:0008150	unk: Breast cancer suppressor candidate	9
Chloride intracellular channel 1	Clic1	Q542F1	5.3	2.1	1.6	2.8	2.2	GO:0005247	voltage-gated chloride channel activity	17
Glutamate dehydrogenase 1	Glud1	Q3TSQ7	9.0	1.9	2.2	2.2	2.1	GO:0006520	cellular amino acid metabolic process	14
Heat shock protein 1	Hspb1	D3YZ06	5.3	2.0	2.1	nq	2.1	GO:0000502	proteasome complex	5
Nucleoside diphosphate kinase A	Nme1	P15532	9.3	2.0	1.7	2.1	1.9	GO:0007399	nervous system development	11
Valyl-tRNA synthetase	Vars	Q790I0	4.0	2.2	1.6	1.9	1.9	GO:0000166	nucleotide binding	17
Chaperonin containing Tcp1, subunit 3	Cct3	Q3U4U6	4.0	2.2	1.2	2.2	1.9	GO:0051082	unfolded protein binding	3
Ribosomal protein SA, pseudogene 10	Rpsa	D3YTT7	6.3	1.0	0.9	3.7	1.9	GO:0030529	ribonucleoprotein complex	3
Ezrin	Ezr	Q4KML7	7.0	2.3	1.1	2.1	1.8	GO:0008092	cytoskeletal protein binding	17
Tubulin beta-5 chain	Tubb5	Q80ZV2	39.3	1.1	1.3	3.1	1.8	GO:0005200	structural constituent of cytoskeleton	17
Protease, serine, 1 (trypsin 1)	Prss1	Q9Z1R9	13.0	1.7	1.4	2.3	1.8	GO:0006508	proteolysis	6
Aldolase A, fructose-bisphosphate	Aldoa	A6ZI44	11.3	2.6	1.6	0.8	1.7	GO:0006096	glycolysis	7
EH-domain containing 2	Ehd2	Q8BH64	2.7	2.1	1.3	1.6	1.7	GO:0030866	cortical actin cytoskeleton organization	7

Table 1. The top-25 over-expressed proteins from the murine Ts16 cerebral cortex. **Name**, the name of the detected protein. **Symbol**, the UniProt symbol of the identified protein. **UniProt ID**, the ID number used to generate Gene Ontology annotations. **Pep (95%) Mean**, the number of distinct peptides from three replicate experiments having at least 95% confidence. Multiple modified and cleaved states of the same underlying peptide sequence are considered distinct peptides because they have different molecular formulas. **114:115 E1-E3,** The ratio of protein expression between trisomic and normal cell line in separate replicate experiments. DS samples (CTb) were labeled with iTRAQ tag 114 and normal samples (CNh) with tag 115. **114:115 Mean**, the mean expression ratio, the average ratio for the protein based on triplicate data. **114:115 Std Dev**, The standard deviation of expression ratios across three replicates. **GO ID**, Gene Ontology identification number. **GO Function**, biological process based on GO ID searching. **Chr**, the chromosome number on which the protein's gene resides. Identified proteins were considered significantly up- or down-regulated when the protein expression level between trisomic and normal cells was ≥1.5 and ≤0.6 respectively and at least two peptides at greater than 95% confidence contributed to the protein identification.

Table 2:

Name	Symbol	UniProt ID	Pep (95%) Mean	114:115 E1	114:115 E2	114:115 E3	114:115 Mean	GO ID	GO Function	Chr
Eukaryotic translation elongation factor 1 d	Eef1d	P57776	2.7	0.2	nq	0.2	0.2	GO:0003746	translation elongation factor activity	15
Interferon-induced guanylate-binding protein 2	Gbp2	Q4FK03	2.7	0.4	0.3	0.1	0.3	GO:0006184	GTP catabolic process	3
Eukaryotic translation elongation factor 1 b2	Eef1b2	O70251	2.7	0.1	0.5	0.4	0.3	GO:0003746	translation elongation factor activity	1
Annexin A5	Anxa5	P48036	5.3	0.4	0.4	0.3	0.3	GO:0005544	calcium-dependent phospholipid binding	3
Nucleophosmin 1	Npm1	Q5SQB0	10.7	0.5	0.5	0.1	0.4	GO:0051726	regulation of cell cycle	11
Acidic nuclear phosphoprotein 32	Anp32b	Q9EST5	3.0	0.2	0.6	nq	0.4	GO:0005634	nucleus	4
Vimentin	Vim	Q3TFD9	57.7	0.3	0.5	0.3	0.4	GO:0031252	cell leading edge	2
Myoferlin (Fer-1-like protein 3)	Myof	Q69ZN7	16.0	0.4	0.4	0.4	0.4	GO:0034605	cellular response to heat	19
Putative uncharacterized protein	Txnl1	Q3UNH3	3.7	0.6	0.3	0.4	0.4	GO:0045454	cell redox homeostasis	18
Dync1h1 dynein 1 heavy chain 1	Dync1h1	Q923F5	21.3	0.5	0.6	0.3	0.4	GO:0007018	microtubule-based movement	12
K/Na-transporting ATPase, alpha-1	Atp1a1	Q8K2R2	4.0	0.6	0.5	0.2	0.4	GO:0006754	ATP biosynthetic process	3
Y box protein 1	Ybx1	P62960	6.3	0.5	0.5	nq	0.5	GO:0006397	mRNA processing	4
Importin 5	Ipo5	Q8BKC5	4.7	0.3	0.6	0.5	0.5	GO:0006886	intracellular protein transport	14
UDP glucuronosyltransferase 1 family	Ugt1a7c	Q6ZQM8	5.3	0.5	0.4	0.5	0.5	GO:0016740	transferase activity	1

