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Quantitative Proteomic Analysis Reveals Similarities between Huntington's Disease (HD) and Huntington's Disease-Like 2 (HDL2) Human Brains

Tamara Ratovitski^{*,†}, Raghothama Chaerkady[‡], Kai Kammers[§], Jacqueline C. Stewart[†], Anialak Zavala[†], Olga Pletnikova^{||}, Juan C. Troncoso^{||}, Dobrila D. Rudnicki[†], Russell L. Margolis^{†,⊥}, Robert N. Cole[‡], and Christopher A. Ross^{*,†,⊥,#}

[†]Division of Neurobiology, Department of Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, 600 North Wolfe Street, CMSC 8-121, Baltimore, Maryland 21287, United States

[‡]Mass Spectrometry and Proteomics Facility, Department of Biological Chemistry, Johns Hopkins University School of Medicine, 733 North Broadway Street, Suite 371 BRB, Baltimore, Maryland 21205, United States

[§]Department of Biostatistics, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland 21205, United States

^{||}Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21287, United States

[⊥]Department of Neurology and Program in Cellular and Molecular Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21287, United States

[#]Departments of Pharmacology and Neuroscience, Johns Hopkins University School of Medicine, Baltimore, Maryland 21287, United States

Abstract

The pathogenesis of HD and HDL2, similar progressive neurodegenerative disorders caused by expansion mutations, remains incompletely understood. No systematic quantitative proteomics studies, assessing global changes in HD or HDL2 human brain, were reported. To address this

*Corresponding Authors C.A.R.: caross@jhu.edu. *T.R.: tratovi1@jhmi.edu. Fax: 410-614-0013.

ASSOCIATED CONTENT

Supporting Information

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Supplementary Figures S1–S5, Supplementary tables legends, IPA networks, and pathways shapes key. (PDF) Supplementary Table S1. List of proteins, present in at least one iTRAQ experiment for HD versus control data set. (XLS)

Supplementary Table S2. List of proteins, significantly changed in abundance in HD relative to control brain tissues, annotated by IPA for functional analysis. (XLS) Supplementary Table S3. List of proteins, present in at least one TMT experiment for HDL2 versus control data set. (XLS)

Supplementary Table S4. List of proteins, significantly changed in abundance in HDL2 relative to control brain tissues, annotated by IPA for functional analysis. (XLS) Supplementary Table S5. Comparison of our data to previously published data sets. (XLS)

The authors declare no competing financial interest.

deficit, we used a stable isotope labeling-based approach to quantify the changes in protein abundances in the cortex of 12 HD and 12 control cases and, separately, of 6 HDL2 and 6 control cases. The quality of the tissues was assessed to minimize variability due to post mortem autolysis. We applied a robust median sweep algorithm to quantify protein abundance and performed statistical inference using moderated test statistics. 1211 proteins showed statistically significant fold changes between HD and control tissues; the differences in selected proteins were verified by Western blotting. Differentially abundant proteins were enriched in cellular pathways previously implicated in HD, including Rho-mediated, actin cytoskeleton and integrin signaling, mitochondrial dysfunction, endocytosis, axonal guidance, DNA/RNA processing, and protein transport. The abundance of 717 proteins significantly differed between control and HDL2 brain. Comparative analysis of the disease-associated changes in the HD and HDL2 proteomes revealed that similar pathways were altered, suggesting the commonality of pathogenesis between the two disorders.

Graphical Abstract

Keywords

Huntington's disease; neurodegenerative disorder; proteomics; iTRAQ; TMT; human brain

INTRODUCTION

Huntington's disease (HD) is a progressive autosomal dominant neurodegenerative disorder caused by a CAG expansion mutation in the gene *huntingtin*.¹ While numerous cellular pathways have been found to be disrupted in HD, mechanisms of HD cellular pathogenesis remain largely unknown.^{2,3} Huntingtin (Htt) protein is ubiquitously expressed throughout most tissues; however, brain pathology is the hallmark of HD. Quantitative proteomics of HD human brain tissues directly addresses the changes in protein abundances that occur in the disease state, thus potentially providing insight into the disease mechanism, as well as identifying potential biomarkers for HD.

Another goal of this study was to determine if there are common pathogenic pathways between HD and Huntington's disease-like 2 (HDL2). HDL2 is a rare autosomal dominant, progressive, adult onset neurodegenerative disorder that is genetically, clinically, and pathologically similar to HD and is also caused by a triplet repeat expansion.^{4–10} Both disorders are manifested by a striking cortical and striatal neurodegeneration and the presence of neuronal protein aggregates, which appear similar in structure and are detectable

with antiubiquitin antibodies and with antibodies specific for expanded polyglutamine tracts.⁸ HDL2 is caused by a CTG/CAG repeat expansion (to 40–59 triplets, compared with normal range of 6–28 triplets) in an alternatively spliced exon of the gene *junctophilin-3* (*JPH3*).⁴ As in HD, longer repeats are associated with an earlier onset age.⁵

Proteomics changes in brain tissues have been described in a number of neurodegenerative disorders, as previously reviewed,¹¹ including Alzheimer's disease,^{12–15} Parkinson's disease,^{16,17} amyotrophic lateral sclerosis, and Prion diseases.¹⁸ Previous proteomics studies of HD include the analysis of Htt protein interactome in mouse and cell models^{19–21} and mouse brain proteomics studies.^{22–24} These analyses identified a number of cellular processes and pathways affected in HD, including energy production and metabolism, gene transcription, protein translation, RNA processing, cytoskeleton dynamic, and protein trafficking. There have been reported only a few proteomics studies conducted on human HD brain tissues, including two studies employing a combination of 2D gel electrophoresis and nonquantitative mass spectrometry.^{25,26} In another study, the protein expression changes in human substantia nigra in Alzheimer's disease, HD, and multiple sclerosis were detected using label-free quantitative proteomic analysis.²⁷ However, to date, there are no systematic studies conducted on multiple human brain tissues designed to access and quantify global changes in HD brain proteome using modern methods of quantitative mass spectrometry (MS).