Annexin A1	Anxa1	P10107	14.7	0.6	0.6	0.3	0.5	GO:0042127	regulation of cell proliferation	19
Cofilin-1	Cfl2	P45591	6.0	0.7	0.6	0.1	0.5	GO:0005856	cytoskeleton	12
34 kDa protein	Ywhaq	P27348	7.3	0.6	nq	0.4	0.5	GO:0045892	negative regulation of transcription	12
Dynein, light intermediate chain 2	Dync1li2	Q3UGJ3	3.7	0.3	0.5	0.7	0.5	GO:0003774	motor activity	8
Elongation factor 1-gamma	Eef1g	Q8R1N8	6.7	0.4	0.5	0.6	0.5	GO:0003746	translation elongation factor activity	19
Cofilin 2, muscle	Cfl2	P45591	5.3	0.4	0.4	0.8	0.5	GO:0005856	cytoskeleton	12
Ubiquitin-conjugating enzyme E2 L3	Ube2l3	P68037	3.7	0.6	0.7	0.3	0.5	GO:0008283	cell proliferation	16
Moesin	Msn	P26041	9.0	0.6	0.7	0.3	0.5	GO:0008092	cytoskeletal protein binding	X
Nuclear transport factor 2	Nutf2	P61971	1.3	0.5	0.9	0.1	0.5	GO:0015031	protein transport	8
AHNAK nucleoprotein isoform 1	Ahnak	Q09666	56.7	0.4	0.6	0.6	0.5	GO:0007399	nervous system development	19
Stathmin 1	Stmn1	D3Z5N2	4.0	0.8	nq	0.3	0.5	GO:0031115	regulation of microtubule polymerization	4

Table 2. The top-25 over-expressed proteins from the murine Ts16 cerebral cortex. **Name**, the name of the detected protein. **Symbol**, the UniProt symbol of the identified protein. **UniProt ID**, the ID number used to generate Gene Ontology annotations. **Pep (95%) Mean**, the number of distinct peptides from three replicate experiments having at least 95% confidence. Multiple modified and cleaved states of the same underlying peptide sequence are considered distinct peptides because they have different molecular formulas. **114:115 E1-E3,** The ratio of protein expression between trisomic and normal cell line. DS samples (CTb) were labeled with iTRAQ tag 114 and normal samples (CNh) with tag 115. **114:115 Mean**, the mean expression ratio, the average ratio for the protein based on triplicate data. **114:115 Std Dev**, The standard deviation of expression ratios across three replicates. **GO ID**, Gene Ontology identification number. **GO Function**, biological process based on GO ID searching. **Chr**, the chromosome number on which the protein's gene resides. Identified proteins were considered significantly up- or down-regulated when the protein expression level between trisomic and normal cells was ≥1.5 and ≤0.6 respectively and at least two peptides at greater than 95% confidence contributed to the protein identification.

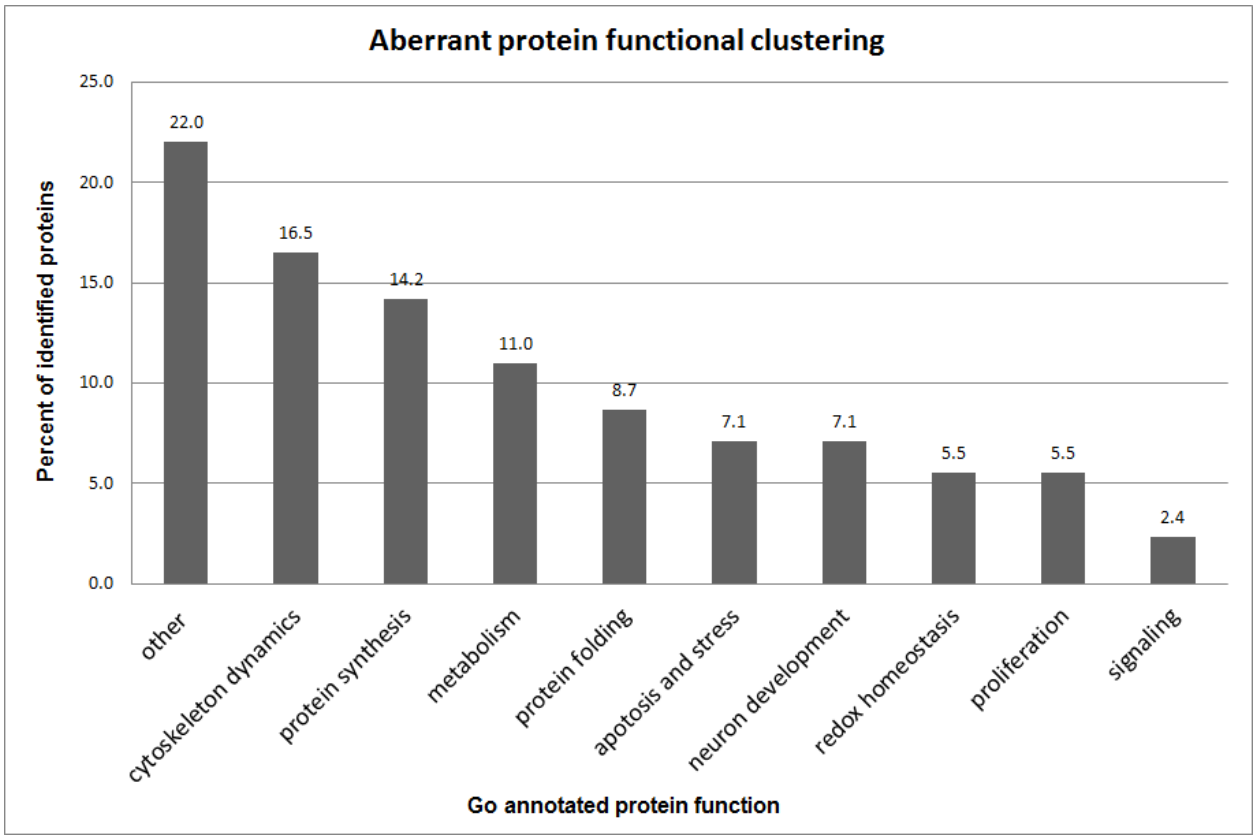

Figure 1. Gene Ontology groupings of the aberrantly expressed proteins. The functional clustering of aberrantly expressed proteins based on Gene Ontology annotations (GO) retrieved from the Mouse Genome Database (MGD) using UniProt protein IDs.

Oxidation-reduction

Aldo-keto reductases A4 (Akr1A4), B3 (Akr1B3) and B8 (Akr1B8) are enzymes with alditol NADP+ 1-oxidoreductase activity and all exhibited increased expression within the CTb cell line compared to the CNh control. Akr1A4 is known to be involved in ascorbic acid biosynthesis and Akr1B3 in the positive regulation of JAK-STAT cascade.

Cytoskeleton

Microtubule associated proteins

iTRAQ analysis confidently identified beta-5 and alpha-1B tubulins both of which exhibited increased protein levels. The microtubule-based motor protein dynein demonstrated low levels of the cytoplasmic heavy chain-1 subunit in the trisomic cell line. However other subunits did not show significant differential expression. No aberrant expression was noted for kinesin, the other microtubule-based motor protein.

Microfilament associated proteins

Although the actin subunits of microfilaments did not show clear changes in expression, many microfilament associated proteins did exhibit differential expression in the trisomic cell line. Ezrin, a microfilament-plasma membrane crosslinking protein, and plastin-3, a microfilament-microfilament crosslinking protein, was significantly up-regulated in the CTb cell line. Cofilin-1, a protein involved in the polymerization and depolymerization of actin subunits, displayed decreased expression.

Intermediate filament associated proteins

Intermediate filament proteins making up the nuclear lamina and vimentin networks were differentially expressed in the trisomic cell line when compared to the control line line. Lamin, which provides nuclear structure and stability, were over-expressed, while vimentin, the most widely distributed intermediate filament protein, was significantly under-expressed.

Nuclear proteins

Nucleophosmin-1 is involved in histone binding and exchange and was down-regulated in the trisomic cell line. A protein involved in nuclear transport, isoform 1 of importin-5, also displayed aberrant expression and was under-expressed in the Ts16 line.

Proteins involved in RNA processing and translation

iTRAQ identified the nucleolar ribosome synthesis and maturation proteins, nucleolin and nucleophosmin, as having decreased expression in the trisomic samples. A trend towards down regulation was noted for the components of the eukaryotic translational machinery within the trisomic cell line. Two translation initiation factors (Eif4a1, Eif5) and 4 elongation factors (Eef1b2, Eef1d, Eef1g, Eef2) all exhibited decreased expression. Increased expression was noted for a valyl-tRNA synthetase. However, of the other tRNA synthetases that were confidently identified, none showed significant differential expression.

Heat shock proteins, molecular chaperones and protein folding

As might be expected, several proteins involved in stress responses and possessing chaperone activities were aberrantly expressed in the trisomic cell line. Strong up-regulation was seen for the constitutively expressed heat shock protein 105 kDa isoform beta (HSP105), and the glucose-regulated heat shock protein A5. Heat shock protein beta-1 isoform B was also consistently over-expressed in the trisomic cells.