Quantitative MS-based proteomics analysis makes it possible to measure the relative amounts of the proteins present in complex biological samples.²⁸ Labeling methods using isobaric mass tags enable sample multiplexing for identification and direct comparison of the relative amounts of equivalent peptides from proteins present in normal and HD brains. Both iTRAQ (Isobaric Tags for Relative and Absolute Quantitation)²⁹ and TMT (Tandem Mass Tag)³⁰ methods are based on amine-reactive isobaric tagging reagents that label peptides in a mixture of digested proteins. These procedures allow a single MS analysis with sample multiplexing, which can be up to 8-plex in a single iTRAQ or up to 10 in a single TMT experiment. In a recently reported analysis of human brain tissues, the iTRAQ-based approach was successfully used to identify proteomic signatures of different human Prion diseases.¹⁸

In the current work, we used iTRAQ-based quantitative proteomics to assess the changes in protein abundances in the frontal motor cortex of 12 HD and 12 age-matched control cases. The quality of the post-mortem tissues was assessed using two different criteria, enabling us to select only well-preserved tissues. Within each iTRAQ experiment relative protein abundances were quantified by a recently described robust median sweep algorithm,^{31–33} and statistical inference between study groups was addressed using an empirical Bayes framework,^{33,34} allowing the analysis of multiple iTRAQ experiments simultaneously. Our study yielded identification and quantification of 4789 proteins from the human proteome. The MS quantitation has been verified for selected proteins using Western blotting. As a result, we discovered protein expression changes in cellular pathways that have been previously implicated in HD pathogenesis, including Rho-mediated signaling, actin cytoskeleton dynamics, mitochondrial dysfunction, axonal guidance, integrin signaling, vesicular transport, protein folding, DNA/RNA processing, and gene expression. An

additional analysis of HDL2 and control brain samples, using TMT-based quantitation, revealed a significant overlap between HD and HDL2, supporting the idea of the commonality of pathogenesis between the two disorders.

EXPERIMENTAL SECTION

Quality Control of Human Brain Tissues Using Western Blotting for Detection of Htt and β -Tubulin

Total cell homogenates from human superior frontal gyrus (100 mg of frozen brain tissue) of normal controls, HD, and HDL2 cases were prepared by the Dounce homogenization in Triton lysis buffer, containing 50 mM Tris, pH 7.0, 150 mM NaCl, 5 mM EDTA, 50 mM MgCl₂, 0.5% Triton X100, 0.5% Na deoxycholate, Protease Inhibitor Cocktail III (Calbiochem), and Halt Phosphatase Inhibitor Cocktail (Thermo Scientific), followed by centrifugation at 13 000g. Protein concentrations were estimated using BCA method (BioRad). Lysates were precleared by incubating with Protein G-Sepharose beads (GE Healthcare) for 1 h at 4 °C, followed by the incubation (overnight, at 4 °C) with the primary MAB 2166 antibody (Millipore) to immunoprecipitate (IP) normal Htt or with MW1 antibody (a gift from late Paul Patterson) to IP expanded Htt-, and then were incubated with Protein G-Sepharose for 1 h at 4 °C. The IPs were washed three times with the lysis buffer, and protein complexes were eluted from the beads with 2X SDS Laemmli sample buffer (BioRad), fractionated on NuPAGE 4–12% bis-tris polyacrylamide gels (PAGE, GE Healthcare), transferred to PVDF membranes, and probed with antibodies to Htt (MAB2166 or N17, a gift from Ray Truant) and β -tubulin. Immunoblots were developed with peroxidase-conjugated secondary antibodies (GE Healthcare) and enhanced chemiluminescence (ECL-Plus detection reagent, GE Healthcare). Protein bands were visualized using Molecular Imager Gel Doc XR System (BioRad).

Sample Preparation for Proteomic Analysis

The superior frontal gyrus tissues from selected 12 HD and 12 control cases (shown in Table 1) were homogenized and sonicated in a buffer containing nonionic detergents and centrifuged to collect supernatants of the total soluble proteins (as described above). 400 μ g of total protein (as determined using BCA analysis) was precipitated using a 2-D clean-up kit (GE Healthcare). For additional quality control (QC), aliquots of the prepared material were fractionated on NuPAGE 4–12% bis-tris polyacrylamide gels and stained with Coomassie protein stain to ensure the lack of protein degradation during the procedure. These samples (12 HD and 12 controls) were randomized to three 8-plex iTRAQ experiments, permitting quantitative comparison among all of the different samples. For the analysis of HDL2 and control tissues, the superior frontal gyrus tissues from selected 6 HDL2 and 6 control cases (Table 1, in bold) were prepared as described above. These samples were analyzed using TMT labeling.

iTRAQ and TMT Labeling and LC-MS(/MS) Proteomics Analysis

After proteolysis using trypsin, 100 μ g of each proteins sample was dried to 37 μ L, and peptides were labeled with an isobaric tag by adding an iTRAQ reagent (dissolved in 50 μ L of isopropanol) at room temperature for 2 h. After labeling, all samples were mixed and

dried to a volume of 200 μ L and fractionated by basic reverse phase (bRP) liquid chromatography (LC) on an Agilent 1200 Capillary HPLC system using an XBridge C18, 5 μ m 100 $\times$ 2.1 mm analytical column. Each bRP fraction was dissolved in 0.2% formic acid and separated on a C18 column with an 8 μ m emitter tip using 5–40% B (90% acetonitrile, 0.1% formic acid) gradient over 60 min at 300 nL/min. Peptides were fractionated by reverse-phase HPLC on a 75 μ m $\times$ 15 cm PicoFrit column with a 15 μ m emitter (PF3360-75-15-N-5, New Objective, www.newobjective.com) in-house packed with Magic C18AQ (5 μ m, 120 Å, www.unichrom.com) using 0–60% acetonitrile/0.1% formic acid gradient over 70 min at 300 nL/min. Eluting peptides were sprayed (at 2.0 kV) directly into Q-exactive Orbitrap mass spectrometer (www.thermoscientific.com) interfaced with Easy NanoLC 1000 nanoflow system. Survey scans (full MS) were acquired from 350 to 1800 m/z with up to 15 peptide masses (precursor ions) individually isolated with a 1.2 Da window and fragmented (MS/MS) using a collision energy of 31 and 30 s dynamic exclusion. Precursor and the fragment ions were analyzed at 70 000 and 17 500 resolutions, respectively. Peptide sequences were identified from isotopically resolved masses in MS and MS/MS spectra extracted with and without deconvolution using Thermo Scientific MS2 processor and Xtract software. Data were searched against Refseq human 2012 database, specifying sample's species, trypsin as the enzyme allowing one missed cleavage with variable modifications of oxidation on methionine, deamidation on residues N and Q, and 8-plex iTRAQ on tyrosine, and fixed modifications of methylthiomethane on cysteine and 8-plex iTRAQ on lysine and N-term (fixed) using Mascot software (version 2.2, www.matrixscience.com/) interfaced in the Proteome Discoverer 1.4 (<http://portal.thermo-brims.com/>) workflow. Amine reactive 6-plex tandem mass tag reagents (TMT, Thermo Scientific) were used to analyze a second group of samples, including six HDL2 and six control brain tissues. Labeling protocol, identification, and quantitation are essentially the same as described above for iTRAQ labeling, except 6-plex TMT reagents were prepared in 41 μ L of anhydrous acetonitrile and 100 μ L of tryptic digests was added (100 μ g protein from each sample). At the end of 1 h, 8 μ L of 5% hydroxylamine was added to quench the reaction. Data were searched using Refseq human 2012 database, specifying sample's species, trypsin as the enzyme allowing one missed cleavage with oxidation on methionine, deamidation on residues N and Q (variable modifications), and methylthiomethane on cysteine and 6-plex TMT on lysine and N-term (fixed modifications).