Miscellaneous proteins

Three proteins belonging to the large family of calcium dependent phospholipid binding annexins were aberrantly-regulated. Both annexin-1 and annexin-5 exhibited reduced expression, while annexin-2 displayed increased expression in the trisomic samples. Catalase, an antioxidant protein involved in the degradation of hydrogen peroxide within cells, was consistently up-regulated, while a protein involved in metabolism and detoxification of endogenous and xenobiotic compounds, UDP glucuronosyltransferase was down-regulated. Additional over-expressed proteins included peroxiredoxin-6, chloride intracellular channel protein-1 and programmed cell death-6 interacting protein. Other proteins exhibiting decreased expression included sodium/potassium-transporting ATPase subunit alpha-1, interferon-induced guanylate-binding protein-2, bridging integrator-1 isoform-2, myoferlin and AHNAK nucleoprotein.

Discussion

Aberrant protein expression can produce serious phenotypic effects in all organisms. Because of the lack of obvious correspondence between RNA transcripts and protein levels in various cell type[28], by studying proteins a better understanding of the effects of genetic anomalies such as Down syndrome (DS) can be achieved. Previous studies have examined the levels of selected, individual proteins in DS and revealed the over-expression of proteins encoded by genes on human chromosome 21 (Chr21; mouse Chr16). Moreover, it has often been assumed that Chr21 gene products are expressed at 50% excess in DS due to the presence of the third Chr21[29,30,31,32]. It is also widely thought that the majority of genes responsible for the DS phenotype are located in the DS Critical Region on Chr21[33]. More recent expression analysis of chromosome 21 has indicated that gene-dosage compensation may take place and only genes responsible for the DS phenotype are actually over-expressed[34,35] although neither of these two studies gave significant discussion regarding significant under-expression. In the present study, however, we have used a more global and unbiased approach that is not limited to examination of proteins encoded by a specific locus, or even on a specific chromosome, in order to study the dynamics of a complicated expression system. While proteomics methods have been used to study the DS proteome[36], the work presented in the current manuscript employed differential protein expression analysis using isobaric tags for relative and absolute quantification (iTRAQ) coupled to mass spectrometric identification and digital database searching. Our study has added to the body of evidence that suggests DS cannot be fully characterized by limiting the study to regions of only one chromosome, or by examining only over-expressed proteins. The results show that at least 71 proteins were significantly over-expressed and that another 56 proteins were significantly under-expressed in the fetal Ts16 mouse cerebral cortex cell line when compared to its normal counterpart. While this paper does not quantitatively study the so called gene dosage effect observed in DS, the chromosomal locations of each aberrantly expressed protein was mapped and shown in Table 1 and Table 2. No gene-dosage bias toward a particular chromosome (including Chr 16) was found based on a gene per chromosome or gene per chromosome normalized for size in base-pair calculation (data not shown).

Over-expression

Neuron structure and function

Because the brain in DS is adversely affected during development[37] leading to the presence of mental disability in all DS cases, it was of interest to identify abnormally-expressed proteins that could influence brain development. Glutamic acid (Glu) is a potent neuro-transmitter and -activator with long-term effects on the function and structure of neurons[38]. Our iTRAQ analysis showed increased expression of glutamate dehydrogenase (Glud1), a mitochondrial enzyme that enables the production and eventual storage of glu in synaptic vesicles[39] in the trisomic mouse cerebral cortex. The effects of long-term exposure to excessive Glu in the central nervous system were recently demonstrated through transgenic expression of Glud1 in mice[40], which provided evidence of altered synaptic plasticity and neuronal vulnerability. Over-expression of Glud1 and the resulting Glu excess produced mice with neuronal, dendritic spine and nerve ending losses in variable regions of the brain, as well as a pronounced resistance to anesthetic agents that act on glutamatergic synapses. Additionally, the survival of *Glud1* mice into early aging allowed the study of Glud1 over-

expression with increasing age. Age-associated neuronal losses in select brain regions, alterations in dendrite and synaptic structures, abnormal dendrite spine density and diminished long-term potentiation (LTP) of synaptic activity were observed. Altered LTP has previously been noted in DS models, such as the Ts65Dn segmental trisomic mouse[41]. Because LTP is central to long-term memory and the mechanism of learning[42], the alteration of LTP may contribute to the many types of cognitive difficulties experienced by patients with DS, ranging from the simple classical conditioning to higher-level cognition[43].

Endoplasmic reticulum stress and suppression of apoptosis

Heat shock protein A5 (HSPa5, also known as GRP78/BiP), a highly conserved member of the HSP70 family of chaperones[44], was over-expressed in the trisomic cerebral cortex. Abnormal expression of this chaperone has been implicated in mental dysfunction and neurodegeneration[45]. HSPa5 is localized to the endoplasmic reticulum (ER)[46] where it reversibly binds to regions of hydrophobic amino acid residues that are exposed in unfolded lumenal proteins in an effort to restrict aggregation and assist with proper polypeptide folding[47]. Additionally, HSPa5 works in coordination with a number of other proteins to act as a sensor and regulator of the unfolded protein response (UPR)[48], a set of adaptive pathways that is triggered by a disruption of normal ER function and production of misfolded proteins[48]. The UPR alleviates ER stress by arresting general protein translation, up-regulating production of chaperones and folding enzymes, and degrading misfolded proteins[49]. If ER stress cannot be alleviated, the cell proceeds through an apoptotic pathway to protect surrounding tissues from the potential toxic effects of uncontrolled necrosis[50]. An ER-stressed state and activated UPR pathway was also noted to be important in the development of Alzheimer's disease where the accumulation of amyloid B and hyperphosphorylated tau proteins (neurofibrillary tangles) are known to trigger the UPR[51]. This is consistent with the fact that patients with DS develop early onset Alzheimer's by the fourth decade of life. The likelihood of UPR activation in the DS cerebral cortex may be supported by the fact that two translation initiation factors (Eif4a1, Eif5) and 4 elongation factors (Eef1b2, Eef1d, Eef1g, Eef2) were all found to be down-regulated in our study (Table 2). When UPR is activated, the assembly of ribosomal initiation complexes is inhibited resulting in a reduced level of protein translation[48]. While ER stress and UPR have been observed and correlated to neurodegenerative disease such as Alzheimer's and Marinesco–Sjögren syndrome, this is the first report of protein over-expression in DS that suggests that UPR is occurring. In support of the hypothesis that DS neurons experience an ER stressed state, we observed a number of other over-expressed proteins associated with protein folding including Pdia3[52], HSP90ab1[53] and HSP105.

Increased levels of a second heat shock protein, HSP105 (HSP110), were also observed in the trisomic cerebral cortex cell lines when compared to controls. HSP105 is a member of the HSP110 family of molecular chaperones, is highly expressed in the brain[54], is localized to the cytosol and is capable of preventing aggregation of denatured protein both *in vivo* and *in vitro*[55]. HSP105 is thought to be a nucleotide exchange factor for HSP70, a well-studied chaperone and facilitates the release of peptide substrate from HSP70 in an ATP-dependent manner, thereby driving the energy dependant protein folding cycle of the HSP70 chaperone forward[56]. Researchers have also shown that HSP105 inhibits apoptosis induced by heat-stress, serum deprivation, hydrogen peroxide, etoposide and actinomycin D[57]. Yeast two-hybrid screening has identified several other HSP105-interacting proteins, including a-tubulin, dynein light chain 2A, and HSPa5 (discussed above)[58]. Since HSPa5 is involved in

ER function and the UPR, HSP105 may be particularly involved in chaperoning events in the ER, especially responses to ER stress. In the case of a-tubulin interactions, it has been demonstrated that HSP105 can prevent the disorganization of microtubules caused by heat-stress as well as facilitate the reorganization of microtubules *in vivo*[58]. This cytoskeletal organizational role is supported in our study by the discovery of a number of aberrantly expressed cytoskeletal proteins (discussed below).

An additional over-expressed protein identified by our iTRAQ analysis suggests that the trisomic cerebral cortex cells work to prevent apoptosis. Programmed cell death 6-interacting protein *(*PDCD6IP) is thought to participate in programmed cell death as studies using mouse cells have shown over-expression of this protein can block apoptosis. In addition, the product of this gene binds in a calcium-dependent manner to the product of the *PDCD6* gene, a protein required for apoptosis[59]. PDCD6IP also binds to endophilins, proteins that regulate membrane shape during endocytosis.

The presence of up-regulated UPR markers, heat shock proteins and apoptosis suppressors coupled with down-regulated translation factors and ribosomal subunits indicate that the DS neuron senses ER stress and activates UPR to address unfolded protein accumulation while also synthesizing other factors in an effort to maintain an anti-apoptotic state.

Under-expression

Until recently, it was widely accepted that Down syndrome is characterized by the over-expression of genes present on the extra copy of Chr21 (the dosage effect). However, recent studies are producing an emerging body of evidence that gene down-regulation is also responsible for a portion of the Ts21 phenotype and that proteins encoded by chromosomes other than Chr21 also have an influence. A current example with exciting therapeutic potential was shown in studies reporting reduced levels of the transcription factor methyl-CpG-binding protein 2 (MeCP2) in the DS brain due to the action of miR-155 and miR-802, two of the five known microRNAs (miRNAs) that are encoded on Chr21. Decreased MeCP2 expression resulted in the down-regulation of *MEF2C/Mef2c*, a gene that controls neuronal plasticity and development[60]. Silencing of these miRNAs *in utero* resulted in normal range brain MeCP2 levels after only seven days. In the present study we have uncovered a number of down-regulated proteins that may also play a role in the mental retardation and neural dysfunction suffered by all patients with Down syndrome. Levels of these proteins may be targeted by miRNA silencing as potential strategies for intervention.