Protein Quantitation

The peptides with a confidence threshold 1% false discovery rate (FDR) were considered for analysis. (FDR was identified based on a concatenated decoy database search.) Statistical calculations were performed using R version 3.2.2 (R Core Team, 2015, <https://www.R-project.org/>). In a first step, reporter ion spectra with isolation interference 30% were excluded. Protein log 2 relative abundances were estimated using the method of Herbrich et al.³² In this algorithm, the log 2 reporter ion intensities for each spectrum were “median-polished”; that is, the spectrum median log 2 intensity was subtracted from the observed log 2 intensities. The relative abundance estimate for a protein was calculated as the median of these median-polished data, using all reporter ion intensity spectra belonging to this protein. Adjustments for different sample preprocessing and amounts of material loaded in the channels were carried out by subtracting the channel median from the relative abundance

estimates, normalizing all channels to have median zero. In a final step, proteins that were identified and quantified by reporter ion intensities from only one peptide were excluded.

Statistical Inference

For the HD versus control tissues based on three 8-plex iTRAQ experiments, we fitted for each protein the following linear regression model

$$Y = a + \beta \times X_1 + \gamma \times X_2 + \varepsilon$$

where Y denotes the $\log_2$ relative abundances of protein, X_1 is the tissue type ($X_1 = 0$ for control and $X_1 = 1$ for HD samples), X_2 is the experiment ID, and ε is the error term. The parameter of interest is β , representing the expected difference in $\log_2$ relative abundances of a protein when comparing HD to control tissue samples from the same experiment.

Analogously, for the second analysis of six HDL2 and six control brain tissue samples, we fitted a similar regression model for each protein (here $X_1 = 1$ denotes HDL2 samples). Statistical inference for the slope parameters β was assessed with moderated test statistics and p values (developed by Smyth³⁴ and extended to quantitative proteomics experiments by Kammers et al.³³). Here the observed protein samples variances were shrunk toward a pooled variance estimate to obtain more stable variability estimates. For multiples comparison correction, we calculated q values from the observed p values to control the FDR.³⁵ If a protein has a q value of 0.05, we expect to see 5% among the proteins that show smaller p values to be false-positives. Proteins with calculated q values <0.05 between different tissues types were declared statistically significant.

Functional Enrichment Analysis

Statistically significant ($q < 0.05$) changes were further functionally analyzed using Ingenuity Pathway Analysis (IPA, <http://www.ingenuity.com/>) with Ingenuity Knowledge Base reference set (Genes only) to determine the functional relevance of proteins, either increased or decreased in HD and HDL2 brains compared with match controls. We have determined the enrichment of particular canonical functions or pathways based on the observed number of proteins (whose expression has changed in a diseased brain) for each pathway, relative to the number expected by chance. We also used IPA Pathway Designer and Network analysis tools with overlaying expression values for individual proteins to determine and visualize cellular pathways and networks potentially altered in HD and HDL2 brain. Network analysis included direct and indirect relationships with set 35 molecules per network and 25 networks per analysis. We have also used DAVID Functional Annotation Tool (<https://david.ncifcrf.gov>) for enrichment analysis to highlight the most relevant Gene Ontology (GO) terms associated with proteins altered in HD brain versus controls.

Western Blot Analysis for Verification of Differentially Abundant Proteins

Total cell homogenates from frozen superior frontal gyrus of normal controls and HD cases were prepared by the Dounce homogenization in Triton lysis buffer (described above), followed by centrifugation at 13 000g. Protein concentrations were estimated using BCA method (BioRad). Lysates were fractionated on NuPAGE 4–12% bis-tris polyacrylamide

gels (GE Healthcare), transferred to nitrocellulose membranes, and probed with primary antibodies for 1 h at room temperature. The antibodies against the following proteins were used: glial fibrillary acidic protein (GFAP, R&D Systems); Histone H3 and Histone H2A (Cell Signaling Technology); and Ras homologue family member G (RHOG), serpin peptidase inhibitor, clade A member 3 (SERPINA3), NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 10 (NDUFA10), and β -tubulin (TUBB, Santa Cruz Biotechnology). Immunoblots were developed with peroxidase-conjugated secondary antibodies (GE Healthcare), and enhanced chemiluminescence (ECL-Plus detection reagent, GE Healthcare). Protein bands were visualized using Molecular Imager Gel Doc XR System (BioRad) and quantified using ImageJ software. The mean intensity values were normalized for β -tubulin as a loading control for each group.

RESULTS

Tissue Selection and Quality Control

We have conducted dissections of motor frontal cortex for 32 HD cases, 18 control cases, and 8 HDL2 cases (all available cases) and used two different criteria to assess the quality of the post-mortem brain tissues to select the cases suitable for proteomics analysis.

As our first criteria, we evaluated the integrity of Htt protein using Western blotting after IP of Htt (Figure 1A). We have developed a procedure for IP and detection of endogenous Htt from human brain. Our data indicate that 2166 antibody to Htt shows preference for binding nonexpanded Htt, whereas MW1 antibody (polyQ-specific) selectively binds full-length expanded Htt from HD brains. Therefore, in our QC experiments, 2166 antibody was used to IP normal Htt from control tissues, and MW1 antibody was used to pull down expanded Htt from HD tissues. Htt proteins were then detected with either 2166 antibody (Figure 1A) or with an antibody to the N-terminal of Htt (N17, Figure 1B). We based our selection of cases primarily on the integrity of the high molecular Htt band, which was robustly detected in ~50% of the cases tested, as shown on a representative blot on Figure 1A. In addition, we have also evaluated the integrity of β -tubulin, which in most cases correlated with Htt preservation; however, it appears to be more stable than Htt protein. Thus, in some cases we have observed intact β -tubulin bands, while Htt proteins showed partial degradation. Notably, we did not observe any obvious correlation between the post-mortem delay (see Table 1) and the integrity of Htt protein in brain tissues analyzed. Figure 1B shows 12 HD and 12 control cases, selected for further proteomics experiments. Table 1 summarizes the selected HD, HDL2, and control cases.

As another measure of nonspecific post-mortem protein degradation, we have implemented a new strategy for QC of the samples on an ongoing basis in the MS experiments. Trypsin is used to produce peptides from the protein samples in the MS workflow; however, random proteolytic degradation from post-mortem autolysis may occur at nontryptic sites. We performed database searches specifying a semitryptic enzyme (only one terminus must be a tryptic site) and specifically monitored the relative amount of semitryptic peptides (with the carboxyl terminus not an arginine or lysine) identified from Htt and β -tubulin proteins. This allowed us to compare the relative amount of protein degradation arising from autolysis (nontryptic cleavage) between samples. This approach constitutes an MS-derived definition

of degradation, which can be compared with the Western blotting analysis. Figure S1 shows a comparison of the relative amounts of tubulin β 4A peptides with nontryptic carboxyl termini. Each of the three panels shows distributions of peptide ratios within each of the three iTRAQ experiments. Fold changes contributed by semitryptic peptides were compared across 24 different samples. None of the samples showed increased degradation of β -tubulin. On the basis of the amount of spectral data obtained for β -tubulin in each of three iTRAQ experiments, only 2–5% of it arose from semitryptic peptides, and 95–98% was contributed by tryptic peptides, used for quantification. This reflects a very low degree of protein degradation due to post-mortem autolysis in the brain samples, which have been previously prescreened using a Western blot assay described above. No nontryptic peptides were identified from Htt; however, monitoring another protein, β -actin, showed comparable results in the same samples as for β -tubulin (data not shown).

Protein Abundances Changes in HD versus Control: Quantification and Significance Analysis

The superior frontal gyrus tissues from selected 12 HD and 12 control cases (shown in Table 1) were homogenized and prepared as described in the Experimental Section. For additional QC, aliquots of the prepared material were fractionated on SDS-PAGE and stained with Coomassie protein stain to ensure the lack of protein degradation during the procedure (Figure S2). The samples were randomized to three 8-plex iTRAQ experiments, permitting quantitative comparison among all of the different samples: four HD samples and four control samples were allocated to each iTRAQ experiment. MS, protein quantitation, and statistical analysis were carried out as described in the Experimental Section. In total, 4789 proteins (present in at least one iTRAQ experiment) were identified and quantified based on more than one peptide with a confidence threshold 1% FDR based on a concatenated decoy database search (Table S1). 1211 proteins showed statistically significant changes in protein abundance at FDR of 5% ($q < 0.05$) in HD group relative to control tissues. Figure 2 depicts the results in a volcano plot showing the estimated log 2 fold changes versus $-\log_{10}(p \text{ value})$ for each protein in the HD versus control brain comparison. To evaluate variations within HD and control groups and to visualize strong patterns in our data sets, we performed principal component analysis (PCA) and hierarchical clustering of protein abundances (Figure 3). PCA was performed on 2713 proteins identified and quantified in all three iTRAQ experiments (Figure 3A), and hierarchical clustering was based on the proteins significantly changed in abundances between HD and control within this data set (Figure 3B). Both analyses demonstrate that HD and control tissues are separated into two clusters (except one HD and one control case that cluster together, separately from the rest of the samples), confirming the differential abundances of these proteins in the two categories.

Verification of the Changes in Protein Abundances in HD versus Control Using Western Blot

To validate the quantitative proteomics results, we performed Western blotting for a selected group of proteins using HD and control superior frontal gyrus tissues, randomly selected from the pool of tissues used for MS (Figure 4). For verification, we chose two proteins with the highest fold change between HD and control brains: Glial fibrillary acidic protein (GFAP) and serpin peptidase inhibitor, clade A (SERPINA3). In addition, we chose proteins

representing top pathways enriched in proteins significantly changed in HD cortex versus control (see next sections of Results): histones H3 and H2A, known to be involved in regulation of gene transcription; ras homology family member G (RHOG), representing Rho family GTP-ases signaling pathway; and NADH:ubiquinone oxidoreductase subunit NDUFA10, a known subunit of mitochondrial complex I. The results demonstrate a good correlation between MS-based quantitation and Western blotting analysis (Figure 4C).

Functional Enrichment Analysis of Changes in Protein Abundances in HD versus Control

1211 proteins, which showed statistically significant changed in abundances between HD and control groups, were further analyzed using IPA to determine their functional grouping. The list of proteins annotated by IPA for functional analysis (1167 proteins) is shown on Table S2. Notably, 121 of these proteins were classified by IPA as related to HD (“Diseases and Functions” analysis, Figure S3). The IPA functional analysis with Ingenuity Knowledge Base reference set (genes only) determines the enrichment of particular canonical pathways or cellular function in the proteins from the data set (in this case, differentially abundant in HD brain), relative to the number expected by chance. We used “Canonical Pathways” analysis to determine whether proteins, changed in abundance in HD brain, belong to predefined pathways. Figure 5 shows the top pathways identified by the IPA sorted by the enrichment *p* values, which are calculated by IPA using Fisher’s exact test and are reflecting enrichment in differentially abundant proteins within each pathway. Activation *z* score, calculated by IPA, is a statistical measure of the match between expected relationship direction and observed changes in protein expression (*z* score >2 or <-2 is considered significant). This analysis suggests that signaling by Rho proteins, actin cytoskeleton signaling, and mitochondrial dysfunction pathways appear to be most altered in HD. Notably, our previous proteomics analysis of HD-induced pluripotent stem cells (iPS) cells showed alterations of an overlapping set of pathways³⁶ (and unpublished data), including actin cytoskeleton, integrin and Rho-mediated signaling, axon guidance and semaphorin pathways, mitochondrial dysfunction, and 14-3-3-mediated signaling.

Next we analyzed only proteins that were found to be statistically significant less abundant in HD. “Diseases and Functions” analysis by IPA pinpoints “Metabolic Disease” category as a top disease, most significantly enriched in these proteins. Figure 6 shows a network of proteins related to mitochondrial disorder, complex I deficiency, and oxidative phosphorylation deficiency, which are less abundant in HD brain (shown in green). These proteins include 12 mitochondrial complex I NADH dehydrogenase components and several other mitochondrial-related proteins, as determined by IPA. Mitochondrial dysfunction has been previously implicated in HD pathogenesis.^{37–41} Protein transport and exocytosis proteins are also deficient in HD cortex (Figure 7), consistent with evidence that Htt facilitates vesicular trafficking and protein transport.^{3,42,43}

We also used IPA Pathway Designer and Network analysis tools with overlaying expression values for individual proteins to determine and visualize cellular pathways and networks potentially altered in HD brain. Network analysis included direct and indirect relationships with 35 molecules per network and 25 networks per analysis. Examples of such analysis are described below. “Cell death and protein folding network” is particularly enriched in

proteins more abundant in HD cortex, consistent with evidence that protein misfolding contributes to HD pathogenesis,³ possibly as a part of compensatory changes in HD (Figure 8). Gene transcription and RNA processing have been identified as key pathways potentially disrupted in HD.^{20,44–50} A related network, including proteins involved in DNA replication and repair and RNA processing (Figure 9), is prominently changed in HD (up-regulation of histone family members and down-regulation of heterogeneous nuclear ribonucleoproteins (HNRNPs)). Interestingly, three closely related RNA-binding proteins, FUS/TLS (Translocated in liposarcoma), EWSR1 (Ewing sarcoma breakpoint region 1), and TAF15 (TATA-binding protein-associated factor 15), each harboring both RNA-recognition motif (RRM) and prion domains and recently implicated in amyotrophic lateral sclerosis (ALS) and other neurodegenerative disorders,^{51–54} are more abundant in HD.