Cytoskeletal dysfunction

Control of neurogenesis and cell morphology and establishment of interactions with the extracellular environment, require an assortment of cytoskeletal proteins and regulators. Proper development of the cerebral cortex relies fundamentally on the orchestrated interaction of these molecules to control the extremely dynamic neuronal cytoskeleton. Precise control of microtubules, microfilaments, and intermediate filaments is required for proper neuron and glial remodeling which provide normal brain plasticity and provision of the capacity for learning and memory. In the current study, we have identified a number of down-regulated proteins that together normally contribute to the characteristically dynamic structure and function of the neuronal cytoskeleton. A number of syndromes associated with mental retardation, such as Rett and Down syndrome, are characterized by abnormal dendritic structure including reduced dendritic length and decreased dendritic spine density, which

affect the neurons of the cerebral cortex[61]. Our findings help validate results of another recent proteomic study of the DS cerebral cortex, which employed 2-dimensional gel electrophoretic separation of brain tissue and mass spectrometry and identified two down-regulated cytoskeletal proteins. The identified proteins, b-tubulin and septin7, were proposed to have a potential role in neuronal dysgenesis and hampered synaptic function[36]. In corroboration of these findings, our study has also uncovered an additional set of important down-regulated cytoskeletal proteins that could also contribute to neurodysfunction in patients with DS. One of these, moesin (membrane-organizing extension spike protein), is a member of the Ezrin/Radixin/Moesin (ERM) protein family. ERM proteins are localized to the membranous protrusions important for cell-cell recognition and signaling where they act as cross-linkers between plasma membranes and actin-based cytoskeletons. A particularly dynamic region of the neuronal cytoskeleton is found in dendrites, locations where neurons receive and process signals from multiple presynaptic inputs. Both the shape of the dendritic trees and the density and shape of their spines can undergo significant changes during the development and life of a neuron[62]. Dendritic spines are composed mainly of actin microfilaments that undergo a continuous remodeling central to the neuronal circuits that enable memory and learning. Upon activation, moesin undergoes a conformational change that allows binding to both plasma membrane proteins and actin filaments, a process necessary for the remodeling of dendritic spines[63]. It has been shown that a loss of activated moesin significantly suppresses aspects of neuronal development, including dendritic spine initiation and outgrowth[64]. Recent studies have also illustrated the importance of actin-binding proteins such as moesin in steroid hormone regulation of neuronal cell morphology. Through the recruitment of moesin and similar proteins, steroid hormones are able to rapidly signal the actin cytoskeleton and control morphological changes[65]. Supporting research has demonstrated that moesin-related signal transduction pathways initiated by binding of Estrogen Receptor-alpha contribute to the extension and branching of actin filaments during remodeling of the cytoskeleton and membrane that accompanies dendritic spine formation in cortical rat neurons[66]. Due to the relevance of Moesin to cortical neuron development and function, its under-expression in our study may indicate that it could be a possible therapeutic target for *in utero* application.

Dyneins form molecular motors that slide along microtubules and are essential for numerous cellular functions including organelle trafficking, mitosis, virus transport, and cell migration[67,68,69]. Dynein forms a massive multi-subunit complex composed of heavy chains containing motor domains and variable numbers of associated intermediate and light chains. This complex associates with the dynactin protein complex of actin and mediates the attachment of cytoplasmic dynein to cellular cargo[70]. In our study a dynein heavy chain subunit was under-expressed in Ts16 cerebral cortex cells. This improper ratio of the subunits required to form a functional dynein complex could subsequently impair cargo transport. In neurons, dynein is responsible for the rapid transport of membrane-bound organelles, such as signaling endosomes, from the axon terminus to the neuron cell body. Signaling endosomes are formed when target-derived growth factors bind to and activate neuronal receptors and it has been shown that retrograde transport of these organelles is required for neuronal survival and axonal plasticity[71]. Due to its role in the neuron, aberrant dynein expression and its influence on cytoskeletal dysfunction could contribute to the decreased synaptic plasticity and deficient memory and learning in patients with DS. Normal dynein expression is also required for proper flow of spinal fluid in the central nervous system. Ependymal epithelial cells lining the brain ventricles carry motile cilia that generate a laminar flow of cerebrospinal fluid through the cerebral aqueduct and require functional cytoskeletal components for normal

function. In dynein-mutant mice, triventricular hydrocephalus occurs during early postnatal brain development due to a lack of ependymal flow and closure of the aqueduct[72]. While hydrocephalus rarely accompanies DS, a number of case studies are reported in the literature[73,74] and a DS-hydrocephalus mouse model exists[75] which highlights the importance of precise dynein expression for the structural and functional integrity of the brain. Another recent study may link the under-expression of Dynein that we observed in our study with the early onset Alzheimer's disease suffered by almost all DS patients[76]. These researchers demonstrated that siRNA-induced dynein dysfunction caused endocytic pathology and increased levels of Rab GTPases, which is seen in aged monkey brains, as well as significantly disrupted exosome release. Importantly, the induced dynein under-expression also resulted in endosomal β-amyloid precursor protein accumulation. These findings suggest that dynein dysfunction may underlie age-dependent endocytic dysfunction that ultimately leads to Alzheimer disease pathology[77].