In addition to IPA, we used the Database for Annotation, Visualization and Integrated Discovery (DAVID) Functional Annotation Tool to highlight the most relevant Gene Ontology (GO) terms associated with proteins altered in HD brain (Figure 10). Both Functional Annotation Chart (Figure 10A) and Functional Annotation Clustering report (which groups/displays similar annotations together, Figure 10B) highlight axonal formation, cytoskeletal actin binding, mitochondrial function, vesicular transport, and protein folding as major functions that may be altered in HD. Consistent with IPA Network analysis (Figure 9), Functional Annotation Clustering analysis, which includes only proteins with fold change above 1.2, reports chromatin/nucleosome assembly and organization (which includes ~10% of proteins analyzed) as the most enriched cluster (enrichment score 3.31, *p* values range from 10^{-8} to 10^{-5}).

By comparing our data to previously published gene profiling study of human cortex,⁴⁴ we found several similar biological processes and pathways showing significant enrichment in dysregulated proteins. As shown in table 5 of the above study,⁴⁴ the most significantly enriched GO biological processes with the highest proportion of dysregulated probe sets in BA4 cortex include molecular transport, microtubule-based movement, metabolism, glycolysis, protein transport, glucose metabolism, and endocytosis. The same pathways appear to be also dysregulated in our study (Figures 5–7)

Protein Abundances Changes in HDL2 versus Control

To compare relative protein abundances between the six HDL2 and six control cases, we prepared superior frontal gyrus tissue (Table 1, in bold) as described for HD/control comparisons. These samples were labeled with TMT reagents and subjected to MS. Protein quantification and statistical analysis were performed as described in the Experimental Section. 5567 proteins were detected in at least one TMT experiment, based on more than one peptide with a confidence threshold 1% FDR based on a concatenated decoy database search (Table S3). Figure 11 depicts the results in a volcano plot showing the estimated log₂ fold changes versus $-\log_{10}$ (*p* value) for each protein in the HDL2 versus control brain comparison. The abundance of 717 proteins was significantly different at 5% FDR (*q* < 0.05) in HDL2 cortex compared with control cortex. PCA, performed on 3101 proteins identified and quantified in all TMT experiments (Figure 12A), and hierarchical clustering, based on the proteins significantly changed in abundances between HDL2 and control within

this data set, demonstrate that HDL2 and control tissues are well-separated into two clusters (Figure 12B).

The functional grouping of 717 proteins that significantly differed between HDL2 and control cases ($q < 0.05$) was determined using IPA. The list of proteins annotated by IPA for functional analysis (707 proteins) is shown in Table S4. IPA “Canonical Pathways” analysis (Figure S4) shows that the top pathways were similar to those detected in HD: partially overlapping integrin, actin cytoskeleton, and Rho-mediated pathways as well as axonal guidance and endocytosis, suggesting that these pathways might be altered in both disorders

Comparative Analysis of the HD and HDL2 Proteomes

A total of 2341 proteins common to both HD and HDL2 cases were detected (Figure 13A), and 457 of these common proteins were significantly changed relative to control in both disorders (Figure 13B): 245 were up-regulated and 198 were down-regulated in both HD and HDL2 (Figure 13C,D). This is equivalent to 96% of concordant changes observed among the proteins that changed in abundance in both disorders relative to control.

The large extent of the overlap between the two disorders was also evident from the comparative functional analysis (using IPA) of the changes observed in HD and HDL2. Figure S5A highlights the top canonical pathways defined by proteins that differed in abundance from control in both HD and HDL2, sorted by p values that determine the likelihood of pathway enrichment relative to chance. Figure S5B depicts canonical pathways sorted based on the activation z score (z score >2 or <-2 is considered significant). Most (but not all) of the shown pathways are predicted to be either activated or inhibited concordantly in HD and HDL2 groups. The “Upstream analysis” (Figure S5C), designed to predict which regulators caused changes in protein expression and the directional state of the regulator, also demonstrates a large degree of functional overlap between HD and HDL2. For example, the BDNF is predicted to be inhibited, while p53 and CREB are predicted to be activated in both disorders.

Figure 14 depicts a closer look at the actin cytoskeleton pathway (the most enriched in the proteins that differed from control in both HD and HDL2) with overlaying expression changes of individual proteins in HD (Figure 14A) and HDL2 (Figure 14B). We observed a prominent overlap in HD and HDL2 proteins altered in this pathway, with some notable differences, for example, (WAVE)-complex member WASF2 is up in HD while it is unchanged in HDL2, whereas Rho GTP-ase signaling components BAI1-associated protein BIAP2 and cytoskeletal adaptor protein abl-interactor 2 (ABI2) are down in HDL2 and unchanged in HD. These differences, however, do not change the overall IPA predictions of the activation of this pathway in both disorders.

DISCUSSION

In this study we quantified and compared relative protein abundances in cortical post-mortem tissues from HD, HDL2, and normal control cases. The control of tissue quality is an important concern in the quantitative proteomics analysis of post-mortem tissues, as post-mortem autolysis might lead to biases favoring more intact samples or more stable proteins.

We used a few approaches that enabled us to select well-preserved tissue. First, all tissues were prescreened for the presence of intact Htt protein using Western blotting (Figure 1). Because Htt is a fairly large protein, its integrity presumably would indicate less protein degradation overall. Notably, only ~50% of the tissues tested passed this initial screen, with no obvious correlation to the post-mortem delay. This suggests the value of the initial evaluation of the quality of post-mortem tissues. As another QC step, the integrity of β -tubulin was also evaluated, which in most cases correlated with Htt protein preservation. In some cases we have observed intact β -tubulin bands, while Htt proteins showed partial degradation. To ensure the lack of protein degradation during the preparation of samples for MS, aliquots of the prepared material were fractionated on SDS-PAGE and stained with Coomassie protein stain (Figure 2S). In addition, an MS-based QC method was implemented to assess the relative degree of protein degradation across all samples (Figure S1). None of the samples, previously prescreened using a Western blot assay and selected for this study, showed increased degradation of β -tubulin, measured as the relative amount of semitryptic peptides present. Thus, our QC workflow has ensured that only high-quality material was used for quantitation of protein abundances.

Another important issue in quantitative proteomics is normalization of protein abundances to overcome experimental variability in sample preparation and in the detection of the peptides by mass spectrometer. This normalization is especially crucial when several proteomics experiments are needed for the analysis of multiple samples. We have calculated relative protein abundances in each iTRAQ/TMT experiment separately using a robust “median-sweep” approach proposed by Herbrich et al.^{31,32} Effectiveness of the quantitative proteomics approach used in this study was validated by Western blotting for a selected group of proteins, demonstrating a good correlation between iTRAQ-based quantitation and Western blotting analysis. Supporting relevance of our studies, proteomics analysis of HD versus control cortex revealed changes in protein abundances within several cellular pathways that have been previously implicated in HD pathogenesis, including metabolic dysfunction, cytoskeleton signaling, molecular trafficking, RNA processing, and gene transcription.^{2,3}

We have also directly compared our list of proteins significantly changed in HD (Table S2) to previously published data sets from several previous gene profiling, proteomics and Htt interactions studies (Table S5). Table S5 lists around 200 proteins that changed in abundances in our study and in at least one of the three transcriptomics^{44,55,56} or two proteomics studies,^{26,27} or have been identified as Htt interactors in one out of two previous Htt interaction studies.^{21,57} The most comparable to our work is the gene profiling study by Hodges and coworkers⁴⁴ performed on several human brain regions, including cortex. We have directly compared our data set with the top 30 differentially expressed mRNAs⁴⁴ and found 18 proteins in our data set that changed in the same direction and 6 that changed in the opposite direction, as in the above mRNA list. The recently published integrated genomics and proteomics study by Langfelder et al.⁵⁵ is focused on HD mouse models and therefore is not directly relevant to our study; however, it includes a list of 25 genes⁵⁵ with concordant changes observed in the mouse and at least three out of four previously published human patient data sets. Out of these 25 genes, we found 11 corresponding proteins that changed concordantly. In addition, the most recent RNA-seq study, performed on comparable human

brain region,⁵⁶ includes 224 differentially expressed genes in HD versus controls.⁵⁶ The common genes between the above and our study are also included in Table S5. We also include comparisons to the only two relatively relevant proteomics studies on human HD brain.^{26,27} Out of 18 proteins found differentially expressed in HD cortex by Sorolla and coworkers,²⁶ 6 proteins are also present in our data set with concordant changes. Interestingly, 21 proteins (or their subunits/isoforms) out of 102 Htt primary interactors found in yeast two-hybrid (Y2H) and affinity purification screens⁵⁷ and 135 out of 576 Htt interactors found in HD mouse brain²¹ significantly changed in abundance in our study between HD and controls. Overall, these comparisons demonstrate a fairly substantial degree of overlap between our data and previous “omics” studies of HD brain.

Our functional analysis suggests that mitochondrial dysfunction pathway appears to be one of the most altered in HD. Mitochondrial dysfunction in HD has been well-documented.^{37–41} Mutant Htt directly associates with mitochondria, impairs mitochondrial dynamics, and decreases mitochondrial function in affected in HD brain regions.^{40,41,58} In line with these reports are our previous findings that expanded Htt complexes from striatal cell line were particularly enriched in interacting proteins with mitochondrial function and localization.²⁰ Among these we have identified several components of mitochondrial complexes I, III, and IV. Several previous studies from other laboratories showed deficiency of mitochondrial complex II in the striatum of HD patients.^{59,60} Complex II defects were also demonstrated in cultured striatal neurons and in HD animal models expressing N-terminal fragments of mutant Htt.^{61,62} In our current proteomics analysis we found that components of mitochondrial complex I (NADH dehydrogenase) were significantly less abundant in HD post-mortem brain compared with controls (Figure 6 and Table S2). These include several core subunits (NDUFA 3, 9–11, NDUFB 3 and 8, and NDUFV1) and assembly factors (NDUFAF1, 3, 4, and 7 and NDUF5 1–3, 7, and 8). The results were confirmed by Western blotting for NDUFA10. Mutations in the complex I enzymes causing mitochondrial deficiencies have been linked to Leigh syndrome and other early onset neurodegenerative disorders.⁶³ In addition, we also found that a subunit of complex II (succinate dehydrogenase), SDHA, subunits of complex III (cytochrome *bcl*), UQCRC1 and 2, and a component of cytochrome *c* oxidase, SCO2 (complex IV), were also less abundant in HD cortex relative to controls. Overall, the observed changes in the protein levels in HD brains are consistent with the current view of the mechanisms of mitochondrial dysfunction in HD, such as a decrease in the enzymes of pyruvate dehydrogenase complex (PDHC),⁶⁴ inhibition of the respiratory chain enzymes (described above), increased oxidative stress⁶⁵ (superoxide dismutase 1, SOD1, is increased), decrease in brain-specific creatine kinase (CKB),⁶⁶ and inhibition of mitochondrial protein import by mutant Htt⁶⁷ (TIMM44 and TIMM8A, components of presequence translocase-associated motor complex, PAM, are decreased). The observed changes in six family members of the motor protein kinesin and dynein and changes in abundance of mitochondrial fission factors MFF, MFN2, and FIS1 are also consistent with the idea that mutant Htt may dysregulate mitochondrial dynamics and trafficking.^{40,41}

We found that among the top pathways most significantly enriched in differentially abundant proteins (between HD and control) are interconnected integrin, actin cytoskeleton and Rho proteins signaling pathways (Figure 5). A number of neurodegenerative disorders have been

linked to the dysfunction of the cytoskeleton.⁶⁸ Numerous cytoskeleton-associated proteins have been previously found among Htt interactors, including Htt-associated protein 1 (HAP1),⁶⁹ Httinteracting protein 1 (HIP1),⁷⁰ tubulin,⁷¹ dynactin,^{72,73} and F-actin.⁷⁴ Htt was also demonstrated to be directly involved in nuclear actin remodeling and to localize within nuclear cofilin–actin rods during the stress response.⁷⁵ A comprehensive analysis of Htt protein interaction network derived from the yeast 2-hybrid screens demonstrated that Rho, actin cytoskeleton, and integrin were among the most significant pathways enriched in Htt interactors.^{57,76} In addition, integration of Htt protein interaction with gene expression data from postmortem HD brain⁴⁴ showed that Rho and actin signaling pathways were also enriched in dysregulated genes in HD and highlighted the role of Rho family GTP-ase signaling proteins in HD pathology. Rho GTP-ase signaling components BAI1-associated protein (BAIAP2), ezrin (EZR), phosphatidylinositol 3-kinase regulatory subunit alpha (PIK3R1), p21 protein (Cdc42/Rac)-activated kinase 2 (PAK2), and Ras-related C3 botulinum toxin substrate 1 (RAC1) were also found to modify mutant Htt toxicity in striatal HD cells.⁵⁷ Our proteomics analysis indicates that changes in the HD brain are consistent with the dysfunction of actin cytoskeleton remodeling, as several Rho family key proteins involved in this pathway were found to be dysregulated in HD cortex (Figure 14A), including increased PAK2, Wiskott-Aldrich syndrome protein family (WAVE)-complex member WASF2, and actin-related protein 2/3 complex (ARPC5). Actin polymerization-regulating proteins profilin1 (PFN1 and 2) and cofilin (CFL1) and proteins linking cytoskeleton to the plasma membrane ezrin (EZR), radixin (RDX), and moesin (MSN) were all found to be more abundant in HD brain. Our previous proteomics analysis of the HD iPS cells also showed that actin cytoskeleton, integrin, and Rho-mediated signaling and related axon guidance pathways were enriched in the proteins that changed levels in HD cells relative to control³⁶ (and unpublished data).