A highly conserved phosphoprotein, nucleophosmin-1 (NPM), exhibited reduced expression in our study. NPM is found in nucleoli[78] and plays a key role in ribosome biogenesis, providing the necessary export signals that are required to transport components of the ribosome from nucleus to cytoplasm[79]. In addition, NPM appears to be intimately linked to the processing and assembly of ribosomes based on its nucleus-cytoplasmic shuttling properties and inherent RNAse activity[80]. In addition, it binds nucleic acids[81] and acts as protein chaperone[82]. Its role in the development of maturing ribosomes and regulation of the protein translational machinery[79] suggests that NPM is centrally involved in the orchestration of protein synthesis and proliferation. Additional research has shown that NPM is essential for embryonic development and the maintenance of genomic stability. *Npm* deletion mutants have aberrant organogenesis and die between embryonic day E11.5 and E16.5. It was shown that *Npm1* inactivation leads to unrestricted centrosome duplication and genomic instability, while *Npm1*$^{+/-}$ mice develop features of human myelodysplastic syndrome (MDS), a hematological condition[83]. In addition to MDS, mutated and improperly-localized NPM was found in leukaemic blasts in a high proportion of patients with acute myeloid leukaemia (AML)[84]. Combined, these findings define an essential developmental role for NPM and associate its functional loss with tumorigenesis and MDS pathogenesis. The aberrant expression of NPM we observed in the DS cerebral cortex may correlate to the high occurrence of leukemia[85,86] and the presence of MDS in DS[87].

Experimental Section

Trisomic cell lines

Cerebral cortex cell lines from fetal mice were obtained from Dr. Pablo A. Caviedes, Molecular and Clinical Pharmacology, Faculty of Medicine, University of Chile, (Santiago, Chile). These cell lines were previously established from both normal and Ts16 fetal mice by growth in tissue culture medium supplemented with rat thyroid cell line UCHT1 conditioned medium[16]. Immortalized clones were obtained after 7-8 months in culture, producing the cell lines CNh (derived from normal cerebral cortex), and CTb (derived from Ts16 cerebral cortex), each from an individual animal.

Tissue culture and protein solublization

Each murine cell line was grown in Dulbecco's modified Eagles/high glucose medium (D-MEM, GIBCO, Cat #SH30003.03), containing 25 mM HEPES, 44 mM $NaHCO_3$, 2.86 mM 2-mercaptoethanol, 2 mM L-glutamine, 50 units mL-1 penicillin-streptomycin, and 2 mM sodium pyruvate, at 37º C, in a humidified 5% CO_2 in air atmosphere. Once confluent, the cells were dislodged from the flask surface after treatment with trypsin (GIBCO, Cat #25200-072), at 37º C for 15 min. Trypsinized cells were washed and suspended in growth medium (up to 22.1 mL) and 100 µL of the suspension were removed for counting viable cell number using a Neubaur hemocytometer. Two mL from each sample were seeded into new flasks (for continued growth of the cell lines) and the remaining 20 mL were centrifuged (5,000 x g, 15 min, RT), the supernatant removed and the cells washed by centrifugation with PBS. The pellet was re-suspended in chilled lysis buffer (4.5 M urea, 0.2% SDS) to the appropriate volume to give 2 x 10^7 cells/mL. The cells were sonicated on ice for 1 min at a setting of 1.0 on a model 100 Sonic Dismembranator (Fisher Scientific) fitted with a 5 mm diameter sonic probe. The sonicated samples were microcentrifuged (8,000 x g, 5 min, 4º C) to remove insoluble materials and the lysates were frozen at -80º C until further use. On three different occasions over a three month period, cell lysates were prepared as described from both control and trisomic cell lines.

Protein quantitation, digestion and peptide labeling

Cell lysates were thawed from -80º C and protein concentrations were measured for each sample using a bicinchoninic acid kit (BCA1-1KT, Sigma-Aldrich, St. Louis, MO) with bovine serum albumin as the protein standard. Proteins (100 µg) were precipitated from each sample by addition of nine volumes of ice cold acetone and overnight incubation at 4º C. Following acetone removal, the pellet was re-suspended in 30 µL 0.5 M triethyl ammonium bicarbonate/ 0.2% SDS and the proteins were digested with proteomics grade porcine trypsin (Cat No. V511A, Promega, Madison, WI). The resulting tryptic peptides were labeled with isobaric tags (iTRAQ® Reagents Multiplex Kit, Cat. No. 4352135, Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's instructions. Peptides from proteins of trisomic CTb cells were labeled with isobaric tag 114 and from proteins of control CNh cells with isobaric tag 115. The labeled peptide samples were combined to form a single biological replicate and the process was repeated in two additional instances for a total of 3 biological replicates.

Strong cation exchange high performance liquid chromatography

Peptide mixtures (pooled iTRAQ labeled peptides) were subjected to strong cation exchange (SCX) high performance liquid chromatography (HPLC) before MS analysis. A Vision Workstation (AB, Foster City, USA) was equipped with a polysulfoethyl A, 100 mm X 2.6 mm, 5 µm, 300 Å SCX column (Poly LC, Columbia, MD). The labeled peptide mixtures were adjusted to 2 mL in buffer A (10 mM KPO_4, pH 2.7/ 25% ACN) and injected onto the column. The column was equilibrated for 20 min in buffer A, then a buffer gradient (0-35% buffer B; 10 mM KH_2PO_4, 25% ACN, 0.5 M KCl) was applied over 30 min at a flow rate of 0.5 mL/min. Fractions were collected at one minute intervals after injection, reduced in

volume in a Speed-Vac vacuum concentrator (Savant Instruments, Holbrook, NY) and transferred to autosampler vials (LC Packings, Amsterdam).

Liquid chromatography and tandem mass spectrometry (LC-MS/MS)

Analysis by LC-MS/MS was performed using an integrated Famos autosampler, Switchos II switching pump and UltiMate micro pump (LC Packings, Amsterdam) system coupled to a Hybrid Quadrupole-TOF LC-MS/MS Mass Spectrometer (QStar Pulsar i) equipped with a nano-electrospray ionization source (Proxeon, Odense, Denmark) and fitted with a 10 µm fused-silica emitter tip (New Objective, Woburn, MA). Chromatographic separation was achieved using a 75 µm x 15 cm C18 PepMap Nano LC column (3 µm, 100 Å, LC Packings, Amsterdam). A 300 µm x 5 mm C18 PepMap guard column (5 µm, 100 Å, LC Packings, Amsterdam) was put in place before switching in line with the analytical column and the MS. Water/acetonitrile (98:2 v/v) containing 0.05% formic acid made up the mobile phase (solvent A) for sample injection and equilibration of the guard column at a flow rate of 100 µL/min. Upon switching the trapping column in line, a linear gradient was created by mixing solvent B consisting of acetonitrile/water (98:2 v/v) with 0.05% formic acid, and the flow rate was reduced to 200 nL/min for high resolution chromatography and introduction into the MS. Samples were adjusted to 20 µL with 5% ACN and 3% formic acid then transferred to autosampler vials (LC Packings, Amsterdam). Ten µL of sample were injected into 95% solvent A and equilibrated on the trapping column for 10 min to wash away contaminants. A linear gradient of 95-40% solvent A was developed for 40 min upon switching in line with the MS, followed by an increase of the mobile phase composition to 20% A over 5 min, then a 15 min equilibration with 95% A before the next sample injection. MS data were acquired automatically through Analyst QS 1.0 software Service Pack 8 (ABI MDS SCIEX, Concord, Canada), using an information dependent acquisition method consisting of a 1 second TOF-MS survey scan of mass range 400-1200 amu and two 2.5 second product ion scans of mass range 100-1500 amu. For fragmentation, the two most intense peaks over 20 counts with charge states 2-5 were selected. A 6 amu window was used to prevent peaks from the same isotopic cluster from being fragmented again and, once selected for MS/MS fragmentation, an ion was put on an exclude list for 180 seconds. Curtain gas was set to 23, nitrogen was used as the collision gas and the ionization tip was set to 2700 V. If the A_{215} was > 0.1 for any collected SCX fraction, a 2.5 hour gradient (95-50% solvent A) was used to compensate for the higher peptide concentration in that fraction.

Data processing and analysis

Using ProteinPilot V3.0 (Applied Biosystems Inc,. Foster City, CA, USA), the protein sequences in a *M. musculus* protein database (IPI Mouse ver. 3.71) were trypsin digested *in silico* and the MS/MS fragmentation patterns were predicted for all the resulting peptides. By comparing the predicted peptide fragmentation patterns with those observed during the iTRAQ experiments, the tryptic peptides (and parent proteins) were identified[88]. Protein identification was considered to be confident with an unused Protscore ≥ 1.3 (Conf. ≥ 95%). Greater confidence for the protein identification was gained when a protein was found in more than one biological replicate. For individual peptides, ProteinPilot software calculated the relative abundance of each of the iTRAQ reporter ion fragment masses, which was used to establish a ratio for peptide amount in samples from trisomic versus normal cell lines. The

cumulative signal of the reporter ions from all peptides assigned to a specific protein gave the relative abundance of the given protein when one sample was compared to the other.

Gene ontology (GO) analysis

Identified protein names and IPI accession numbers obtained from the *M. musculus* protein database (IPI Mouse ver. 3.71) were converted to UniProt IDs and symbols with resources (UniProt Knowledgebase) at the UniProt Consortium website, http://www.uniprot.org89. UniProt IDs were used to generate Gene Ontology annotations (GO) and chromosome locations using the Mouse Genome Database (MGD): the Mouse Genome Database Group, http://www.informatics.jax.org[90] (see Figure 1).

Conclusion

Based upon the trisomy 16 mouse model used in this study, aberrant protein expression in the Down Syndrome neuron appears to effect many aspects of cell biology including cytoskeletal development and synaptic plasticity. In addition the neuron protein translation machinery seems to be compromised indicating a delicate balance between ER stress and prevention of apoptotic pathways. The data presented here will allow future hypothesis-driven studies of individual neuronal proteins in an effort to elucidate protein targets which may be useful for the development of DS therapies. Therapies involving such targets may be applied *in utero* or neonatally to improve the health and mental capacity of individuals with DS.

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