Htt is known to regulate cytoskeletal trafficking, including vesicle transport, partially through its interactions with HAP1, Htt-associated protein of 40 kDa (HAP40), and dynein.⁴² Normal function of Htt is thought to include facilitation of microtubule-dependent vesicular transport of brain-derived neurotrophic factor (BDNF), and this process was reported to be disrupted in HD neurons.⁴³ The deregulation of BDNF gene transcription was also reported in HD neurons.⁷⁷ As a result, BDNF levels appear to be reduced in HD patients, as reviewed elsewhere.⁷⁸ Consistent with documented BDNF deficiency in HD, our “Upstream Regulator Analysis” (using IPA) showed that BDNF pathway is predicted to be inhibited because 17 of 25 proteins expression change (HD vs control) consistent with inhibition of BDNF (z score -2.39 , overlap p 1.8×10^{-6}). In addition, the observed decrease in three dynein subunits and changes in the abundance of six kinesins support the previously suggested role of expanded Htt in the regulation of retrograde and anterograde transport of BDNF vesicles in neurons.⁴³

Gene transcription, RNA processing, and splicing abnormalities have been previously associated with HD based on several expression profiling studies in various HD models.^{20,44–50} Here we found a striking up-regulation of 10 histone proteins and down-regulation of 7 HNRNPs (Figure 9 and Table S2). Another interesting finding is that three proteins of FET family, FUS/TLS, EWSR1, and TAF1, containing both RNA-recognition motif and prion domain, are more abundant in HD brain. These DNA/RNA-binding proteins

have been shown to function in both RNA polymerase II-mediated transcription and pre-mRNA splicing, possibly connecting these two processes.⁵⁴ FET proteins interact with general transcription factors and subunits of RNA polymerase II via their amino termini while interacting with splicing factors and components of spliceosome via their carboxy-termini.⁵⁴ Recently, a prion-related mechanisms have been suggested in the pathogenesis of neurodegenerative disorders linked to protein misfolding and aggregation, including Alzheimer's disease, Parkinson's disease, and HD.⁵² Notably, proteins containing prion-like domains were shown to be enriched in proteins that also harbor RRM RNA-recognition motif.⁵² This group of proteins, which include FUS/TLS, EWSR1, and TAF1, is rapidly emerging in connection with ALS, frontotemporal lobar degeneration (FTLD), and other neuro-degenerative diseases.^{51–53}

One of the goals of this study was to explore potential common mechanisms of pathogenesis shared between HD and HDL2 disorders. HDL2 is often considered as a genocopy of HD, because the genetic, clinical, and neuropathological features of the two diseases are similar, although they are associated with different genetic mutations.^{4–10} Emerging evidence obtained using cellular and animal models and human post-mortem tissue indicates that the pathogenesis of both HD and HDL2 disorders may include multiple factors and gain- and loss-of-function mechanisms. Partial loss-of-function mechanisms were proposed for both HDL2 and HD pathogenesis. Normal function of JPH3 protein, mutated in HDL2, includes facilitation of junction structures between plasma membrane and endoplasmic reticulum to provide a crosstalk between cell surface and intracellular ion channels.^{79,80} JPH3 impairment due to the expansion of the CUG repeat and reduced JPH3 expression was suggested as one of the molecular mechanisms that contribute HDL2 pathogenesis;^{79,81} however, the loss of JPH3 causes motor deficits but not intranuclear inclusions or neurodegeneration,⁷⁹ therefore suggesting that impairment of JPH3 function alone cannot account for all features of HDL2 pathogenesis. In HD, partial loss of normal Htt function in facilitation of molecular transport has been demonstrated as well. However, the central hypothesis of HD pathogenesis is a gain of toxic function of an expanded polyglutamine protein, Htt, expressed from a CAG-expanded transcript.³ Polyglutamine protein toxicity was also demonstrated in BAC-HDL2 mouse model.⁸² Thus, the gain-of-function mechanism mediated by expanded polyglutamine may be shared between the two disorders. Notably, an antisense transcript containing CUG repeats was also detected at HD locus, and it was demonstrated to regulate *HTT* expression.⁸³

Another common pathogenic mechanism suggested for both HDL2 and HD involves RNA toxicity of CAG/CUG expanded transcripts. RNA transcribed from CUG JPH3 sense strand was shown to form RNA foci that can sequester a splicing regulator protein muscleblind-like 1 (MBNL1) and interfere with its function.^{84,85} RNA-triggered mechanism of toxicity is also likely to be involved in HD, including the formation of hairpin structures demonstrated for *HTT*CAG-expanded transcript, causing sequestration of RNA-binding proteins, such as MBNL.^{86–90} In agreement with the contributory role of RNA toxicity, we found that in both HD and HDL2 a number of heterogeneous ribonucleoproteins, involved in RNA processing, were less abundant relative to controls.

Reflecting the common aspects of HD and HDL2 pathogenesis, described above, it is likely that shared pathways may be disrupted in both disorders. In fact, our proteomics analysis revealed several pathways enriched in proteins that changed concordantly in both HD and HDL2, compared with control, such as integrin, actin cytoskeleton, and Rho-mediated pathways as well as axonal guidance and semaphorin pathways, endocytosis, and DNA/RNA processing. Notably, one difference is that widespread dysregulation of the proteins related to mitochondrial dysfunction was observed in HD but not in HDL2 brains. Thus, our analysis highlights the points of convergence as well as differences between HD and HDL2, which may help us to elucidate the pathogeneses of both disorders.

CONCLUSIONS

We have demonstrated that post-mortem brain tissue is suitable for quantitative proteomics analysis, emphasizing the importance of QC steps to minimize variability due to post-mortem autolysis. We conducted analysis of multiple samples randomly distributed among several proteomics experiments and consolidated quantitative proteomic data from multiple instrument runs using a new statistical method.^{31–33} Our proteomics data were verified using Western blotting for a selected group of proteins. Our findings provide partial confirmation for previously identified pathogenic pathways in HD, suggest additional pathways of potential pathogenic significance, and support the value of isolating pathogenic pathways common to HD and HDL2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Quality control of human brain tissues using Western blotting. (A) Total cell homogenates from frozen superior frontal gyrus of normal controls and HD cases were prepared by homogenization and sonication, as described in the Experimental Section.

Immunoprecipitations (IPs) of Htt proteins were carried out using 100 mg of frozen brain tissue and MAB 2166 or MW1 antibodies to IP normal Htt or expanded Htt. Blots were probed with antibodies to Htt (MAB2166, top panels). β -Tubulin expression was also evaluated in the lysates (bottom panels). The cases shown in red were selected for mass spectrometry. Representative gels are shown. (B) 12 HD and 12 control cases selected for

further proteomics experiments. The brain tissues were processed as in panel A using MAB2166 and MW1 antibody for IP of Htt proteins and N17 antibody (Truant Lab) for detection.

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Figure 2.
“Volcano-plot” HD versus control. Inference based on three 8-plex iTRAQ experiments with four HD and four control samples each (frontal motor cortex) and 4789 proteins in total. The volcano plot shows the estimated log 2 fold changes (x axis) versus $-\log_{10} p$ values (y axis) for each protein.

Figure 3.

Principal component analysis (PCA) and hierarchical clustering of protein abundances in HD versus control group. PCA was performed on 2713 proteins identified and quantified in all three iTRAQ experiments (A), and hierarchical clustering was based on 1156 proteins significantly changed in abundances between HD and control within this data set (B).

Figure 4.

Validation of expression of selected proteins by Western blotting. (A) Total cell homogenates from frozen human superior frontal gyrus of normal controls and HD cases were prepared as described in the Experimental Section. Lysates were fractionated on NuPAGE 4–12% bis-tris polyacrylamide gels, transferred to nitrocellulose membranes, and probed with the indicated antibodies. Protein bands were visualized using Molecular Imager Gel Doc XR System and quantified using ImageJ software. (B) Graphs show the mean intensity values ($\pm$ SEM), normalized for β -tubulin as a loading control, for each group. The number of cases used in each group is indicated (n). (C) Comparison of the fold change HD

versus control obtained using Western blotting and quantitative MS (iTRAQ) for indicated proteins.

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Figure 5.

IPA analysis of top canonical pathways altered in HD. Top cellular pathways (as determined by IPA) most significantly enriched within the proteins that changed in abundance (both up and down) in HD brain versus control. $-\log_{10}$ overlapping p values are calculated by IPA using Fisher's exact test and are reflecting the enrichment of particular canonical pathways based on the observed number of proteins (whose expression has changed in HD brain) for each pathway, relative to the number expected by chance. Activation z score, calculated by IPA, is a statistical measure of the match between expected relationship direction and observed changes in protein expression (z score >2 or <-2 is considered significant). Only

proteins with statistically significant changes at a FDR of 5% ($q < 0.05$) were included in the analysis.

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Figure 6. Proteins related to “Metabolic Disease” are less abundant in HD brain. “Metabolic Disease” identified as a top disease in “Diseases and Functions” analysis (IPA), which includes the proteins significantly less abundant in HD (FDR of 5%, $q < 0.05$), shown in green. The key to IPA pathways and networks shapes is included in the Supporting Information.

Figure 7. Proteins related to “Protein Transport” are less abundant in HD brain. “Diseases and Functions” analysis (IPA), which includes the proteins significantly less abundant in HD (FDR of 5%, $q < 0.05$), shown in green. The key to IPA pathways and networks shapes is included in the Supporting Information.

Figure 8.

Proteins involved in cell death and protein folding are more abundant in HD brain. IPA “Network Analysis” including direct and indirect relationships with set 35 molecules per network and 25 networks per analysis. “Cell death and protein folding network” is significantly enriched in proteins more abundant in HD brain. Only proteins statistically significantly more abundant in HD versus control (FDR of 5%, $q < 0.05$), shown in red, were included in analysis. The key to IPA pathways and networks shapes is included in the Supporting Information.

Figure 9.

Proteins involved in gene expression and RNA processing are dysregulated in HD brain. IPA Network analysis including direct and indirect relationships with set 35 molecules per network and 25 networks per analysis. “DNA replication and repair and RNA processing”—Two merged networks enriched with proteins altered in HD are shown. Proteins increased in abundance are in red, and decreased are in green. Proteins (not changed in our data set) predicted by IPA to be activated are in orange; predicted to be inhibited are in blue. Only proteins with statistically significant changes at a FDR of 5% ($q < 0.05$) were included in the analysis. The key to IPA pathways and networks shapes is included in the Supporting Information.

Figure 10.

DAVID Functional Annotation Tool enrichment analysis of the proteins that changed in abundance (both up and down) in HD brain versus control. Functional annotation Chart (A) and Functional Annotation Clustering report (B) demonstrating $-\log_{10}$ of p value for enrichment of GO terms listed and % of proteins involved in this term relative to all proteins analyzed. The overall enrichment score for the group of terms is shown at the bottom of panel B. Selected GO terms are shown. Only proteins with statistically significant changes at a FDR of 5% ($q < 0.05$) were included in the analysis.

Figure 11. “Volcano-plot” HDL2 versus control. Inference based on three 6-plex TMT experiments with two HDL2 and two control samples each. The volcano plot shows the estimated log 2 fold changes (x axis) versus $-\log_{10} p$ values (y axis) for each of the 5567 proteins.

Figure 12.

Principal component analysis (PCA) and hierarchical clustering of protein abundances in HDL2 versus control group. (A) PCA, performed on 3101 proteins identified and quantified in all TMT experiments, and (B) hierarchical clustering, based on 1004 proteins with statistically significant changes in abundances at a FDR of 5% ($q < 0.05$) between HDL2 and control within this data set.

Figure 13.

Venn diagrams. Comparison of the changes in protein abundances observed in HD versus control and HDL2 versus control experimental groups. (A) Overlapping proteins detected in all iTRAQ experiments (for HD) and in all TMT experiments (for HDL2). (B) Overlapping proteins that changed in abundance within HD and HDL2 groups (relative to control). (C) Overlapping proteins that increased in abundance within HD and HDL2 groups (relative to control). (D) Overlapping proteins that decreased in abundance within HD and HDL2 groups (relative to control).

Figure 14.

Actin cytoskeleton pathway is enriched with proteins concordantly changed in the abundances in both HD and HDL2, relative to control. Shown are overlaying expression changes of individual proteins in HD (A) and HDL2 (B) relative to control. Proteins increased in abundance are in red, and decreased are in green. Proteins (not changed in our data set) predicted by IPA to be activated are in orange; predicted to be inhibited are in blue. Only proteins with statistically significant changes in abundances at a FDR of 5% ($q < 0.05$)

between HD and control (A) or HDL2 and control (B) were included in analysis. The key to IPA pathways and networks shapes is included in the Supporting Information.

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Table 1Summary of Control and HD/HDL2 Cases Selected for Analysis^{91a}

case	diagnosis	PMD (h)	VS grade	age	sex	race
HD057	HD	0	2	65	M	W
HD058	HD	17	3	44	M	W
HD061	HD	6	3	69	M	W
HD068	HD	17	2	56	F	W
HD095	HD	7	4	32	M	W
HD098	HD	8	3	69	M	W
HD262	HD	6	3	53	F	W
HD283	HD	22	4	51	F	W
HD288	HD	9.5	3	57	F	W
HD290	HD	10	3	52	M	W
HD306	HD	6	3	67	M	W
HD309	HD	7	3	59	M	W
HD205	HDL2	24	4	49	M	W
HD237	HDL2	8.5	4	57	M	B
HD240	HDL2					
HD244	HDL2	11.5	3 to 4	58	M	B
HD261	HDL2	3	3	41	F	B
HD304	HDL2	29.5	3	46	M	
C003	control	24		46	F	B
C004	control	19		42	M	W
C006	control	24		54	F	W
C008	control	17		50	M	B
C009	control	6		48	M	B
C011	control	15		57	M	W
C012	control	11		64	M	W
C013	control	20		59	M	B
C384	control	14		68	M	W
C702	control	6		40	M	W

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case	diagnosis	PMD (h)	VS grade	age	sex	race
C994	control	12		49	M	W
C2234	control	12		68	F	W

^aPMD, post-mortem delay; VS, Vonsattel grade of the severity of neuropathological changes, 91 Cases used for comparative analysis of HDL2 and control groups are in bold.